ABSENCE OF AN IMMUNODOMINANT MHC CLASS Ia-RESTRICTED EPITOPE
ELICITS A COMPENSATORY MHC CLASS Ib-RESTRICTED CD8+ T CELL
RESPONSE TO MOUSE POLYOMAVIRUS CNS INFECTION

A Thesis in
Anatomy
by
Amrita Jaiprakash

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The thesis of Amrita Jaiprakash was reviewed and approved* by the following:

Aron E. Lukacher
Professor of Microbiology and Immunology
Chair of the Department of Microbiology and Immunology
Thesis Advisor

Patricia McLaughlin
Professor of Neural and Behavioral Sciences
Director of the Graduate Program in Anatomy

James Connor
Distinguished Professor of Neurosurgery, Neural and Behavioral Sciences, and Pediatrics

Todd Schell
Professor of Microbiology and Immunology

* Signatures are on file in the Graduate School
ABSTRACT

CD8+ T lymphocytes are the major immunocytes responsible for host defense against viral infection. The ligand for a CD8+ T cell receptor consists of a short peptide derived from a viral protein bound to a heterodimeric molecule encoded within the major histocompatibility complex (MHC). Most MHC molecules are highly polymorphic, a property responsible for the extensive repertoire of peptides available for recognition by T cells; these MHC molecules are designated MHC-Ia. Given the dynamic interplay between a host’s immune system and viruses, viruses often mutate antigenic peptides to prevent binding to MHC-Ia molecules and thereby evade recognition by antiviral CD8+ T cells. Recently, non- and oligo-morphic MHC-Ib molecules have been described as adept presenters of microbial peptides to CD8+ T cells across most outbred members of a given species. Our group recently uncovered a protective MHC-Ib-restricted CD8+ T cell response to mouse polyomavirus (MuPyV), a natural, persistent mouse pathogen; however, this response was only detected in mice lacking MHC-Ia molecules. In this study, we asked whether ablation of the dominant MHC-Ia-restricted viral peptide epitope in MuPyV-infected immunocompetent mice would allow emergence of CD8+ T cells specific for a minor MuPyV capsid protein derived epitope, Q9:VP2.139-147. This restriction element is part of the MHC-Ib family and is shared across inbred mouse strains with varying MHC-Ia alleles. To test the proposed hypothesis, we inoculated C57BL/6 mice intracerebrally (i.c.) with a mutant MuPyV lacking the dominant CD8+ T cell epitope D^b:LT359. The i.c. route of inoculation causes robust recruitment of anti-MuPyV T cells to the brain, an organ targeted by the human JC polyomavirus to cause the often-fatal demyelinating disease, progressive multifocal leukoencephalopathy. Using this LT359null mutant virus and assessing T cell responses by intracellular cytokine staining (ICS), we uncovered a strong MHC-Ib-restricted CD8+ T cell response to the Q9:VP2.139 epitope. Notably, VP2.139+ CD8+ T cells were observed in the infected brain, but not the spleen, although virus
levels were similar in both organs. In contrast, MuPyV-specific CD8\(^+\) T cells recognizing subdominant MHC-Ia epitopes were detected in both the brain and spleen. The compensatory increase by a non-classical MHC-Ib-restricted CD8\(^+\) T cell population suggests that MHC-Ib-restricted CD8\(^+\) T cells may serve as a backup host defense mechanism for polyomaviruses infecting the CNS that mutate viral peptides presented by MHC-Ia molecules.
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<table>
<thead>
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<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>β2m</td>
<td>Beta 2 microglobulin</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>dpi</td>
<td>Days post infection</td>
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<tr>
<td>f.p.</td>
<td>Footpad</td>
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<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<tr>
<td>i.c.</td>
<td>Intracerebral</td>
</tr>
<tr>
<td>IFN-</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IRIS</td>
<td>Immune reconstitution inflammatory syndrome</td>
</tr>
<tr>
<td>JCV</td>
<td>John Cunningham Virus</td>
</tr>
<tr>
<td>K.O.</td>
<td>Knockout</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LT</td>
<td>Large T antigen</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MHC-I</td>
<td>Major Histocompatibility Complex class I</td>
</tr>
<tr>
<td>MHC-Ia</td>
<td>Major Histocompatibility Complex class Ia</td>
</tr>
<tr>
<td>MHC-Ib</td>
<td>Major Histocompatibility Complex class Ib</td>
</tr>
<tr>
<td>MHC-II</td>
<td>Major Histocompatibility Complex class II</td>
</tr>
<tr>
<td>MT</td>
<td>Middle T antigen</td>
</tr>
<tr>
<td>MuPyV</td>
<td>Mouse Polyomavirus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>NCCR</td>
<td>Non-coding control region</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>p:MHC</td>
<td>Peptide-major histocompatibility complex</td>
</tr>
<tr>
<td>pAPC</td>
<td>Professional antigen presenting cell</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post infection</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque-forming units</td>
</tr>
<tr>
<td>PML</td>
<td>Progressive multifocal leukoencephalopathy</td>
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<tr>
<td>PyV</td>
<td>Polyomavirus</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SLO</td>
<td>Secondary lymphoid organs</td>
</tr>
<tr>
<td>ST</td>
<td>Small T antigen</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal transducer and activator of transcription 1</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TCRβxδ/-</td>
<td>T cell receptor β x δ knockout</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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Finally, I must thank Lucius and Ophelia, for welcoming me into the refuge of their fur and purrs after long days in the lab.
Chapter 1: Introduction

I. Primary immune response to viral infection

Parasitism has been eminent in the evolution of what is now a remarkable host defense system against pathogens. Of the most commonly encountered lethal microorganisms, obligate parasites such as viruses challenge the immune system of virtually every organism. The inadequacy of innate immunity in combating intracellular viral infection pressures the system to include specialized, adaptive lymphocytes and diversified antigen-receptor genes. The meticulous communication network of cytokines, chemokines, protein messengers, and receptors exemplifies the affiliation of the two arms of the immune system, the innate and adaptive, and their joint efforts in upholding host viability.

Viral denomination encompasses a number of properties that vary between viral families. As a whole, viruses contain genomic proteins enclosed in a protein shell. The varying features include the nature of the genome - whether it is single or double stranded DNA or RNA, symmetry of the capsid, the presence of an envelope, and the dimensions of the virion. Without individual replicative machinery, these microscopic entities are unable to survive and multiply alone, accordingly requiring and depending on a host organism. The hijacking of host cells contributes greatly to disease manifestation. Viruses are the causative agents of a multitude of human diseases. The evolution of the human immune system is the product of stressors, provoking the defense systems to learn how to identify and challenge pathogens that threaten a host’s functional integrity.

Initial infection launches an acute immune response by members of the innate immune system. Host cells, once infected, have an array of defensive mechanisms to prevent hijacking of replicative machinery, or halt DNA replication or protein production en masse. Signaling cascades in such instances prompt the release of protein messengers, chemokines, and cytokines (e.g., TNF-
α, IL-1, IL-6, and type I interferons) that have a paracrine influence on the local microenvironment to initiate a pro-inflammatory antiviral response. The extravasation of neutrophils, natural killer (NK) cells, and peripheral dendritic cells (DC) from the vasculature into infected tissues begins the direct elimination of infected cells and processing of pathogenic particles, a step that further amplifies inflammation of the site. Along with direct lysis of infected cells, NK cells secrete IFN-γ, a major antiviral cytokine with immunomodulatory properties, including enhancement of viral antigen processing/presentation and cell surface expression of major histocompatibility complex (MHC) class I (MHC-I) and MHC class II (MHC-II) molecules. Immature local and recruited antigen presenting cells (APC) are vital players of the primary immune response that use an alternative approach in aiding viral clearance. Macrophages and DCs are heavily involved during acute phases of infections. However, DCs have exceptional efficiency in endocytosing proteins and creating antigenic peptides that puts them at the forefront of initiating antigen-specific T cell responses. Viral components with the ability to bind toll-like receptors and NOD-like receptors on DCs and macrophages triggers their maturation, subsequent phagocytosis of compromised cells, and proteolysis of the virus and its particles. Their release of endogenous pyrogens (e.g., TNF-α, IL-1β, and IL-6) and presentation of viral antigens on their surface MHC-I or MHC class II molecules activate the acute-phase response, communicating viral presence to the adaptive immune system.

The MHC is an essential feature of a cell's innate ability to communicate its functional integrity to the immunological environment. MHC-I are molecules presented on the surface of all nucleated cells and fall under two subclasses: MHC class Ia (MHC-Ia) that are polymorphic among members of a given species, and MHC-Ib, which are oligo- or non-polymorphic. The diverse spectrum of peptide binding that classical MHC-Ia molecules exhibit is an important characteristic
of their subcategory of proteins and is fundamental to their role in adaptive immunity as activators of antigen-specific CD8\(^+\) T cells. All MHC-I molecules are heterotrimers, consisting of a heavy chain, \(\beta_2\)-microglobulin, and an octa-, nona-, or decameric peptide within the antigen binding groove. Within the antigen binding groove are a varying number of anchoring pockets that, based on the allele, can be stably occupied by a fixed residue or set of residues that are characteristically similar.\(^6,7\) Such residue sets can be exemplified by the MHC-Ia K\(^b\) restriction element which has two anchor pockets at positions five and eight. The anchor pocket within the \(\alpha_1\) domain will stably bind either phenylalanine and tyrosine, which are two of three aromatic amino acids, as a residue in position five of the nonameric peptide sequence.\(^7\) As they are highly polymorphic due to hypervariability in their antigen-binding groove, the repertoire of antigenic peptides that MHC-Ia molecules can bind and present is tremendously large. Modifications of the amino acid residues within the antigen-binding groove determine which short peptide segments can be bound and expressed to CD8\(^+\) T cells. Each T cell receptor (TCR) on the surface of a CD8\(^+\) T cell is only able to recognize unique epitope combinations, leaving the bone marrow and thymus to, remarkably, differentiate and mature a vast number of T cells.\(^8\)

Unlike their classical counterparts, the non-classical MHC class Ib (MHC-Ib) proteins are characteristically oligomorphic, having a selective, more narrow pattern of expression despite being structurally similar to MHC-Ia. Wide-range expression on cells, but not ubiquity, aids its roles within innate and acquired immunity, such as (but not limited to) CD8\(^+\) T cell/NK cell activation and indirect immunoregulation.\(^8,9\) MHC-Ib epitope presentation includes peptides derived from TCR variable regions, intracellular proteins and lipids, and, primarily, MHC Ia-signal peptides. Receptors of other leukocyte subsets act as secondary activators for the innate immune system.\(^9\)
The exploration of the role of MHC-Ib molecules in cell-mediated immunity has revealed their role in host defense against viruses and bacteria, such as human cytomegalovirus, influenza virus, herpes simplex virus (HSV), *Mycobacterium tuberculosis*, and *Salmonella typhimurium*.\(^{10-13}\) The contribution of oligomorphic MHC class I molecules introduces the possibility of a fail-safe defense system against conventional MHC-Ia-restricted CD8\(^+\) T cell-evading pathogens. Their contribution also creates the possibility of new vaccine strategies that cover a broader range of MHC haplotypes. Overall, the addition of non-classically-restricted CD8\(^+\) T cells diversifies cell-mediated antiviral immunity.

MHC-Ib genes are homologous to their MHC-Ia counterparts within the same species. Such genes between humans and mice are considered functionally orthologous, in that they bind peptides with equivalent lengths, have similar MHC anchor residues, and are capable of interacting with receptors on NK cells and CD8 T cells.\(^9\) H-2 MHC-Ib molecules characterize the antigenic peptide binding of HLA-E, -F, and -G.\(^8\)

### II. The adaptive immune response

Despite being delayed by several days, the adaptive immune response, mediated by T and B lymphocytes, launches an intensive pursuit for circulating and covert virus that concludes only once cognate antigen is no longer present at high levels, or completely cleared.\(^{14}\) Antiviral clearance primarily involves T lymphocytes whose absence or defective functioning can result in higher viral loads and diseases for the host.\(^{15}\) Specifically, CD8\(^+\) T cells are the indispensable contributors to antigen-specific elimination. B lymphocytes and their neutralizing antibodies (Abs)
emerge during primary infection with similar kinetics to the CD8+ T cell response, but play a more significant role in prevention of viral spread during secondary viremia.2,16

The migration of recently-matured DCs to secondary lymphoid organs (SLO), such as the spleen and lymph nodes, after encountering virus is crucial for the activation of naive CD4+ and CD8+ T cells, and the overall adaptive immune response. A chemotactic gradient of CCR7 ligands, CCL19, and CCL21 from SLOs influence DCs to migrate via lymphatic vasculature.17 Within the SLOs, DCs present their MHC class I/II-antigenic peptide complexes to naive T cells that coincidentally reside in lymphatic tissue that drains infected areas. Naïve T cells require at least three signals for complete activation: binding of their TCR to cognate antigen presented on a class I or II MHC molecule on the surface of a professional APC (pAPC), which can fine-tune T cell priming by its signal strength and duration; costimulation of the pAPC ligands (e.g., CD28, CD40 ligand) to respective cell surface receptors on the lymphocyte surface (e.g., CD80, CD86) for heightened signal transduction; and inflammatory cytokine stimulation for greater effector functioning, survival, and memory population integrity.4,18–21 Successful activation induces their proliferation and differentiation into mature effector CD8+ and CD4+ T cells.

CD8+ T cells are not dependent on CD4+ T cells during their acute response to viral infection. However, the absence of CD4+ T cells promotes extensive attrition of CD8+ T cells during low-level viral persistence. Impaired differentiation of, and recall-incompetent, memory CD8+ T cells are generated in MHC class II-deficient mice, provoking flawed CD8+ T cell-mediated immunity that is further marred by a diminished ability to recruit and maintain de novo primed virus-specific CD8+ T cells during chronic infection.22 Contraction of CD8+ T cells after the primary response to an acute viral infection, even in the presence of CD4+ T cells, is grossly paralleled with viral clearance. Cells that persevere into memory phenotypes require binding of
IL-7 to receptors expressed on their surfaces, and IL-15 for prolonged survival. The recall of memory CD8+ T cells during secondary infection is rapidly induced in conjunction with neutralizing Ab to efficiently defend against the invading virus.

While most viral pathogens in nature can be quickly cleared by a host’s acute immune response, a handful, such as human immunodeficient virus (HIV), polyomaviruses (PyV), and HSV, are able to maintain their pathogenic potential while in dormant, non-lytic states. The need for CD8+ T cells to identify and eliminate compromised host cells is a grueling feat that poses the risk of causing loss of functionality and deletion with recurrent antigen encounter. This phenomenon, termed T cell exhaustion, more often occurs to CD8+ T cells recruited and primed early in acute infection than in de novo primed cells during the persistent phase. Memory CD8+ T cells recruited during the smoldering phase of infection possess greater effector capabilities than their acute-phase counterparts, surviving at higher frequencies and eliminating virus more appreciably. Even so, the cytotoxic capacity of these cells is harmful to more than just compromised host cells regardless of infection stage; non-compromised cells and tissue regions may be subjected to necrosis caused by an inflammatory microenvironment. CD8+ T cells have the challenge of minimizing immunopathology while simultaneously clearing infected cells.

The fundamental role of MHC-Ia and -Ib-restricted CD8+ T cells in immunosurveillance and viral clearance provides the logical basis to peptide-based and T cell vaccination. Under the premise of limited polymorphisms in murine and human non-classical MHC molecules, understanding of human antiviral immunity can be derived from murine-based investigations. For example, H-2Q9, a frequently characterized non-classical mouse MHC molecule, can identify with non-classical human HLA-E, -F, -G, and classical HLA-C. The discovery of a CD8+ T cell population restricted by Q9, a member of the Qa-2 locus, and specific for murine polyomavirus
(MuPyV) grants the opportunity for an in-depth analysis of the role of such CD8+ T cell subsets in cell-mediated immunity within low-level, persistent viral infections, and suggests novel approaches to viral vaccine therapies.

III. Polyoma virus

Polyoma-viridae (PyV) are a family of non-enveloped, icosahedral, double-stranded DNA tumor viruses. The virus, only 40-45 nm in diameter, has a wide host range, including humans, primates, mice, and birds. Most mammalian PyV’s persist as lifelong obligate parasites without causing severe, acute disease in immunocompetent hosts. The first two human PyV’s, BK and JC virus (JCV), were coincidentally reported in 1971 by two different groups. The latter virus was identified in the brain tissue of a patient, John Cunningham, who suffered from Hodgkin's lymphoma and, later, progressive multifocal leukoencephalopathy (PML) as a result of immunocompromised status from the primary diagnosis. At present day, thirteen strains of human polyomaviruses exist, but not all are associated with disease.

The route of natural transmission of JCV has not been identified, but considering its persistent infection of the kidneys and detection of JCV DNA in urine and feces, a fecal-oral route is now considered more likely than a respiratory one. However, other routes of entry may include transplacental, organ transplantations, blood transfusions (possibly secondary to bystander inoculation by cutaneous polyomaviruses), and respiratory. The variance of efficient dissemination in different communities also implies that some cultural aspects may influence transmission.

Initial infection with JCV causes an asymptomatic viremia followed by low-level persistence in the kidneys and bone marrow. Opportunistic in nature, JCV does not demonstrate
its pathogenic potential until the immune system begins to fail, developing gaps in its defensive capabilities. The infrequency of PML development is accentuated by the necessity for the virus to travel to the brain and establish permissive infection in host cells, a clandestine feat involving evasion of peripheral- and neuro-immunosurveillance.\textsuperscript{33,34} It has been speculated that astrocytes and oligodendrocytes are targeted.\textsuperscript{34}

Anti-human PyV Abs are detected in most healthy, asymptomatic people. All JCV strains belong to a single serotype, but fall under the classification of Type A, B, or C based on genetic variations. Strain-specific infection is distinguishable by geographical locations across Europe, Asia, and Africa. Age is also a factor in JCV seroepidemiology;\textsuperscript{35,36} >80\% of adults are positive for JCV-reactive VP1 Abs.\textsuperscript{31,37} Primary infection does not produce any recognizable disease in immunocompetent hosts.\textsuperscript{38} Susceptibility to developing PML is heightened in HIV-1/AIDS patients prior to highly antiretroviral therapy (HAART) HIV-1/AIDS, and in those who receive immunomodulatory therapies, organ transplants and suffer from hematological malignancies.\textsuperscript{39} The pre-HAART era had an incidence rate of 3.3 cases per 1000 person-years for HIV patients. Although treatment with HAART has reduced the risk to 1.3 PML cases per 1000 person-years, positive HIV status and the duration of such infection remains a major predisposing disorder for PML.\textsuperscript{40} More recently, immunotherapies for treatment of autoimmune diseases has provoked vulnerability in a new population of patients. The highest risk for PML in patients receiving Natalizumab, with an incidence of 11.1 cases per 1000 patients, are those who are positive for anti-JCV Abs with prior use of immunosuppressants and a current duration of Natalizumab therapy for a minimum of 48 months.\textsuperscript{41} The increased incidence of PML since 2005 can be attributed to the greater use of such immunomodulatory therapies.\textsuperscript{37} Natalizumab and other such monoclonal antibody-based medications prevent T and B cell extravasation into the brain by binding to their
surface α-integrin molecules, thus compromising neuro-immunosurveillance and indirectly leaving the brain vulnerable to PML manifestation.\textsuperscript{41,42}

The clinical diagnosis of PML is often achieved through MRI, histology, and PCR using cerebrospinal fluid.\textsuperscript{42,43} PML lesions by MRI exhibit hyperintense discontinuities in tissue and usually do not display edema. Additionally, lesions are often in the frontal or parieto-occipital regions, though they can vary in location within the subcortical white matter. The pathological lesions of PML are typically small, irregularly-shaped, demyelinated plaques that coalesce into larger foci. Their ill-defined boundaries are bordered by macrophages, ‘bizarre’ astrocytes, and oligodendrocytes with enlarged nuclei.\textsuperscript{34} The aggressive growth and confluency of lesions are attributed to the unabating necrotic lysis (less frequently, apoptosis occurs as well) of oligodendrocytes which serve as one of several permissive cellular hosts for the virus. The loss of myelin concomitantly causes a loss of function that can aid in distinguishing PML from other neurologic diseases. Cognitive impairments, disorientation, behavioral changes, motor dysfunction, ataxia, and hemianopsia are just a few of the symptoms of worsening PML.\textsuperscript{42}

No vaccines are available against JCV; several therapeutic agents have successfully decreased JCV replication \textit{in vitro}, but none have been effective in clinical studies.\textsuperscript{34} Current treatments mainly focus on reconstituting the immune response. The initiation of HAART in HIV-1 patients and the cessation of immunomodulatory therapies such as Natalizumab in patients diagnosed with autoimmune diseases and PML is a definitive first step towards abating neurodegeneration. However, reconstitution of the immune system can drive an influx of heterogeneous infiltrates and is associated with amplified tissue injury and cerebral edema. Due to an abundant amount of viral structural proteins indicative of replicating virus, many oligodendrocytes bordering lesions have enlarged nuclei.\textsuperscript{39} The aggravation of PML as a
complication of immune reconstitution, or “overshooting” of the immune response, is referred to as PML - Immune Reconstitution Inflammatory Syndrome (IRIS), and is almost always seen in PML patients who discontinue their immunosuppressive agents, and in approximately 30% HIV-patients who initiate HAART after PML diagnosis.\textsuperscript{44,45} Plasma exchange in immunomodulatory-associated cases provides better prognosis. Successful reconstitution of the immune system after Natalizumab cessation has documented 21% mortality, but no surviving patients escape neurological deficiencies. In HIV related cases, 50 - 80% of patients may survive with HAART therapy.\textsuperscript{34}

IV. The immunologic response to mouse polyomavirus

The discovery of MuPyV by Gross, Stewart, and Eddy in the mid-20th century led to the coining of the polyomavirus family designation, an appropriate name for a virus with significant oncogenicity. The PyV genome, which is contained in a single molecule of circular, double-stranded DNA, approximating only 5 kbp in size, is divided into three major regions: of the polyomavirus family designation, an appropriate name for a virus with significant oncogenicity. The PyV genome, which is contained in a single molecule of circular, double-stranded DNA, approximating only 5 kbp in size, is divided into three major regions: the non-coding control region (NCCR), early coding region, and late coding region (Figure 1).\textsuperscript{46} The genome codes for six protein: three structural and three non-structural. The early coding region
encodes three of the six proteins, the small, middle, and large tumor antigens, while the late coding region encodes remaining three, the VP1, VP2, and VP3 viral capsid proteins. Overall, tumor antigens (T-Ag) are responsible for interference with cell cycle regulation including initiation of late viral mRNA transcription, and tumor formation through cellular transformation. Specifically, large T-Ag (LT) is involved in disabling its hosts’ cell cycle arrest machinery and small T-Ag (ST) promotes cellular transformation. Middle T-Ag (MT) plays a major role in viral assembly, regulation of viral DNA replication and RNA transcription, and tumor induction. MT also works in cooperation with the small T-Ag (ST) to oppose apoptosis caused by the p53 tumor suppressor gene.\textsuperscript{47,48} Of the three late viral capsid proteins, the major structural protein comprising more than 70% of total virion protein content is VP1. Virions consist of 72 pentamers of VP1, each associated with a single protein of the two other structural proteins, which are termed the minor structural proteins.\textsuperscript{28} The interaction between VP1 and the terminal saccharide on many glycolipids - sialic acid - is fundamental for virion entry into one of many distinct host cell types.\textsuperscript{16} Encoding of all T-Ag proteins occurs regardless of a cell's productive or non-productive status, but its permissiveness for viral replication determines whether the structural capsid proteins will be transcribed. Successful splicing of the late regions RNA transcripts produces structural proteins that will drive productive infection, virus assembly, and the dissemination of virions through cellular lysis.\textsuperscript{49}
The adaptive immune response is a necessary consequence of viral infection, especially infections that are persistent. MuPyV-infected mice with severe combined immunodeficiency (SCID), which lack T and B lymphocytes, are prone to death by two weeks post-infection (p.i.) by an acute myeloproliferative disease. Humoral protection is limited to viral clearance by neutralization, as shown in T-cell deficient (TCR β x δ chain knockout [KO] mice [TCRβxδ−/−]) mice that maintain high levels of protective anti-MuPyV IgG and IgM in serum. The task of withstanding oncogenesis, therefore, is the primary responsibility of the T lymphocyte mediated adaptive immune response. Even single depletion of either CD8+ or CD4+ T cells causes a slight increase in the occurrence of tumors, and the virus may exist in a limited number of organs aside from the kidney and spleen, which are frequently infected in mice with intact T cell responses. Dual depletion of CD8+ and CD4+ T cells, however, leave mice with 29% higher susceptibility to tumor development, and persistent virus is harbored in multiple organs (bone, gonads, liver, skin, etc.) for up to six months p.i. This has also been demonstrated in β2-microglobulin (β2m)−/− mice which have no MHC-Ia or MHC-Ib expression on cell surfaces. To a greater extent, these mice are similarly prone to tumor growth and persistent viral infection in an array of organs than CD8−/− mice. The interaction between MHC-I-restricted viral epitopes and TCRs from CD8+ T cells is perhaps the most essential communication required for prevention of virus-induced neoplasia.

MHC-Ia molecules derived from H2-Db and H2-Kb are most frequently observed during MuPyV infection, concomitantly increasing the prevalence of CD8+ T cells restricted to such proteins. At the peak of CD8+ T cell expansion (8 days post infection (dpi)), a hierarchy of epitope-specificity can be distinctly observed by tetramer staining. Tetramers, an artificially synthesized
complex of four peptide:MHC (p:MHC) complexes (including β2m), streptavidin, and a fluorophore, are commonly used to bind to and quantify cells exhibiting a specific MHC type and peptide (Figure 2).

Use of this technique has shown that approximately 75% of brain isolated CD8$^+$ T cells from intracerebrally (i.c.) infected mice are restricted to the D$^b$:LT359-368 viral epitope. Tetramer staining on splenocytes isolated from footpad (f.p.) infected B6 mice concludes approximately 5% to be K$^b$:MT246-specific, and 1% D$^b$:LT638-specific (Table 1).

Regardless of i.c. inoculation dose being high (2 x 10$^6$ PFU) or low (5 x 10$^3$ PFU), the magnitude of CD8$^+$ T cell expansion and hierarchy of epitope immunodominance remains the same during the acute phase response. These CD8$^+$ T cell populations, which only differ in their recruitment by varying antigen dose, contract by day 12 p.i., a delay in expansion kinetics between the two doses notwithstanding. This indicates the beginning of the persistent phase of infection and a low viral load, sufficient for CD8$^+$ T cell surveillance.

<table>
<thead>
<tr>
<th>Epitope</th>
<th>MHC Restriction</th>
<th>Frequency of CD8$^+$ T cells in MuPyV Infected C57BL/6J mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT359</td>
<td>D$^b$</td>
<td>75% Brain, 30% Spleen</td>
</tr>
<tr>
<td>MT246</td>
<td>K$^b$</td>
<td>10% Brain, 5% Spleen</td>
</tr>
<tr>
<td>LT638</td>
<td>D$^b$</td>
<td>5% Brain, 1% Spleen</td>
</tr>
<tr>
<td>VP2.139</td>
<td>Q$^b$</td>
<td>? Brain, ? Spleen</td>
</tr>
</tbody>
</table>

Table 1. Hierarchy and frequency of MuPyV-specific viral epitopes. Frequencies were determined through tetramer staining of brain lymphocytes and splenocytes in either i.c. or f.p. infected B6 mice, respectively, 8 dpi.
escape. Despite the progressive decline of competent memory CD8$^+$ T cells due to functional exhaustion and reduced observance of cognate Ag during chronic infections, the quantification of IFN-γ-producing LT359$^+$ CD8$^+$ T cells and LT359 tetramer-stained cells are similar. This suggests that functional avidity is maintained by the surviving memory CD8$^+$ T cells, albeit a decline from the acute phase. These cells, along with de novo primed MuPyV-specific CD8$^+$ T cells that are recruited to offset ineffective and functionally exhausted cells, sustain a consistent long-term antiviral response.

Curiously, K$^{b/-}$D$^{b/-}$ C57BL/6J (B6) mice are resistant to tumors induced by MuPyV and successfully control viral load as efficiently as wildtype (WT) B6 mice. In efforts to elucidate the ability and inability of K$^{b/-}$D$^{b/-}$ and β2m$^-$ mice, respectively, to resist tumorigenesis, a Q9-restricted MHC-Ib-restricted peptide was identified. The identification of the Q9:VP2.139-147 epitope is an unusual finding given the scarcity of non-classical MHC involvement in viral immunity. VP2.139$^+$ CD8$^+$ T cells expand 8 dpi, similar to conventional MHC-Ia-restricted MuPyV-specific CD8$^+$ T cells. However, they exhibit an extended, escalating response over three months and terminate the progressive growth in a high-volume plateau phase. 40% and 80% of splenic and blood CD8$^+$ T cells, respectively, were specific for VP2.139 80 dpi through an initial f.p. infection. Another distinguishing factor is that VP2.139$^+$ CD8$^+$ T cells are minimally (10 - 14%) contributed by de novo primed CD8$^+$ T cells recruited during the concurrent protracted expansion and persistent phase of infection, and only require cognate Ag for proliferation but not maintenance. A large fraction of these cells are in a low- or non-proliferative state during the expansion phase, and a small fraction stain positive for Annexin V, a protein that binds to phosphatidylserine to identify apoptotic cells. These features of distinguish VP2.139$^+$ CD8$^+$ T cells from MHC-Ia-restricted MuPyV-specific CD8$^+$ T cells.
The unique kinetics and unconventional behavior of non-classical-restricted CD8\(^+\) T cells reinforces the multifaceted nature of the CD8\(^+\) T cell defense system. Although the Q9:VP2.139-specific CD8\(^+\) T cell population presents distinctive, persistent infection-phase kinetics that could tackle MuPyV infection, these cells are minimally represented in the total MuPyV-specific CD8\(^+\) T cell pool in mice infected with the A2 strain of MuPyV.\(^{18}\) By site-directed mutagenesis of the LT359 epitope to replace D\(^b\)-binding amino acids with non-binding residues, N. Andrews created a mutant MuPyV to investigate the reserve capacity of subdominant viral-epitope-specific CD8\(^+\) T cells,\(^{54}\) a sui generis approach that has revealed an unexpected compensatory re-ranking of the MuPyV epitope hierarchy. The removal of such an antigen allows inquiry into the extent to which, or if at all, antiviral immunity can be achieved. This thesis will explore the outcome of removing the immunodominant D\(^b\):LT359 epitope in MuPyV in regards to the ability of CD8\(^+\) T cells to control infection and resist pathogenesis, and the scope of MHC Ib-restricted CD8\(^+\) T cell contribution. I predict that the absence of such an epitope will diminish overall MPyV-specific CD8\(^+\) T cell numbers and viral clearance in the brain and spleen.
Chapter 2: Materials and Methods

Mice

C57BL/6J (B6) female mice were purchased from the National Cancer Institute at the Frederick Cancer Research and Development Center (Frederick, MD). Their housing at the Pennsylvania State University College of Medicine was in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Viruses and virus inoculation

B6 mice were infected i.c. in the right frontal lobe with 1 x 10^6 PFU of either parental MuPyV (A2 strain) or the mutant A2.LT359null virus in 30 μL DMEM with 5% FBS. Mice were infected between 6 – 10 weeks of age. The stock A2.LT359null virus was generated using the previously described PCR-based site-directed mutagenesis to change amino acid 363 in LT from arginine to threonine.54

Synthetic peptides

D^b:LT359-368 (SAVKNYCSKL), K^b:MT246-253 (SNPTYSVM), D^b:LT638-646 (MGVANLDNL), and Q9:VP2.139-147 (HALNVVHDW) peptides were purchased from Peptide 2.0 Inc. Peptide stocks were solubilized in DMSO or PBS and stored at -20°C, and were thawed and diluted in assay medium immediately before use.

DNA Isolation and quantitative PCR
DNA isolation was performed on spleens, brains, and kidneys using the Maxwell® 16 System DNA Purification Kits (Promega), and the resulting DNA isolate was quantified using the ND-1000 Spectrophotometer (Thermo Scientific). Real time qPCR (Applied Biosystems) was run using the previously described primers and amplification parameters in the works of C. Kemball.\textsuperscript{14}

\textit{Lymphocyte isolation and flow cytometry}

Tetramers for H-2D(b):LT359, H-2D(b):LT638, H-2K(b):MT246, and Q9(K\textsuperscript{b}):VP2.139 (H-2Q9-H-2K(b) chimera) were obtained from the NIH Tetramer Core Facility at Emory University (Atlanta, GA).

Mice were sacrificed day 8 p.i. Single-cell suspensions were derived from brains and RBC-lysed spleens. For brain isolation, mice were perfused with 10% heparin in PBS and the brain was digested by collagenase and DNase and layered on a Percoll gradient of 44% in DMEM with 2% FBS and 66% in PBS. After centrifugation, the myelin layer was removed from the cellular suspension. Cells were plated in 96-well round-bottom plates and surface stained for FITC-conjugated anti-CD8\textalpha{} (Invitrogen, CT-CD8\textalpha{}); AF700-conjugated (BioLegend) or V500-conjugated (BD Bioscience) anti-CD44 (IM7); APC-conjugated LT359, LT638, and MT246 tetramers; and BV421-conjugated VP2.139 tetramer. Splenocytes were isolated per standard protocol and stained identically with the brain lymphocytes.

\textit{Intracellular staining}

Cells were stimulated directly ex vivo with 1 μM of either LT359, LT638, MT246, or VP2.139 peptide and 0.2% brefeldin A (GolgiPlug), diluted in 0.2 ml/well Isocove’s Media containing 10%
FBS and 1% L-glutamine (Gibco, 200 mM) for 4.5 h in 96-well round-bottom plates. Cells were then stained for 30 minutes with Abs for the aforementioned cell surface markers. After washing, cells were permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) and stained intracellularly with APC-conjugated anti-IFN-γ (BioLegend, XMG1.2); PE-conjugated (BD Bioscience) or PE-Cy7-conjugated (eBioscience, TN3-19) anti-TNF-α; and PE-Cy7-conjugated anti-IL-2 (BioLegend, JES6-5H4) for 45 minutes.

**Statistics**

Statistics of data was calculated using statistical software (Prism; GraphPad Software, Inc.). All non-parametric data analyses used the Mann-Whitney U test. Statistical significance was considered when $P \leq 0.05$. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Error bars indicate mean ± SD.
Chapter 3: Results

D\textsuperscript{b}:LT359 epitope-specific CD8\textsuperscript{+} T cells are the dominant virus-specific CD8\textsuperscript{+} T cell population to MuPyV infection in C57BL/6 mice, contributing >70\%.\textsuperscript{53} We sought to ask whether CD8\textsuperscript{+} T cells control MuPyV infection in the brain. A mutant MuPyV lacking expression of this dominant epitope was isolated and utilized to ascertain deficits in viral control. The asparagine codon for the major D\textsuperscript{b} binding residue of the LT359-368 sequence was changed to threonine, thereby rendering the virus unable to elicit a LT359\textsuperscript{+} CD8\textsuperscript{+} T cell response upon infection. This virus, designated A2.LT359\textsuperscript{null}, forces the host to either adapt its MuPyV-specific CD8\textsuperscript{+} T cell population or be overcome by unchecked viral replication. We had previously shown that CD8\textalpha KO mice can generate a CD4\textsuperscript{−}CD8\textsuperscript{−} D\textsuperscript{b}:LT359-specific T cell response in the spleen and in the brain (E.L. Frost, unpublished data), and that antibody-mediated CD8\textsuperscript{+} T cell depletion is incomplete. Ablating the LT359 epitope in the virus itself rather than globally depleting the CD8\textsuperscript{+} T cell compartment in the host provided a strategy to specifically gauge the impact on viral control in an organ so heavily dominated by CD8\textsuperscript{+} T cells of a particular specificity. An alternative possibility is that infection by A2.LT359\textsuperscript{null} (hereby referred to as LT359\textsuperscript{null}) virus result in compensation by CD8\textsuperscript{+} T cells directed to the subdominant K\textsuperscript{b}:MT246 and/or D\textsuperscript{b}:LT638 epitopes; if this prediction was validated, virus infection in the brain may be controlled.

Determining the hierarchy of D\textsuperscript{b}- and K\textsuperscript{b}-restricted MuPyV-specific CD8\textsuperscript{+} T cells

To ascertain whether MuPyV-specific CD8\textsuperscript{+} T cells control viral infection and possibly observe their populations adaptive capabilities, adult B6 mice were inoculated i.c. with either A2 or A2.LT359\textsuperscript{null} virus. Brain lymphocytes and splenocytes were isolated at day 8 p.i., and MHC-
Figure 3. LT359null virus-infected B6 mice infected have similar numbers of activated CD8\(^+\) T cells and the ability to control virus with the same efficacy as A2 infected mice. Isolated brain lymphocytes and splenocytes were stained with surface marker Abs and MuPyV-specific MHC-Ia tetramers and analyzed after flow cytometry. (A) Frequency of tetramer-specific activated brain- (left) and spleen-derived (right) T CD8\(^+\) T lymphocytes 8 dpi (n = 4 – 26 mice for each indicated tetramer; Data pooled from five separate experiments). (B) Dot plots of tetramer-specific CD44\(^+\) T cells in the brain (left) and spleen (right) were gated on CD8\(^+\) cells. Values represent the percentage of cells within the indicated gate. (C) Frequency of activated CD8\(^+\) T cells in the brain and spleens at 8 dpi (n = 25 – 37 mice for each indicated organ and experiment arm). (D) Tissue samples from the brain, spleen, and kidney were isolated 8 dpi for DNA. Genome copies of MuPyV per µg of tissue was determined through real time qPCR. The line represents the LOD (limit of detection) (n = 5 – 10 mice for each indicated organ and experiment arm). ****, P < 0.0001; ***, P < 0.001; *, P < 0.01. Data are shown mean +/- SD.
Ia epitope specific CD8+ T cell epitopes were assay by staining with MHC-Ia tetramers directly ex vivo. FACS analysis of lymphocytes revealed a significant increase in the number of LT638+ CD8+ T cells and an insignificant increase in MT246+ CD8+ T cells (Figure 3a). Both epitope-specific CD8+ T cell populations significantly increased in the spleen (Figure 3a). Unexpectedly, the total number of CD8+ CD44hi T cells in the brain and spleen did not vary between A2- and LT359 null-infected groups (Figure 3c). Viral load was similar in the brain, spleen, and kidney (Figure 3d), suggesting that these cells are still able to control virus. However, despite an increase, the percentage of MT246-specific and LT638-specific CD8+ T cells did not summate to 100%. I hypothesized that another viral epitope-specific CD8+ T cell population was contributing to viral clearance.

**Accountability of the Q9(Kb):VP2.139 epitope in contributing to antiviral immunity**

Previous literature describing the influence of the non-classical MHC-Ib Q9:VP2.139 epitope on viral clearance inspired the investigation of its role in the A2.LT359 null virus mouse model. To determine whether VP2.139+ CD8+ T cells contribute to clearance of virus, brain lymphocytes and splenocytes from mice infected with either LT359 null virus or A2.H145A, a mutant virus in which the VP2.139 epitope is killed, were stimulated ex vivo with the VP2.139 peptide and stained for IFN-γ. Lymphocytes isolated from mice infected with A2.H145A did not produce IFNγ when stimulated with the VP2.139 peptide (M.D. Lauver, unpublished data). However, as shown in Figure 4a, at 8 dpi there was significantly increased IFN-γ production by VP2.139 peptide-stimulated cells in lymphocytes isolated from the brains of mice infected with LT359null virus. The output observed with VP2.139 peptide stimulation was nearly equal to that of cells stimulated with LT638 peptide, suggesting that such peptide-stimulated populations have
Figure 4. Absence of the immunodominant epitope promotes a compensatory increase in Q9:VP2.139-specific CD8+ T cells. Brain and spleen T lymphocytes were isolated from mice 8 dpi. Cells were stimulated with either LT359, LT638, or VP2.139 peptide for 4.5 hours and IFN-γ output was quantified in graphically (A) (n=9-27 mice per peptide). Gates in (B) represent the IFN-γ+ CD44+ CD8+ population. Values represent frequency of CD8+ CD44+ cells producing IFN-γ. ****, P < 0.0001; ***, P < 0.001; *, P < 0.01. Data are shown mean +/- SD. Data are pooled from a minimum of three separate experiments.
Figure 5. MuPyV-specific CD8+ T cells are dual-producers of effector cytokines. Brain (top) and spleen (bottom) T lymphocytes stimulated with either LT359, LT638, or VP2.139 peptide were permeabilized and stained for intracellular cytokines. The upper right quadrant represents co-production of IFN-γ and TNF-α. Values represent frequency of cells. n=4 mice per plot.
the capacity to offset the absence of effector function from LT359+ CD8+ T cells. The increased IFN-γ production seen in the brain was also seen in the spleen, but to a lesser degree (Figure 5). These findings are aligned with those of Philip Swanson, who observed that splenic VP2.139 tetramer-specific CD8+ T cells from subcutaneous (s.c.) infected Kb−/−Db−/− mice were functionally impaired, with >50% unable to produce intracellular IFN-γ after VP2.139-peptide stimulation at any given time.18 However, in stark contrast, this study has found that not just splenic, but also brain-derived VP2.139 peptide-stimulated CD8+ T cells were able to produce IFN-γ and TNF-α during the early stages of primary infection (day 8 p.i.) (Figure 5), with similar expression intensities in both the A2 and LT359null viruses.
Chapter 4: Discussion

In this study, it was demonstrated that the absence of the immunodominant LT359 epitope in MuPyV does not affect the ability of CD8+ T cells to clear virus. Mice subjected to i.c. infections with mutant virus lacking the immunodominant LT359 epitope could mount an antiviral response of CD8+ T cells specific for two MHC-Ia- and MHC-Ib-restricted epitopes, LT638 and VP2.139 epitopes, respectively. Considering the minimal frequencies of virus-specific CD8+ T cells specific for these epitopes in parental A2 infection models, it was interesting to note their substantial contribution to viral clearance. Brain CD8+ T cells stimulated with VP2.139 peptide produced similar levels of IFN-γ compared to cells stimulated with LT638 peptide, suggesting impressive cytokine effector competence and functional integrity. In contrast to previous literature describing a lack of IFN-γ production in splenocytes stimulated directly ex vivo with 1 µM of VP2.139 peptide (cells were derived from Kb-/-Db-/- mice infected s.c. with 10^6 PFU of A2 virus), a greater percentage of MHC-Ia-restricted splenocytes in this study produced IFN-γ than were specific for tetramer. This contrast may be explained by the inoculation route; as opposed to an intraperitoneal (i.p.) infection, i.c. infection might limit the antigen dose in the spleen. Whether the discrepancy between the percent of VP2.139+ CD8+ T cells in the spleen and their ability to produce IFN-γ is attributed to intrinsic functional deficits or to the small-scale CD8+ T cell response consequence of MuPyV i.c. infections, is not currently known. The MT246 peptide was not tested through ICS assay since MT246-tetramer staining revealed a statistically insignificant difference between tetramer+ frequencies from the two viruses, suggesting that this population does not compensate for the absence of the LT359 epitope.

Discrepancy of summation
The lack of data on the contribution of MT246+ CD8+ T cells in this LT359null virus i.c. infection model leaves a gap in the knowledge of MuPyV antigens conferring viral immunity. The frequencies of overall MuPyV-specific IFN-γ-producing cells do not summate to 100% despite the abundance of IFN-γ garnered from VP2.139 peptide-stimulated CD8+ T cells. Although lack of summation is also seen in A2 i.c. infections, the extent of inexplicable viral immunity observed with LT359null infections is more substantial. MT246- or another subdominant epitope-specific CD8+ T cell population, likely restricted to MHC-Ia because of its prevalence in viral immunity, not accounted for in this study may have compensatory increases even greater than that of VP2.139. Similar to this study, the emergence of a cryptic, immunorecessive epitope upon loss of dominant antigens has previously been described in SV40, suggesting that such an occurrence may also be possible with the loss of the MuPyV-specific Dβ:LT359 epitope.55

The dose-dependent relationship between MuPyV and MuPyV-specific CD8+ T cell recruitment highlights the notion that lower doses of inoculation may allow the recruitment of antigen-nonspecific CD8+ T cells into the brain; non-specific contributors to viral clearance may account for the gap in virus-specific CD8+ T cell summation.50 Such non-specific populations can be activated from their naïve state through strong cytokine-driven stimulation (e.g. IL-7, IL-15) that overcomes the need for TCR signaling.56 Indeed, data from Elizabeth Frost53 has shown that B6 mice inoculated with 2 x 10^6 PFU/mL of A2 virus have ¾ of virus-specific CD8+ T cells in the brain restricted to LT359, while data from this study shows a disproportionate decrease to approximately 45% with a lower dose. The percent contribution of the subdominant MT246, LT638, and VP2.139 epitopes has yet to be published, and findings from this study suggests that approximately 30% of A2-infected virus-specific cells are restricted to MT246, and 5% to LT638. The idea that there is a set quantity of CD8+ T cells able to be recruited into the brain during acute
infection allows the possibility that if a lower quantity is recruited, there may be greater room for other non-lymphocytic or antigen-nonspecific CD8⁺ T cells to contribute to the inflammatory environment. Phenotyping brain CD8⁺ T cells using canonical expression patterns of markers such as CD69, CD62L, KLRG1, PD-1, and CD103 can distinguish infiltrating CD8⁺ T cells from central memory, effector memory, tissue resident memory (T_RM), and antigen-nonspecific CD8⁺ T cells.⁵⁷

**Tetramer insufficiency**

Tetramers for LT359, MT246, and LT638 were initially used as a method of quantifying CD8⁺ T cell specificity by flow cytometry. However, the VP2.139 tetramer, mutated to substitute the α3 chain of H-2Kb for Q9 due to insufficient binding with the wildtype Q9 tetramer, was unable to provide accurate data due to its inefficacy, and conclusive quantification and phenotyping via tetramer staining was not possible.⁵⁸ Previous literature defining the dominance of T_RM cells and effector LT359⁺ CD8⁺ T cells in the brain describes high antigen sensitivity with high affinity TCRs critical for clearance of virus during low-level infection.⁵³ The belated kinetics of VP2.139⁺ CD8⁺ T compared to MuPyV-specific MHC-Ia-restricted CD8⁺ T cells allows the possibility of the non-classically-restricted CD8⁺ T cell population to express low affinity TCRs. The evolution of the TCR from low to high affinity, as the T cell population expands during acute infection and plateaus during persistent infection may involve characteristics that could not be seen at 8 dpi. In fact, the day 8 time point has been used in MuPyV studies as ideal for observing the LT359, MT246, and LT638, but not VP2.139-specific CD8⁺ T cells immediately after peak expansion. The two-dimensional micropipette adhesion-frequency assay may provide insight into the responsiveness of VP2.139⁺ CD8⁺ T cells. Juxtaposition of the TCR and the p:MHC from a pAPC creates a two-dimensional membrane junction upon binding. The on- and off-rate of the p:MHC:TCR complex in relation to TCR density determines the kinetics and affinity of the TCR,
and can identify antigen-specific populations below the affinity threshold of tetramers.\textsuperscript{53,59} Utilization of this technique may successfully quantify the VP2.139\textsuperscript{+} CD8\textsuperscript{+} T cell population, which will subsequently disclose IFN-\(\gamma\) effector competency.

The effective control of viral pathogens is closely affiliated with TCR diversity. It should be noted that the VP2.139 tetramer was constructed under the (likely) assumption that CD8\textsuperscript{+} T cells specific to the epitope had \(\alpha\beta\)-TCRs. Per contra, the \(\delta\gamma\)-TCR phenotypic possibility had not been refuted and poses the prospect of an unconventional CD8\textsuperscript{+} T cell population increasing its appearance in LT359\textsuperscript{null} virus-infected mice. Moreover, VP2.139-specific CD8\textsuperscript{+} T cell receptors have less affinity for cognate antigen compared to its MHC-Ia-specific counterparts due to the impaired binding capacity of the CD8 coreceptor to the AB loop of Q9, which is directed at a position almost 12 Å away from where CD8 would normally bind to MHC-Ia molecules. The rigid structure of Q9 limits conformational change, unlike the flexible MHC-Ia class, and curbs its ability to contact the coreceptor.\textsuperscript{60} Its inefficiency constrains the TCR repertoire and demands a heightened TCR affinity of VP2.139\textsuperscript{+} CD8\textsuperscript{+} T cells to bind successfully. Yet, the massive production of IFN-\(\gamma\) by these CD8\textsuperscript{+} T cells suggests a strong effector capacity.

**Antiviral cytokine inaccuracies**

It is prudent to also consider IFN-\(\gamma\) production as a confounding variable. Its ability to directly and indirectly, through influence on TAP1/2 and other steps of MHC-viral antigen processing, upregulate MHC-I and -II expression, activate macrophages, convert CD4\textsuperscript{+} T cells to type 1 T helper cells,\textsuperscript{61} and augment lymphocyte recruitment concomitantly triggers a positive feedback loop, exponentially increasing the release of IFN-\(\gamma\) from a variety of cells.\textsuperscript{62} Its quantification in this study as a maximal functional capacity readout of viral peptide-specific CD8\textsuperscript{+} T cells and an indirect estimation of the hierarchy of viral epitopes based on frequency therefore
may be skewed. Additionally, direct ex vivo stimulation is an artificial system of evoking antiviral cytokine release; the magnitude of IFN-γ quantified through ICS assays may not be indicative of its output in vivo.

Unpublished data from our lab has suggested that IFN-γ may not be the major cytokine responsible for viral clearance. A2 viral replication is unchecked in STAT1−/− and, specifically only during acute infection, in IFN-αR−/− mice on a B6 background, whereas IFN-γR−/− B6 mice maintain similar viral loads to WT B6 mice. Activated CD8+ T cell and LT359+ CD8+ T cell frequencies remain unchanged in all three conditions, and microglial activation was significantly lower in STAT1−/− mice. Additional data has also shown that dual depletion of CD8+ and CD4+ T cells from the brain and periphery allows uncontrolled viral replication. This data and similar CD8+ T cell frequencies between the A2 and LT359null virus-infected B6 mice from this study suggests that the presence of CD8+ T cells in the brain is critical for, but limited in, controlling the virus.

The ability of CD8+ T cells to destroy or induce apoptosis in infected cells directly or indirectly, respectively, has the potential for a dual-edged role in viral control and immunopathology. CD8+ T cells can secrete cytotoxic molecules such as perforin and granzymes, along with effector cytokines that have been previously described. Previous literature describing the viral clearance potential of respiratory syncytial virus-specific CD8+ T cells deficient in IFN-γ has shown significantly reduced CD8+ T cell-mediated pathology and viral control in the lung. Alternatively, CD8+ T cells deficient in perforin and TNF-α production did not alter normal viral control or immunopathology. The effect of such cytotoxic molecules in regards to CD8+ T cells and MuPyV CNS infection has been largely unexplored. Ascertainment of their role in immunopathology in this mouse model, which includes features such as demyelination, vasculitis, ventriculitis, cerebral edema, and neuronal death (T.E. Mockus, unpublished data), could aid in
conclusively determining whether the pathological hallmarks of MuPyV CNS infection are attributed to their cytotoxicity or virus-associated cellular lysis. This question has yet to be answered.

This study has provided a novel observation of a robust compensatory increase in a non-classical, exceedingly subdominant MuPyV-specific CD8+ T cell population. Despite their atypical kinetic and phenotypic features, the heightened presence of VP2.139+CD8+ T cells as part of the antiviral defense system in the absence of an immunodominant, classical MHC-Ia epitope is unprecedented.

Mutations of the immunodominant viral epitopes have been known to allow evasion of virus-specific CD8+ T cells, as seen in HIV-1 and simian immunodeficiency virus.64 As such, vaccines should be designed to account for dynamic CD8+ T cell responses and viral evolution. Considering the exclusivity of the Q9:VP2.139 epitope to MuPyV, it is relevant to identify non-classical, mammalian polyomavirus equivalents; HLA-G has been considered as the closest functional human homologue.65 The significance of a non-classical, Qa-2 MHC class I molecule of limited polymorphisms being implicated in the control of MuPyV gives rise to the possibility of developing peptide-based vaccine strategies that can confer protection against a broad range of MHC haplotypes.
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