CD8 T Cell Immunity to Viral Infection:

A Balance Between Protective and Pathological Responses

by

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ABSTRACT

Vaccination remains one of the most effective means for preventing infectious diseases. During viral infection, activated CD8 T cells differentiate into cytotoxic effector cells that directly kill infected cells and produce anti-viral cytokines. Further T cell differentiation results in a population of memory CD8 T cells that have the ability to self-renew and rapidly proliferate into effector cells during secondary infections. However during persistent viral infection, T cell differentiation is disrupted due to sustained antigen stimulation resulting in a loss of T cell effector function. Despite the development of vaccines for a wide range of viral diseases, efficacious vaccines for persistent viral infections have been challenging to design. Immunization against virus T cell epitopes has been proposed as an alternative vaccination strategy for persistent viral infections, such as HIV. However, vaccines that selectively engage T cell responses can result in inappropriate immune responses that increase, rather than prevent, disease.

Quantitative models of virus infection and immune response were used to investigate how virus and immune system variables influence pathogenic versus protective T cell responses generated during persistent viral infection. It was determined that an intermediate precursor frequency of virus-specific memory CD8 T cells prior to LCMV infection resulted in maximum T cell mediated pathology. Increased pathology was independent of antigen sensitivity or the diversity of TCR in the CD8 T cell response, but was dependent on CD8 T cell production of TNF and the magnitude of initial virus exposure. The threshold for exhaustion of responding CD8 T cells ultimately influences the precursor frequency that causes enhanced disease.

In addition, viral infection can occur in the context of co-infection by heterologous pathogens that modulate immune responses and/or disease. Co-infection of two unrelated viruses in their natural host, Ectromelia virus (ECTV) and Lymphocytic

Choriomeningitis virus (LCMV) infection in mice, were studied. ECTV infection can be a lethal infection in mice due in part to the blockade of antiviral cytokines, including Type I Interferons (IFN-I). It was determined that ECTV/LCMV co-infection results in decreased ECTV viral load and amelioration of ECTV-induced disease, presumably due to IFN-I induction by LCMV. However, immune responses to LCMV in ECTV co-infected mice were also lower compared to mice infected with LCMV alone and biased toward effector-memory cell generation. Thus, providing evidence for bi-directional effects of viral co-infection that modulate disease and immunity. Together the results suggest heterogeneity in T cell responses during vaccination with viral vectors may be in part due to heterologous virus infection or vaccine usage and that TNF-blockade may be useful for minimizing pathology while maintaining protection during virus infection. Lastly, quantitative mathematical models of virus and T cell immunity can be useful to generate predictions regarding which molecular and cellular pathways mediate T cell protection versus pathology.

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LIST OF ABBREVIATIONS

dsRNA	Double stranded RNA
HIV	Human Immunodeficiency virus
HCV	Hepatitis C virus
HBV	Hepatitis B virus
HDV	Hepatitis D virus
RSV	Respiratory syncytial virus
NK	Natural killer cell
DCs	Dendritic cell
PAMPs	Pathogen associated molecular pattern
TLR	Toll like receptor
RIG-I	Retinoic acid inducible gene-I receptor
IFN-I	Type I interferon
ISGF3	Interferon stimulated gene factor 3 complex
OAS	2’5’ oligoadenylate synthase
Ig	Antibody
BCR	B cell receptor
TCR	T cell receptor
APC	Antigen presenting cell
ssRNA	Single stranded RNA
GP	Glycoprotein
NP	Nucleoprotein
BCR	B cell receptor
Ig	Antibody
V	Variable

D	Diversity
J	Joining
MHC	major histocompatibility complex
LCMV	Lymphocytic choriomeningitis virus
ECTV	Ectromelia virus
Treg	Regulatory CD4 T cells
Th1	CD4 T helper 1
Th2	CD4 T helper 2
Tfh	CD4 T follicular helper
T17	CD4 T helper 17
CTL	Cytotoxic T lymphocyte
IFNγ	Interferon gamma
TNF	Tumor necrosis factor
IL	Interleukin
PD-1	Programmed cell death-1
ip	Intraperitoneal
iv	Intravenous
ic	Intracranial
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4

CHAPTER 1

INTRODUCTION

Historical perspectives of immune memory

The concept of immune memory was utilized long before the understanding of microbiology or immunology. Early understanding (400 BCE) of immune memory was due to the observation that people who survived a disease rarely became sick from the same disease more than once(1, 2). In the 16th century the first expansive attempts at purposeful induction of immune memory against smallpox was attempted through the process of variolation(3). Variolation consisted of subcutaneously introducing smallpox pustules from infected patients into healthy individuals to prevent mortality during smallpox epidemics. However, variolation resulted in disseminated smallpox in a small proportion of recipients resulting in 2-3% mortality following inoculation(1, 3).

In 1796, the English physician Edward Jenner utilized his knowledge that dairymaids exposed to cowpox appeared to be immune to smallpox in order to deliberately transmit protection from one person to another. Jenner inoculated an 8–year-old boy with a lesion from a dairymaid infected with cowpox, waited two months, and then inoculated the boy with smallpox. The boy did not demonstrate any disease symptoms leading Jenner to conclude cowpox infection mediated protection from smallpox. Jenner named the procedure vaccination after the Latin word for cowpox, vaccinia(4, 5). The English farmer Benjamin Jesty has now been recognized as the first to vaccinate against smallpox using cowpox lesions in 1774(6). However, Edward Jenner is widely acknowledged as the pioneer of vaccination due to his lifelong pursuit to scientifically promote smallpox vaccination.

The modern definition of immunological memory is the ability of a diverse population of purposely developed and pre-programmed immune cells to respond to secondary infection of a pathogen faster, and with greater magnitude, resulting in protection from either infection or disease(7-9). One of the most relevant contributions of immunology to human health is the ability to utilize immune memory for the design of vaccines against infectious diseases(7, 10). Four of the top ten leading global causes of premature death in humans are diseases due to infectious pathogens(11). However, in the past decade there has been a global shift away from premature death due to infectious diseases, except in Africa where 70% of calculated years of life lost are due to infectious diseases, maternal, neonatal, and nutritional causes(11). One of the main reasons for this transition is that expanded vaccination coverage has significantly enhanced our ability to elicit effective memory immune responses to fight and prevent human diseases caused by infectious pathogens(12, 13). Vaccines designed against smallpox, polio and measles are only a few examples of the remarkable impact vaccination has made towards the reduction of the global disease burden(1, 14).

Despite the success of current vaccines, there are many remaining challenges for vaccines designed to prevent viral infections, such as human immunodeficiency virus (HIV)(15-17), hepatitis C virus (HCV)(18) and respiratory syncytial virus (RSV)(19). In 2013, 35 million people were estimated to be living with HIV resulting in an estimated 1.5 million deaths due to HIV-related causes(11). Effective antiretroviral therapy has increased the quality and longevity of life in HIV-infected individuals(20). However, in 2013, 2.1 million people worldwide became newly infected with HIV(11), emphasizing the need for a vaccine that prevents infection in order to eliminate HIV/AIDS in humans(21).

The traditional approaches for vaccine design against HIV have been hampered by the safety concerns for immunizing with attenuated HIV and the lack of efficacy of vaccines designed using inactivated virus (17, 21-23). Vaccination for T cell responses using recombinant vectors has been proposed as an alternative strategy to elicit immune responses and continues to be the focus of future vaccine candidates(21, 24, 25).

Therefore, it is important to continue to study the intricacies of immunological memory in order to inform the safe design of vaccines through defining the magnitude, functionality, and specificity of immune responses to viral infection(9, 12). The generation of immunological memory to infectious pathogens involves the interaction and communication of almost all of the different types of cells in the immune system(26). In order to study adaptive memory responses, the function of the innate immune system must be understood. The innate immune system is critical for the detection of invading agents, initial control of overwhelming replication of pathogens, and informing adaptive immune cells(27).

Fundamentals of innate immunity

The immune system is composed of two very different yet equally important parts labeled as either innate or adaptive components. Innate immunity is characterized by a rapid, but fixed, response to a large, but limited number of stimuli. Innate immunity is composed of physical, chemical and biological barriers including specialized cells and soluble molecules that are present in all healthy individuals regardless of past exposure to pathogens(27). The main effector cells of the innate immune system are macrophages, granulocytes, natural killer (NK) cells, and dendritic cells (DCs)(16, 28, 29).

The main function of these cells is phagocytosis of invading pathogens, release of inflammatory cytokines and chemokines, activation of complement proteins, and presentation of foreign antigen to adaptive immune cells(30-32).

Innate immune cell effector mechanisms are stimulated by detection of specific foreign molecular structures ubiquitous in microorganisms, such as lipopolysaccharides and nucleic acids, collectively termed pathogen associated molecular patterns (PAMPs)(31, 33, 34). PAMP recognition by specific receptors known as pattern recognition receptors (PRRs), including the families of Toll like receptors (TLRs) and retinoic acid inducible gene I (RIG-I) like receptors, result in signal transduction and activation of innate cell effector functions such as phagocytosis and production of inflammatory proteins(32, 33, 35, 36). One of the most important innate responses to PAMP detection of viral pathogens is the synthesis of type I interferons (IFN-I)(37, 38).

Type I interferons mediate antiviral defense

Type I interferons (alpha/beta) are a multi-member cytokine family whose function is to signal the presence of intracellular infections and facilitate communication among cells that provide defense against viruses and intracellular bacteria(39, 40). The preservation of type I interferons throughout the vertebrate lineage suggest that IFN-I has a vital role in anti-viral defense(37). In both mice and humans there are more than 20 interferon alpha genes and one interferon beta gene. IFN-I provides a rapid and direct response to invading pathogens within hours, limiting viral replication days before activation of a pathogen-specific adaptive immune response(41). Two specific receptor families are primarily responsible for coupling viral recognition via detection of viral nucleic acids and synthesis of IFN-I.

These families include TLRs expressed on innate immune cells and the ubiquitously expressed cytosolic receptors exemplified by RIG-I and MDA-5(42, 43).

Several members of the TLR family of receptors expressed in macrophages and dendritic cells detect the presence of viral nucleic acids in the endosome after phagocytosis(44, 45). One example, TLR3, recognizes a common feature of viral infection not normally present in healthy cells, double-stranded RNA (dsRNA)(46). The intracellular expression of TLR3 in the endosome allows TLR recognition of viral nucleic acids entering from outside the cell, separating viral infection from viral recognition in order to produce interferon(44, 46). Almost all nucleated cells respond to viral infection via the production of IFN-I(37). In addition to TLR recognition of viral nucleic acids by macrophages and dendritic cells, ubiquitous expression of cytosolic receptors exemplified by RIG-I and MDA-5 allow for the detection of viral nucleic acids in infected non-immune cells(36, 42, 47). RIG-I and MDA-5 both utilize the adapter protein IPS-1 leading to IRF3 phosphorylation and dimerization resulting in activation of transcription of the IFNγ gene(36, 48-50) **(Figure 1.1A)**.

IFN-I produced by infected cells can signal in both an autocrine and paracrine manner to indicate the presence of viral infection within the cell(37). IFN-I binds to a heterodimeric receptor composed of IFNAR1 and IFAR2 subunits (IFNAR) on neighboring cells(51). Receptor binding leads to activation of Janus protein tyrosine kinases leading to the formation and activation of the heterotrimeric interferon stimulated gene factor 3 complex (ISGF3) that ultimately binds to interferon stimulated response elements in the nucleus promoting the transcription of hundreds of interferon-stimulated genes(52, 53). Signaling via IFNAR in uninfected cells results in the establishment of the “anti-viral” state(54) **(Figure 1.1B)**.

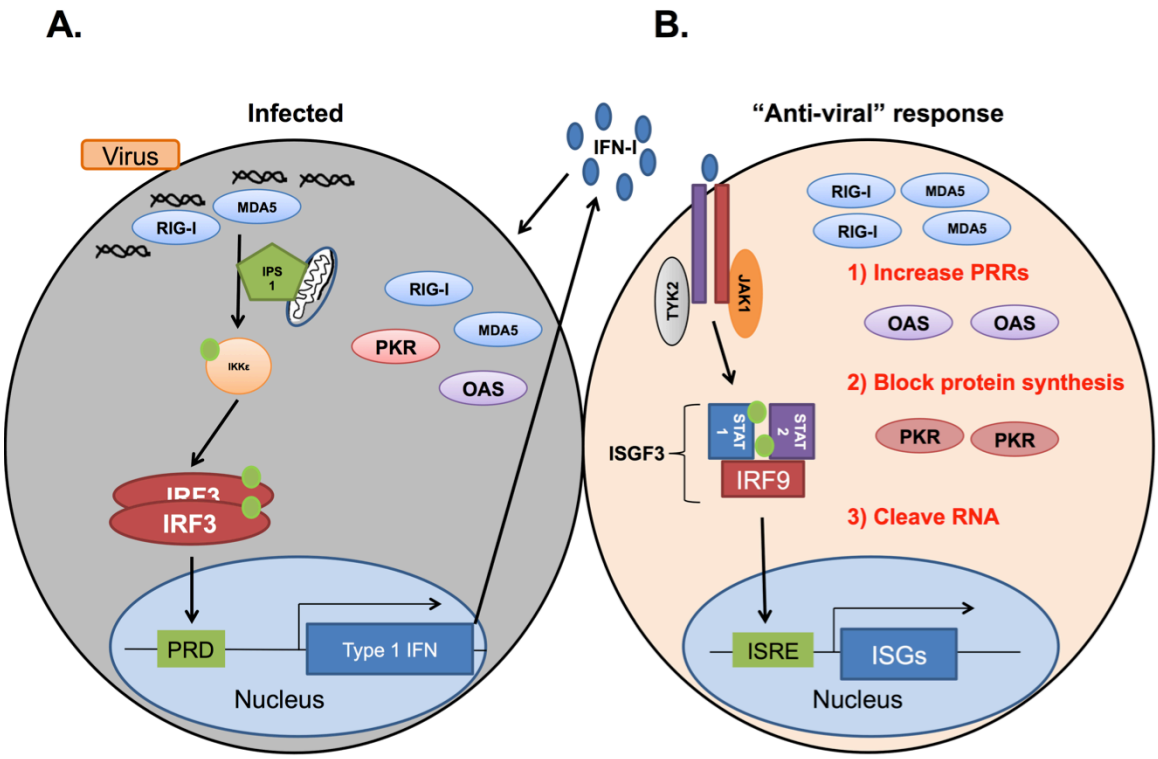

Figure 1.1: Viral detection leads to production of Type I interferons to promote anti-viral defenses in neighboring cells. [A] Viral nucleic acid is detected by cytosolic pattern recognition receptors such as RIG-I or MDA-5 in infected cells leading to activation of IRF-3 and production of IFN-I. **[B]** IFN-I ligation to IFNAR-1 results in signaling via ISGF3 complex leading to induction of pattern recognition receptors, induction of proteins to block protein synthesis (PKR) and induction of proteins to cleave viral RNA (OAS) resulting in an anti-viral state.

IFN- induced proteins with broad anti-viral effects include 2'5' oligoadenylate synthase (OAS) that, upon activation via viral dsRNA, in turn activate the nuclease RNase L leading to degradation of viral RNA transcripts(55). IFN-induced protein PKR can undergo activation via viral dsRNA leading to eIF2a phosphorylation resulting in the obstruction of translation of viral and cellular mRNA(56). Additionally, the viral nucleic acid sensors RIG-I and MDA-5 are IFN inducible, thereby increasing the sensitivity of neighboring cells to detect viral infection(57). Collectively, the action of IFN-I signaling is to sensitize the cell to apoptosis upon subsequent viral infection. This action results in inhibition of virus replication, elimination of virally infected cells, and prevention of viral spread(54).

In addition, IFN-I also has a critical role in the functional linkage of innate cell detection and adaptive cell response(40, 58). The finding that all vertebrates contain genes that encodes IFNα and IFNβ in addition to the presence of NK cells and adaptive T and B cells supports a connection between interferon and effector cell generation(37). Other than the cell intrinsic effects of IFN-I, two important effector cell populations are regulated by IFN-I, IFN-I can directly activate NK cells to enhance cytotoxic activity(59) and it can influence differentiation and function of CD8 T cells(60). Consequently, IFN-I has both pro-apoptotic and anti-apoptotic activities since IFN-I can directly impact viral replication in infected cells (pro-apoptotic) and provide stimulation of anti-viral immune responses (anti-apoptotic), depending on the differentiated state of the signaled cell(58). Therefore, innate immune cells have greater significance than only the early detection and control of invading pathogens. Innate immunity is critical for the subsequent generation of adaptive immune responses, which are the primary focus of the following investigations.

Adaptive immunity provides immunological memory

Adaptive immune cells are responsible for providing immunological memory to pathogens after previous exposures. The adaptive immune system is composed of three separate but cooperative compartments: B cells, CD4 helper T cells and CD8 cytotoxic T cells(61). It was not until the mid 1960s that B and T lymphocytes were determined to be primarily responsible for the basic functions of antibody production and cell mediated immune responses(62). The first scientists to suggest a division of labor between cells responsible for antibody production and cell mediated delayed type hypersensitivity were Max Cooper and Robert Good. They found that cells derived from the chicken bursa of fabricius were required for antibody production (B cells), while cells mediating graft versus host reactions (T cells) required an intact thymus(63).

B-lymphocytes produce antibodies

B cells are modestly defined as a population of lymphocytes that express clonally diverse cell surface immunoglobulin receptors, B cell receptor (BCR), that recognize specific antigenic epitopes(64). Antibodies (Ig) produced by activated B cells are simply the secreted version of the membrane bound BCR(65). Antibodies consist of two different polypeptide chains designated as heavy (50kDa) and light (25kDa). Antibodies are roughly “Y-shaped” in structure with two matching antigen binding sites due to each Ig consisting of two identical heavy chains connected by disulfide linkages in addition to one identical light chain linked to each heavy chain by disulfide bonds(66, 67). In order to generate a diverse array of B cell receptors or antibodies capable of recognizing any potential pathogen, a developing B cell in the bone marrow undergoes combinatorial rearrangements of multiple gene segments in the heavy and light chain loci.

The heavy chain rearranges variable (V), diversity (D) and joining (J) gene segments and the light chain locus rearranges only V and J segments prior to linkage with a selected constant(C) region resulting in the generation of unique and diverse antigen binding sites on individual B cells(68, 69). Moreover, the discovery of somatic gene rearrangements to generate diverse Ig receptors by Susumu Tonegawa made such an enormous contribution to the understanding of immunology that he was awarded the Nobel Prize in physiology or medicine in 1987(64).

Pre-existing antibodies are immensely important for immune protection against extracellular pathogens because B cells, unlike T cells that require pathogen antigens to be presented on host proteins for recognition, directly bind to the invading free virus particle, bacteria or parasite(70). B cell activation after BCR antigen recognition results in dynamic changes to the responding B cell including localization to the germinal center in secondary lymphoid tissues, clonal expansion, class switch recombination, somatic hypermutation and affinity maturation(71, 72). CD4 follicular helper cells and follicular dendritic cells in the germinal center are particularly important to trap antigens and provide co-stimulatory signals leading to the generation of memory B cells and long lived plasma cells that produce very high affinity antibodies of the desired isotype(73, 74). Neutralizing antibodies are important for the prevention of viral infection at the sites of viral entry, especially in mucosal tissues(8, 75)

T-lymphocytes significantly influence control of viral infection

The clinical observation that patients without functioning T cells experience an increased frequency and severity of viral infections, suggests T cells are critical for anti-viral defense(76-78).

Additionally, children with Bruton's a-gammaglobulinemia present with undetectable serum antibody titer, yet resist subsequent infections to the majority of viruses(79-82). Thus, suggesting T cell responses are critical for the control, clearance, and subsequent memory of viral infections.

Similar to B cells, in order to generate a T cell response to a broad array of infectious pathogens, T cells express clonally distributed T cell receptors (TCR) on their cell surface(83). The structure of the TCR consists of a membrane bound heterodimer of T cell alpha and beta polypeptide chains(84). In order to generate a broad repertoire of TCR capable of responding to any and all invading viral pathogens, T cells undergo combinatorial rearrangements of gene segments in both the alpha (V and J) and beta (V, D, J) loci joined to a randomly selected constant region during T cell development in the thymus(85). In contrast to the BCR, a TCR has only one antigen-binding site that primarily contains the regions of the alpha and beta chains encoding the V-J or V-D-J junctions. This is due to the diversity generated by selection of random gene segments and by the random incorporation or removal of junctional nucleotides during somatic gene rearrangement(83, 85, 86). Both the alpha and beta chains form the TCR antigen-binding site(84). Therefore increased diversity is also due to random alpha and beta chain pairing in each T cell(87). The diversity of potential T cell receptors is estimated to range from 10^7 to 10^{15} unique TCR(85, 88-90).

T-lymphocytes require recognition of both self-protein and foreign peptide

In contrast to B cells, T cell recognition of pathogen infection requires foreign antigens to be bound to host proteins on the cell surface. T cells simultaneously recognize both foreign peptides and host major histocompatibility complex (MHC) proteins.

Foreign peptides can be presented by infected cells or “professional” antigen presenting cells (APCs), such as dendritic cells and macrophages(91). CD8 T cells, primarily responsible for cytolysis of infected cells, recognize short (8-10 amino acid) foreign peptides presented on MHC-class I (MHC-I) proteins that consist of a single polypeptide associated with the B2M structural protein that are expressed on virtually all cells types(92). Whereas CD4 helper T cells recognize slightly longer foreign peptides (12-16 amino acids) presented on MHC-class II proteins (MHC-II) that consist of two associated polypeptides, alpha and beta, expressed primarily on “professional” antigen presenting cells(93). A unique characteristic of MHC proteins is that they are both polygenic and polymorphic. Humans have three genes that express classical MHC class I proteins, HLA-A, HLA-B and HLA-C, and three genes that express classical MHC class II proteins, HLA-DR, HLA-DQ and HLA-DP(94). In addition, different peptide-binding grooves due to polymorphisms of MHC proteins result in variations in antigen presentation and immune responses between individuals(95). The Nobel Prize wining discovery of MHC restriction in 1974 by Rolf Zinkernagel and Peter Doherty revealed that CD8 T cells could only lyse virally infected target cells that presented the same type of MHC as the originating mouse strain(96, 97).

One of the main causes for the functional differences between CD8 and CD4 T cell subsets is due to the divergent origins of the foreign peptide that bind to MHC-I and MHC-II proteins(92). CD8 peptides that bind to MHC-I are generated from foreign proteins degraded by cytoplasmic proteasomes(98), which are then transported to the endoplasmic reticulum, via TAP protein, where they bind MHC-I(99). Furthermore, MHC-I:peptide complexes undergo vesicle transportation to the cell surface in order to activate TCR on CD8 T cells, allowing adaptive immune detection of intracellular infections(100).

In contrast, CD4 peptides that bind to MHC-II are generated from foreign proteins taken up from the extracellular space into intracellular vesicles(101). Phagosome fusion with endosomes, containing proteases activated by acidification, generate peptide fragments that are subsequently loaded onto MHC-II. MHC-II:peptide complexes are then transported to the cell surface in order to activate TCR on CD4 T cells, allowing adaptive immune detection of extracellular infections(102, 103).

There are also circumstances in which CD8 T cells can recognize peptides derived from extracellular sources. Mike Bevan initially observed this phenomenon in 1976 when he found that CD8 T cells could recognize peptides derived from other cells (extracellular sources) presented on MHC-I proteins(104). Cross-presentation of extracellular sources of foreign proteins can happen either when phagosomes disintegrate (depositing proteins into the cytoplasm) or phagosomes fuse with the ER and foreign proteins are transported back to the cytoplasm by the host protein Sec61(105, 106). In both cases, extracellular derived proteins are then processed into peptides by cytoplasmic proteases and undergo MHC-I loading(107). Cross presentation is a critical component in the induction of CD8 T cell immune responses since activation of naïve CD8 T cells requires antigen presentation via professional APCs that may or may not be directly infected. Thus, APCs must acquire exogenous antigens from infected cells and present them on MHC-I using cross-presentation. Dendritic cells positive for CD8 expression have been identified as the main type of cross-presenting cell during *in vivo* T cell activation(108).

Additionally, CD4 T cells can respond to peptides derived from intracellular pathogens via autophagy(109). Autophagy is a cellular process normally used for the degradation or recycling of host cytoplasmic proteins(110).

In the event of a viral or intracellular bacterial infection, foreign proteins in the cytoplasm can be collected into vesicles via autophagy followed by similar peptide generation and loading onto MHC-II proteins seen after phagocytosis(103, 109).

T cells undergo positive and negative selection during development

T cell development in the thymus involves an initial stepwise process of TCR alpha and beta chain rearrangement followed by positive selection for host MHC binding and negative selection for auto reactive T cells(111). After successful TCR expression on the cell surface, double positive T cells expressing both CD8 and CD4 co-receptor proteins ($CD8^+$ $CD4^+$) undergo "positive" selection for their ability to recognize host MHC-I or MHC-II proteins expressed on cortical epithelial cells(112). The majority of double positive T cells express a recombined TCR that interacts poorly with host MHC proteins failing to provide the necessary intracellular signaling to promote cell survival(113). Depending on the strength of the TCR interaction to MHC-I or MHC-II, double positive T cells become lineage committed to either CD8 or CD4 populations(113, 114). Expression of CD8 or CD4 co-receptor proteins is critical for mature T cells due to the importance of co-receptor engagement with MHC to strengthen MHC:peptide:TCR interactions(115, 116).

Following successful TCR:MHC interaction, lineage committed CD8 and CD4 T cells undergo "negative" selection during which auto-reactive cells are deleted(117). Strongly reactive T cells to self-peptides presented on MHC proteins expressed by medullary epithelial cells or bone marrow derived macrophages and dendritic cells undergo apoptosis and are eliminated from the T cell repertoire(118, 119).

Weak TCR ligation with self-peptide after positive selection in the thymus results in continued survival and migration of mature T cells to secondary lymphoid tissues(117). Central tolerance of T cells reactive to self-peptides is critical to limit potentially harmful autoimmune reactions to self-proteins(120, 121).

T cell activation requires co-stimulation

T lymphocytes in the periphery that recirculate between secondary lymphoid tissues and the blood consist mainly of two functionally discrete subpopulations: CD4 helper T cells and CD8 cytotoxic T cells. In order for a T cell to respond to an infection, it must first undergo three signals of activation by dendritic cells migrating from sites of infection to draining lymph nodes(122). The first step of T cell activation is recognition between the TCR and its cognate antigen presented on MHC by APCs. TCR and peptide:MHC ligation results in the formation of the immunological synapse resulting in the activation of ITAM motifs on the zeta chain of the TCR associated protein CD3, leading to the activation of Zap-70(123-125). Zap-70 activation results in the phosphorylation of the adaptor protein LAT, leading to the recruitment of different components of several signaling pathways and ultimately activating the transcription factors NFATc, AP-1 and NF-kB(126-129).

In addition, T cells need a second signal, such as co-stimulation via CD28 ligation to CD80 or CD86 expressed on the activating APC that results in potentiation of TCR signaling above a threshold in which full activation occurs(130, 131). This leads to IL-2 driven survival and proliferation(130, 132).

Furthermore, a third signal via pro-inflammatory cytokines such as IL-12 or IFN-I is required for differentiation into effector T cell subsets and sustained proliferation(133-136). TCR ligation without co-stimulation leads to a state of unresponsiveness defined as T cell anergy(137, 138).

CD4 helper T cell differentiation

CD4 T cells further differentiate into distinct subpopulations after activation that play a major role in mediating immune response via the secretion of specific cytokines and expression of co-stimulatory proteins including CD40-L(139-141). CD4 T cell subsets, such as T-helper 1 (Th1), T-helper 2(Th2), T-helper 17 (Th17) and T follicular helper (Tfh), carry out a broad array of functions(140, 142, 143). These functions include activation of innate immune cells, stimulation of B cells to undergo isotype switching and somatic hypermutation, and stimulation of CD8 T cells(139, 142, 144-147). Additionally, natural regulatory CD4 T cells (Treg) and inducible Treg subsets are critical components of immune regulation that are capable of suppressing immune responses(148-150).

Naïve CD4 differentiation into distinctive helper cell lineages is dependent on the specific cytokines secreted by the activating APCs, which in turn are determined by the particular PRR activated by the infecting pathogen(139, 151). Differential cytokine signaling in the naïve CD4 T cell during TCR:peptide:MHC ligation results in the induction of specific transcription factors that lead to a defined cytokine profile of the responding helper cell(142, 151, 152) . For example, intracellular PAMP receptor signaling via detection of viral nucleic acids in the endosome condition DCs to secrete IL-12 resulting in the differentiation of Th1 helper cells(140, 153).

IL-12 binding to the IL-12 receptor on the CD4 T cell induces the activation of STAT4, leading to the activation of the transcription factor T_{bet} that promotes the production of IFNγ(146, 147, 154). IFNγ is a critical cytokine for the control of intracellular pathogens because it can directly inhibit viral replication and activate macrophages to fuse endosomes with lysosomes, resulting in enhanced microbial killing and activation of NK cells and CD8 T cells to directly lyse infected cells(155, 156). CD4 helper T cells are a complex and interconnecting element of the immune system due to their broad ability to modulate innate cell subsets, antibody production and CD8 cytotoxic responses to invading pathogens(142, 151).

CD8 T cell effector function

In contrast to the defined subpopulations of CD4 helper T cells, the primary effector function of CD8 T cells is to kill pathogen infected or transformed tumor target cells(157, 158). Activated CD8 T cells exit lymph nodes and migrate into infected tissues or infiltrate tumor(159, 160). There, TCR ligation of foreign peptides presented by MHC-I causes directed cytolysis of the target cells via two different mechanisms(161). One mechanism is accomplished by the directed release of granules containing the effector proteins perforin and granzyme B(162). Perforin mediated entry of granzyme B into target cells results in the activation of caspase 3 and BID proteins and the subsequent release of mitochondrial cytochrome C, leading to fragmentation of cellular DNA and apoptosis(163-166). The second mechanism of directed cytolysis requires engagement of T cell expressed Fas-ligand to Fas resulting in FADD mediated caspase activation leading to DNA fragmentation and apoptosis(167, 168). In both scenarios, CD8 T cell cytolysis is antigen-specific, controlled by TCR recognition, and does not damage surrounding cells(169, 170).

CD8 T cells can also directly inhibit viral infection and stimulate immune responses via production of the inflammatory cytokines IFNγ and TNF(157, 171). Tumor necrosis factor (TNF) was originally discovered as the soluble factor mediating tumor regression in mice after LPS stimulation(172). TNF signaling is currently recognized as causing two very different effects since it can either induce apoptosis via caspase activation or promote differentiation and proliferation via activation of transcription factor NF-kB(173-175). TNF has two identified receptors with varying levels of expression between cell types(176). TNFR1 has been identified on all human cell types whereas TNFR2 is mainly expressed on immune cells and endothelial cells(177). However, genetic deletion of either receptor eliminates the majority of signals transduced by TNF. Furthermore, TNFR1 may mediate apoptotic signals and TNFR2 may mediate proliferation(178-180). In addition, TNF and TNFR1 have both been discovered to be critical for the protective effects of TNF during infection. The genetic-deficiency of TNF or TNFR1 in mice leads to increased susceptibility of intracellular bacteria infection(181, 182). However, deletion of TNF signalizing also provides resistance to LPS-mediated septic shock, indicating a crucial balance between the protective potential of TNF produced during infection by effector CD8 T cells and the unintended damage of tissues due to overwhelming inflammation(183-185).

CD8 T cells also produce the type II interferon, IFNγ that can either directly limit viral replication or modulate multiple components of the immune response(186). IFNγ signaling through the heterodimeric IFNGR1 receptor can result in the expression of viral resistance genes that increase a cell's ability to recognize viral infection and signal infection to the immune system(187-189). Additionally, IFNγ signaling can stimulate the development of immune effector cells via activation of macrophages, increased antigen presentation and MHC protein expression, and activation NK cell cytolysis(156, 190).

CD8 T cells are important for the control of viral infections due to their sensitivity to respond to an array of viral structural and non-structural proteins and ability to directly limit viral replication by elimination of virally infected cells(191). However, the direct killing of infected cells combined with the release of large amounts of inflammatory cytokines by CD8 T cells can lead to unintended and severe immunopathology(192-194). Immunopathology is especially common during infection with non-cytolytic viruses that do not directly kill host cells, thus clinical disease symptoms are a direct result of the immune response and not due to pathogen burden(195). To ensure host survival there must be a balance between an effective anti-viral response and limiting unintended immunopathology.

Dynamics of the T cell response during acute viral infection

Naïve antigen-specific CD8 T cell frequency has been estimated to be around 1 in every 200,000 CD8 T cells in a mouse spleen(196). A major component to the effector phase of the T cell response after activation is clonal expansion of stimulated antigen-specific naive cells(197, 198). This leads to a greater frequency of antigen-specific effector cells during the peak response. Antigen-specific naïve T cells can divide up to 14 times before reaching the peak of their expansion, which usually occurs between 7-10 days post infection(199). The greater than 10,000 fold expansion results in antigen-specific effector cells reaching a potential frequency of 1 in every 2 CD8 T cells in the spleen(200). The massive expansion of antigen-specific T cells after stimulation is one of three distinct phases of the T cell response during a viral infection(159, 201) **(Figure 1.2)**.

Following the peak response, 90-99 percent of effector cells die during a contraction phase of the response until the remaining 1-10 percent of responding cells become long-lived CD8 memory cells(9, 160, 202, 203). The ratio of the number of cells at the peak of the response versus the number of formed memory cells characterizes the extent of contraction(202).

At the peak of the effector T cell response, there is a heterogeneous population of both terminally differentiated effector cells and memory precursors(9, 160, 203). The contraction of effector T cells is not impacted by the kinetics of pathogen clearance but can be influenced by the magnitude of inflammation in the environment during priming(204, 205). The homeostatic cytokines, IL-7 and IL-15, promote memory cell formation(206, 207) while the contraction of effector cells may be co-regulated by IL-2-family members, TNF-family members, perforin and IFNγ(9, 203). Contraction of short-lived effector cells is required to maintain flexibility in the T cell compartment but it must be limited in order to increase the precursor frequency of memory antigen-specific cells for potential secondary exposures(202).

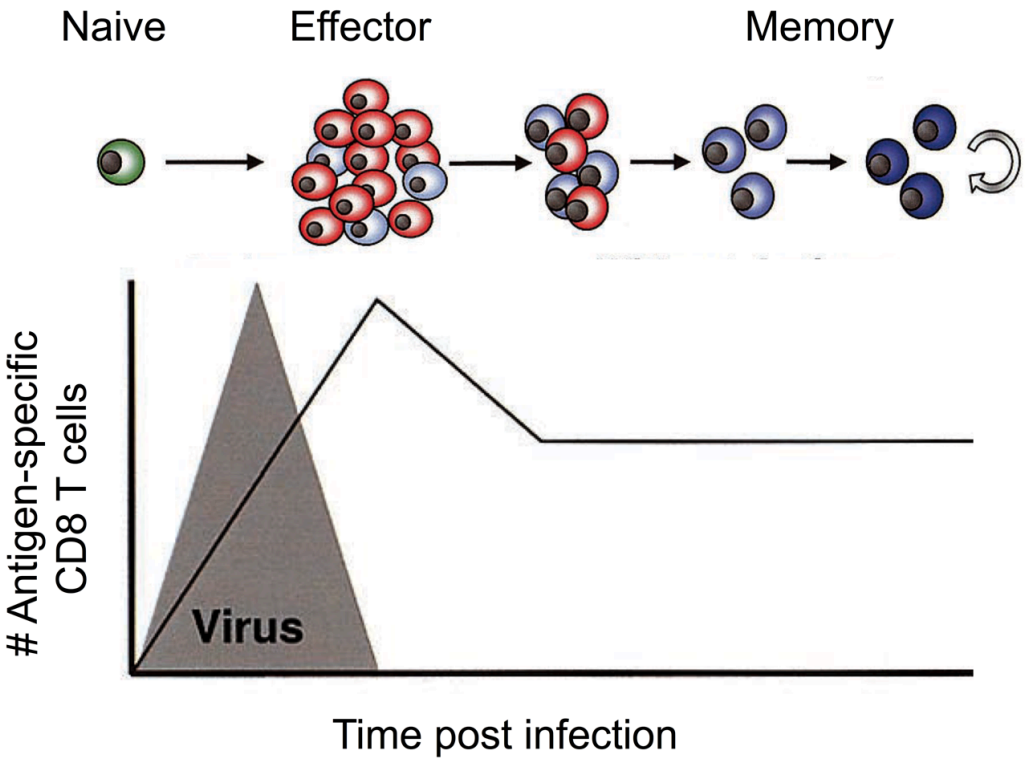

Figure 1.2: Dynamics of CD8 T cell response during viral infection. Naïve CD8 T cells clonally expand into effector cells during viral infection. Following viral clearance, the majority of effector cells die during a contraction phase that culminates in the formation of memory T cells. Memory cell differentiation is a linear process resulting in long-term viral-specific cells maintained the in central lymphoid tissues and the periphery without continued antigen stimulation.

CD8 T cell memory differentiation

It has been found that the subset of memory precursor effector cells that preferentially become long-lasting memory CD8 cells after acute infections are characterized by high expression of IL-7αR and low expression of KLRG1(9, 160, 208). Furthermore, the transcription factor T-bet has been found to be critical for the regulation of effector and memory cell differentiation. Overexpression of T-bet in effector cells leads to enhanced generation of short-lived terminal effectors(209, 210). One of the major determinants of the short lived effector versus memory precursor cell fate decision is the level of inflammation that CD8 T cells are exposed to during priming. In particular, IL-12 can modulate expression of T-bet resulting in more short-lived effector cells with high expression of KLRG1 and low expression of IL-7R. Accordingly, lower levels of IL-12 promote the formation of $KLRG1^{low}/IL\text{-}7R^{high}$ memory precursors(160, 211, 212).

Additionally, epigenetic modifications such as DNA methylation or histone acetylation occur in CD8 T cells during the effector phase that are maintained after memory cell formation(211). Epigenetic modifications of genes involved in T cell survival, metabolism, proliferation and effector functions are the molecular basis for the propagated ability of CD8 T cells to rapidly respond to antigen stimulation, even after antigen-independent homeostatic cell division of memory cells(209, 213). For example, genes for IFNγ, IL-2, granzyme B and perforin become transcriptionally up-regulated due to the proximal promoter region losing repressive epigenetic markers resulting in these loci becoming epigenetically poised for polymerase accessibility and transcriptional activation in memory cells(214, 215).

Thus, there is a distinct transcriptional profile between short-lived effector and memory precursor cells during the effector phase that impacts contraction and memory formation. This is, in part, regulated by restriction of access to chromatin via DNA and histone modifications(209, 213-215).

CD8 T cells require CD4 helper responses to form effective functional memory cells that can rapidly respond to secondary infection(216). Memory CD8 T cells primed in the absence of CD4 T cells do not the lose protective ability to clear acute viral or bacterial infections, but do demonstrate reduced expansion following secondary acute infections with or without continued CD4 T cell help(216-219). The requirement for CD4 T cell help during priming to generate fully functional differentiated memory cells highlights the importance of CD4 T cell responses during primary infection due to their production of inflammatory cytokines, activation of APCs, or direct CD40:CD40L interactions between CD4 and CD8 T cells(142, 216, 217). Memory CD8 T cells and CD4 T cells are one of the key components of protective immunity against viral infections. CD8 memory T cell numbers are stable and long-lasting after infection, whereas CD4 T memory cells slowly erode, as measured by tetramer-monitoring of CD8 T cell clones and CD4 T cell clones 20 to 900 days post infection(201, 220).

Memory CD8 T cells undergo additional changes in phenotype, function and gene expression during differentiation 1-2 months after infection. This results in the establishment of a pool of antigen-specific T cells that can be maintained without continued antigen stimulation(9). Homeostatic turnover of memory T cells driven by IL-7 and IL-15 results in the slow and steady cellular division of antigen-specific T cells that can respond faster to subsequent infection(221, 222). Memory CD8 T cells can rapidly respond to secondary infection primarily due to a simple increase in precursor frequency of antigen-specific cells and the retained potential to rapidly kill infected cells(223).

Memory CD8 T cells can release perforin/granzyme B and produce large amounts of IFNγ and TNF after exposure to an antigen without the activation and proliferation via traditional stimulation from APCs in draining lymph nodes(9, 211, 224). *Chapter 2 focuses on memory CD8 T cell secondary responses to viral infection. Specifically, the impact of the precursor frequency and the number of epitopes targeted by memory virus-specific CD8 T cells on disease outcomes including protection from disseminated viral infection and resulting T cell mediated pathology.*

Heterogeneity exists in memory CD8 T cell populations

As in effector cells during expansion, there is heterogeneity in the population of memory cells formed after an acute infection(225). There are two main subsets of memory CD8 T cells, central and effector memory CD8 T cells, defined by the expression of cell surface proteins and tissue distribution(9, 160, 226). Central memory (T_{CM}) cells are defined by the high expression of lymph node homing receptors CD62L and CCR7. T_{CM} are known to have increased proliferative capacity as well as the ability to rapidly produce IFNγ, TNF and IL-2 after antigen stimulation. In contrast, the effector memory (T_{EM}) cell subset is defined by low expression of CD62L and CCr7(227). Therefore, T_{EM} are found enriched in non-lymphoid tissues and are important for the rapid recall of effector function as a first line of dense at peripheral sites of infection(228). Effector memory cells also have the ability to rapidly perform effector functions after antigen stimulation, but they do not have the same proliferative potential of central memory subsets(227, 229).

Central memory cells are associated with better protective immunity due to their ability to proliferate into a larger pool of secondary effector cells(230). However, both subsets contribute to protective immunity that is dependent on the nature and route of infection(228). Moreover, memory cell subsets do not arise as separate linages, but rather they move along a continuum of differentiation. Over time, CD62L expression on "effector" subsets increases(9, 231, 232). This results in a greater proportion of central memory cells that can self-renew via homeostatic turnover and rapidly proliferate into effector cells during secondary infections. *Chapter 3 investigates, in part, the outcome of T cell expansion and memory differentiation of viral specific CD8 T cells in the context of viral co-infection. Specifically, the differences in effector memory and central memory CD8 T cell formation of virus specific T cells due to variations in inflammation during T cell activation via the secondary virus's modulation of type I interferon production and signaling.*

Differences in T cell response following persistent viral infection

The fundamentals of T cell expansion, contraction and memory formation described above were elucidated from studies of the T cell response to acute viral infections. Acute viral infections such as influenza virus are characterized by short time-points of high antigen load (viremia) in the host followed by viral clearance(233). However, there are persistent viral infections such as hepatitis B virus (HBV), or HIV in which the virus is not controlled in the host following primary infection(234-236). This results in chronic antigen stimulation and persistent inflammation due to continued viral replication over time(234, 237).

Under conditions of chronic antigen stimulation, CD8 T cells do not undergo traditional differentiation into functional memory cells(232, 238). There are multiple alterations to the T cell response that occur under situations of chronic antigen stimulation such as T cell localization, functionality, and breadth of response(232, 239). For example, there may be preferential localization of antigen-specific T cells in non-lymphoid tissues due to sustained high antigen loads in infected organs or alteration in the expression of homing molecules on the CD8 T cell(232).

Persistent viral infection induces CD8 T cell exhaustion

One of the key differences between the T cell response to chronic and acute viral infection is the impaired effector function of responding T cells. The dysfunction of responding T cells during chronic viral infection due to sustained antigen stimulation is referred to as an exhaustive state of the T cell. The state of exhaustion of CD4 or CD8 T cell responses has been observed in a number of clinical and experimental viral infections in humans, non-human primates, and mice, such as HIV, HCV and HBV(240-247). Additionally, bacterial infections, parasitic infections, and progressive tumor settings have all described responding T cells demonstrating a state of dysfunction(240-246). This suggests that exhaustion is a conserved mechanism for limiting T cell immunopathology during antigen persistence**.**

A unique molecular signature markedly different from naïve, effector or memory T cells, can characterize exhausted CD8 T cells. However, T cell exhaustion is not an all-or-nothing phenomenon. During chronic infection, CD8 T cells progressively lose effector functions with the degree of dysfunction directly correlating with the levels of virus or antigen present and the absence or loss of CD4 T cell help (**Figure 1.3**) (238).

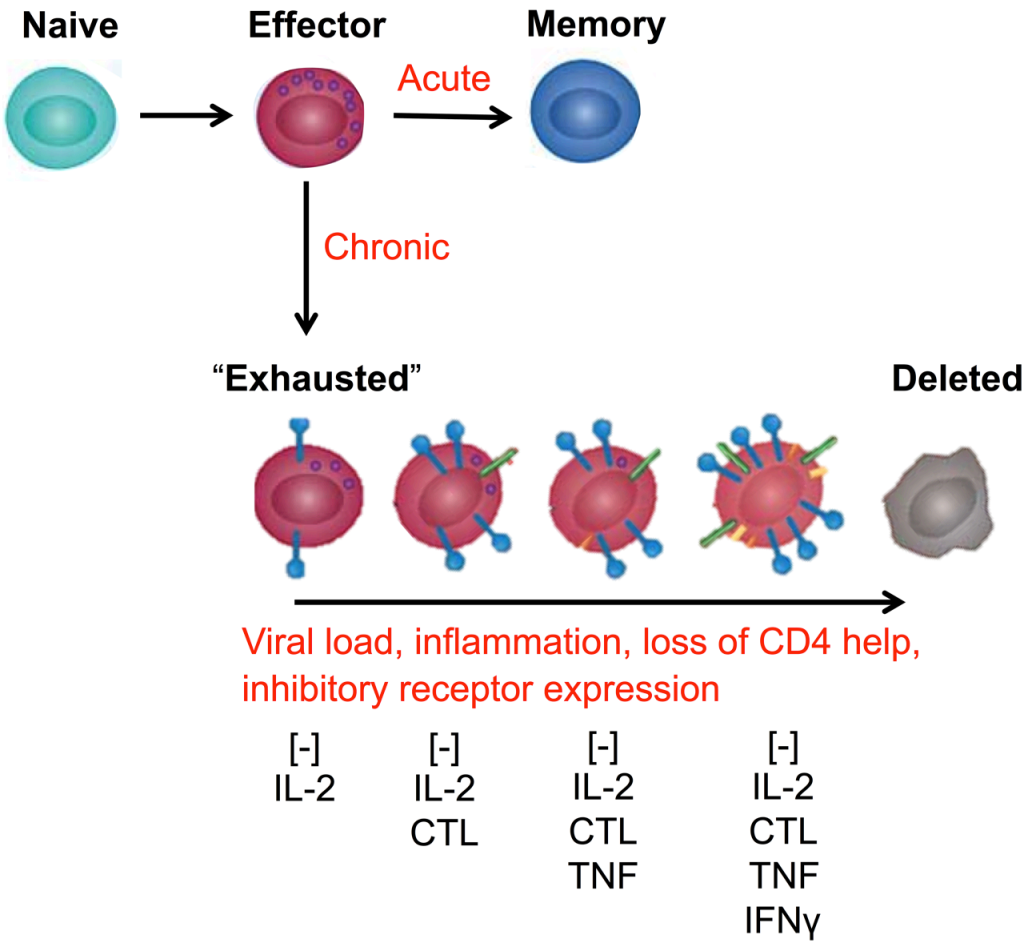

Figure 1.3: Progressive increase in T cell exhaustion during viral infection. Persistent viral infection can result in dysfunction CD8 T cell differentiation in which chronic antigen stimulation drives exhaustion of CD8 T cells. As infection or antigen load increases, T cells undergo higher degrees of dysfunction. Initially, T cells lose the ability to produce IL-2 and proliferate, followed by lose of cytotoxic ability and inflammatory cytokine production. Higher expression of inhibitory receptors correlates with the degree of dysfunction of the exhausted cell. Lastly, virus-specific T cells can undergo apoptosis and be eliminated from the response.

Initially, T cells lose the ability to proliferate in response to antigen stimulation concurrent with the loss of production of the T cell growth factor, interleukin-2 (IL-2). Subsequently, T cells lose the ability to secrete the inflammatory cytokine tumor necrosis factor (TNF).

The highest level of T cell exhaustion correlates with impaired cytolytic killing of infected target cells and the inability to produce the important anti-viral cytokine interferon-γ (IFN-γ)(248, 249). Lastly, critically exhaustive T cells may undergo clonal deletion as a final step of the exhausted phenotype summarized in **Figure 1.3**. Clonal deletion may further result in a change in the hierarchy of viral epitope-specific T cells responding to persistent viral infection as compared to an acute viral infection. This is due to the findings that CD8 T cells responding to immunodominant viral epitopes may be more susceptible to T cell exhaustion and deletion, resulting in the skewing of the T cell response to subdominant viral epitopes(238, 250).

Exhausted CD8 T cells maintain phenotypic and functional properties associated with exhaustion even after transfer into antigen-free hosts(251-253). Therefore, maintenance of the exhausted state must be mediated in part by regulatory mechanisms independent of external cues. The progressive loss of function during chronic viral infection is exacerbated by the loss of CD4 T cell help(250, 252). This increases with continuous antigen stimulation, indicating there must also be regulation by external cues. The varying levels of T cell exhaustion are regulated both by extrinsic and intrinsic mechanisms(249). Extrinsic mechanisms include the inhibition of CD8 T cells by regulatory CD4 T cell subsets and signaling via the inhibitory cytokines IL-10 and transforming growth factor-beta (TGF-β)(254, 255). Intrinsic mechanisms include the loss of expression of cytokines/cytokine receptors and increased expression of surface inhibitory receptors (249, 256).

The transient expression of inhibitory receptors such as Programmed cell death-1 (PD-1) or cytotoxic T lymphocyte-associated antigen (CTLA-4) after T cell activation is presumably a mechanism used to control T cell responses in order to prevent hyper-activation and autoimmunity(257). CTLA-4 competes with CD28 on T cells for the co-stimulatory ligand B7 on APCs to limit T cell activation. Whereas, PD-1/PD-L1 signaling regulates T cell function via direct attenuation of functional and proliferative capacity by the repression of TCR signaling and induction of genes such as BATF that regulate T cell function(258). Additionally, PD-1 expression has been implicated for the ability to limit T cell mobility, preventing target cell cytolysis in infected tissues(258). In the context of continued antigen stimulation, loss of T cell function usually coincides with increased expression of the inhibitory surface receptor PD-1. PD-1 and other inhibitory receptors such as LAG-3, 2B4 and Tim-3 can act synergistically due to non-redundant signaling pathways helping to establish the loss of function in responding T cells(259).

Evidence has emerged indicating PD-1 as a major inhibitor of T cell function in the exhaustive state. Exhaustive T cells in the context of chronic viral infection have epigenetic variations in the *Pdcd1* locus, resulting in long-term high expression of PD-1 on virus specific T cells(260, 261). Additionally, antibody blockade of PD-1/PD-L1 has demonstrated a therapeutic benefit by increasing T cell function *in vivo* resulting in an increase in viral control in animal models of chronic viral infections(262). The effectiveness of PD-1 antibody blockade depends of the level of PD-1 expression of the exhaustive cell population since only T cells with intermediate PD-1 expression experienced reversible exhaustion, whereas PD-1^{high} terminally differentiated cells did not(263).

T cell exhaustion is neither an irreversible terminal differentiated state nor a simple unresponsive T cell state, such as anergic T cells. The complete functional capacity of exhaustive cells *in vivo* has been difficult to determine due to the hierarchical loss of function during the progression of chronic viral infections but also due to the subtle variation in exhausted cells found during different contexts of infection(249). In studies of persistent antigen, T cell exhaustion can be defined by the inability of T cells to control pathogen infection or tumor progression *in vivo or* as the inability of T cells to exert effector functions measured during *in vitro* assays(264).

These distinctions do not inherently differentiate between T cells that are continuously exerting effector functions *in vivo*, and have not had sufficient time to recover prior to analysis in *ex vivo* assays, versus T cells undergoing programmed T cell tolerance resulting in a lack of *in vivo* function.

While exhaustion may be an adaptive state of hyper-responsiveness that is insufficient to control disseminated viral infection, it may also provide the host with the limited ability to control overwhelming viral replication without destructive T cell mediated immune pathology. The limited functional capacity of exhaustive T cells is supported by selection of T cell escape viruses in HIV patients, despite exhaustion of virus-specific T cells suggesting that these cells continue to exert some selective pressure during chronic infection(265). Furthermore, depletion of CD8 T cells during chronic viral infection of mice can result in a rapid increase of viremia(266, 267). Regulation of virus-specific T cell function by extrinsic factors such as regulatory T cells, IL-10, and TGF-β suggest that these are required to suppress ongoing T cell effector responses(249, 254, 268).

Lymphocytic choriomeningitis virus model system

Mouse models of viral infection have lead to the elucidation of a remarkable number of immunological properties. Specifically, infection of mice with Lymphocytic choriomeningitis virus (LCMV) has allowed the study of the immunological responses that contribute to the control of viral infection and disease(269). Animal studies utilizing the LCMV mouse model of infection have led to the description of the basis for MHC restriction of viral antigens(96, 270, 271), cross-presentation of epitopes by MHC proteins(272), how T cell effector mechanisms function to control virus infection(200, 273, 274), the generation and maintenance of T cell memory(9, 221, 222), and exhaustion of T cell responses during persistent infection(238, 239, 249, 275, 276).

Charles Armstrong unintentionally discovered LCMV during an outbreak of encephalitis in St Louis in 1933(277). It was further discovered that isolates of LCMV from infected humans were identical to isolates from naturally infected house mice (*Mus musculus*) in the United States and Europe highlighting the zoonotic potential of LCMV(269). LCMV is the prototypic Old World virus of the family *Arenaviridae(278)*. The LCMV particle consists of an enveloped, bi-segmented ambisense single-stranded RNA (ssRNA) genome(278). The two segments of the LCMV genome contain only four genes and are designated as the long (L) segment and short (S) segment due to their respective lengths(279). The 7.2kb L segment encodes the viral RNA-dependent RNA polymerase (L) and a small structural or regulatory polypeptide that contains a zinc finger motif (Z)(280). The 3.4kb S segment encodes the structural proteins including the viral nucleoprotein (NP) and the glycoprotein precursor (GP-C) that is ultimately post-transcriptionally cleaved into the mature GP-1 and GP-2 proteins(281, 282).

The ambisense coding strategy of arenaviruses requires that the NP and L coding regions located at the 3' ends of the L and S segments are directly transcribed into genomic-complementary mRNA prior to translation, similar to other negative sense RNA viruses(278). In contrast, the GP and Z coding regions located at the 5' ends of the L and S segments are transcribed into genomic-sense mRNA off of anti-genomic RNA generated during viral replication after NP and L translation occurs(283). LCMV GP-1 interacts with the cellular receptor α-Dystroglycan (α-DG) and the affinity of LCMV GP-1 binding to α-DG has been implicated to determine viral tropism and the outcome of infection, acute or chronic, in mice (284). The LCMV model is particularly useful to study T cell responses due to the generation of transgenic mice encoding virus specific T cell subsets. The P14 transgenic mouse that generates CD8 T cells that express only TCR specific for the D^{b}GP33-41 co-dominant epitope of LCMV is especially valuable in order to manipulate the number of antigen-specific naïve or memory CD8 T cells prior to viral challenge(285-288). Additionally, SMARTA transgenic mice that encode CD4 T cells specific for the GP61 epitope of LCMV(289) and B cell transgenic mice expressing the LCMV neutralizing KL25 antibody have been developed(290).

Immune response to acute LCMV infection

There are several variants of LCMV that have been discovered in research laboratories that result in distinct pathological outcomes in laboratory mice after infection as illustrated in **Figure 1.4**(291, 292). The following chapters employ two of the most popular strains utilized in mouse models for immunological research are the Armstrong and clone 13 strains. LCMV Armstrong is an acute infection after intraperitoneal (ip) or intravenous (iv) inoculation in adult mice.

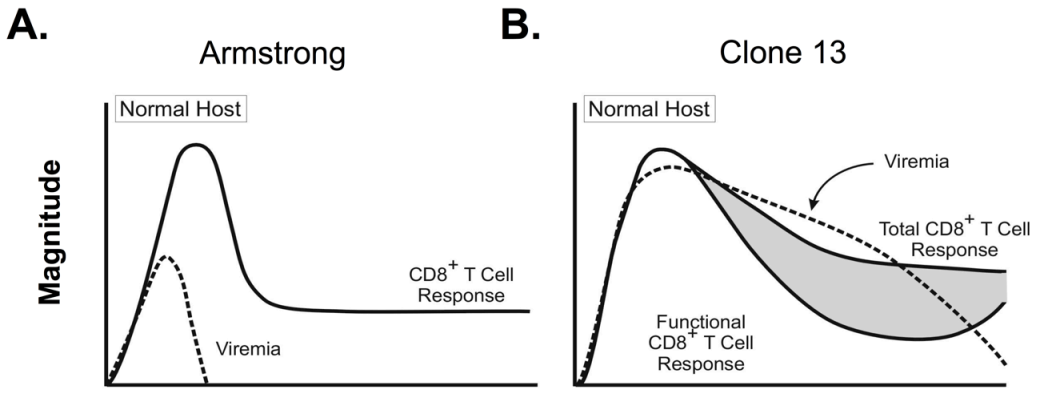

Figure 1.4: The LCMV Armstrong and Clone 13 variants result in very different outcomes of infection in mice. **[A]** LCMV Armstrong infection of immune competent mice produces no overt clinical disease with undetectable viral titers in the serum by day 8 post infection due to the rapid and vigorous expansion of CD8 T cells followed by the formation of a stable LCMV specific memory population. **[B]** LCMV clone 13 infection of C57Bl/6 mice is characterized by diminished viral control resulting in high viral titers detectable in serum up to 60 days post infection. Persistence is due in part to dysfunctional CD8 T cell differentiation leading to exhaustion of the responding immunodominant CD8 T cells.

LCMV Armstrong infection of immune competent mice produces no overt clinical disease with undetectable viral titers in the serum by day 8 post infection due to the rapid and vigorous expansion of CD8 and CD4 T cells followed by the formation of a stable LCMV specific memory population(8, 200, 221). CD8 T cells are critical and necessary to control acute LCMV infection since mice deficient in the production of CD8 T cells are unable to control viral replication after infection(293). Additionally, antibody production is presumably unnecessary for LCMV Armstrong viral clearance after primary infection because there is no measurable difference in the kinetics of viral clearance in B cell deficient mice(294, 295)

The majority of the responding CD8 T cells to LCMV Armstrong recognize either the immunodominant LCMV epitope from the viral nucleoprotein ($H2D^b$, NP396-404) or the viral glycoprotein ($H2\text{-}D^b$, GP33-41)(238). Although, the measured response to the GP33 peptide in *in vitro* assays is the total response of two separate populations because the GP33 peptide can also stimulate responding CD8 T cells that are specific for the GP34-41 ($H\text{-}2K^b$) epitope of LCMV. **Table 1.1** summarizes additional GP or NP derived peptides such as GP276-286, GP118-125, GP92-101, and NP205-212 that have been measured to induce production of the greatest amount of IFNγ by CD8 T cells at the peak of the response post LCMV Armstrong infection(296). Further analysis of T cell responses to the entire LCMV Armstrong proteome has revealed measurable responses to 28 total LCMV epitopes including 15 subdominant responses to the viral polymerase(297). The resulting population of LCMV-specific CD8 T cells, after infection, is a broadly responsive polyclonal pool of memory cells.

Table 1.1: Summary of LCMV CD8 T cell epitopes and their relative immunodominance. Peptides utilized in this study are highlighted, percentage of LCMV T cell response for each peptide was derived from (Masopust, 2007).

Peptide	Sequence	% IFNγ+ of CD8 T cells*	
		day 8	day 90
GP33/GP34	KAVYNFATM	27.54	5.29
NP396	FQPQNGQFI	23.63	3.81
GP276	SGVENPGGYCL	7.99	1.84
NP166	SLLNNQFGTM	7.77	1.14
GP118	ISHNFCNL	6.43	1.57
NP205	YTVKYPNL	5.85	0.99
GP92	CSANNSHHYI	1.17	0.25
NP235	NISGYNFSL	1.07	-
GP70	GVYQFKSV	0.77	0.27

However, the identical T cells responsible for controlling virus replication after ip inoculation in naive mice are also responsible for mediating lethal leptomeningitis when the virus is replicating in the brain post-intracranial (ic) inoculation leading to mortality 6-8 days post infection(298-300). Pathology may be due to inflammatory conditions propagated by responding T cells either directly killing infected cells and producing cytokines such as TNF or the production of chemokines that result in the recruitment of innate immune cells such as macrophages and neutrophils that lead to increased vascular injury and lethal meningitis(301). The rapid control of viral replication in the brain after ic challenge due to the response of memory LCMV-specific CD8 T cells protects mice from lethal meningitis(302, 303). Thus demonstrating the importance of the kinetics in which virus can be eliminated from the host that can be affected by initial viral load, replication rates, and the effectiveness of responding T cells in order to limit severe pathology in critical tissues.

Pathology observed during LCMV infection in mice may be dependent on virus strain, inoculum size and route of infection but ultimately pathology is mediated by the immune response (303-305). Congenital carrier mice with life-long LCMV infection in the absence of a LCMV-specific T cell response demonstrate limited pathology (273). This suggests that immune responses to LCMV, capable of generating protective responses to intracranial challenge or parental challenge with persistent stains, promote both virus clearance as well as immunopathology.

Immune response to persistent LCMV infection

In contrast, the persistent LCMV clone 13 strain results in a protracted viral infection in wild type C57Bl/6 mice(292). Rafi Ahmed discovered LCMV clone 13 in 1984 after observing sustained viral loads and suppression of LCMV-specific cytolytic activity in adult immunocompetent mice that received transfer of splenocytes from 2-month-old Balb/C WEHI mice infected at birth with LCMV Armstrong(292). Persistent LCMV clone 13 infection in C57Bl/6 mice is characterized by diminished viral control, resulting in high viral titers detectable in serum up to 60 days post infection(238, 292). This is due to, in part, dysfunctional CD8 T cell differentiation leading to exhaustion of the responding immunodominant CD8 T cells (**Figure 1.4B**) (238). In the absence of CD4 T cell help, CD8 T cell dysfunction is exacerbated, resulting in life-long chronic infection in mice with long-term elevated viral titers in serum and tissues (218). Furthermore, the pathogenic difference between LCMV Armstrong and LCMV clone 13 infections is propagated by only three amino acid residue mutations between the two viruses(306). Two mutations occur within the viral spike GP-1 on the S segment at amino acid position 176 and 260 and one occurs within the viral polymerase on the L segment at amino acid position 1076(307) as illustrated in **Figure 1.5A**.

The F260L mutation in the GP-1 protein in the LCMV clone- 13 variant results in an increased affinity for the cellular receptor, α-Dystroglycan, resulting in an increase in the relative infectivity of the virus. The K1079Q mutation in the viral polymerase of LCMV clone-13 results in an increase in replication rate of the virus (308). Importantly, these mutations that alter virus tropism and replication rates lead to stark differences in viral dissemination in the host, as illustrated in **Figure 1.5B,** yet they do not affect viral T cell epitopes(276).

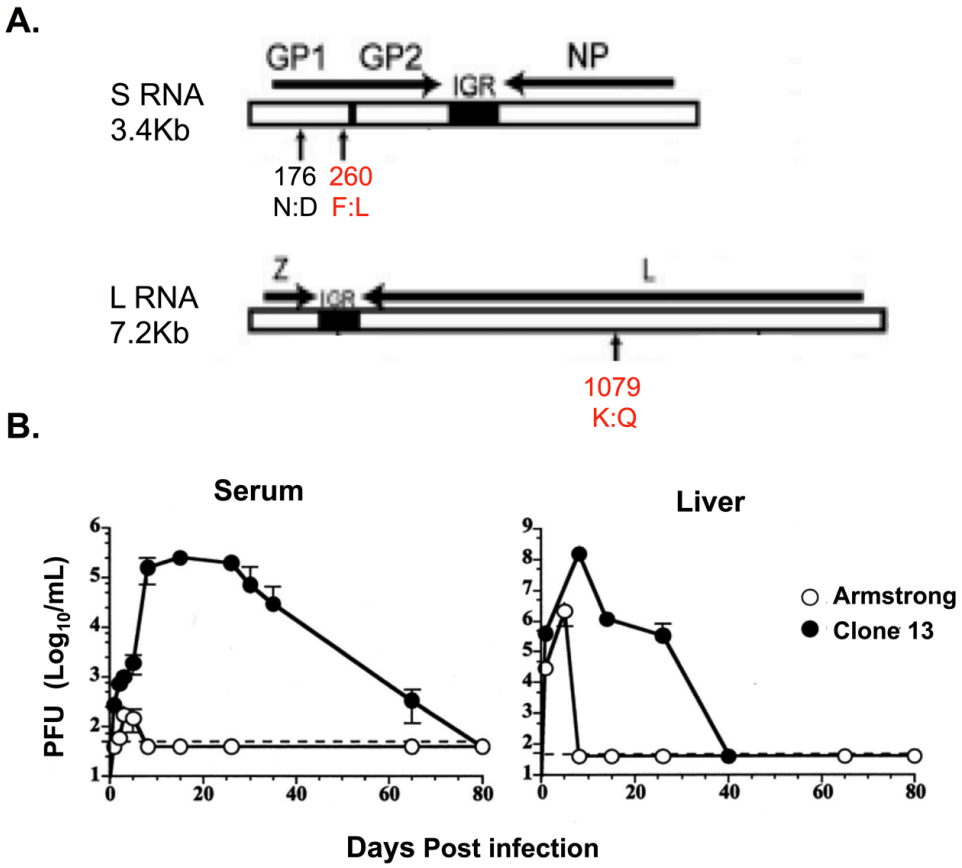

Figure 1.5: LCMV clone 13 has two amino acid mutations that are critical for the pronounced difference in viral dissemination *in vivo*. [A] The F260L mutation in the GP-1 protein in the LCMV clone- 13 variant results in an increased affinity for the cellular receptor, α-Dystroglycan, resulting in an increase in the relative infectivity of the virus. The K1079Q mutation in the viral polymerase of LCMV clone-13 results in an increase in replication rate of the virus. **[B]** LCMV Clone 13 mutations that alter virus tropism and replication rates lead to stark differences in viral dissemination. High virus is detectable in the serum and liver greater than 30 days post infection in Clone 13 infected mice.

The selection of the LCMV clone 13 genetic variant may have been a consequence of virus-host evolution towards balanced pathogenicity, since LCMV CTL activity is critical not only for viral control, but also fatal immunopathology mediated by responding CD8 T cells after intracranial infection of adult mice.

Naïve C57Bl/6 mice exhibit clinical disease symptoms after high dose ($2x10^6$ pfu) intravenous LCMV clone 13 challenge but they do not experience lethal immunopathology. Persistent but non-lethal infection is in part due to deletion of effector cells, such as T cells responding to the dominant NP396 peptide(238) or the progressive decrease in effector functions of exhausted effector cells, due to prolonged viral stimulation(239). Primary CD8 T cell responses to the persistent variant of LCMV can also result in fatal immunopathology due to alterations in the magnitude of T cell exhaustion. If the extent of exhaustion of the activated T cells is altered, due to either an increase in the number of naïve LCMV-specific CD8 T cells present before challenge(239) or a decrease in the initial viral load via a reduction in magnitude of the viral inoculum(275), it can result in two very different pathological outcomes.

The increase in precursor frequency through the adoptive transfer of 10^6 LCMV-specific CD8 T cells prior to high dose clone 13 challenge results in a robust effector response, increasing the kinetics of viral control, preventing T cell exhaustion and eliminating disease entirely. In contrast, a moderate increase in precursor frequency via adoptive transfer of 20,000-10^5 naïve LCMV-specific T cells prior to high dose clone 13, results in only partial exhaustion of the responding T cells. This can then cause fatal immunopathology via cytolysis and the production of inflammatory cytokines in the lungs.

A smaller increase in precursor frequency through the adoptive transfer of less than 10,000 LCMV-specific CD8 T cells prior to high dose 13 challenge results in weaker magnitude responses, complete exhaustion, and elimination of increased immunopathology due to the absence of effector function of the responding cells(239, 276).

Consistent with the pathological outcomes due to varying precursor frequency of antigen-specific T cell subsets, the same phenomenon was observed after infection of naïve C57Bl/6 mice with decreasing magnitudes of LCMV. Infection with a 100-fold decrease of LCMV clone 13 ($2x10^4$ pfu) resulted in strong effector responses, early viral control, prevention of T cell exhaustion, and elimination of disease. LCMV clone 13 challenge with an intermediate dose ($2x10^5$ pfu) resulted in only partial exhaustion of the responding T cells, yet without complete viral control allows the development of severe pathology, leading to death by 12 days post-infection. Lastly, high dose infection does not cause immunopathology due to the greater extent of exhaustion of responding T cells, resulting in loss of effector functions that mediated lethal lung and liver pathology(275).

Memory CD8 T cell mediated immunopathology

The immunopathology mediated by LCMV specific CTL described above is not exclusive to the primary immune response. Memory CD8 T cells generated via immunization(303) or after heterologous infection(309, 310) have been shown to induce as severe immunopathology during secondary responses after viral challenge. Earlier studies by Oehen reported that narrow vaccination against one protein of LCMV (either NP or GP) followed by intracranial challenge with LCMV-WE, resulted in increased pathology as measured by mouse mortality (303).

Researchers predicted the observed pathology was a consequence of a narrow vaccination and could be avoided by induction of a broader immune response against more viral epitopes. Additionally, it has been reported that stimulation of immunized mice (with high levels of LCMV-specific CD8 T cells) with cognate peptide resulted in lethal immunopathology largely dependent on CD8 T cell production of TNF (305).

The LCMV model system allows the isolated investigation of specific T cell factors, such as phenotype and effector function, that may contribute to virus control or pathology during an immune response. The easy manipulation of LCMV viral parameters such as replication rate and infectivity via use of LCMV variants allows the direct comparison and investigation of immune responses during acute or persistent viral settings. Furthermore, LCMV Armstrong can be used as a vaccine strain to generate memory T cell responses reactive to LCMV clone 13 epitopes that allow investigation into the characteristics of protective or pathologic memory T cell responses after exposure to chronic viral infections.

Mathematical models predict immunopathology during LCMV infection

Although considerable progress has been made towards understanding the molecular and cellular basis for immunopathogenesis during viral infection, it is often difficult in such situations to predict the outcome of infection due to exponentially expanding virus and immune cell populations(311-314). Mathematical models can help decipher these complex interactions, in part, by making predictions about which cells or pathways mediate immunopathology versus immune protection. LCMV infection of mice is also a useful system in which to mathematically model immunological responses, such as T cell exhaustion, that contributes to the control of viral infection versus disease (315).

This is due to the observation that CD8 T cell responses are required to mediate viral clearance (239). However, since LCMV is a non-cytolytic virus, the pathology observed during infection is mostly due to responding T cells.

An empirically based mathematical model of LCMV infection in mice has been developed using observations of T cell responses to LCMV. The ordinary differential equation model takes into account changes in virus, T cell responses, and exhaustion (276). The developed model accurately describes both the viral dynamics and the immune response to infection. In particular, the model replicates the key features of persistent infection in which high numbers of virus-specific CD8 T cells rapidly control infection, while low numbers of virus-specific CD8 T cells are unable to control viral replication and become functionally exhausted(239, 276). Furthermore, the model reproduces the outcome that maximum T cell mediated pathology, without virus control, occurs when an intermediate number of naive virus-specific T cell precursors are present(239). The developed model incorporates immunopathology by modeling T cell production of TNF, resulting in severe vascular leakage, as seen in many hemorrhagic fever virus infections(316, 317).

The developed model makes several key predictions about which immune or virus parameters are most important for resulting T cell mediated pathology. Specifically the model predicts that immunopathology, due to either infected cell cytolysis or cytokine production, is insensitive to the enhanced ability of memory CD8 T cells to respond to secondary infection. Additionally, the model predicts that enhancement of pathology is unaffected by the breadth of memory CD8 T cells responding. The model also suggests that the number of T cells that cause maximum immunopathology is proportional to the initial virus inoculum and that changes in virus inoculum will result in concomitant changes in the number of T cells that cause maximum disease. *Chapter 2*

directly tests the predictions of the developed mathematical model to determine how virus and immune system variables affect vaccine-enhanced disease during persistent viral infection.

Historical perspective of vaccine-mediated immunopathology

The outcome of vaccination ordinarily results in either protective or non-protective immunity after subsequent exposure to a pathogen. Previous attempts to design vaccines for viral infection highlight the potential for vaccination (or prior exposure to a pathogen) to result in unintended immune mediated pathology (276, 303, 318-322). The mechanism of vaccine induced immune mediated pathology can vary due to the type of immunizing agent utilized, demonstrated by the historical efforts of developing a vaccine for respiratory syncytial virus (RSV) (323). An experimental vaccine designed in 1966-67 consisting of intramuscular injection of concentrated formalin inactivated RSV to infants and children (2 months-9 years) resulted in a failure to offer protection during a subsequent outbreak of RSV. In addition to the vaccine demonstrating limited efficacy, an exaggerated clinical response was seen in 80 percent of young vaccine recipients, including two deaths (320, 324, 325). Follow up studies in animal models indicated that the inducement of T cells is crucial for the immunopathogenesis of vaccine enhanced RSV-disease.

Particularly, the formalin inactivated RSV vaccine induces a Th2 bias that can result in increased lung inflammation and eosinophilia following subsequent infection (326). Surprisingly, researchers attempting to control the immune response towards nonpathogenic responses by selectively priming T cell subsets with recombinant RSV expressing prototypic Th1 cytokine IFNγ also reported enhanced weight loss and immunopathology due to pulmonary influx of RSV-specific CD8 T cells (327).

Whereas, mice that received a recombinant RSV expressing prototypic Th2 cytokine IL-4 vaccine suffered no additional weight loss but demonstrated lung pathology and eosinophilia as expected (327). Moreover, further studies have demonstrated RSV-specific CD8 T cell induction of immunopathology during acute RSV infection after depletion of T_{regs} in mice (328).

Other experimental settings have demonstrated vaccines designed to employ a cell-mediated immune response may also result in an unintended increase in disease (318, 319, 322). Vaccine-mediated immunopathology occurs specifically when elicitation of only T cell responses or inappropriate versions of these responses to a pathogen occur. Therefore, determination of the circumstances in which memory T cell responses result in protection from persistent viral burden versus those that mediate immunopathology is critical for the design of safe and effective vaccines against diseases such as HIV/AIDS.

Viruses have evolved to modulate host immunity

Variola virus (VARV) is responsible for causing one of the most destructive diseases in human history. VARV, the causative agent of smallpox, is one of many double-stranded DNA viruses of the *Poxviridae* family that is best characterized by their large size, cytoplasmic replication and production of immune modulatory proteins(329). The World Heath Organization (WHO) declared smallpox eradicated in 1980 following the most successful vaccination program against a human pathogen in our history(330). The restricted use of VARV in scientific laboratories required the development of laboratory animal models of orthopoxvirus infection in order to study viral immune modulation and pathogenesis.

Poxviruses have been widely used to understand how the immune system responds to infection(331) and are currently being investigated for use as potential vaccine vectors(25) for many important human pathogens such as HIV(21). Recently, poxviruses have been broadly studied for use in oncolytic therapies and to reduce GVHD after bone marrow transplants(332).

Poxvirus infection initiates a race between viral replication, transmission and the host's immune response. Poxviruses have evolved several mechanisms to circumvent immune surveillance and subsequent immunological responses to infection(329). Type I interferons are potent antiviral cytokines responsible for production of a broad range of antiviral proteins and directly modulate adaptive immune responses(40). Consequently, poxviruses have evolved multiple strategies to suppress IFN-I production, modulate IFN-I signaling, and block the action of anti-viral proteins. One such mechanism is the sequestration of virally produced dsRNA in order to inhibit detection by host pattern recognition receptors that detect viral infection and initiate IFN-I production(333). The poxvirus protein E3L contains a highly conserved double-stranded RNA (dsRNA)-binding domain that has been shown to be required for Vaccinia virus interferon resistance and pathogenesis in mice(334).

In addition to intracellular blockade of IFN-I production, poxviruses also encode proteins to sequester extracellular IFN-I to limit IFN-I anti-viral effects in neighboring cells. Poxviruses produce a type I interferon binding protein (T1-IFNbp) that can bind to IFN-I with high affinity either in solution or associated with cell membranes to prevent IFN-I produced during infection to ligate with host type I interferon receptors(335). Intracellular and extracellular modulation of IFN-I is critical for poxvirus virulence as the deletion of either of these proteins reduce in vivo pathogenesis(333, 335).

Ectromelia virus model system

Ectromelia virus (ECTV) was first discovered in the 1930s to cause a fatal disease in laboratory mice that mimicked smallpox disease in humans(336, 337). Similar to VARV in humans, ECTV is infectious at low doses and causes severe disease in mice with high mortality rates(338, 339). Experimental inoculation of susceptible mice with ECTV results in an acute, lethal disease corresponding with high viral replication in the liver and hepatic damage(337, 340). Inbred strains of laboratory mice are either susceptible or resistant to ECTV infection depending on specific genetic factors such as MHC genotype that ultimately influences the adaptive immune response after infection(341, 342). Resistant C57Bl/6 mice have more rapid and stronger NK and CTL responses than susceptive mouse strains(336, 340).

In addition to enhanced adaptive immune responses in resistant mice, production of IFNα/β has been shown to be important for the recovery of C57Bl/6 mice after ECTV infection(5, 338). Thus is supported by the observation that antibody blockade of ECTV T1-IFNbp in susceptible mice leads to increased survival(343). Interestingly, deletion of ECTV T1-IFNbp drastically reduces virulence in mice, but the ECTV T1-IFNbp has been found to only block the biological activity of mouse IFNα(5). However, ECTV is resistant to IFNβ treatment in vitro, suggesting that the ECTV E3L homolog is an important overlapping mechanism to circumvent the anti-viral affects of IFN-I production(344). The importance of IFN-I blockade during poxvirus infection is highlighted by the severe decrease in viral pathogenicity and replication *in vivo* after deletion of only one of the poxvirus immunomodulatory genes that block IFN-I(335). However, there is no known immunomodulatory protein that is shared by all poxviruses due to each virus species encoding a unique combination of proteins necessary to evade the immune response of its natural host(329).

Moreover, certain modulatory proteins are specifically limited to interact with host molecules(336, 345). ECTV infection of laboratory mice is useful in order to study orthopoxvirus immune modulation in its natural host(343).

The immune system has evolved to maintain homeostasis with commensal microbiota

Significant progress has been made using a reductionist approach to understand host-microbe interactions in which specific cell populations and proteins are classified and dissected to determine their relative impact on immune responses. Therefore, the majority of microbiological research is performed in specific-pathogen-free, clean, and controlled environments that limit potential co-infection by heterologous pathogens.

However, most "real-world" infections likely occur in the context of co-infection by heterologous pathogens or host microbiota(346). The collection of bacteria, viruses, parasites and fungi that compose the human microbiome is estimated to number over 100 trillion microbes(347). Co-evolution between mammalian hosts and microbial communities has resulted in a symbiotic relationship. Commensal microbes, that do not normally cause disease, enhance digestion while benefiting from stable nutritional resources(348). Although, in order to prevent opportunistic invasion the human immune system has evolved to maintain homeostasis with host microbiota(349, 350). For example, germ-free mice that are born in a sterile environment do not develop lymphoid follicles in the small intestine, fail to secrete IgA antibodies, and lack intraepithelial lymphocytes(351), suggesting that co-evolution has also resulted in the dependence on host microbiota to influence immune development and immune cell regulation(348).

Alterations in host microbiota have been shown to influence, not only gut associated lymph tissue, but also influence peripheral immune responses. Parasitic helminths facilitate long-term survival in hosts by the regulation of immune responses via induction of regulatory CD4 T cell subsets and anti-inflammatory cytokines(352). Parasitic induced immune regulation extends beyond parasite-specific responses, since helminths infection can lower immune response to BCG vaccination(353) and reduce T cells responses during HCV infection(354). Parasitic modulation of systemic immunity highlights the potential consequences of microbial co-infection due to immunomodulatory mechanisms that can influence non-specific immune response and potentially alter disease(346). Therefore, implicating the potential alteration in immune responses during viral co-infection, due to virus evolution of an array of immune modulatory mechanisms.

Consequences of viral co-infection of global health

Viral co-infection can occur by simultaneous exposure of two heterologous viruses that may share the same route of transmission or chronically infected individuals can become co-infected after independent exposure to circulating viral strains. Due to the success of highly active antiretroviral therapy in HIV-infected individuals that has decreased AIDS-related morbidity and mortality, HIV co-infection with Hepatitis virus has emerged as an additional source of morbidity and mortality(355). Co-infection with HCV and HIV is a relatively common event occurring in 15-30% of all HIV-infected individuals and 5-10% of all HCV-infected individuals(356, 357) (**Figure 1.6**).

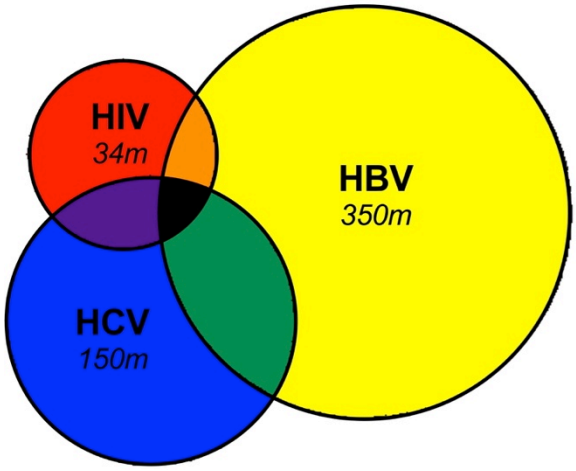

Figure 1.6. Global prevalence of HIV and Hepatitis virus co-infection. Venn diagram illustrates prevalence of HIV, HBV or HCV co-infection. Area of circles and intersections is a to-scale representation of population prevalence. Sub-Saharan Africa accounts for ~70% of global burden of HIV, and that around 10% of all those with HIV are HBV co-infected.

HIV co-infection is associated with weaker HCV adaptive immune responses, increased liver fibrosis, higher HCV RNA levels, reduced response rates to anti-viral treatment, and worse HCV disease progression(356, 358). The differences in immunity and disease in co-infected individuals may be due to alterations in the intrahepatic cytokine milieu as a result of HIV infection(359, 360). Detection of cytokine mRNA in HIV/HCV co-infected patients revealed lower levels of TNF, IL-8, and IL-10 mRNA and increased levels of TGF-β compared to individuals infected with HCV alone(360). In addition, HBV co-infection has been reported to occur in up to 36% of all HIV-infected individuals in Africa, which currently accounts for over 70 percent of global HIV-infection(357, 361). HIV co-infection with HBV also correlates with higher rates of HBV persistence and increased risk of liver-related morbidity and mortality(355, 357, 362).

Another potential consequence of viral co-infection is co-evolution in which one virus may supply ancillary functions or suppress immune functions for another(345). Such a relationship has previously been described for Hepatitis B (HBV) and Hepatitis D (HDV) viruses. HDV cannot form mature virions without the presence of the Hepatitis B structural proteins(363). Furthermore, co-infection of HBV and HDV or super infection of persistently infected HBV patients with HDV also results in increased liver pathology and poorer prognosis in patients due to altered immune responses and type I interferon signaling in the host(364, 365). *Chapter 3 investigates if alterations in innate signaling due to heterologous viral co-infection in a natural host will influence CD8 T cell responses and disease. Furthermore, chapter 4 assesses the consequence of viral co-infection with persistent viral strains known to induce dysfunctional CTL response will alter CD8 T cell differentiation and potential CD8 T cell immunopathology.*

Objectives

Our current approach to develop a quantitative understanding of the dynamics of CD8 T cell responses during persistent viral infection involves the utilization of LCMV infection of mice. The LCMV model allows the study of specific components of the immunological responses that contribute to the control of infection versus those that mediate enhanced disease after vaccination, due to the capability of adoptive transfer of defined virus-specific T cell subsets into naive mice. The manipulation of T cell parameters (phenotype, functional capacity, breadth of response) and viral parameters (replication, infectivity) allow isolated investigation of multiple factors that may contribute to virus control and the reduction of pathology during an immune response.

Our objective is to further inspect how the CD8 T cell response, responsible for viral clearance and/or immunopathology, can change due to alteration of the breadth of targeted epitopes, T cell effector function, and initial viral load. In addition, the consequence of viral co-infection during vaccination or infection on the resulting CD8 T cell effector response, memory differentiation, and protective capacity after secondary challenge will be examined.

CHAPTER 2

A QUANTITATIVE UNDERSTANDING OF THE BALANCE BETWEEN PATHOLOGIC AND PROTECTIVE MEMORY T CELL RESPONSES DURING VIRAL INFECTION

ABSTRACT

Despite the development of vaccines for a wide range of viral diseases, efficacious vaccines for persistent viral infections have been elusive in part due to safety concerns for use of attenuated viruses and lack of efficacy for killed virus. Immunization against virus T cell epitopes has been proposed as an alternative vaccination strategy for persistent viral infections. However, vaccines that selectively engage T cell responses can potentially result in inappropriate immune responses that increase, rather than prevent, pathology after subsequent infection. Using dynamic quantitative models of virus infection and immune responses, we investigated the quantitative basis for pathologic versus protective T cell responses during disseminated viral infection, including which virus and immune system variables affect vaccine-enhanced disease. We found that an intermediate number of memory CD8 T cells prior to LCMV infection resulted in maximum T cell mediated pathology. Increased pathology at intermediate numbers of T cells was independent of the sensitivity or breadth of the T cell response but was dependent on T cell production of TNF and the magnitude of initial virus inoculum. In particular, abrogation of TNF signaling resulted in decreased pathology but no change in viral clearance, suggesting that TNF-blockade may be useful for minimizing pathology while maintaining protection during virus infection. Thus, mathematical models of virus and T cell immunity can be used to make useful predictions regarding which molecular and cellular pathways differentially mediate T cell protection versus pathology.

IMPORTANCE

Vaccines remain the most effective method for preventing or treating viral diseases. Unfortunately, vaccines for many persistent viral infections have not shown efficacy. One strategy that has been widely employed to generate vaccines for persistent viral infections are recombinant vaccines expressing pathogen T cell epitopes. To better understand the quantitative relationship between memory T cell numbers and protection versus pathology resulting from such responses, we have developed quantitative mathematical models of virus infection and immune responses and used these to predict which molecular and cellular interactions mediate each of these outcomes. The following investigation demonstrates that memory T cell mediated pathology is independent of TCR sensitivity or breadth of the epitopes targeted, but is dependent on the magnitude of virus inoculum and T cell production of TNF. The blocking TNF signaling during disseminated virus infection, was able to abrogate pathology while maintaining T cell protection. These data suggest that TNF blockade intervention strategies may be useful for enhancing host survival during virus infection without preventing T cell protection.

INTRODUCTION

Four of the top ten leading global causes of premature death in humans are diseases due to infectious pathogens(11). Total deaths from HIV (1.6 million), tuberculosis (1.1 million) and malaria (627,000) infections totaled more than 3 million in 2012(11). However, in the past decade there has been a global shift away from premature death due to infectious diseases, except in Africa where 70% of calculated years of life lost are due to infectious diseases, maternal, neonatal and nutritional causes(11).

One of the main reasons for this transition is that expanded vaccination coverage has significantly enhanced our ability to elicit effective memory immune responses to fight and prevent human diseases caused by infectious pathogens(12, 13). From 2000-2010 an estimated 2.5 million deaths were prevented each year among children less than 5 years old by the use of measles, polio and diphtheria-tetanus-pertussis vaccines(366).

The vast majority of currently used vaccines against infectious diseases employ attenuated or killed versions of pathogens to induce protective immunity(367). Such vaccines induce antibodies that circulate the body as a first line of defense or induce populations of memory T and B cells(367, 368). Memory T cells are better able to rapidly respond to secondary infection via production of inflammatory cytokines and cytolysis of infected host cells to decrease the severity of disease(12, 13, 369-371). However, such T cell responses also have the potential to increase disease severity by production of inflammatory cytokines, in particular TNF, which can result in increased vascular leakage or cell death(316).

Despite the success of current vaccines, inherent dangers associated with attenuated vaccines and ineffectiveness of killed vaccines for persistent viral infections has limited prevention of these diseases(17, 23, 24, 318, 372). One solution to this problem is the use of recombinant vectors expressing pathogen T cell epitopes as a means of inducing protective immunity(21, 22, 315, 373). Paradoxically, the production of a large number of memory T cells also has the potential to cause increased immunopathology and exacerbate disease compared to that observed in unvaccinated individuals(302, 318, 320, 321, 323-325, 374, 375). This was originally observed by Oehen et al., in which vaccination with recombinant viral vectors expressing lymphocytic choriomeningitis virus (LCMV) proteins resulted in enhanced disease, rather than protection, during subsequent virus challenge(303).

Previous studies have quantitated the relationship between naïve antigen-specific T cell precursors and the outcome of virus infection, showing that maximum pathology, but not protection, occurs at intermediate T cell numbers during chronic LCMV infection(239, 276). T cell enhancement pathology after viral infection has been reported in a variety of other animal models of virus infection including, LCMV(275, 276, 303, 304, 376), influenza(377, 378) and respiratory syncytial virus(327, 379). Thus, a major goal of vaccination should be induction of immune responses that maximize elimination of infectious organisms while minimizing immunopathology. Therefore, determination of the circumstances in which memory T cell responses elicited by vaccination result in protection from virus infection versus those that mediate immunopathology is critical for the design of safe and effective recombinant vaccines against persistent viruses such as HIV.

Although, considerable progress has been made towards understanding the molecular and cellular basis for immunopathogenesis during viral infection, it is often difficult in such situations to predict the outcome of infection due to exponentially expanding virus and immune cell populations(311-314). Mathematical models can help decipher these complex interactions by making predictions about which cells or pathways mediate immunopathology versus immune protection. Mathematical models that qualitatively describe previously observed dynamics of virus infection and naive T cell responses have been developed (276). These models make several key predictions about which immune or virus parameters are most important for T cell mediated pathology. Specifically, the models predict that immunopathology due to either cell loss or cytokine production is insensitive to the enhanced ability of memory T cells to respond to infection or to the breadth of the T cell response.

However, the models do suggest that the number of T cells that cause maximum immunopathology is proportional to the initial virus inoculum dose. The following investigation brings the models into risky contact with experimental data and show that these predictions are supported in the LCMV mouse model of virus infection.

The previously described ordinary differential equation mathematical model takes into account changes in virus, T cell responses, and exhaustion and recapitulates the dynamics of virus and CD8 T cell response during acute and persistent LCMV infection(276). In particular, the model replicates the key features of chronic infection in which high numbers of virus-specific CD8 T cells rapidly control chronic LCMV infection while low numbers of virus-specific CD8 T cells are unable to control viremia and become functionally exhausted(239, 276). Furthermore, the model reproduces the outcome that maximum T cell mediated pathology, without virus control, occurs when an intermediate number of virus-specific T cell precursors are present(239). Based on experimental results, immunopathology has been incorporated by modeling T cell production of TNF, resulting in severe vascular leakage, as seen in many hemorrhagic fever virus infections(316, 317).

Further simulations of the developed model make key predictions regarding aspects of the virus and immune response that challenge current vaccinology dogma. Specifically, the model predicts that: 1) enhancement of pathology is unaffected by TCR sensitivity, 2) enhancement of pathology is unaffected by the breadth of the T cell response and 3) changes in virus inoculum dose result in concomitant changes in the number of T cells that cause maximum disease. The following investigation tests each of these predictions using the developed experimental model of LCMV infection of mice.

MATERIALS AND METHODS

Mice: 6-8 week old C57BL/6, TNFR$^{-/-}$ (p55$^{-/-}$x p75$^{-/-}$) and IFNγR$^{-/-}$ mice were purchased from Jackson laboratories (Bar Harbor, ME) and bred in our ASU animal facilities. P14 transgenic mice, in which CD8 T cells express TCR specific for the D^{b}GP33-41 epitope of LCMV, were obtained from Dr. Rafi Ahmed and bred in our animal facilities. All mice were maintained under specific-pathogen free conditions at The Biodesign Institute and experiments were performed in compliance with institutional guidelines as approved by Institutional Animal Care and Use Committee of Arizona State University.

Cells: BHK cells were maintained in complete Eagles' MEM (5% fetal bovine serum (FBS), 2mM L-glutamine (L-Q), 100 U/mL penicillin, 100 μg/ml streptomycin). Vero and MC57 cells were maintained in complete DMEM (10% FBS, 2mM L-Q, 100 U/ml penicillin, 100 μg/mL streptomycin).

Viruses and infections: Lymphocytic choriomeningitis virus clone-13 and Armstrong stocks were kindly provided by Rafi Ahmed (Emory University, Atlanta, GA) and produced in BHK cells as previously described (380). Viral stocks and serum viral titers were determined by plaque assay on Vero cell monolayers as previously described(292).

Memory cell generation and adoptive transfer: Memory P14 cells were generated by adoptive transfer of 10^5 naive P14xThy1.1 cells into naïve Thy1.2 C57Bl/6 (B6) donor mice followed by intraperitoneal (ip) immunization with $2x10^5$ pfu LCMV Armstrong. After stable memory formation (typically >45 days post immunization), memory CD8 T cells were purified from splenocytes using Thy1.1 MACS magnetic beads purchased from Miltenyi Biotech (San Diego, CA) and adoptively transferred into Thy1.2 B6 recipient mice.

Recipient mice were then challenged intravenously (iv) with $2x10^6$ pfu LCMV clone-13 or varying doses as indicated. Mice were monitored daily for morbidity and mortality via clinical scoring (hunched posture, ruffled fur, non-motility) and weight loss.

Peptides: Lymphocytic choriomeningitis virus CD8 T cell epitopes NP396, GP33, GP276, GP118, GP92 and NP205 were purchased from Genscript (Piscataway, NJ). Ex vivo peptide stimulations were done at a concentration of 1ug/ml as previously described(200).

Cell surface antibody staining: Single cell suspensions were prepared from splenocytes as previously described(200). Erythrocytes were lysed with ammonium chloride lysis (ACK) buffer purchased from Lonza (Allendale, NJ and FACS staining was done as previously described(381) in 96 well plates with flurochrome-labeled monoclonal antibodies: anti-CD8 (clone 53-6.7), anti-Thy1.1 (clone X), anti-CD44 (clone IM7), antii-PD-1 (clone J43), anti-CD4 (clone GK1.5). Samples were then fixed in 1% paraformaldehyde solution and immediately acquired on a BD LSR II Fortessa flow cytometer (San Jose, CA) and analyzed using FlowJo Software (Tree-Star, Ashland, OR). All surface and intracellular monoclonal antibodies were purchased from BD Pharmigen (San Diego, CA) or eBiosciences (San Diego, CA).

Intracellular cytokine staining: For quantification of LCMV-specific T cell responses, splenocytes (10^6/well) were stimulated with 1μM LCMV peptide as previously described(200). After 5 hours of stimulation, cells were permeabilized according to manufacturer's instructions using the Cytofix/Cytoperm kit (BD Pharmigen) and intracellular cytokine producing cells were detected by staining with anti-IFNγ (clone XMG1.2) and anti-TNF (clone MP6-XT22) as previously described(200). The samples were acquired and analyzed as described above.

Histology: formalin fixed liver and lung tissue were cut into 4μm thick sections on a microtome. Liver and lung samples were stained with hematoxylin and eosin as previously described(382). 40x images were taken with a Nikon Eclipse E600 microscope and evaluated by a veterinary pathologist.

Statistics: Prism software (Graphpad, La Jolla, CA) was used to calculate t-test p values to determine significance or log-rank test to determine survival curve significance (* = $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$).

RESULTS

The increased sensitivity of memory CD8 T cells to viral antigens does not limit vaccine-induced pathology. Naïve and memory CD8 T cells differ in their requirements for activation, proliferative capacity and *in vivo* migration; memory CD8 T cells have an increased sensitivity to antigen stimulation allowing them to respond more rapidly to subsequent infections(223, 224). Additionally, memory CD8 T cells may be more tightly regulated than naïve CD8 T cells during disseminated LCMV infection causing them to be more prone to T cell exhaustion as a potential safety mechanism to limit pathology during recall responses(383). As is often that case with complex biologic systems, it is difficult to intuit how this might impact resulting immunopathology during virus infection. Using the developed quantitative models, we make the prediction that the increased sensitivity of memory T cells to antigen is unlikely to affect the enhancement of disease at intermediate T cell responses (mathematical simulation results are relatively insensitive to the parameter that describes sensitivity).

The overall strategy for testing whether intermediate numbers of memory CD8 T cells mediate maximum pathology during persistent viral infection is shown in **Figure 2.1**. Briefly, memory P14 CD8 T cells specific for the GP33/34 epitope of LCMV were generated by LCMV Armstrong immunization. After a period of at least 45 days, P14xThy1.1 memory CD8 T cells were isolated and varying numbers were adoptively transferred into naïve mice two days prior to LCMV clone-13 challenge. As expected, mice receiving high numbers ($>10^5$) of memory P14 CD8 T cells followed by LCMV clone-13 infection did not prevent initial viral seeding. LCMV viral titers in the serum at day 5 post-infection were detectable in all groups tested (**Figure 2.2**).

However, as predicted by the quantitative model, transfer of an intermediate number of memory P14 CD8 T cells prior to LCMV challenge resulted in maximum immunopathology. Greater than 85% (7/8) of mice that received $7x10^4$ P14 memory CD8 T cells, and 60% (7/12) of mice that received 10^5 memory P14 CD8 T cells prior to LCMV clone-13 infection perished by 13 days post infection. In contrast, mice that received high ($>10^5$), low numbers (<20,000) and no p14 memory cells had no increase in mortality following LCMV clone 13 challenge (**Figure 2.3**). Additionally, mice that received a large number of memory p14 cells had markedly reduced disease in contrast to mice that received an intermediate number of memory p14 cells that had decidedly increased disease as measured by weight loss (**Figure 2.4A**) and subjective clinical scores (**Figure 2.4B**).

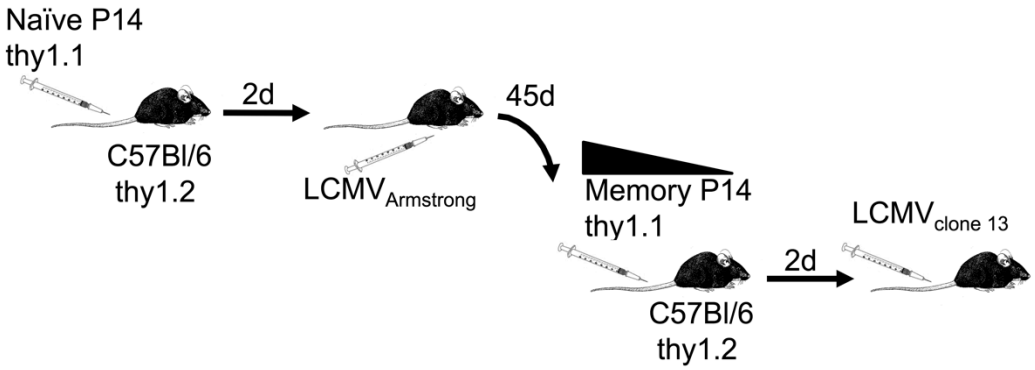

Figure 2.1: Overall strategy to test memory CD8 T cell capacity to mediate immunopathology during persistent viral infection. Memory P14 cells were generated by transfer of naïve p14 x thy1.1 cells into recipient C57Bl/6 x thy1.2 mice followed by immunization with $2x10^5$ pfu LCMV Armstrong. After a period of at least 45 days, P14 memory CD8 T cells were isolated and varying numbers were adoptively transferred into naïve C57Bl/6 mice followed two days post transfer with challenge of $2x10^6$ LCMV clone-13.

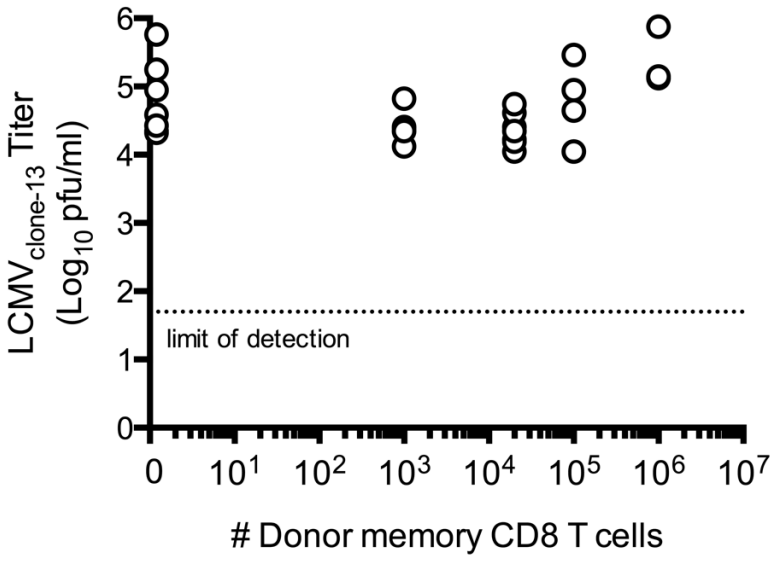

Figure 2.2: Adoptive transfer of LCMV-specific memory CD8 T cells does not prevent initial LCMV replication. Memory P14 cells were generated by transfer of naïve p14xthy1.1 cells into recipient C57Bl/6 thy1.2 mice followed by immunization with 2x105 pfu LCMV Armstrong. After a period of at least 45 days, C57Bl/6 mice received adoptive transfer of varying numbers of memory p14 CD8 T cells followed by challenge with $2x10^6$ LCMV clone-13. LCMV clone 13 viral titer in serum of C57Bl/6 recipient mice at day 5 post- infection (n= 3-5 mice per group).

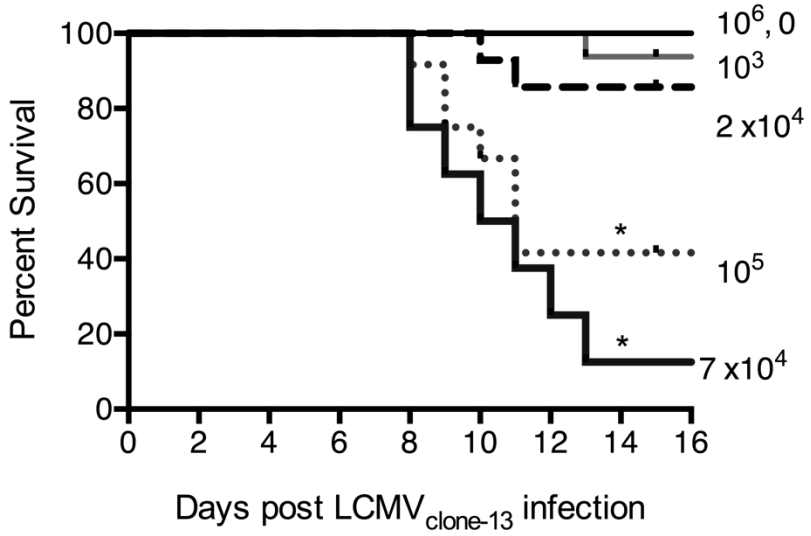

Figure 2.3: Intermediate numbers of LCMV-specific memory CD8 T cells cause maximum pathology after subsequent infection with LCMV clone-13. Kinetics of survival of C57Bl/6 mice after adoptive transfer of varying numbers memory p14 CD8 T cells followed two days post transfer by LCMV clone-13 infection (n=3-5 mice/group, three independent experiments).

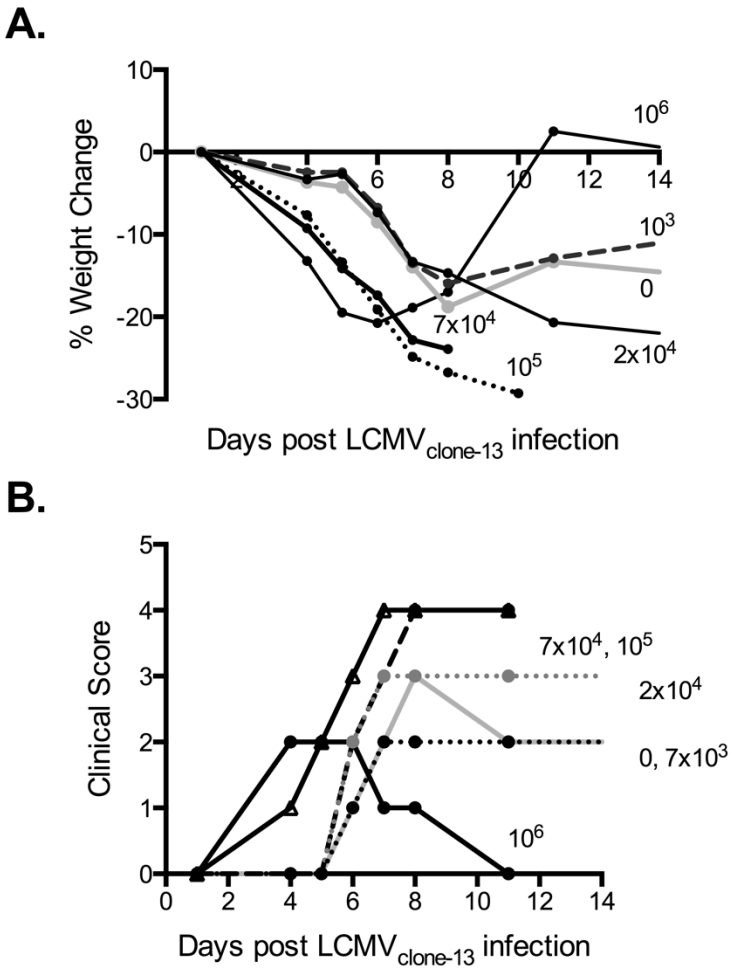

Figure 2.4: Varying precursor frequency of LCMV-specific memory CD8 T cells alters pathology after subsequent infection with LCMV clone-13. C57Bl/6 mice received adoptive transfer of varying numbers of memory p14 CD8 T cells followed by challenge with $2x10^6$ LCMV clone-13. **[A]** Representative example of weight loss of challenged mice that received varying numbers of p14 memory CD8 T cells measured as a percentage of weight change from weight at day 0 post infection. **[B]** Representative example of calculated clinical score of challenged mice that received varying numbers of p14 memory CD8 T cells determined by mouse morbidity, motility, respiration and overall appearance.

The observed reduction of disease in mice that received a high number of memory p14 cells correlated with controlled viremia at day 8 post infection. Although, transfer of intermediate or low numbers of memory p14 CD8 T cells prior to LCMV clone 13 challenge resulted in non-protective effector responses since all mice failed to control viremia at day 8 post infection regardless of an observed increase in pathology (**Figure 2.5**). Additionally, LCMV persisted in the serum for up to 30 days in infected mice that received low dose transfer, indistinguishable from infected controls receiving no additional cells. Recipient mice that had a high precursor frequency of memory p14 cells after adoptive transfer maintained viral clearance up to 30 days post infection, with no detectable viral recrudescence (**Figure 2.6**).

Interestingly, LCMV viral variants that contain a mutated GP33 epitope are generated in 50 percent of mice that receive adoptive transfer of large numbers of naïve p14 cells prior to LCMV clone 13 infection. Generation of viral variants that are not recognized presumably occurs due to selective pressure by the responding donor p14 cells(239). The absence of viral recrudescence after high dose memory p14 transfer suggests that the capacity of memory p14 cells to more rapidly produce effector responses after viral infection limits the potential generation of LCMV escape variants resulting in long term viral clearance.

The increase in immunopathology resulting in mortality due to memory p14 transfer prior to LCMV clone 13 challenge was indistinguishable from survival data after transfer of an intermediate number of naïve P14 cells that has previously been shown to result in increased immunopathology(239, 276) (**Figure 2.7**).

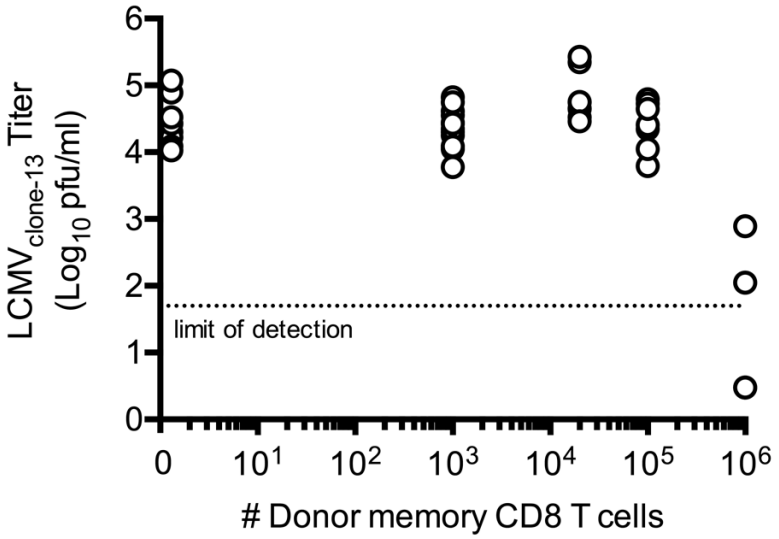

Figure 2.5: High precursor frequency of LCMV-specific memory CD8 T cells protect mice from viral persistence after LCMV clone 13 infection. LCMV clone 13 viral titer in serum of memory p14 recipient mice at day 8 post infection (n=3-5 mice per group, three independent experiments).

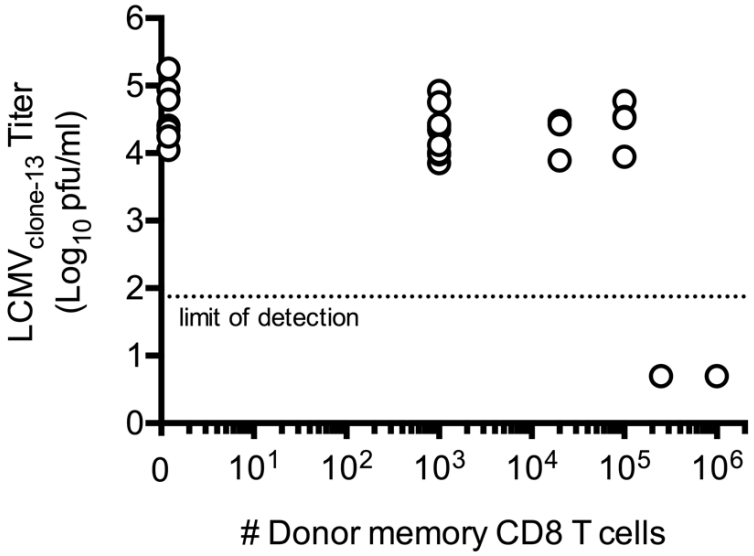

Figure 2.6: High precursor frequency of LCMV-specific memory CD8 T cells does not select for LCMV viral escape variants after LCMV clone 13 infection. LCMV clone 13 viral titer in serum of memory p14 recipient mice at day 30 post infection (n=3-5 mice per group, three independent experiments).

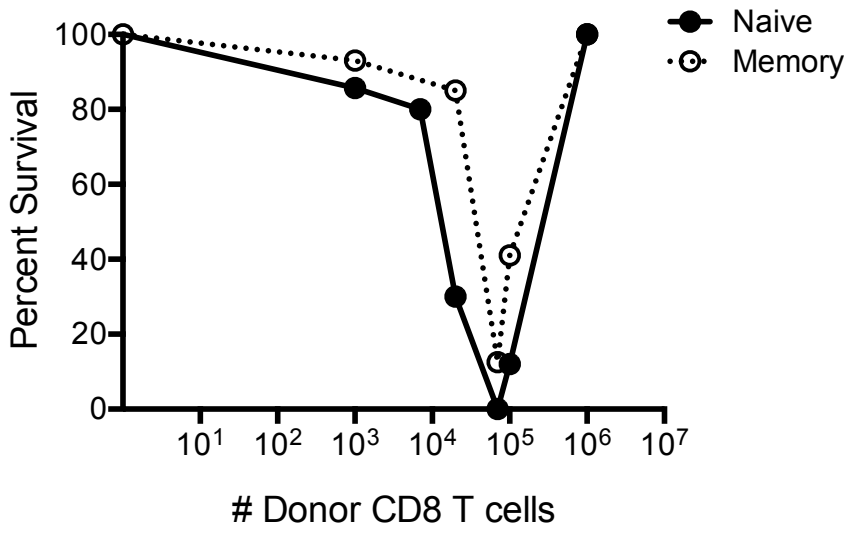

Figure 2.7: The increased sensitivity of memory CD8 T cells to viral antigens does not limit vaccine-induced pathology. Survival of C57Bl/6 mice after adoptive transfer of varying numbers of either memory p14 or naïve p14 CD8 T cells followed two days post transfer by LCMV clone-13 infection (n=3-5 mice/group, three independent experiments).

Intermediate numbers of both memory and naïve p14 CD8 T cells cause increased pathology after persistent LCMV infection. Memory and naïve CD8 T cells differ in their requirements for activation, thus the sensitivity of the CD8 T cell to respond to antigen stimulation does not change the observed pathology. The observed similar increase in disease from varying precursor frequency of naïve or memory LCMV specific CD8 T cells support the previously described mathematical model prediction that TCR sensitivity is independent of the observed pathology. A key consideration highlighted by these experiments is why mice receiving lower doses of memory LCMV specific CD8 T cells do not suffer a similar enhancement of disease.

Lose of CD8 T cell effector function and high PD-1 expression limits immunopathology at low precursor frequency of memory CD8 T cells. Previously it has been shown after naïve CD8 T cell adoptive transfer that exhaustion of the T cell response results in an inability to control infection but also limits T cell mediated immunopathology. Recent reports have shown that memory T cells have a lower threshold for exhaustion(383). Therefore, I investigated whether the exhaustion threshold of donor cells during persistent LCMV clone-13 infection was different in mice that initially received low numbers of donor memory P14 CD8 T cells versus mice that received high numbers of donor memory CD8 T cells. In order to measure exhaustion of donor cells, mice receiving less than $2x10^4$ memory p14 cells or greater than 10^5 memory p14 cells were euthanized 30 days post LCMV clone 13 infection. P14 donor cells were analyzed for persistence and functional capacity to produce inflammatory cytokines after *ex vivo* peptide stimulation.

PD-1 expression of on donor cells was determined since high expression of the inhibitory receptor PD-1 is correlated with the exhaustive phenotype in CD8 T cells responding to LCMV clone 13 infection. Donor memory p14 cells in high dose recipient mice, that controlled LCMV clone 13 viral dissemination by day 8 post infection, persisted long-term. Greater than $8x10^5$ p14 donor cells were detected (**Figure 2.8A**), accounting for more than 3% of total splenocytes in the spleen at day 30 post infection (**Figure 2.8B**, **black bar**). However, less than 10^3 memory p14 donor cells were detected (**Figure 2.8A**), accounting for less than 0.05% of total splenocytes in the spleen of mice that received low dose transfer at day 30 post LCMV clone 13 infection (**Figure 2.8B, white bar**). Thus indicating donor p14 cells underwent clonal deletion in mice initially receiving less than $2x10^4$ memory p14 donor cells prior to LCMV clone 13 infection. High expression of the inhibitory receptor PD-1, greater than 2000 MFI, was observed on detectable donor p14 cells that were not clonally deleted (**Figure 2.9A, white**). In contrast, memory donor cells isolated in mice that received high precursor frequency had very low expression of PD-1, less than 250MFI, with similar PD-1 expression found on functional naïve CD8 T cells (**Figure 2.9A, black**).

Since exhausted T cells lose effector function in a hierarchical manner, the ability of CD8 T cells to produce both TNF and IFNγ in response to antigen stimulation *ex vivo* can be used as a measure of the level of exhaustion of the cell. Isolated donor cells from mice that received low dose transfer were unable to produce TNF in any capacity. Additionally, only 34% of detectable donor cells produced IFNϒ (**Figure 2.9B, white**). In contrast, almost 90% of memory donor p14 cells isolated in mice that received high precursor frequency had full functional capacity to produce both effector cytokines TNF and IFNγ (**Figure 2.9B, black**). Thus, the CD8 T cell response in mice starting with low numbers of memory LCMV-specific CD8 T cells underwent T cell exhaustion.

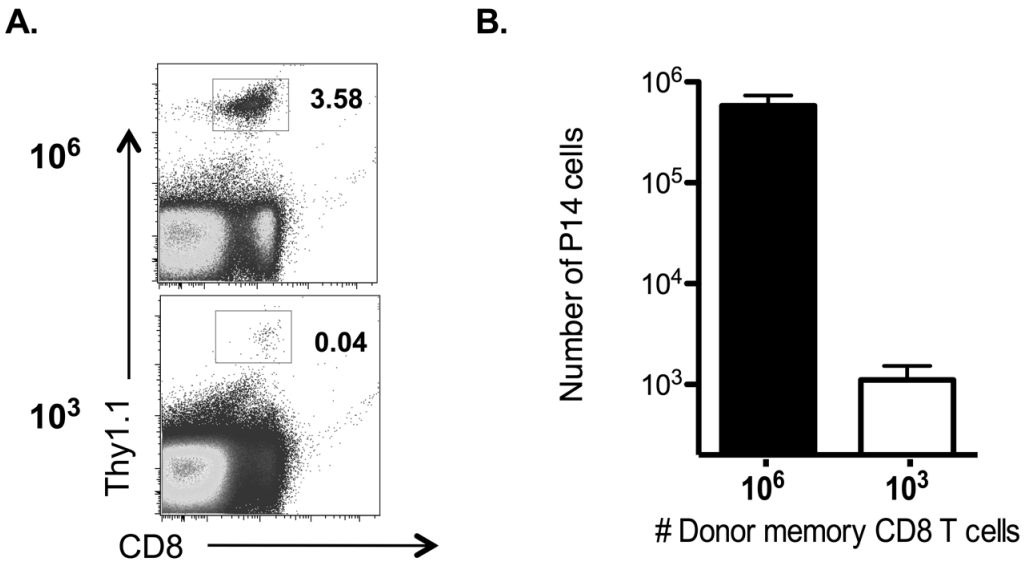

Figure 2.8: CD8 T cell exhaustion limits pathology at low precursor frequency. [A] Representative FACS plot of percentage of donor p14 cells in the spleen at day 30 post-infection in mice that received low dose (10^3) adoptive transfer or high dose (10^6) adoptive transfer of p14 memory cells prior to LCMV clone 13 challenge (n=3-5 mice/group). **[B]** Mean absolute number of memory p14 donor cells recovered from the spleen at day 30 post-infection in mice that received either low dose (10^3) adoptive transfer or high dose (10^6) adoptive transfer of p14 memory cells prior to LCMV clone 13 challenge (n=3-5 mice/group).

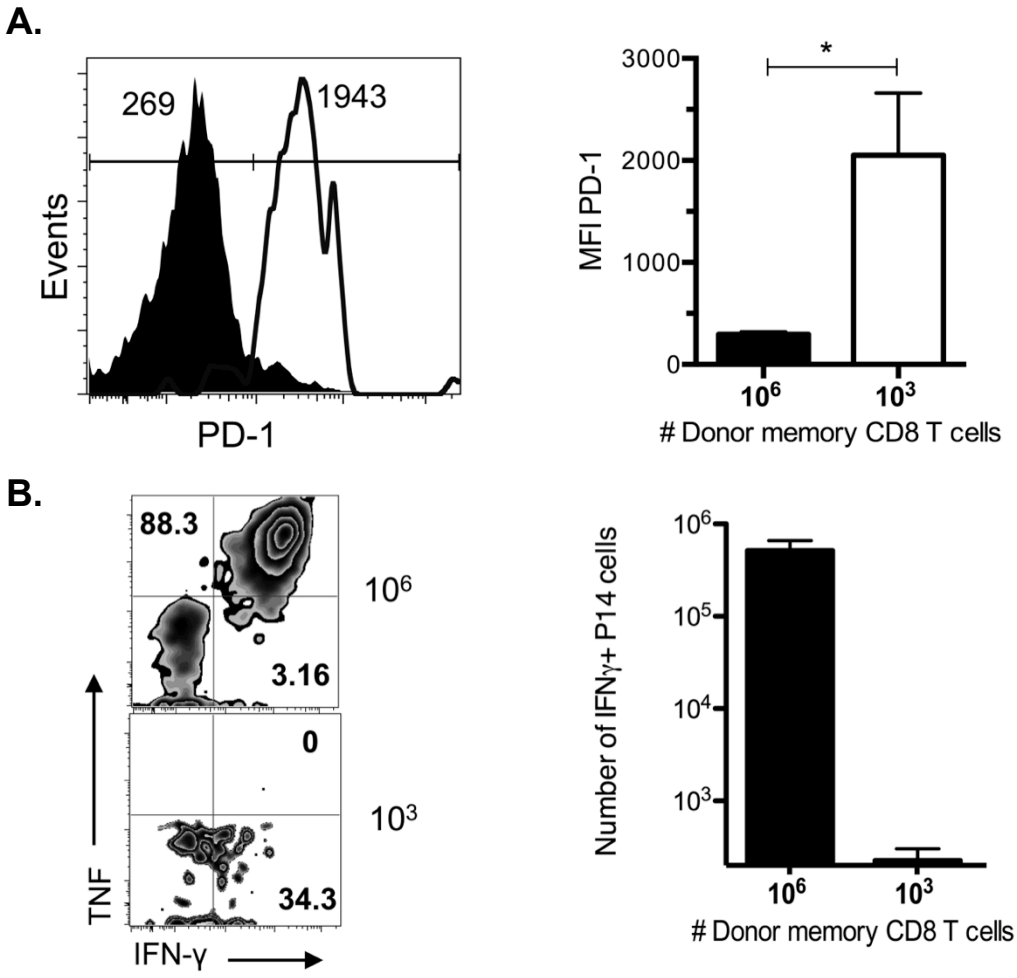

Figure 2.9: High PD-1 expression and loss of cytokine production limits pathology at low precursor frequency. [A] Left panel: Representative FACS histogram of PD-1 expression on donor p14 cells in the spleen at day 30 post-infection in mice that received low dose (10^3) adoptive transfer or high dose (10^6) adoptive transfer of p14 memory cells prior to LCMV clone 13 challenge. Right panel: MFI of PD-1 expression on donor p14 cells (n=3-5 mice/group). **[B]** Left panel: Representative FACS plot of TNF and IFNγ production after peptide stimulation of donor p14 cells in the spleen at day 30 post-infection in mice that received low dose (10^3) adoptive transfer or high dose (10^6) adoptive transfer of p14 memory cells prior to LCMV clone 13 challenge. Right panel: Mean absolute number of IFNγ producing memory p14 donor cells recovered from the spleen at day 30 post-infection (n=3-5 mice/group).

The extent of T cell exhaustion limited the ability of challenged mice to control viral replication, since all mice that received low precursor frequency of donor CD8 T cells could not eliminate virus, as seen by high serum viral titers at day 30 post infection, but the loss of T cell function also limited T cell mediated immunopathology preventing mortality seen in mice that received an intermediate precursor frequency.

Qualitative differences between naïve and memory CD8 T cell mediated pathology. Although mice receiving either memory LCMV-specific CD8 T cells or naïve LCMV-specific T cells both underwent a similar enhancement of pathology at intermediate precursor frequency, there was an observed qualitative difference in the pathology in mice that received memory CD8 T cells versus naïve CD8 T cells. Tissue samples harvested at time of euthanasia were cut to 4 μ m thick formalin fixed sections and stained with hematoxylin and eosin (H&E). Histological analysis of tissue in mice receiving a pathogenic dose of either naïve of memory cells were compared to one another and to healthy mouse tissue. Mice that received a pathogenic dose of donor memory p14 cells displayed necrotic lesions and cell infiltration primarily localized in the liver. Whereas lung sections of mice receiving donor memory p14 cells showed limited vascular leakage and cell infiltration. In contrast, mice that received a pathogenic dose of naïve P14 recipients had extreme lung pathology including breakdown of the vascular architecture, fluid build-up and cell infiltration. However, these mice displayed no detectable necrotic lesions in the liver (**Figure 2.10A**).

In order to investigate if the qualitative difference in tissue damage was due to variation in the recruitment of donor cells to infected tissues, the number of donor cells in each tissue at day 6 post infection (prior to day of required euthanasia) was determined. Since naïve and memory CD8 T cells differ in their proliferative capacity and sensitivity for antigen stimulation, the number of memory or naïve donor cells detected in the liver or lung was compared to the total response of donor p14 cells in the spleen. There was a significant difference in the ratio of donor cells trafficking to the lung and liver between naïve and memory cells.

As expected due to histological analysis, mice receiving a pathogenic dose of naive p14 cells had a greater proportion of the donor response traffic to the lung presumably causing destruction of the vascular architecture. In contrast, mice receiving a pathogenic dose of memory P14 cells had a greater proportion of the donor response traffic to the liver, presumably resulting in the observed necrotic lesions via CTL activity (**Figure 2.10B**). Consistent between naïve and memory donor p14 cells, the greater proportion of the response trafficking to either the lung or the liver correlated with the organ exhibiting maximum pathology. However, it is not known from these experiments whether production of inflammatory cytokines or direct killing of target cells in infected tissues is responsible for mediated the observed lethal pathology.

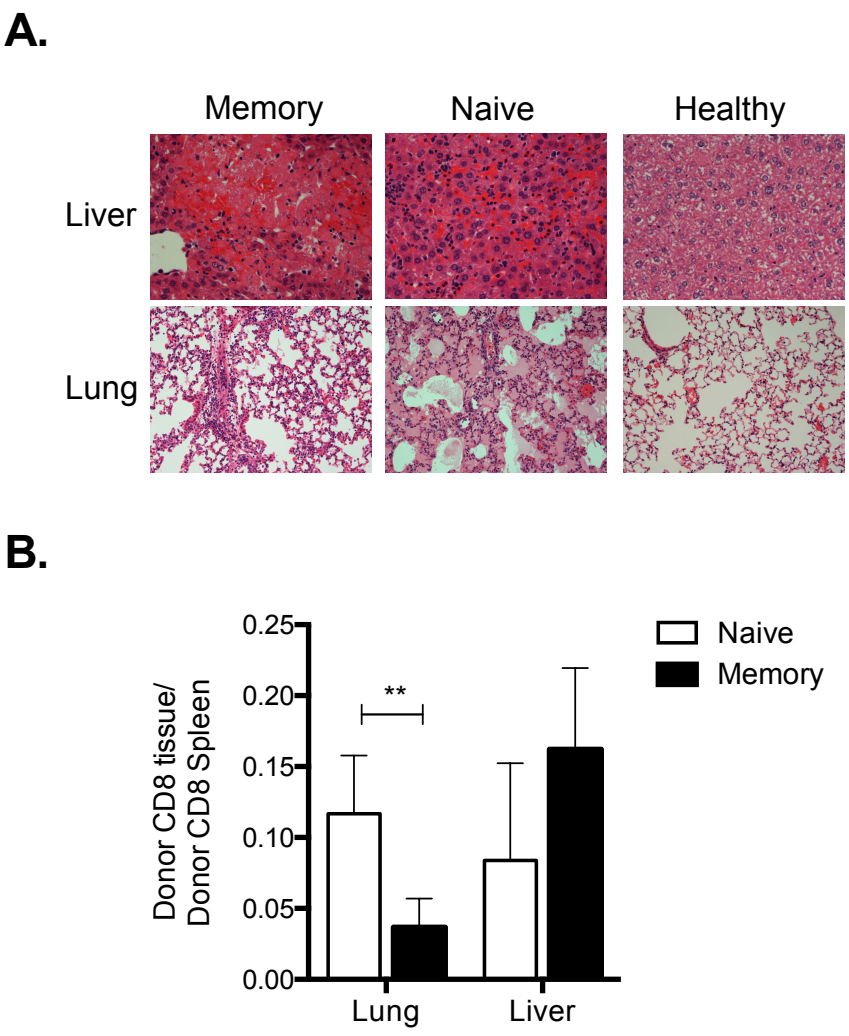

Figure 2.10: Qualitative differences between pathology from memory CD8 T cells and naïve CD8 T cells after subsequent infection with LCMV clone-13. C57Bl/6 mice received adoptive transfer of varying numbers of memory or naive p14 CD8 T cells followed by challenge with $2x10^6$ LCMV clone-13. **[A]** 40X view of hematoxylin and eosin stained 4 μ m thick formalin fixed liver and lung tissue sections collected at time of death for pathogenic doses after transfer of naïve or memory p14 CD8 T cells as compared to healthy mouse tissue [**B**] Ratio of donor p14 memory or p14 naive CD8 T cells harvested from the lung or liver as compared to total donor expansion in the spleen at day 5 post infection (n=3-5 mice per group, two independent experiments).

Pathology is mediated by TNF production. In order to inform the safe design of vaccines, it is necessary to determine which T cell parameters of the CD8 T cell response are responsible for vaccine induced pathology versus those that mediate virus control. To investigate whether pathology in this setting arises from the production of pro-inflammatory cytokines, we transferred LCMV specific memory CD8 T cells into wild type, TNF-R deficient mice (p55-/- x p75-/-) or IFNγ-R deficient mice prior to LCMV clone-13 challenge. Consistent with previous experiments, 75 percent of wild-type recipient mice that received the maximal pathogenic dose ($7x10^4$) of memory p14 cells experienced an increase in disease requiring euthanasia by day 12 post infection. In contrast, recipient mice deficient in both TNF receptors demonstrated no detectable increase in disease after LCMV clone 13 challenge at any dose of memory CD8 T cells tested (**Figure 2.11, open square**).

Surprisingly, we observed identical control of LCMV clone replication by 8 days post infection in both wild-type and TNF-R deficient recipient mice that received high dose transfer of memory LCMV specific CD8 T cells (**Figure 2.12**). This suggests that not only does TNF mediate pathology in this setting; it is also dispensable for virus control due to the CD8 effector T cell response. In stark contrast, mice deficient in the IFNγ-receptor experienced a further enhancement of pathology after LCMV clone 13 infection due to increased precursor frequency of LCMV specific CD8 T cells. The adoptive transfer of greater than 10^3 memory p14 cells resulted in 100% mortality after LCMV clone-13 challenge (**Figure 2.11, open circle**). Furthermore, recipient mice were unable to control infection prior to the day required for euthanasia (**Figure 2.12, open circle**).

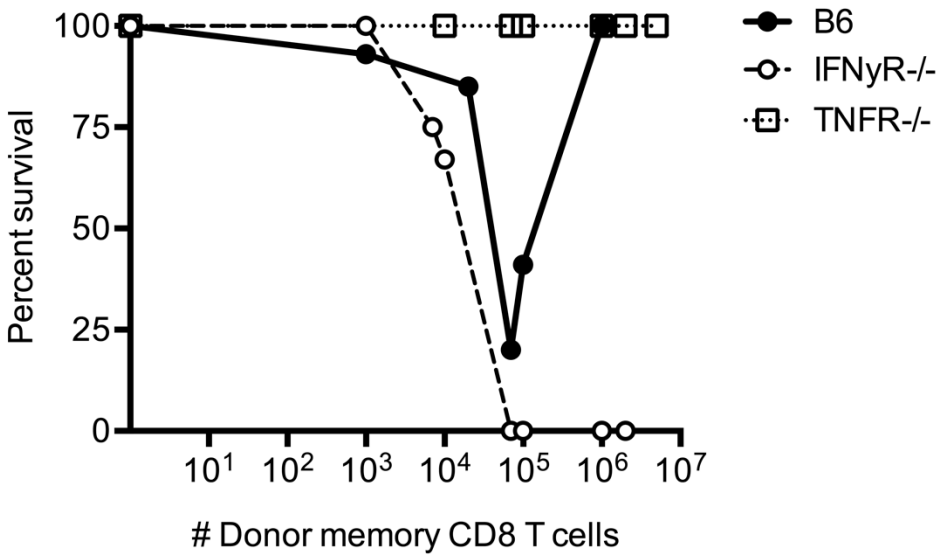

Figure 2.11: Pathology during persistent LCMV infection is dependent on memory T cell production of TNF. C57Bl/6 mice, TNFR-/- mice or IFNγR-/- mice received adoptive transfer of varying doses of p14 memory CD8 T cells followed by $2x10^6$ pfu LCMV clone-13 infection. Percent survival of recipient mice (C57Bl/6 solid circle, IFNγR-/- open circle, TNFR-/- open square) versus number of donor cells transferred after LCMV clone-13 infection (n=3-10 mice/group).

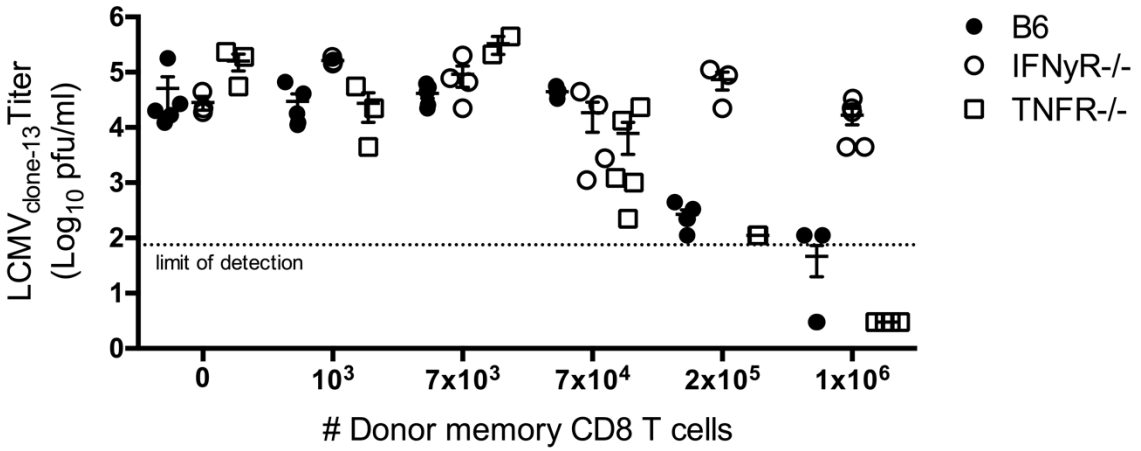

Figure 2.12: High precursor frequency of memory cells protect mice from LCMV persistence in both wild-type and TNF-R deficient mice. C57Bl/6 mice, TNFR-/- mice or IFNγR-/- mice received adoptive transfer of varying doses of p14 memory CD8 T cells followed by $2x10^6$ pfu LCMV clone-13 infection. LCMV viral titer in serum of recipient mice at day 6 (IFNγR/-) or day 8 post infection (TNFR-/- & B6), n=3-5 mice per group, two independent experiments.

Thus, IFNγ production by LCMV specific CD8 T cells is important for CD8 T cell mediated protection from viral dissemination, but is not the major source of pathology in these mice. Taken together, these result show that blockade of different cytokines such as TNF may be possible to prevent pathology while retaining protective effects of vaccine-induced memory T cells.

However, the elimination of pathology could be a result of differences in the *in vivo* expansion of wild-type donor p14 cells in LCMV infected TNF-receptor deficient hosts. In order to confirm the pathogenic dose of wild-type p14 donor cells expanded in a similar fashion after LCMV infection, the magnitude and functional capacity of donor memory p14 cells were determined after LCMV clone 13 infection. There was no statistical difference between the magnitude of the donor memory p14 response between recipient wild-type and TNF-receptor deficient mice in the spleen at day 7 post infection (**Figure 2.13A**). Greater than 10^6 memory p14 donor cells were detected in both wild-type and TNF-R deficient mice. In addition, the functional capacity of the donor cells were similar, over 40 percent of donor cells were able to produce TNF and IFNγ after peptide stimulation (**Figure 2.13B**).

Therefore, the elimination of pathology observed in TNF-R deficient mice is not due to a alteration in donor cell expansion, indicating immunopathology in this model is likely due to CD8 T cell production of TNF. Although, it has previously been hypothesized that vaccine induced CD8 T cell mediated pathology may be due in part to the limited breadth of the T cell response, induction of T cells specific for only 1-2 viral epitopes, following vaccination. Therefore, the impact of the breadth of the LCMV-specific memory CD8 T cell population has on the resulting immunopathology observed during secondary infection needs to be determined.

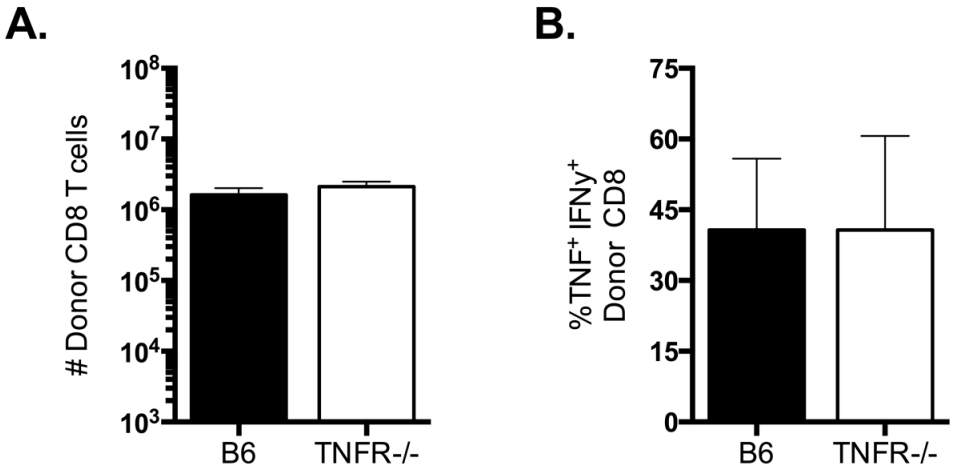

Figure 2.13: Memory CD8 T cell expansion does not change in TNF-R deficient hosts. C57Bl/6 mice or TNF-R-/- mice received adoptive transfer of 70,000 p14 memory CD8 T cells followed by $2x10^6$ pfu LCMV clone-13 infection. **[A]** Number of donor memory cells isolated from wildtype or TNFR-/- recipient mice 7 days post LCMV clone-13 infection. **[B]** Functional capacity of donor memory cells isolated from wildtype or TNFR-/- recipient mice 7 days post LCMV clone-13 infection (n=3-5 mice/group).

Increasing the breadth of the CD8 T cell response does not limit vaccine-induced pathology. In the landmark studies by Oehen et al, vaccination with recombinant vaccinia virus vectors expressing LCMV protein antigens resulted in increased pathology during subsequent infection with LCMV compared to non-vaccinated animals. One main hypothesis from these studies was that T cell mediated immunopathology was a result of the limited breadth of the CD8 T cell response after vaccination using recombinant vectors(303). However, the developed quantitative model predicts that increasing the breadth of the CD8 T cell response should have no impact on resulting immunopathology. The model predicts that, similar to disease caused by donor CD8 T cells from a transgenic mouse specific for one epitope of LCMV, maximum enhancement of disease will occur at intermediate precursor frequency of memory CD8 T cells specific for a multitude of LCMV epitopes.

In order to test the impact of the breadth of the CD8 T cell response on resulting immunopathology during infection, varying numbers of ***polyclonal*** LCMV-specific memory CD8 T cells were adoptively transferred into recipient mice prior to LCMV clone 13 infection. Polyclonal memory LCMV-specific CD8 T cells were generated by immunization of naïve C57Bl/6xThy1.1 congenic mice with LCMV Armstrong (**Figure 2.14A**). The number of polyclonal LCMV-specific memory cells present in the sample of pooled splenocytes harvested from multiple LCMV Armstrong immunized mice was enumerated by intracellular cytokine staining after ex-vivo stimulation with immunodominant LCMV peptides (**Figure 2.14B).** Six LCMV peptides derived from the LCMV glycoprotein or nucleoprotein were used to estimate the number of polyclonal memory CD8 T cells since they have been found to make up over 88 percent of the well characterized CD8 T cell response following LCMV Armstrong infection.

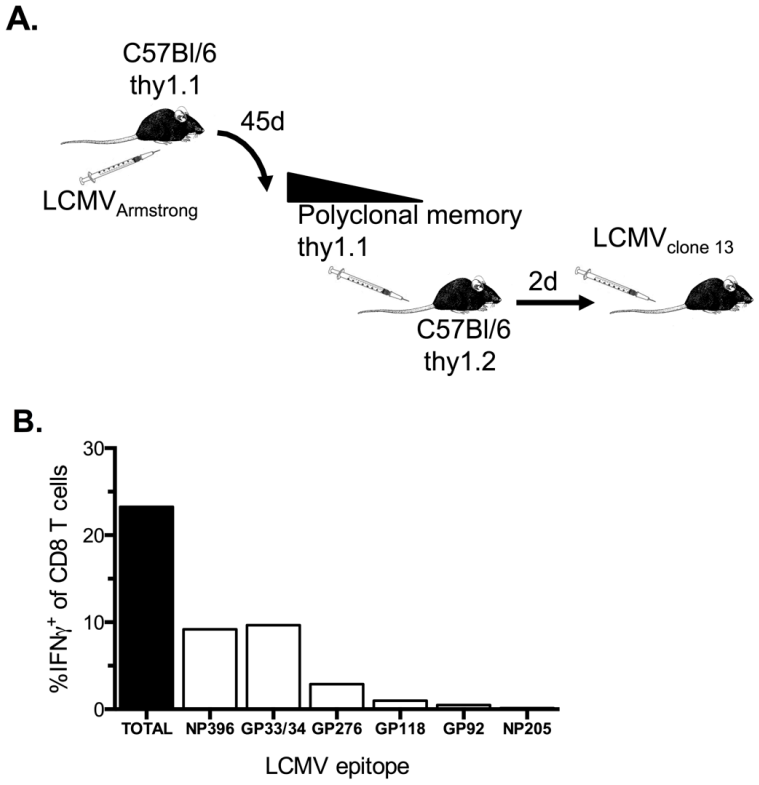

Figure 2.14: General strategy for LCMV polyclonal memory CD8 T cell generation and adoptive transfer. [A] Polyclonal memory cells were generated by immunization of C57Bl/6 Thy1.1 mice with $2x10^5$ pfu LCMV Armstrong. After a period of at least 45 days, C57Bl/6 mice received adoptive transfer of varying numbers of polyclonal memory CD8 T cells followed by challenge with $2x10^6$ LCMV clone-13**. [B]** Input breadth of donor polyclonal memory CD8 T cells as measured by IFNγ production after peptide stimulation.

Consistent with model predictions, mice that received intermediate numbers of polyclonal LCMV-specific CD8 T cells experienced high mortality. Greater than 80% of mice receiving intermediate numbers ($7x10^4$, $2x10^4$) of polyclonal LCMV-specific memory CD8 T cells required euthanasia by day 13 post LCMV clone-13 challenge (**Figure 2.15**). Whereas, no increase in mortality was seen in mice receiving low (<20,000) or high ($>10^5$) numbers of polyclonal memory cells prior to LCMV clone 13 challenge. Similar to results after memory p14 transfer, low precursor frequency of polyclonal LCMV-specific CD8 T cells prior to persistent LCMV infection resulted in a non-protective response and uncontrolled viral replication. LCMV titer, up to 10^5 pfu/mL, was measured in the serum at day 8 post-infection in mice receiving less than $2x10^5$ donor polyclonal memory CD8 T cells. Whereas, transfer of a high number of polyclonal memory CD8 T cells ($>10^5$) prior to LCMV clone 13 resulted rapid viral control by day 8 post infection and minimal disease (**Figure 2.16**). Additionally, LCMV persisted in the serum for up to 30 days in infected mice that received low dose transfer, indistinguishable from infected controls receiving no additional cells. Recipient mice that had a high precursor frequency of polyclonal memory CD8 T cells after adoptive transfer maintained viral clearance up to 30 days post infection, with no detectable viral recrudescence (**Figure 2.17**).

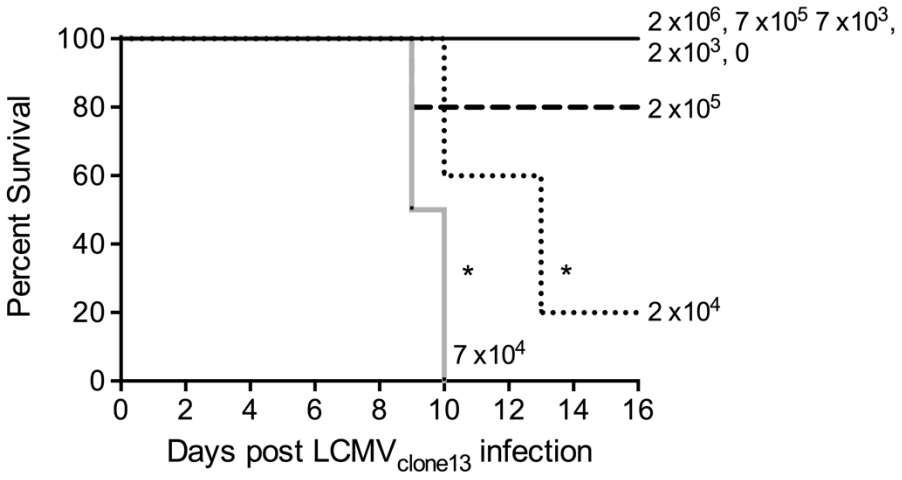

Figure 2.15: Intermediate numbers of antigen specific polyclonal memory CD8 T cells cause maximum pathology after subsequent LCMV clone-13 infection. Percent survival of C57Bl/6 recipient mice of varying numbers of polyclonal memory CD8 T cells followed by LCMV clone-13 infection (n=3-5 mice/group).

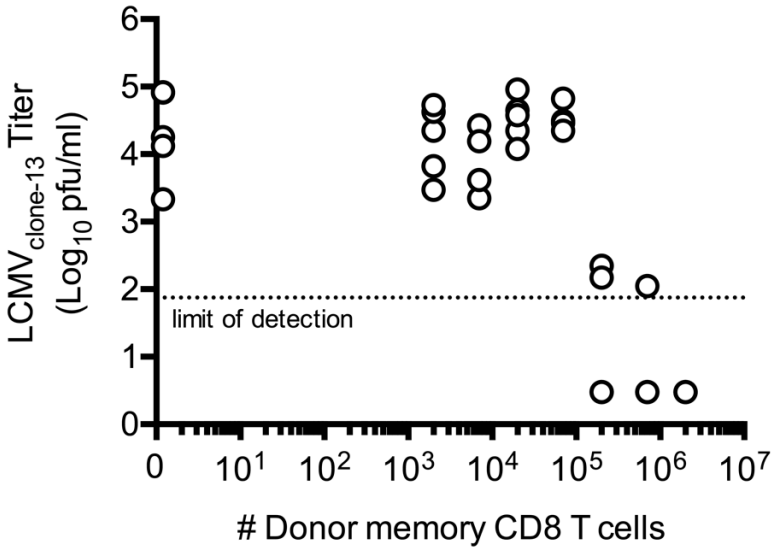

Figure 2.16: High precursor frequency of polyclonal LCMV-specific memory CD8 T cells protect mice from viral persistence after LCMV clone 13 infection. C57Bl/6 mice received adoptive transfer of varying numbers of polyclonal memory CD8 T cells followed by challenge with $2x10^6$ LCMV clone-13. LCMV viral titer in serum of polyclonal memory recipient mice at day 8 post infection (n=3-5 mice/group).

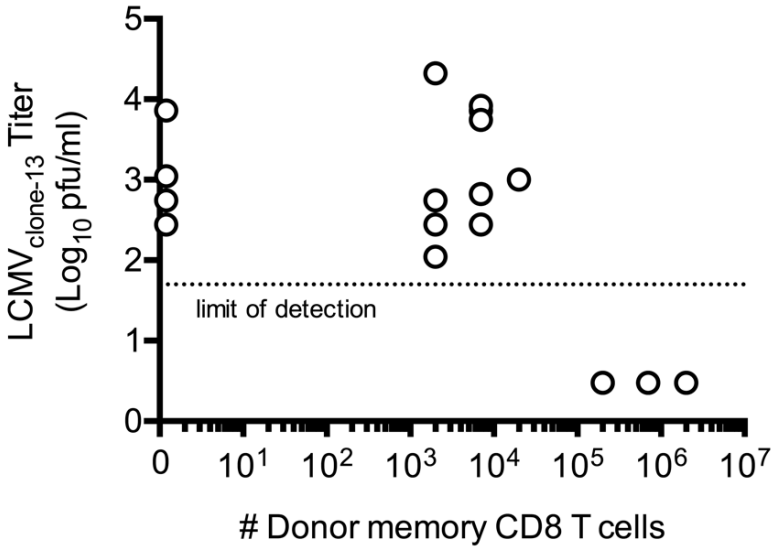

Figure 2.17: High precursor frequency of polyclonal LCMV-specific memory CD8 T cells does not select for LCMV viral escape variants after LCMV clone 13 infection. C57Bl/6 mice received adoptive transfer of varying numbers of polyclonal memory CD8 T cells followed by challenge with $2x10^6$ LCMV clone-13. LCMV viral titer in serum of polyclonal memory recipient mice at day 30 post infection (n=3-5 mice/group).

The increase in immunopathology resulting in mortality due to transfer of polyclonal LCMV-specific memory cells prior to LCMV clone 13 challenge was indistinguishable from survival data generated after transfer of an intermediate number of monoclonal-transgenic memory P14 cells (**Figure 2.18**). Therefore, intermediate numbers of both polyclonal and monoclonal memory CD8 T cell populations can result in unattended increase in disease during persistent LCMV infection. The observed increase in disease from varying precursor frequency of polyclonal or monoclonal memory LCMV specific CD8 T cells support the previously described mathematical model prediction that the breadth of the T cell response induced by vaccination is independent of the observed pathology. Thus, the breadth of the T cell response has no impact on the dose of cells that cause maximum pathology. The breadth of the memory CD8 T cell response does not impact generation of viral escape variants since neither transfer of high dose monoclonal or polyclonal LCMV-specific CD8 T cells resulted in viral recrudescence. However, broader vaccine responses are likely to be useful in preventing virus escape observed for other infections with more mutable viruses. An additional suggestion from the developed model, which was not immediately intuitive, was that inoculation with decreased viral inoculum should result in maximum pathology at lower numbers of T cells and at slightly later times post infection.

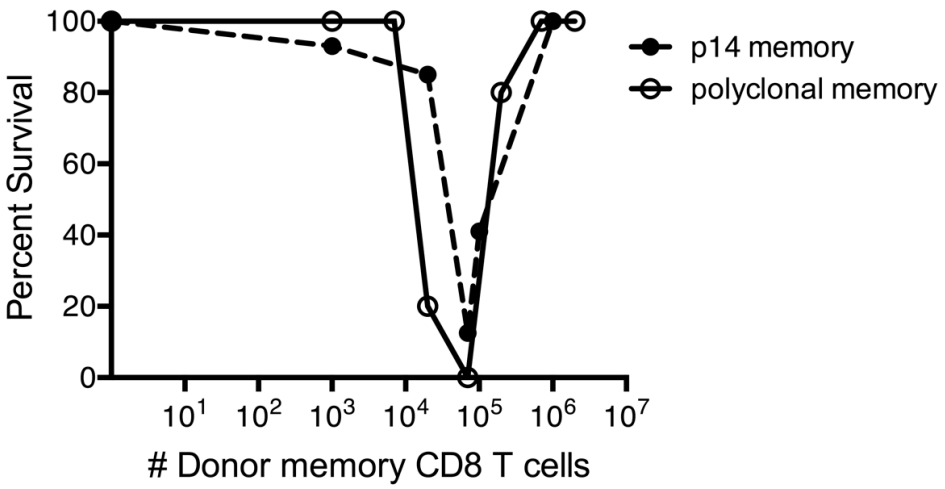

Figure 2.18: Increasing the breadth of the CD8 T cell response does not limit vaccine-induced pathology. Percent survival of C57Bl/6 mice that received varying numbers of either p14 memory or LCMV-specific polyclonal memory CD8 T cells followed by LCMV clone-13 infection (n=5-10 mice/group).

The precursor frequency of memory CD8 T cells that enhances pathology is dependent on the magnitude of initial viral load. The model predicts that inoculation with decreased viral inoculum should result in maximum pathology at lower numbers of T cells. Presumably, this is due to CD8 T cell expansion outpacing virus that quickly reaches carrying capacity in a given host. In order to test how CD8 T cell pathology depends on the initial viral load, varying numbers of LCMV-specific P14 CD8 T cells were transferred into recipient mice followed by infection with increasing or decreasing doses of persistent LCMV clone 13. As shown in **Figure 2.19**, the dose of T cells causing the maximum amount of pathology scaled with the virus inoculum.

Infection with $2x10^5$ pfu LCMV clone 13 (10-fold lower than the standard virus challenge dose) did not induce pathology in mice that received $7x10^4$ donor cells, but instead caused maximum pathology in mice that received 10-fold lower ($7x10^3$) donor cells. Survival of recipient mice decreased from 80% to 50% in mice receiving low ($7x10^3$) precursor frequency of LCMV-specific donor cells after challenge with $2x10^5$ pfu LCMV clone 13. In addition, survival increased from 0% to 100% in mice receiving intermediate ($7x10^4$) precursor frequency of LCMV-specific donor cells after challenge with $2x10^5$ pfu LCMV clone 13. On the other hand, mice challenged with a 5-fold higher dose (10^7) of LCMV clone 13 displayed increased mortality at higher numbers of (10^6) donor cells, (100% to 80% survival) and decreased pathology (0% to 40% survival) at intermediate doses ($7x10^4$) (**Figure 2.19**).

In all cases, maximum pathology occurred at a dose of donor CD8 T cells just below the amount that was able to clear infection at 8 days post-infection (**Figure 2.20**).

This suggests that decreasing the amount of initial virus inoculum resulted in a higher threshold for exhaustion of donor CD8 T cells leading to greater survival at intermediate cell number correlating with increased viral control. Whereas, increasing the amount of the initial inoculum resulted in a lower threshold for exhaustion of donor CD8 T cells leading to greater survival at intermediate cell number due to loss of TNF production. However, leading to decreased survival at high donor CD8 T cell number due to partial exhaustion, lack of early viral control, and T cell production of TNF.

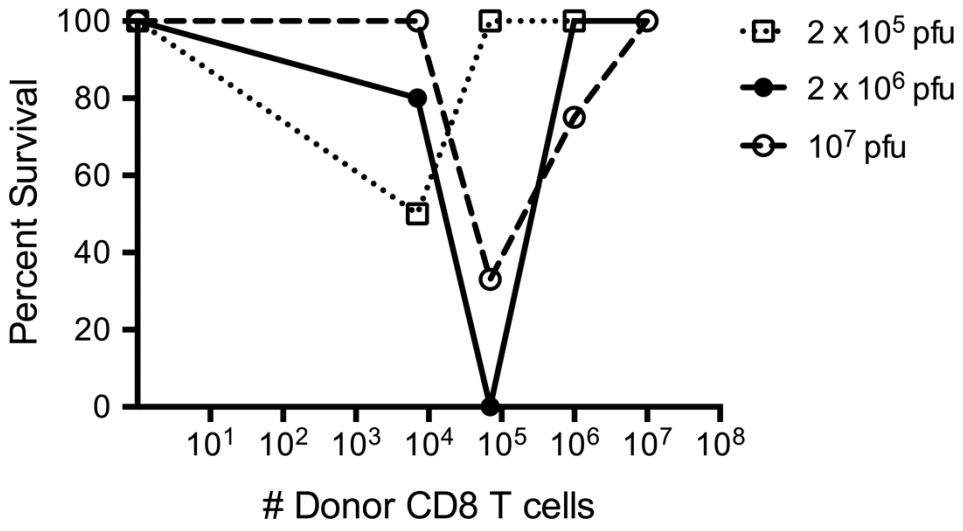

Figure 2.19: Pathology during persistent LCMV infection is dependent on initial viral load. Percent survival of C57Bl/6 mice that received varying doses (10^3-10^7) of naïve p14 CD8 T cells followed by infection with three different doses ($2x10^5$-10^7 pfu) of LCMV clone-13 (n=3-5 mice per group, two independent experiments).

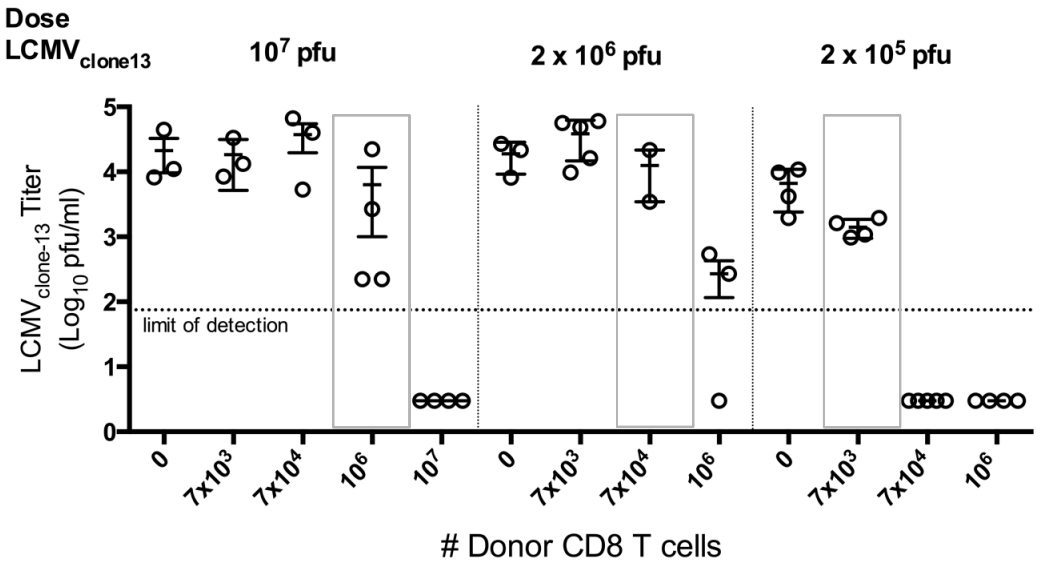

Figure 2.20: Maximum pathology occurs at precursor frequency of antigen-specific CD8 T cells just below the amount that is able to control infection. C57Bl/6 mice received varying doses (10^3-10^7) of naïve p14 CD8 T cells followed by infection with three different doses ($2x10^5$-10^7 pfu) of LCMV clone-13. LCMV clone-13 titer in the serum of recipient mice on day 8 post infection. Grey bars indicate dose that resulted in maximum pathology (n=3-5 mice per group, two independent experiments).

DISCUSSION

A previously developed mathematical model of LCMV infection in mice has generated predictions about which virus and host immune parameters can differentially affect pathology versus protection during persistent viral infection. We have tested these predictions experimentally and our findings indicate that CD8 T cell pathology due to an intermediate precursor of antigen-specific CD8 T cells is largely independent of T cell sensitivity or the breadth of TCR diversity. However, pathology was observed to be dependent on TNF production and initial virus load. These results indicate that variation in precursor frequency of memory CD8 T cells as well as initial viral load drastically alters disease outcome due to changes in the level of T cell exhaustion.

The initial amount of stimulation by varying the magnitude of viral infection alters the frequency at which the responding T cells can result in pathogenic responses. These results are consistent with published data indicating that T cell exhaustion may be a mechanism to limit immunopathology of naïve CD8 T cells(275, 384) and an increase in pathology can occur due to partial exhaustion of the T cell response as mediated by initial viral dose. Our experimental results expand on these findings and support the predictions of the developed mathematical model. Demonstrated by the findings that memory cells (specific to one epitope or multiple epitopes) are also prone to increased pathology dependent on the total number of memory CD8 T cells and the extent of exhaustion these cells experience, due to initial viral load and the magnitude of the response determines pathological or protective outcomes.

Mice that had high numbers of memory CD8 T cells (polyclonal or monoclonal) prior to high dose viral infection did not develop immunopathology because the magnitude of the response was great enough to control viral replication by 8 days post infection. Whereas mice that had an intermediate number of memory CD8 T cells prior to high dose viral infection developed severe pathology due to partial T cell exhaustion of the response resulting in ongoing high viral titers and pathology presumed due to continued production of TNF. The ability of memory CD8 T cells to respond to viral infection with greater sensitivity than naive did not alter the observation that intermediate precursor frequency of antigen specific T cells prior to persistent viral infection can increase disease.

Pathology does not occur at low precursor frequency of memory LCMV specific T cells prior to LCMV clone 13 infection. Analysis of T cell persistence and functional capacity support the hypothesis that T cell exhaustion is a mechanism during persistent viral infection to avoid pathology, since responding T cells lose the ability to proliferate and produce inflammatory cytokines such as TNF. Mice that had low numbers of memory CD8 T cells or no memory CD8 T cells prior to high dose infection did not develop immunopathology because the responding CD8 T cells were highly exhausted and unable to cause pathology. However, when the initial viral dose decreases by one log, to an intermediate viral infection, lower numbers of memory CD8 T cells become only partially exhausted leading to an increase in pathology at a low dose of cells, similar to mice receiving intermediate numbers of CD8 T cells after high dose infection. Indicating there is a fine balance between the magnitude and functionality of the memory CD8 T cell response and initial viral dose, that is not due dependent on TCR diversity of the CD8 T cell response.

Pathology was found to be mediated by CD8 T cell production of TNF since pathology, due to intermediate precursor frequency of LCMV specific memory CD8 T cells, was eliminated in TNF-R mice infected with LCMV clone 13. Although, histological analysis indicated memory CD8 T cells mediated a qualitatively different pathology than that seen mediated by effector responses originating from LCMV-specific naïve CD8 T cells. Pathology due to naïve-originating CD8 T cell responses was found to be primarily localized in the lungs, whereas pathology due to memory-originating CD8 T cell responses was found to be in the liver. The difference in the tissue pathology could be attributed to the kinetics of the T cell response(238). Memory CD8 T cells respond rapidly upon subsequent exposure and may traffic to the liver, an early site of viral replication, whereas naïve cells may traffic to the lungs a secondary site of viral replication(273). Although the reason for the increase in pathology is unchanged, pathology is due to partial exhaustion of the responding T cells.

Furthermore, variation of antigen-specific memory CD8 T cell precursor frequency in TNF-R deficient and IFNγ-R deficient mice indicated that although T cell production of TNF mediates pathology, it is not required for viral control. TNF-R deficient mice that received high dose LCMV-specific memory CD8 T cells were able to control LCMV viral load by day 8 post infection, similar to wild-type mice receiving high dose p14 memory transfer. Whereas, a high precursor frequency of LCMV-specific CD8 T cells in IFNγ-R deficient mice prior to persistent LCMV infection did control viral replication and mice experienced an increase in mortality. Thus, highlighting the importance of the anti-viral effects of IFNγ produced by responding CD8 T cells to control initial viral replication and promote immune response.

Although, precursor frequency of memory or naive antigen specific cells alters disease outcome to the same extent at an intermediate dose resulting in mouse mortality, the same cannot be said after high dose transfer. Mice that receive high numbers of naïve T cells show viral escape 50 percent of the time; with viral recrudescence and high viral loads in the serum 30 days post infection(239). However, we have not observed virus escape after transfer of high dose p14 memory cells (n=15 mice) followed by LCMV clone-13 infection. In addition to a lack of viral escape after transfer of high dose monoclonal memory cells, no escape was seen after polyclonal transfer (n=10 mice). Indicating that immune responses generated from memory CD8 T cells are superior in virus control due to the absence of variant escape viruses. Presumably due to the kinetics of the response, T cells controlling viral loads before selective pressure results in an escape mutant.

Pathology can be due to low precursor frequency or intermediate precursor frequency memory CD8 T cells dependent on the initial viral dose. Therefore, a wide range of precursor frequencies could potentially cause pathology depending on viral dose. Therefore, the strength of the vaccine response for an individual may or may not be pathogenic dependent on the individual's subsequent viral exposure. In order to avoid potential vaccine induced pathology the CD8 T cell response must be above a critical threshold to limit pathology due to early viral control. In addition, vaccines that only elicit one arm of the immune system should be avoided over vaccine candidates that also stimulate antibody production and CD4 T cell help, since the combination of responding immune cells may influence early viral control decreasing the chance of pathology.

Recently it has been published that vaccine induced CD4 memory T cells specific for LCMV can also cause immunopathology and mortality after challenge with LCMV clone-13(385). The investigators found that the pathology was mediated by antigen driven hyper stimulation and activation of LCMV specific memory CD4 T cells that do not undergo traditional T cell exhaustion, seen in our model with LCMV-specific memory CD8 T cells. Pathology was ablated by the addition of high does of P14 cells that resulted in virus control by day 7 post infection and pathology remained after blockade of CD8 T cells during immunization. CD4 mediated pathology was presumably caused by cytokine production, but not by TNF indicated in our results for causing the immunopathology from responding memory CD8 T cells. Our results in conjunction with these results indicate that both memory CD8 and CD4 T cells after immunization can cause immunopathology when the vaccine induced immune response is not sufficient to immediately control virus early after infection. Thereby, allowing the responding cells that do not completely lose functionality due to an exhausted phenotype, continue to exert effector function due to persistent viral load. These studies highlight the importance for future vaccine design to induce both CD4 and CD8 T cell memory responses.

Vaccine induced pathology has been reported as early as the 1960s during an experimental vaccine trial for respiratory syncytial virus (RSV). The candidate vaccine, formalin inactivated RSV given intramuscularly to infants and children, resulted in a failure to offer protection during a subsequent outbreak of RSV. In addition to the vaccine demonstrating limited efficacy, an exaggerated clinical response was seen in 80 percent of young vaccine recipients including two deaths(320, 324).

Thus, a major goal of vaccination should be induction of immune responses that maximize elimination of infectious organisms while minimizing immunopathology. Historical examples of vaccine induced pathology highlight the need to understand the relationship between virus infection, immune responses and immune exhaustion that have been difficult to determine due to the complex non-linear interactions that occur between these variables.

We have developed a model of virus infection and immune responses that can help to decipher these interactions and to make predictions about which features of the virus or immune response contribute to immunopathology. The mathematical framework for analyzing the complex non-linear interactions between immune cells and virus infection can then be useful for maximizing vaccine protection to chronic infections while minimizing enhancement of disease due to elevated immune response, in order to design vaccines that maximize protection while minimizing enhancement of disease.

CHAPTER 3

INTERACTION BETWEEN UNRELATED VIRUSES DURING IN-VIVO CO-INFECTION TO LIMIT PATHOLOGY AND IMMUNITY

ABSTRACT

Great progress has been made in understanding the requirements for immunity to viral infection. However, outside of laboratory experiments, most "real-world" infections occur in the context of co-infection by heterologous pathogens that have the potential to modulate immune responses and/or disease. In order to begin to understand the complex relationships that may occur during heterologous virus co-infection, we have studied co-infection of mice with Ectromelia virus (ECTV) and Lymphocytic Choriomeningitis virus (LCMV), two unrelated viruses that are endemic to mice. Experimental inoculation of mice with ECTV results in a lethal infection with high virus replication in the liver, due in part to a number of ECTV proteins that block the production of and signaling from Type I interferons (IFN-I). Conversely, LCMV Armstrong infection results in an acute viral infection that is rapidly controlled by a potent CD8 T cell response, which is reliant on the induction of Type I interferon. We show that ECTV/LCMV co-infection of mice results in decreased ECTV viral load and amelioration of ECTV-induced disease. Our data suggest that this is due to Type I IFN induction by LCMV that suppresses ECTV replication. However, immune responses to LCMV in ECTV co-infected mice were also lower compared to mice infected with LCMV alone, and biased toward IFNγ producing effector-memory cells. Thus, we provide evidence for bi-directional effects of unrelated viruses during co-infection to modulate disease and immunity. Such observations likely have important implications for the maintenance and spread of these viruses in wild mouse populations.

IMPORTANCE

1. Novel finding of interaction between unrelated viruses during co-infection to limit disease and modulate immunity.

2. Findings suggest that co-infection in wild populations may have important implications for understanding the spread and maintenance of these viruses in endemic populations by increasing host survival and decreasing immunity to facilitate transmission.

3. Data suggest that heterogeneity in responses during vaccination with viral vectors or virus infection may be in part due to heterologous virus infection or vaccine usage.

INTRODUCTION

Tremendous progress has been made in our understanding of the requirements for immunity to viral infection(315, 386). In particular, animal studies utilizing the Lymphocytic Choriomeningitis (LCMV) mouse model of infection have led to the description of numerous fundamental properties of the immune system including the basis for MHC restriction of viral antigens(96, 270, 271), cross-presentation of epitopes by MHC proteins(272), how T cell effector mechanisms function to control virus infection(200, 273, 274), the generation and maintenance of T cell memory(9, 221, 222), and exhaustion of T cell responses during persistent infection(238, 239, 249, 275, 276). Poxviruses have also been widely used to understand how the immune system responds to infection(331) and are currently being investigated for use as potential vaccine vectors(25) for many important human pathogens such as HIV(21).

Despite this progress, an important caveat to such illuminating laboratory experiments is that they are almost always done in isolation, under specific pathogen-free (SPF) conditions. Whereas most "real-world" infections likely occur in the context of co-infection by heterologous pathogens that have the potential to modulate immune responses and/or alter disease(346). Current studies suggest co-infection with different pathogens is a common occurrence that can alter the progression of disease(346, 387-390). One example of such interaction is exacerbation of Listeriosis in mice to lethal disease during co-infection with LCMV(391, 392). Recent studies have also shown that enteric bacterial strains promote infection by poliovirus via mucosal routes(393). Another potential consequence of viral co-infection is viral co-evolution in which one virus may supply ancillary functions or suppress immune functions for another(345). Such a relationship has previously been described for Hepatitis B (HBV) and Hepatitis D (HDV) viruses. HDV cannot form mature virions without the presence of the Hepatitis B structural proteins(363). Co-infection of HBV and HDV or super infection of persistently infected HBV patients with HDV also results in increased liver pathology and poorer prognosis in patients due to altered immune responses and type I interferon signaling in the host(364, 365).

In order to understand the complex relationships that may occur during heterologous virus co-infection, we have studied co-infection of mice with ECTV and LCMV, two unrelated viruses that are endemic to mice. Although previous studies of co-infection with the related vaccinia virus (VACV) and LCMV have shown no alteration in either LCMV CD8 T cell responses or disease, such studies may minimize the role of these interactions(394, 395), as VACV is not endemic to mice. ECTV is a DNA virus of the orthopoxvirus family and encodes a number of proteins that block the production of and signaling by Type I interferons (IFN-I)(5, 338).

Experimental inoculation of mice with ECTV typically results in a lethal infection due to high virus replication in the liver and acute hepatic disease(337, 340). Conversely, LCMV infection of mice results in the rapid expansion of virus-specific CD8 T cells that limit viremia resulting in viral control and clearance within 7 days post infection(238, 396). In striking contrast to ECTV, LCMV induces robust Type I IFN production in mice with peak production during the first 12-48 hours of infection(397, 398). Furthermore, CD8 T cell responses to LCMV in mice are highly dependent on IFN-I signaling for sustained expansion(60, 399); infection of Interferon receptor deficient ($IFNAR^{-/-}$) mice with LCMV results in a defective CD8 T cell response that is unable to control infection(400). Therefore, we hypothesize that co-infection with ECTV and LCMV has the potential for bi-directional effects on disease and immunity by suppression of ECTV replication and disease while limiting LCMV-specific CD8 T cell responses.

We show here that ECTV/LCMV co-infection of mice results in decreased ECTV viral load and ameliorates ECTV-induced disease. Furthermore, we show that this effect is likely due to Type I IFN induction by LCMV that is able to overwhelm ECTV mechanisms for suppression of Type I IFN production and signaling. Conversely, we also show that ECTV partial suppression of type I IFN production during co-infection with LCMV results in diminished CD8 T cell responses to LCMV. Additionally, the LCMV response is biased towards the formation of memory CD8 T cells with a TNF-deficient effector-memory phenotype that has been shown to be less protective in other studies(230, 401, 402). Thus, we provide the first experimental evidence for bi-directional effects of these two unrelated viruses during co-infection to modulate disease and immunity. These findings likely have implications for disease and transmission of these viruses in wild mouse populations, and may in part explain the heterogeneity that is typically observed in LCMV immune responses in the wild(403, 404).

More importantly, our data suggest heterogeneity in responses during vaccination, with viral vectors, or viral infection may be in part due to heterologous virus infection or vaccine usage(405, 406).

MATERIALS AND METHODS

Mice: 6-8 week old female C57Bl/6J mice were purchased from Jackson laboratories (Bar Harbor, Maine). IFNAR1-deficient mice ($IFNAR^{-/-}$) were purchased from Jackson laboratories (Bar Harbor, Maine) and bred in our ASU animal facilities. All studies were conducted according to animal protocol 12-1229R under the approval and guidance of the Arizona State University Institute for Animal Care and Use Committee.

Cells and viruses: BHK cells were maintained in complete Eagles's MEM (5% fetal bovine serum (FBS), 2mM L-glutamine (L-Q), 100 U/mL penicillin, 100 μg/ml streptomycin). Vero and MC57 cells were maintained in complete DMEM (10% FBS, 2mM L-Q, 100 U/ml penicillin, 100 μg/mL streptomycin). LCMV Armstrong and LCMV clone-13 stocks were kindly provided by Rafi Ahmed (Emory University, Atlanta GA) and produced in BHK cells as previously described(380). The titer of LCMV stocks and mouse serum samples were determined by plaque assay on Vero cell monolayers as previously described(380). ECTV expressing the β-gal gene in the CHO locus (US17-βgal) was a gift from Dr. Mark Buller (St Louis University, St Louis MO). ECTV stocks were propagated in Vero cells as previously described(407). ECTV titers in mouse liver homogenates were determined on VERO cell monolayers. Briefly, liver samples were weighed and homogenized in PBS to 10% w/v. Vero monolayers were infected following three freeze-thaw cycles for 1 hour prior to overlay with a 1:1 ratio of 1% agarose and 2X-MEM supplemented with 10% fetal bovine serum.

After three days at 37°C, a second overlay of a 1:1 ratio of 1% Agarose and 2X-MEM supplemented with 10% fetal bovine serum and X-gal (20mg/ml) was applied. Four days after the secondary overlay blue ECTV plaques were counted.

***In vivo* infections:** Unless otherwise stated, LCMV and ECTV stocks were diluted to 10^6 pfu/ml in 1X PBS prior to intraperitoneal infection in a volume of 100ul, delivering a total of 10^5 pfu per mouse. Unless otherwise indicated, co-infected mice received ECTV immediately followed by LCMV inoculation. Mice were monitored daily for clinical disease (hunched posture, ruffled fur, non-motility) and euthanized at the indicated times post infection.

Peptides: Lymphocytic choriomeningitis virus CD8 T cell epitopes GP33 (H-2D^b, KAVYNFATC) and NP396 (H-2D^b, FQPQNGQFI) were purchased from Genscript (Piscataway, NJ).

Cell surface antibody staining: Single cell suspensions were prepared from splenocytes as previously described(200). Erythrocytes were lysed with ammonium chloride lysis (ACK) buffer purchased from Lonza (Allendale, NJ) and FACS staining was done as previously described(200) in 96 well plates with fluorochrome-labeled monoclonal antibodies: anti-CD8 (clone 53-6.7), anti-CD44 (clone IM7), anti-PD-1 (clone J43), anti-CD4(clone GK1.5) and anti-CD62L (clone MEL-14) or APC labeled GP33-tetramer(238). Samples were then fixed in 1% paraformaldehyde solution and immediately acquired on a BD LSR II Fortessa flow cytometer (San Jose, CA) and analyzed using FlowJo software (Tree-Star, Ashland, OR). All surface monoclonal antibodies were purchased from BD Pharmigen (San Diego, CA) or eBiosciences (San Diego, CA).

Intracellular cytokine staining: For quantitation of ECTV-specific T cell responses, splenocytes (10^6/well) were stimulated with uninfected MC57 cells or with ECTV infected MC57 cells (MOI:1, at 24 hours post infection). For quantitation of LCMV-specific T cell responses, splenocytes were cultured alone or with 1mM LCMV peptide epitopes as previously described(200). After 5 hours of stimulation, cells were permeabilized and intracellular cytokine producing cells detected by staining with anti-IFNγ (clone XMG1.2) and anti-TNF (clone MP6-XT22) antibodies purchased from BD Pharmigen (San Diego, CA) or eBiosciences (San Diego, CA). The samples were acquired and analyzed as described above.

Interferon-I ELISA: Mouse interferon beta and interferon alpha ELISA kits were purchased from PBL Assay Science (Piscataway, NJ) and used according to manufacturers instructions to detect serum Type I IFN levels.

Histology: Formalin fixed liver sections were cut into 20μm thick tissue sections on a microtome. Liver samples were stained with x-gal (20mg/mL) using the β-galactosidase reporter gene staining kit purchased from Sigma (St Louis, MO) following the manufacturers instructions. Adjacent sections of formalin fixed liver tissue were stained with hematoxylin and eosin as previously described(382). 20x images were taken with a Zeiss Axioskop (Thornwood, NJ) and evaluated for pathology and punctate blue x-gal staining.

Statistics: Prism software (Graphpad, La Jolla, CA) was used to calculate t-test p values to determine significance or log-rank test to determine survival curve significance (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$).

RESULTS:

ECTV/LCMV co-infection reduces ECTV disease and viral load. We first determined if ECTV/LCMV co-infection alters disease in mice compared to ECTV infection alone, by monitoring physical symptoms and mouse survival. As expected, ECTV-only infected mice exhibited extreme disease symptoms starting at 5 days post infection and required euthanasia by 7 days post-infection (**Figure 3.1**). Surprisingly, 6 week-old ECTV/LCMV co-infected mice exhibited a delay in ECTV-induced disease, with survival between 12-17 days post infection, more than twice that observed in mice infected with ECTV alone.

Orthopoxvirus infections have been shown to be age and immune status dependent, with older mice typically having reduced poxvirus replication and less severe disease(336), we also compared mice infected with ECTV alone to ECTV/LCMV co-infection in 8-week old mice. We observed no difference in ECTV disease in older mice compared to younger mice, presumably due to the high dose of inoculation used, since all mice infected with ECTV alone required euthanasia by 7 days post-infection. In striking contrast, ~70% of 8 week-old ECTV/LCMV co-infected mice exhibited long-term, disease-free survival. Overall, ECTV/LCMV co-infection resulted in 50% survival of mice, with no detectable ECTV in liver homogenates at greater than 60 days post-infection compared to 0% survival after mice infected with ECTV alone. Thus, these results demonstrate that ECTV/LCMV co-infection can ameliorate ECTV disease in mice.

ECTV induced mortality in mice is known to correlate with high viral titers in the liver that result in in acute hepatic failure(336, 343). In order to determine whether the delay in and/or amelioration of ECTV-induced disease in ECTV/LCMV co-infected mice was due to suppression of ECTV viral loads, we next measured ECTV viral titers in the liver of ECTV/LCMV co-infected mice compared to mice infected with ECTV alone.

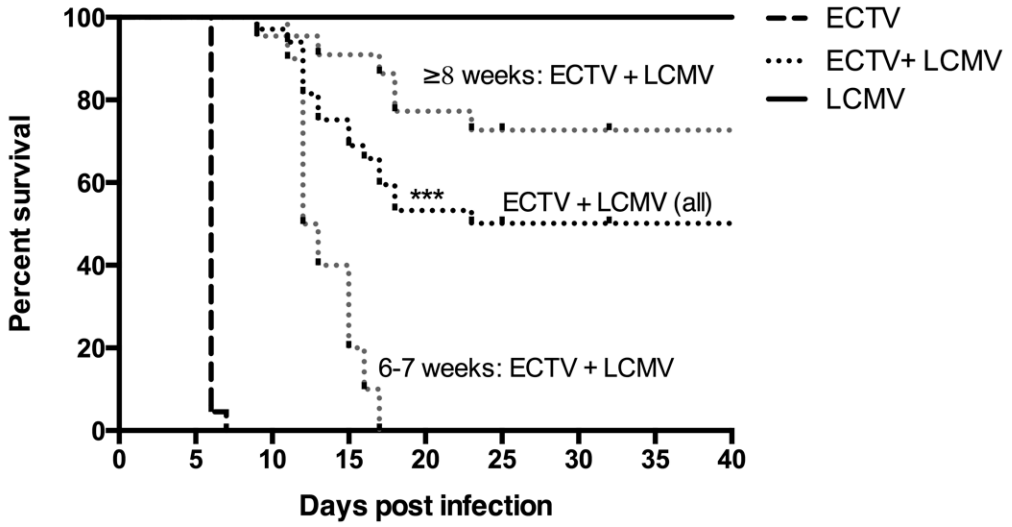

Figure 3.1: ECTV/LCMV co-infection reduces ECTV disease. 6-8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip. All mice were monitored daily for morbidity and mortality. Survival curve of all ECTV infected (n=34), LCMV infected (n=20) and ECTV/ LCMV co-infected (n=47) 6-8 week-old mice. Survival curve breakdown of ECTV/LCMV co-infected by age indicates 8-week and older mice experience complete rescue of mortality.

Mice infected with ECTV alone exhibited high liver viral titers, with up to 10^9 pfu/gram by 6 days post-infection (**Figure 3.2A**). However, ECTV/LCMV co-infected mice exhibited a ~1000 to 100-fold decrease in ECTV liver viral load, remaining below 10^7 pfu/gram up to 9 days post-infection. Thereafter, ECTV/LCMV co-infected mice were either able to completely able to control ECTV infection or succumb to lethal disease.

Notably, in analysis of liver tissue sections directly *ex vivo*, mice infected with ECTV alone displayed punctate x-gal staining across the entire liver section, whereas x-gal staining was undetectable in ECTV/LCMV co-infected mice at day 5 post infection (**Figure 3.2B, upper panel**). These findings were also consistent with histological staining (H&E stain) in which liver tissues at 5 days post infection from mice infected with ECTV alone showed multiple necrotic lesions, whereas liver sections from ECTV/LCMV co-infected mice did not exhibit any detectable necrotic lesions (**Figure 3.2, lower panel**). Taken together, these results show that ECTV/LCMV co-infection reduces ECTV virus load in the liver and therefore ameliorates disease.

In order to investigate if the reduced disease in ECTV/LCMV co-infected mice was due to LCMV prevention of initial ECTV infection of cells, versus suppression of ECTV replication, we tested whether infection of mice with decreasing doses of ECTV during LCMV co-infection had a similar effect on disease. We reasoned that if LCMV inhibited initial seeding of ECTV in the liver we would expect mice receiving lower doses of ECTV to also have less disease and enhanced survival in the absence of LCMV infection. Mice that received up to 1000-fold lower doses of ECTV exhibited similar disease at 5 days post-infection compared to the high dose inoculation used above, and 100% of these mice required euthanasia by 7 days post-infection.

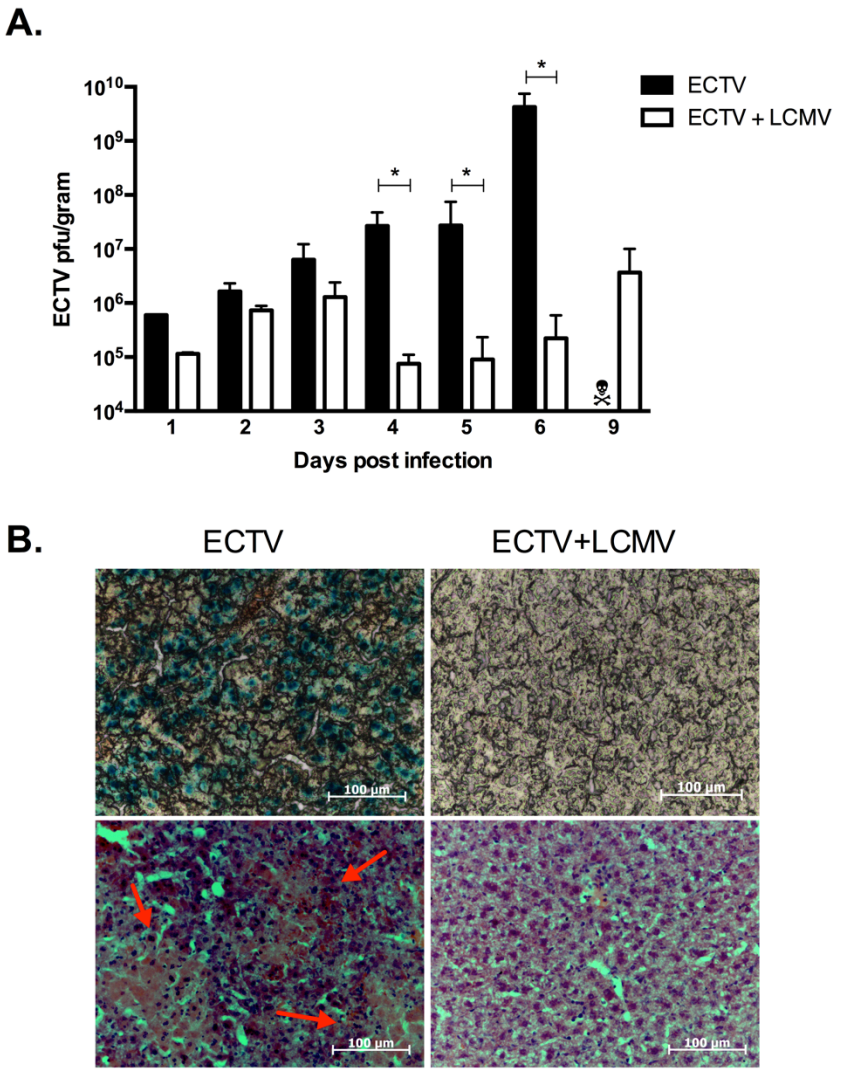

Figure 3.2: ECTV/LCMV co-infection reduces ECTV viral load in the liver. 6-8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip. All mice were monitored daily for morbidity and mortality. **[A]** Mean ECTV viral load (pfu/gram) in the liver of ECTV infected and ECTV/LCMV co-infected mice on days indicated post infection (n=3-5 mice/group, 3 independent experiments). **[B]** Top panel: 20X view of formalin fixed liver section stained with X-gal at day 5 post infection, blue color indicates presence of ECTV infection. Bottom panel: Histological stain (H&E) of formalin fixed liver sections at day 5 post infection, red arrows point to necrotic lesions (n= 3-5 mice/group).

Surprisingly, decreasing the dose of ECTV during ECTV/LCMV co-infection had no impact on suppression of disease: ECTV/LCMV co-infected mice at all doses of ECTV demonstrated delayed disease until day 16 post infection similar to that observed during high dose infection (**Figure 3.3).** These data are consistent with the hypothesis that the observed suppression of ECTV disease in ECTV/LCMV co-infected mice is not due to competition for target cells or blockade of initial ECTV seeding in the liver, but rather suppression of ECTV replication or infection after initial seeding.

The timing of LCMV infection is crucial for reducing disease during ECTV co-infection. We next hypothesized that the observed reduction in ECTV replication and disease during LCMV co-infection may be dependent on the timing (prior exposure or post exposure) of co-infection due to the potential for enhancement of early innate events by LCMV to suppress ECTV infection. Infection with LCMV up to 2 days prior to ECTV infection resulted in similarly reduced ECTV viral loads and disease as observed during concurrent ECTV/LCMV co-infection (**Figure 3.4A).** Mice infected with LCMV 3 days prior to ECTV infection demonstrated a minor delay in disease progression, with mice surviving on average 3 days longer than mice infected with ECTV alone. In striking contrast, mice inoculated with LCMV 1 or 2 days post ECTV infection showed no reduction in ECTV viral loads or disease compared to mice infected with ECTV alone, with 100% of these mice exhibiting lethal disease symptoms by 7 days post-infection (**Figure 3.4B**). Thus, the timing of co-infection for the suppression of ECTV replication and disease suggests that the effects of LCMV on ECTV are to enhance early innate events to suppress ECTV viral infection in the liver.

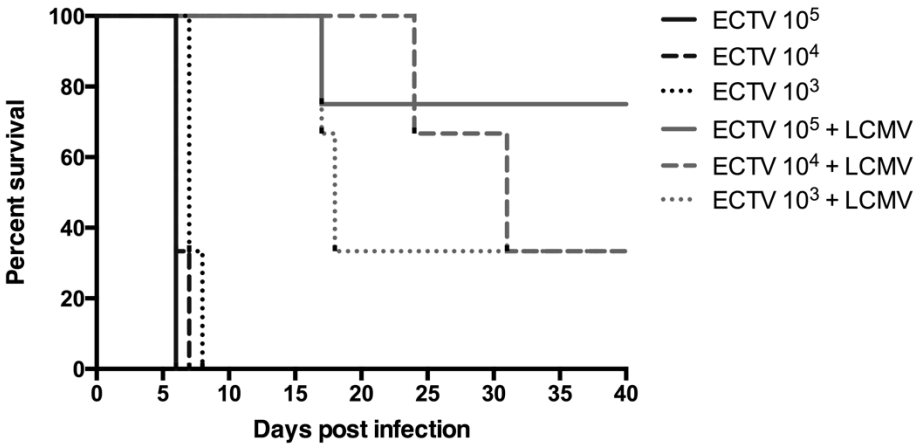

Figure 3.3: LCMV does not prevent initial seeding of ECTV in the liver during co-infection. 8-week old female C57Bl/6 mice infected with 10^5, 10^4 or 10^3 pfu ECTV-US17- β gal ip. Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip. Survival curve of mice infected with ECTV alone or co-infected with ECTV and LCMV at varying infectious doses of ECTV (n= 3-5 mice/group).

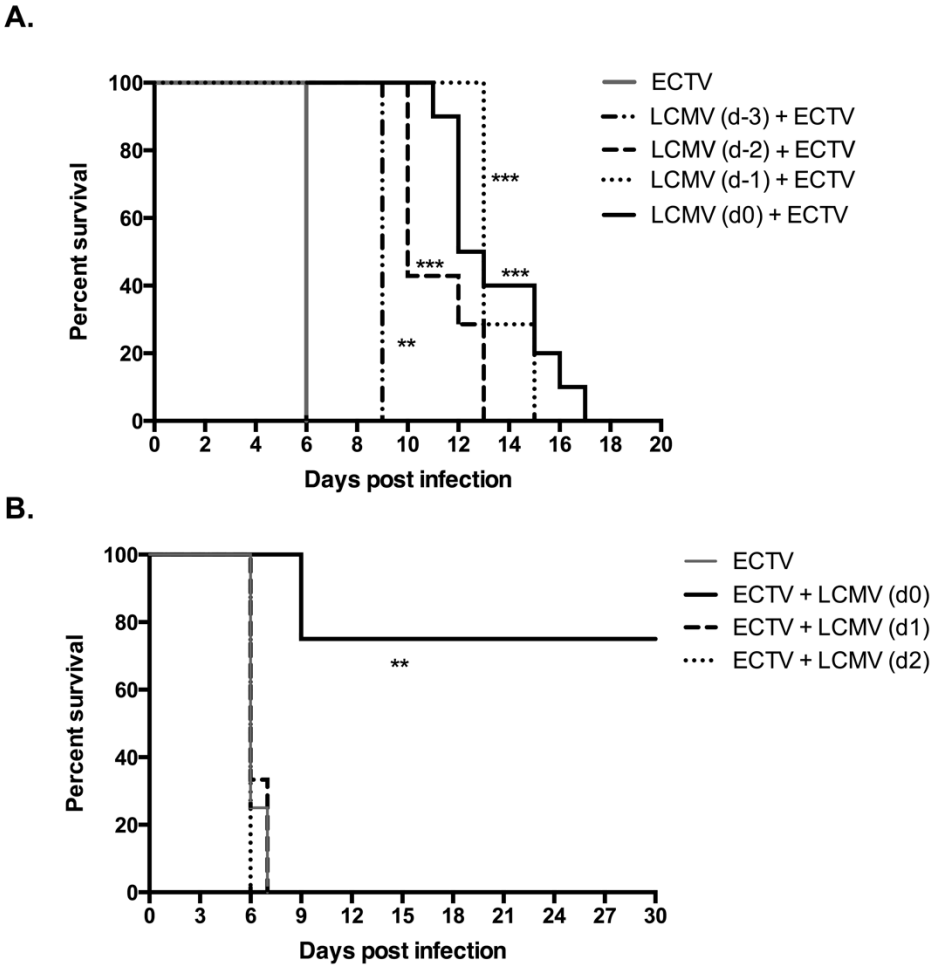

Figure 3.4: The timing of LCMV infection is crucial for reducing disease during ECTV co-infection. [A] Survival of 6 week-old mice infected with 10^5 LCMV Armstrong (ip) 1, 2 or 3 days prior to ECTV-US17-βgal (ip) infection. **[B]** Survival of ECTV-US17-βgal infected 8-week old mice (d0) that received LCMV Armstrong immunization 1 or 2 days post ECTV exposure (n=3-5 mice/group).

ECTV/LCMV co-infection nullifies ECTV abrogation of IFN-I production. Because LCMV is known to induce potent Type I Interferon responses(398), and ECTV is sensitive to Type I Interferons(338, 343), we next hypothesized that the early innate immune suppression of ECTV could be due to an overwhelming Type I IFN response. This would be consistent with the observation that LCMV inoculation 1-2 days prior to ECTV infection resulted in similar reduction in disease and ECTV liver titers, as Type I IFN production is maximal at 1-2 days post LCMV infection and is curtailed thereafter(397).

Serum levels of total IFNα and IFNβ in ECTV infected mice were undetectable, as expected, presumably due to the ability of ECTV viral proteins to completely shut down Type I IFN production(336). In contrast, mice infected with LCMV alone induced potent Type I IFN responses, with >12,000 pg/mL total IFNα and >500 pg/mL IFNβ in the serum of mice infected with LCMV alone by 24 hours post infection. Thereafter, IFNα and IFNβ levels decreased to 9000 pg/mL and 100pg/mL respectively by 48 hours in mice infected with LCMV alone and were low to undetectable thereafter (**Figure 3.5**). ECTV/LCMV co-infected mice exhibited an increase in serum levels of both IFNα and IFNβ, compared to mice infected with ECTV alone (which were undetectable). Serum IFNα levels were not statistically different between LCMV and co-infected animals (**Figure 3.5A**). Whereas, IFNβ levels were lower in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone (**Figure 3.5B**). Thus, these results suggest that although ECTV is able to partially attenuate IFNβ production during LCMV co-infection, sufficient Type I IFN levels remain to suppress ECTV replication and ameliorate disease.

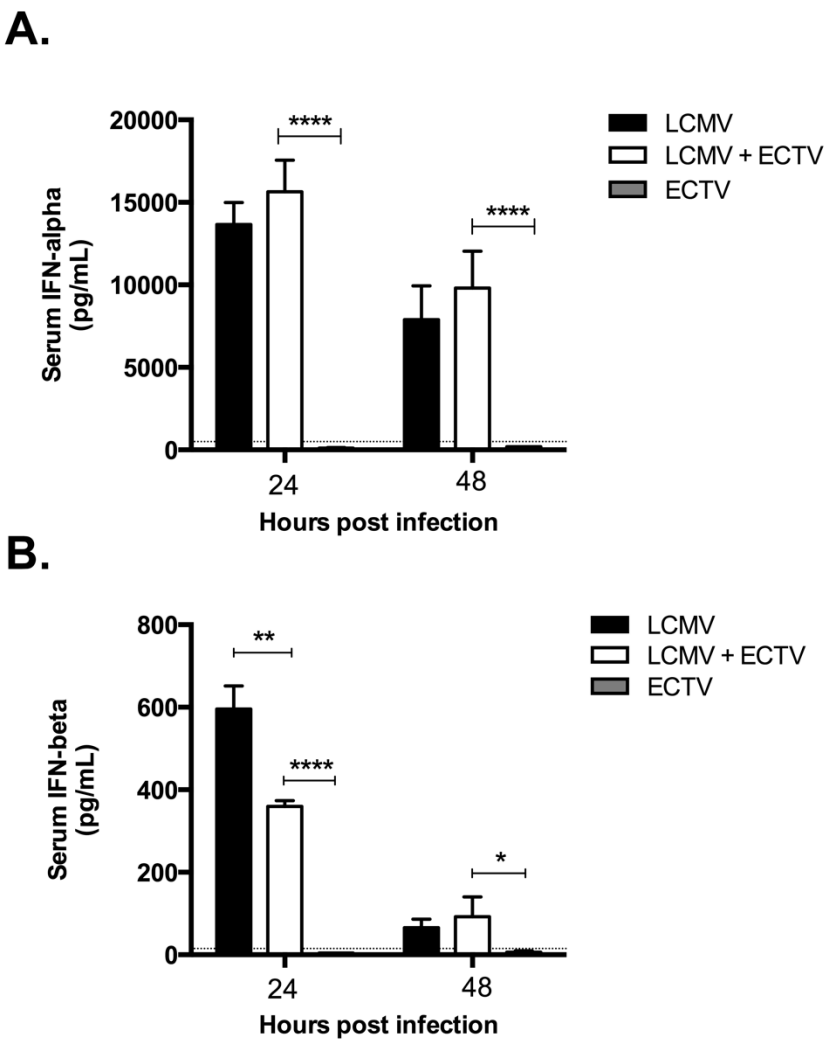

Figure 3.5: ECTV/LCMV co-infection nullifies ECTV abrogation of IFN-I production. 6-8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip (100ul). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip. **[A]** IFNαlevels in serum of ECTV/LCMV co-infected mice as compared to LCMV only and ECTV only control mice at 24 and 48 hours post infection (n=3-5 mice/group). **[B]** IFNβlevels in serum of ECTV/LCMV co-infected mice as compared to LCMV only and ECTV only control mice at 24 and 48 hours post infection (n=3-5 mice/group).

In order to directly test the role of Type I IFN in suppression of ECTV replication and disease during ECTV/LCMV co-infection, we tested whether ECTV/LCMV co-infection could rescue IFNAR$^{-/-}$ mice from disease. Mice infected with ECTV alone and ECTV/LCMV co-infected infected mice had indistinguishable ECTV viral loads and exhibited similar disease, requiring euthanasia by 7 days post-infection **(Figure 3.6**). Thus, LCMV co-infection does not rescue IFNAR$^{-/-}$ mice from lethal ECTV disease. These results suggest that Type I IFN signaling is the main mechanism for LCMV suppression of ECTV replication and disease.

LCMV co-infection does not significantly enhance ECTV-specific CD8 T cell responses. Resistance to mousepox has been shown to correlate with rapid, more enhanced cytotoxic responses(340, 408). Therefore, an alternative explanation for the observed decrease in ECTV disease during LCMV co-infection is that LCMV alters ECTV-specific adaptive immune responses, and thus attenuates immunopathology. It should be noted, that although no studies have shown a dependence on Type I IFN for induction of ECTV T cell responses, as has been shown with LCMV, other orthopoxviruses do not require Type I IFN for induction of CD8 T cell immunity(394). We observed no difference in the total magnitude of the ECTV-specific CD8 T cell response in the spleen, measured by CD8 T cell production of IFNγ after culture with ECTV infected-MC57 cells directly ex-vivo, between mice infected with ECTV alone and ECTV/LCMV co-infected mice.

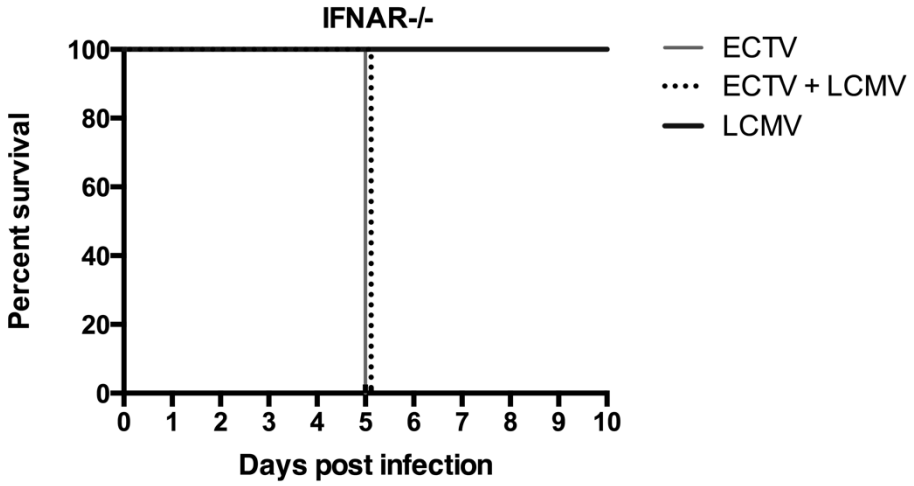

Figure 3.6: LCMV reduction of ECTV mediated disease during ECTV/LCMV co-infection is dependent on Type I interferon signaling. Survival curve of 8-10 week old female IFNAR-/- mice infected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were infected with 10^5 pfu LCMV Armstrong ip (n= 3-5 mice/group).

The ECTV CD8 T cell response was evaluated at a time point preceding presentation of disease symptoms that required euthanasia. Moreover, in both groups of mice the CD8 T cells had equivalent production of IFNγ and TNF (**Figure 3.7A**). Thus, LCMV co-infection does not appear to increase survival in co-infected mice due to alteration of the initial ECTV CD8 T cell response or immunopathology resulting from these cells. Thus, suggesting that the observed effects on reduction of ECTV replication and amelioration of disease due to LCMV co-infection are driven by the increase in innate immune production of Type I IFN. However, the increase in survival seen in co-infected mice past 6 days post infection may allow greater development of the ECTV CTL response, which may further influence ECTV viral replication. In order to investigate the potential bi-directional effects of alterations in IFN-I production during co-infection, we next determined if there were variations in the LCMV-specific T cell response in ECTV/LCMV co-infected mice.

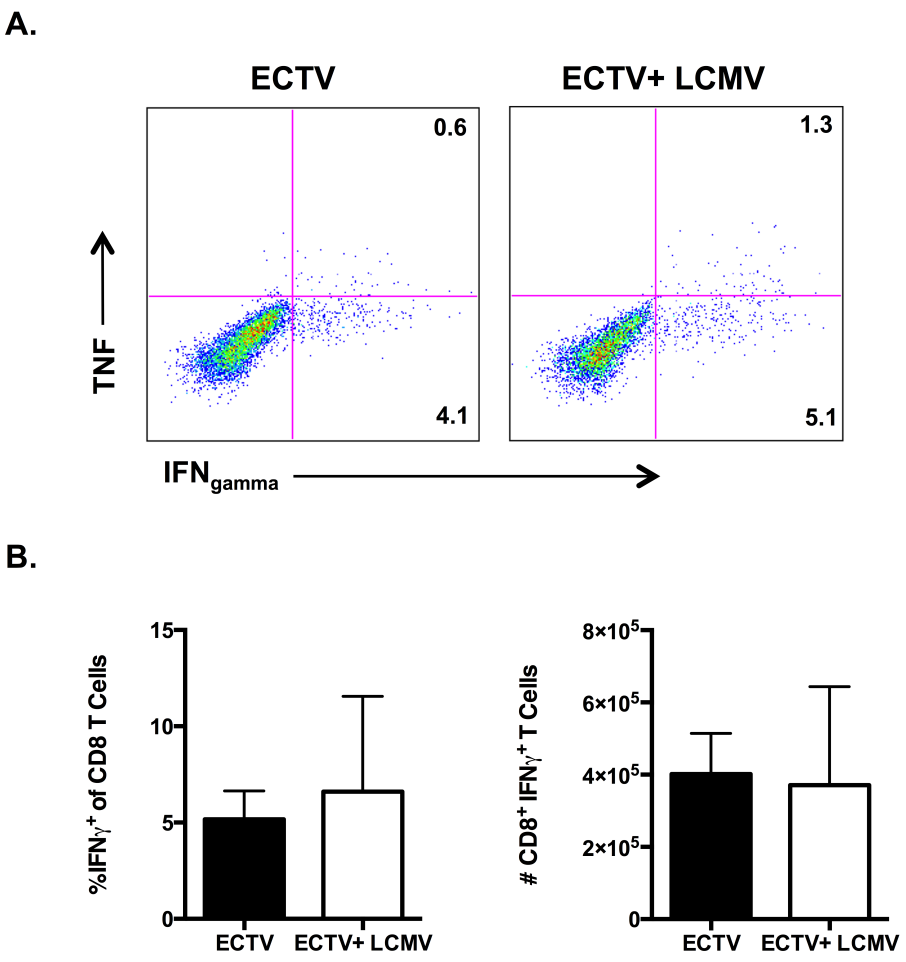

Figure 3.7: ECTV/LCMV co-infection does not significantly enhance magnitude of ECTV-specific CD8 T cell response. 6-8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip (100ul). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip. At the indicated time post infection, splenocytes were harvested, processed to single cell suspensions and stimulated for 6 hours with ECTV infected MC57 cells prior to intracellular cytokine staining. **[A]** Representative FACS plots showing the proportion of CD8 T cells, isolated on day 5 post infection from the spleen of ECTV infected and ECTV/LCMV co-infected mice, capable of producing TNF and IFNγ after stimulation with ECTV-MC57 cells. **[B]** Left panel: mean percentage of ECTV-specific IFNγ producing T cells out of all CD8 T cells in the spleen on day 5 post infection. Right panel: Average number of ECTV-specific CD8 T cells in the spleen on day 5 post infection (n=3-5 mice/group).

Decreased IFN-I during ECTV/LCMV co-infection limits LCMV-specific CD8 T cell expansion. In addition to the effect of LCMV/ECTV co-infection to reduce ECTV replication and disease, we next hypothesized that production of ECTV proteins that suppress Type I IFN production(5) may impair the generation of LCMV-specific CD8 T cell responses that are critically dependent on Type-I IFN for sustained proliferation(60, 399). In order to determine if ECTV/LCMV co-infection impaired LCMV-specific CD8 T cell expansion, we compared LCMV effector CD8 T cell generation between co-infected mice and mice infected with LCMV alone (**Figure 3.8A**). Peak LCMV CD8 T cell responses to the immunodominant GP33 and NP396 epitopes(238) at 9 days post-infection in the spleen of mice infected with LCMV alone were >35% of the total CD8 T cell population, consisting of on average >6 $x10^6$ LCMV-specific cells (**Figure 3.8B, black bars)**. However, ECTV/LCMV co-infection decreased the peak LCMV response by 2-3 fold as GP33 and NP396 epitope-specific CD8 T cells comprised <20% of the total CD8 T cell population with on average <$2x10^6$ LCMV-specific CD8 T cells (**Figure 3.8B, white bars**). This effect was not due to differences in the timing of expansion of the LCMV-specific CD8 T cells, as a similar reduction in the effector T cell response was also seen at day 7 post ECTV/LCMV-infection (**Figure 3.8C**).

Moreover, and consistent with previous reports that show that Type I IFN are necessary for sustained proliferation of CD8 T cells but not the initial activation and early proliferation of these cells(60), we observed no difference in the CD8 T cell response to LCMV between mice infected with LCMV alone and ECTV/LCMV co-infected mice at 5 days post-infection (**Figure 3.8C).** Thus, incomplete ECTV suppression of Type I IFN during LCMV co-infection results in reduced LCMV-specific CD8 T cell expansion.

Additionally, although not statistically significant with the number of mice used in these studies, the percentage of LCMV-specific memory CD8 T cells in ECTV/LCMV co-infected mice was consistently lower than that observed in mice infected with LCMV alone at >35 days post infection. Thus, memory CD8 T cells specific for the immunodominant NP396 and GP33 epitopes appeared to be proportional to peak effector T cell responses in both groups. (**Figure 3.8C**).

Decreased CD8 T cell responses to LCMV during ECTV/LCMV co-infection do not impair control of LCMV or CD8 T cell memory function. We next asked whether the observed reduction in the LCMV-specific CD8 T cell response during ECTV/LCMV co-infection impaired immune control of LCMV. Surprisingly, we observed no difference in serum LCMV titers at 7 days post-infection between LCMV-only and ECTV/LCMV co-infected mice, with neither group having detectable LCMV levels at this time. However, we cannot rule out that there were small differences in the kinetics of control of LCMV. Many studies have suggested that the strength or duration of the initial stimulus has a dramatic impact on the generation and function of CD8 T cell memory(9, 238, 400). Although we observed no impact of ECTV/LCMV co-infection on CD8 T cell mediated control of LCMV, we reasoned that if the diminished CD8 T cell responses in ECTV/LCMV co-infected mice resulted in slight impairment of LCMV-specific immunity, then this might alter the bias generation of LCMV-specific memory T cells or their function. To test whether LCMV-specific memory CD8 T cells in ECTV/LCMV co-infected mice were impaired in their effector functions we next measured the ability of memory cells from mice infected with LCMV alone or ECTV/LCMV co-infected mice to produce the key inflammatory cytokines IFNγ and TNF.

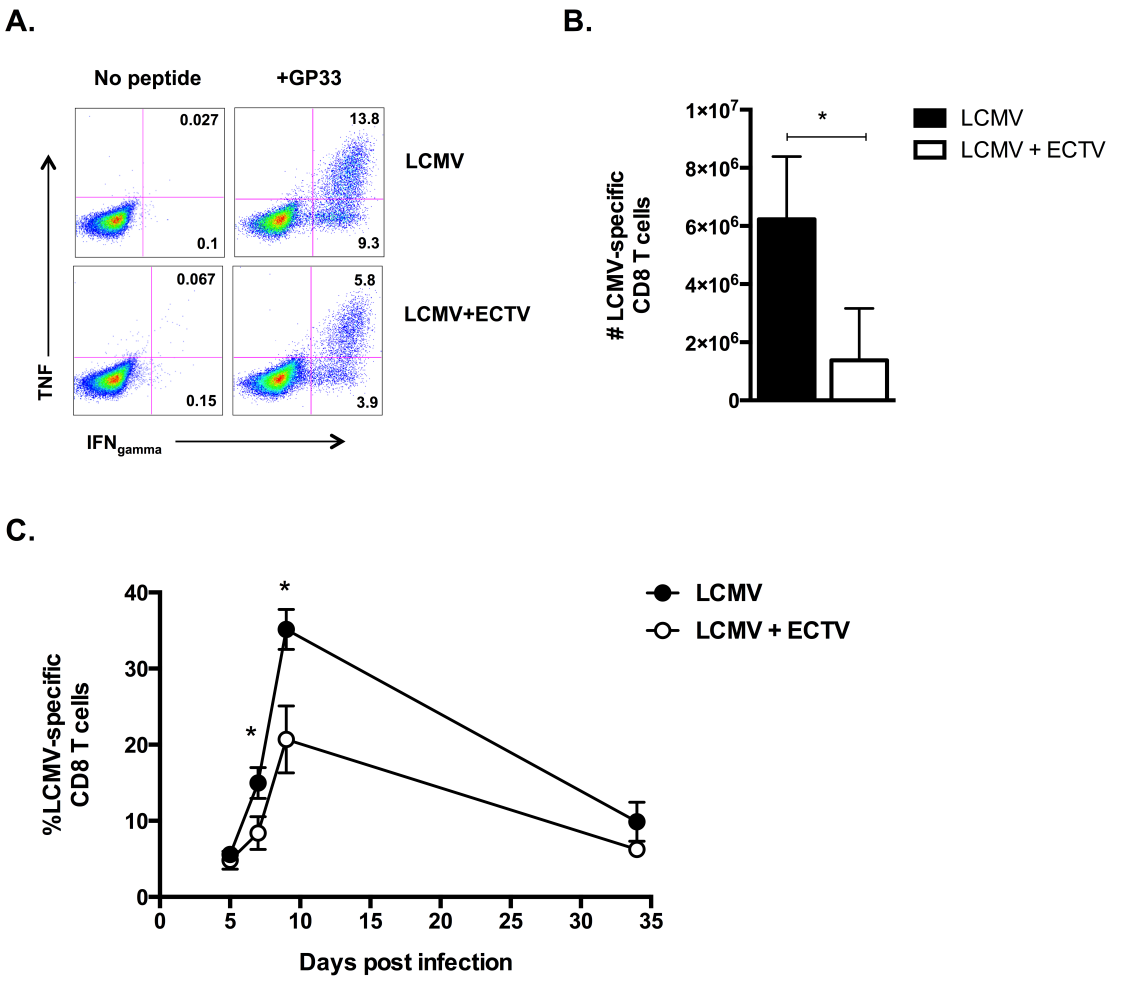

Figure 3.8: Decreased IFN-I during ECTV/LCMV co-infection limits LCMV-specific CD8 T cell expansion. 6-8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip (100ul). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV ip. At the indicated times post infection, splenocytes were harvested, processed to single cell suspensions and stimulated for 6 hours with cognate LCMV peptides prior to intracellular cytokine staining. **[A]** Representative FACS plots of the LCMV-specific (GP33 peptide stimulated) CD8 T cell response in the spleen at day 9 post infection of LCMV infected and ECTV/LCMV co-infected mice. **[B]** Mean total number of LCMV-specific (GP33 and NP396) CD8 T cells in the spleen on day 9 post infection (n=3-5 mice/group). **[C]** Kinetics of LCMV-specific (GP33 and NP396) CD8 T cell response as a percentage of total CD8 T cells in the spleen in ECTV/LCMV co-infected mice and LCMV infected mice (n=3-5 mice/group).

We observed a decrease in the relative proportions of LCMV-specific memory cells, specific for NP396 and GP33 epitopes that were able to produce both IFNγ and TNF after stimulation. 89% of CD8 T cells isolated from mice infected with LCMV alone that produced IFNγ in response to these epitopes were double-positive for TNF. In contrast, only 41% of CD8 T cells in ECTV/LCMV co-infected mice that produced IFNγ also produced TNF (**Figure 3.9A**). The magnitude of LCMV-specific (GP33 and NP396 epitopes) memory CD8 T cells capable of producing TNF and IFNγ in the spleen also decreased from 8% of all CD8 T cells in the spleen of mice infected with LCMV alone to less than 4% of CD8 T cells in the spleen of ECTV/LCMV co-infected mice (**Figure 3.9B**). In addition, the percentage of central-memory ($CD62L^{high}$) CD8 T cells specific for the LCMV epitope GP33 was decreased almost three-fold (>95% $CD62L^{high}$ to ~35% $CD62L^{high}$) in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone (**Figure 3.9C**). Taken together, these results suggest that CD8 T cell control of LCMV is partially impaired in ECTV co-infected mice resulting in fewer multi-functional memory cells biased toward an effector-memory ($CD62L^{low}$) phenotype. Finally, we tested whether the reduced cytokine production and effector-memory bias of CD8 T cell populations in ECTV/LCMV co-infected mice impacted the ability of these mice to control subsequent LCMV infection.

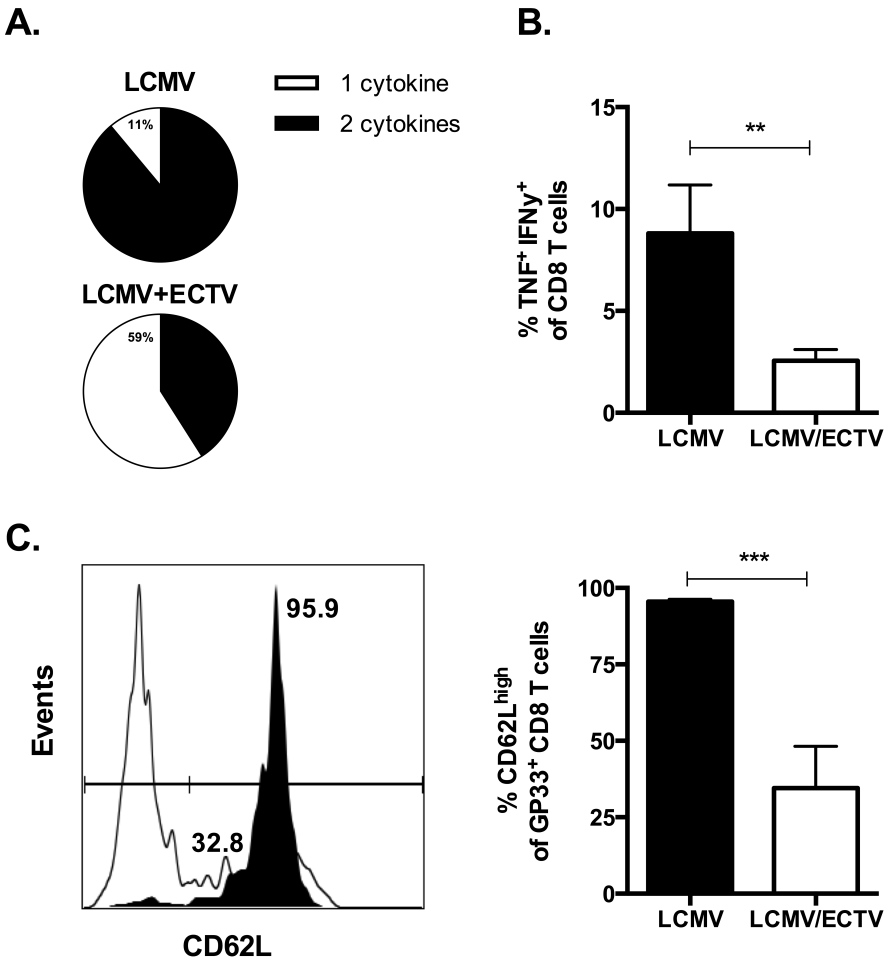

Figure 3.9: Decreased CD8 T cell responses to LCMV during ECTV/LCMV co-infection bias T cell effector memory formation. 6-8 week old female C57Bl/6 mice were infected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV ip. After a period of at least 35 days surviving co-infected mice were euthanized to enumerate LCMV CD8 T cell memory formation and function. **[A]** Functional analysis of GP33 and NP396 memory CD8 T cells at day 35 post infection. White bar indicates ability to produce IFNγ only, black sections indicates ability to produce both TNF and IFNγ after peptide stimulation (n=3-5 mice/group). **[B]** Mean percentage of TNF and IFNγ producing LCMV-specific memory cells in the CD8 T cell splenic population at day 35 post infection. **[C]** Left panel: representative FACS histogram of CD62L expression on GP33-tetramer positive cells in the spleen. Right panel: percentage of GP33-tetramer positive, LCMV-specific central memory ($CD62L^{high}$) CD8 T cells in the spleen in LCMV infected and ECTV/LCMV co-infected mice (n=3-5 mice/group).

We challenged ECTV/LCMV co-infected or mice infected with LCMV at 35 days post-infection with the virulent clone-13 strain of LCMV(284, 292, 308). Non-immunized mice infected with LCMV clone-13 are unable to control LCMV replication and present with high viral titers (~10^5 pfu/ml) in the serum at 7 days post-infection. Additionally, naïve mice challenged with LCMV clone 13 undergo T cell exhaustion resulting in decreased cytokine production and increased expression of the inhibitory receptor PD-1(239). Both groups of immunized mice, whether previously infected with LCMV alone or co-infected with ECTV/LCMV, had undetectable LCMV viral titers in the serum at 7 days post LCMV clone 13 challenge (**Figure 3.10A**).

In addition, LCMV-specific CD8 T cells in ECTV/LCMV co-infected mice did not experience T cell exhaustion, since capacity to produce IFNγ and TNF after stimulation was greater than naive challenged mice and indistinguishable from LCMV immunized mice at day 15 post challenge (**Figure 3.10B**). Furthermore, CD8 T cells isolated from either ECTV/LCMV co-infected or LCMV-immunized mice at day 15 post LCMV clone 13 challenge expressed low levels of PD-1 (**Figure 3.10B**). Thus, suggesting that even though ECTV co-infection reduced the generation, function and central memory phenotype of LCMV specific CD8 T cells, which has been shown to be less protective during LCMV challenge(230, 402), sufficient memory T cells persisted in both groups to mount protective responses to LCMV clone-13 challenge.

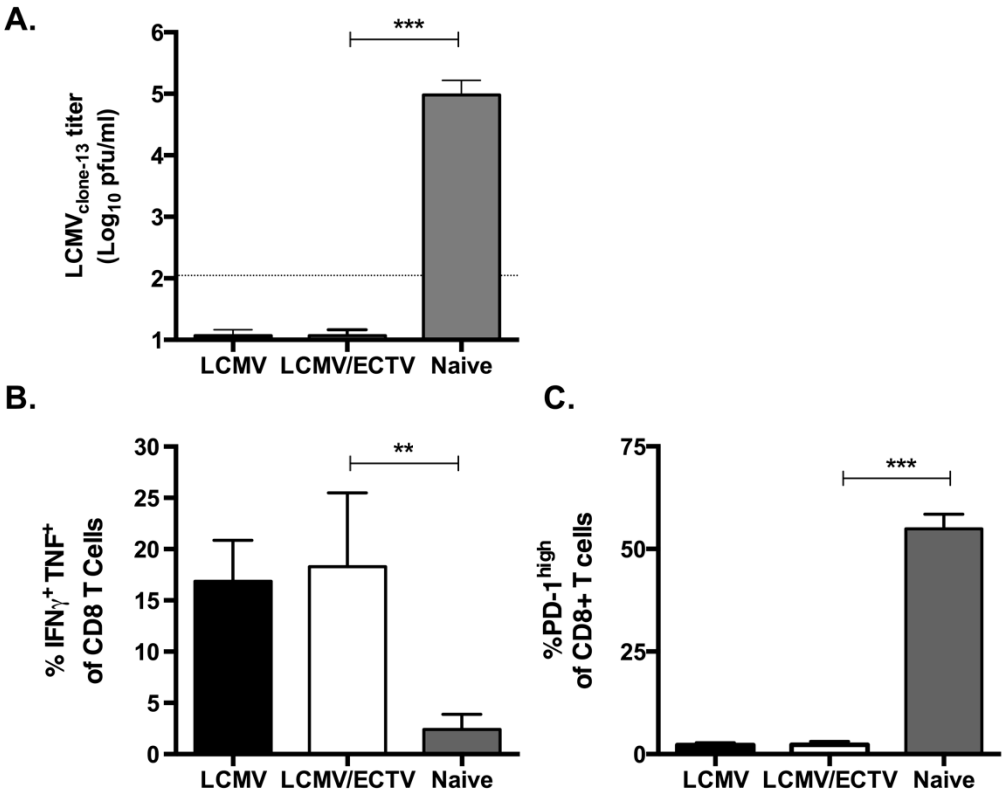

Figure 3.10: ECTV/LCMV co-infection has no impact on secondary responses and protection after LCMV clone-13 challenge. 6-8 week old female C57Bl/6 mice were infected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV ip. After a period of at least 35 days surviving co-infected mice were challenged with $2x10^6$ pfu LCMV clone-13 iv. **[A]** LCMV clone 13 viral load (pfu/mL) in the serum of LCMV clone-13 challenged mice at day 7 post infection. **[B]** Mean percentage of TNF and IFNγ producing secondary effector LCMV-specific CD8 T cells at day 15 post challenge **[C]** Percentage of PD-1^{high} GP33-tetramer positive cells at day 15 post challenge in LCMV immune, ECTV/LCMV co-infected and naïve mice (n=3-5 mice/group).

DISCUSSION

Our results demonstrate interaction between endemic but unrelated viruses during co-infection of mice to limit disease and immunity. Mice co-infected with LCMV and ECTV demonstrated reduced ECTV replication resulting in reduced disease and enhanced survival. Conversely, ECTV co-infection resulted in reduced LCMV-specific CD8 T cell responses. The data suggest that these effects are primarily due to modulation of Type I IFN levels, with primarily lower IFNβ levels in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone and no difference in disease progression during ECTV/LCMV co-infection in IFNAR$^{-/-}$ mice compared to mice infected with ECTV alone. This is further supported by more striking amelioration of disease in ECTV/LCMV co-infected older mice in which Type I IFN signaling has previously been shown to limit Orthopoxvirus replication(343).

Our results implicate Type I IFN production and/or signaling as the main mechanism by which LCMV suppresses ECTV replication and disease in ECTV/LCMV co-infected mice. The kinetics of LCMV infection and induction of Type I IFN responses in this model appears to be critically important as co-infection with LCMV 3 days prior had minimal impact on ECTV disease while inoculation with LCMV >1 day after ECTV infection showed no effect. We suggest in the former case LCMV-induced Type I IFN production is curtailed after 3 days to levels that are effectively blocked by ECTV proteins. In contrast, LCMV infection after establishment of ECTV infection is likely ineffective as ECTV proteins have effectively shut down Type I IFN signaling(336, 341).

Our results also show that ECTV modulation of Type I IFN production during ECTV/LCMV co-infection attenuates LCMV-specific CD8 T cell responses that are dependent on direct signaling via Type I IFN for sustained proliferation. It is unlikely in this context that ECTV is modulating Type I IFN intracellular signaling as ECTV is not known to directly infect CD8 T cells(341). Although we find minimal differences in IFNα in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone, we did observe significantly and consistently lower levels of systemic IFNβ in co-infected mice. Therefore, our data suggests that IFNβ, rather than IFNα, plays a larger role in supporting sustained CD8 T cell proliferation during LCMV infection. In addition to lower overall effector and memory T cell responses in ECTV/LCMV co-infected mice, we also show that ECTV partial suppression of Type I IFN during LCMV co-infection results in decreased memory CD8 T cell functionality and biasing towards an effector-memory phenotype. While we observed no difference in the ability of memory cells in either group to control subsequent LCMV infection, it has been shown that central-memory are better able to control LCMV infection compared to effector-memory cells(9). Taken together with the decreased TNF production by these cells, our results suggest that ECTV co-infection results in a slight impairment of LCMV-specific immunity.

The importance of type I interferon to limit ECTV disease is well known(5, 338). It has previously been shown that antibody blockade of ECTV type I interferon-binding protein, C12R, during ECTV infection drastically reduces ECTV disease(343). Therefore, our results indicate that Type I IFN produced after LCMV infection may be overwhelming the ability of C12R to block signaling, mimicking antibody blockade. It is currently unknown which ECTV-encoded proteins provide the suppressive effects on Type I IFN production that result in attenuation and alteration of LCMV-specific CD8 T cell immunity.

We propose that the most likely candidate in this case is the ECTV dsRNA binding protein (homolog of Vaccinia virus E3L) that would be able to limit type I interferons during co-infection(5, 334, 344). The ECTV IFN-I binding protein has been shown to only block the action of mouse IFNα(5). Our results suggest there is only a decrease in systemic IFNβ, indicating a different mechanism other than ECTV expression of an IFN-I binding protein, is responsible for limiting LCMV-specific CD8 T cell immunity. Interestingly, previous reports that showed no alteration of LCMV-specific immune responses during co-infection with the orthopoxvirus, vaccinia virus, utilized the highly attenuated "Lancy" vaccine strain that has low virulence in mice(394).

The discovery of viral genomes incorporated within mammalian genomes indicates that viral families are much older than previously believed(409). The newly predicted timescale increases the frequency of potential viral co-divergence with hosts in order for the virus to survive(345). In addition, hosts that are infected with multiple viruses or host that are persistently infected and undergo additional infection with a heterologous virus could result in further viral co-evolution. The beneficial interplay via modulation of IFN-I between LCMV and ECTV suggest that there may have been co-evolution of ECTV and LCMV in wild mouse populations due to the potential increase in viral transmission of both viruses.

An important point that these studies raise is whether co-infection alters either ECTV or LCMV transmission in wild mouse populations. ECTV is suspected to be easily transmitted among naturally infected wild populations of mice(336, 339). Multiple ECTV strains with varying disease severity have been isolated from outbreaks in European and North American laboratory mouse colonies(410-412). The delayed progression of ECTV disease during LCMV co-infection that results in continued host survival may lead to greater potential for transmission of ECTV to subsequent hosts.

In addition, decreased CD8 T cell immunity and function during ECTV co-infection could also result in a decreased ability to control infection, potentially leading to increased transmission of both ECTV and LCMV. Although we observed no difference in LCMV control in this experimental setting using the acute Armstrong strain of LCMV, circulating LCMV strains in the wild also include persistent strains (396, 413, 414).

Future experiments will need to address how ECTV modulates Type I IFN during infection during co-infection with persistent strains of LCMV or in established carrier mice infected at birth with the virus. However, recent data suggest that Type I IFNs produced during later time points of persistent LCMV infection actually contribute to persistence by suppressing immune responses (398). Therefore, our results would be consistent with a more important role for ECTV suppression of LCMV Type I IFNs during co-infection resulting in early suppression of immunity. In addition, an important observation in these studies is that ECTV/LCMV co-infection alters the magnitude, function, and phenotype of CD8 T cell responses. One implication of this observation is that heterogeneity in T cell responses during vaccination and/or viral infection (415) may be in part explained by the context of other infections. This is particularly relevant for vaccination studies using recombinant orthopoxviruses as vaccine vectors for other pathogens (25), as individuals with other infections (or other vaccinations) may attenuate the effectiveness of such strategies.

CHAPTER 4

LCMV VIRUS PERSITENCE ABROGATES THE BENEFICAL EFFECT OF IFN-I INDUCTION ON DISEASE DURING ECTV CO-INFECTION

ABSTRACT

Human Immunodeficiency virus (HIV) and Hepatitis C virus (HCV) co-infection is estimated to occur in 15-30% of all HIV-infected individuals and 5-10% of all HCV-infected individuals. In the last decade, due to the success of highly active antiretroviral therapy, HIV/HCV co-infection has emerged as a major source of morbidity and mortality in HIV-infected individuals. HIV/HCV co-infection is associated with weaker HCV immune response, increased liver fibrosis, higher HCV RNA levels, and worse HCV disease progression. Thus, highlighting the potential consequence of co-infection with persistent viruses that may bi-directionally modulate viral persistence and immune responses. We have studied co-infection of mice with persistent and acute variants of Lymphocytic choriomeningitis virus (LCMV) with Ectromelia virus (ECTV). Experimental inoculation of mice with ECTV results in a lethal infection with high virus replication in the liver, due in part to a number of ECTV proteins that block the production of and signaling from Type I interferons (IFN-I). We show that ECTV co-infection of mice with persistent or acute LCMV results in differential impacts on ECTV viral load and amelioration of ECTV-induced disease. CD8 T cell responses to both acute and persistent LCMV in ECTV co-infected mice were attenuated. However, the resulting effect on LCMV viral control and pathology was different between co-infection with either acute or persistent variants of LCMV. Thus, we provide evidence for differential effects of acute versus persistent co-infection that modulate disease and immunity.

INTRODUCTION

Due to the success of highly active antiretroviral therapy in HIV-infected individuals that has decreased AIDS-related morbidity and mortality, HIV co-infection with Hepatitis virus has emerged as a major source of morbidity and mortality in HIV-infected individuals(355). Co- infection with HCV and HIV is a relatively common event, occurring in 15-30% of all HIV-infected individuals and 5-10% of all HCV-infected individuals(356, 357). Viral co-infection can occur by simultaneous exposure of two heterologous viruses, that may share the same route of transmission, or a chronically infected individual can become co-infected after independent exposure to circulating viral strains.

HIV co-infection is associated with weaker HCV immune response, increased liver fibrosis, higher HCV RNA levels, reduced response rates to anti-viral treatment, and worse HCV disease progression(356, 358). The differences in immunity and disease in co-infected individuals may be due to alterations in the intrahepatic cytokine milieu as a result of HIV infection(359, 360). Detection of cytokine mRNA in HIV/HCV co-infected patients revealed lower levels of TNF, IL-8, and IL-10 mRNA and increased levels of TGF-β compared to individuals infected with HCV alone(360). In addition, HBV co-infection has been reported to occur in up to 36% of all HIV-infected individuals in Africa, which currently accounts for over 70 percent of global HIV-infection(357, 361). HIV co-infection with HBV also correlates with higher rates of HBV persistence and increased risk of liver-related morbidity and mortality(355, 357, 362).

In order to understand the complex relationships that may occur during heterologous virus co-infection, we have studied the impact on viral persistence during co-infection of mice with ECTV and LCMV. ECTV is a DNA virus of the orthopoxvirus family and encodes a number of proteins that block the production of and signaling by Type I interferons (IFN-I)(5, 338). Experimental inoculation of mice with ECTV typically results in a lethal infection due to high virus replication in the liver and acute hepatic disease(337, 340). Acute LCMV infection of mice results in the rapid expansion of virus-specific CD8 T cells, viral control and clearance by 7 days post infection(238, 396).

Previous findings highlight the interaction between endemic but unrelated LCMV and ECTV during co-infection of mice to limit disease and immunity. Mice co-infected with the acute strain of LCMV and ECTV demonstrated reduced ECTV replication resulting in reduced disease and enhanced survival. Conversely, ECTV co-infection resulted in reduced LCMV-specific CD8 T cell responses. The data suggest that these effects are primarily due to modulation of Type I IFN levels, with primarily lower IFNβ levels in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone. In striking contrast to acute LCMV Armstrong infection, persistent LCMV clone 13 infection in C57Bl/6 mice is characterized by diminished viral control, resulting in high viral titers detectable in serum up to 60 days post infection(238, 292). This is due to, in part, dysfunctional CD8 T cell differentiation leading to exhaustion of the responding immunodominant CD8 T cells (238). In the absence of CD4 T cell help, CD8 T cell dysfunction is exacerbated, resulting in life-long chronic infection in mice with long-term elevated viral titers in serum and tissues (218). In addition, LCMV clone 13 has also been shown to produce ~4x the amount of IFNβ in the serum(398).

The marked difference between LCMV Armstrong and LCMV clone 13 infections is propagated by only three amino acid residue mutations between the two viruses(306). Two mutations occur within the viral spike GP-1 on the S segment at amino acid position 176 and 260 and one occurs within the viral polymerase on the L segment at amino acid position 1076(307) The identified mutations in the LCMV clone- 13 GP protein results in an increased affinity for the cellular receptor, α-Dystroglycan, resulting in an increase in the relative infectivity of the virus. While the mutation in the viral polymerase of LCMV clone-13 results in an increase in replication rate of the virus (308). The selection of the LCMV clone 13 variant may have been a consequence of virus-host evolution towards balanced pathogenicity, since LCMV CTL activity is critical not only for viral control, but also fatal immunopathology mediated by responding CD8 T cells after intracranial infection of adult mice. Therefore, we hypothesize that co-infection with ECTV and persistent LCMV has the potential for differential effects on disease and immunity due to differences in the speed of replication, induction of T cell exhaustion and greater initial magnitude IFN-I production.

Our results demonstrate that ECTV/CL13 co-infection of mice results in differential outcomes in T cell immunity and disease. We observed a modest 3 day increase in survival during ECTV/Clone 13 co-infection as compared to co-infection with acute LCMV presumably due to IFN-I production by LCMV before progression of ECTV-induced liver pathology. Furthermore, co-infection did not result in control of ECTV replication in the liver past 8 days post infection. We also show that ECTV co-infection results in diminished LCMV-specific CD8 T cell effector expansion and a complementary 10-fold increase in LCMV viral load. Thus, we provide experimental evidence for differential effects of acute versus persistent viruses during co-infection that modulate disease and immunity.

MATERIALS AND METHODS

Mice: 6-8 week old female C57Bl/6J mice were purchased from Jackson laboratories (Bar Harbor, Maine). P14 transgenic mice, in which CD8 T cells express TCR specific for the D^bGP33-41 epitope of LCMV, were obtained from Dr. Rafi Ahmed and bred in our animal facilities. All studies were conducted according to animal protocol 12-1229R under the approval and guidance of the Arizona State University Institute for Animal Care and Use Committee.

Cells and viruses: BHK cells were maintained in complete Eagles's MEM (5% fetal bovine serum (FBS), 2mM L-glutamine (L-Q), 100 U/mL penicillin, 100 μg/ml streptomycin). Vero and MC57 cells were maintained in complete DMEM (10% FBS, 2mM L-Q, 100 U/ml penicillin, 100 μg/mL streptomycin). LCMV Armstrong and LCMV clone-13 stocks were kindly provided by Rafi Ahmed (Emory University, Atlanta GA) and produced in BHK cells as previously described(380). The titer of LCMV stocks and mouse serum samples were determined by plaque assay on Vero cell monolayers as previously described(380). ECTV expressing the β-gal gene in the CHO locus (US17-βgal) was a gift from Dr. Mark Buller (St Louis University, St Louis MO). ECTV stocks were propagated in Vero cells as previously described(407). ECTV titers in mouse liver homogenates were determined on VERO cell monolayers. Briefly, liver samples were weighed and homogenized in PBS to 10% w/v. Vero monolayers were infected following three freeze-thaw cycles for 1 hour prior to overlay with a 1:1 ratio of 1% agarose and 2X-MEM supplemented with 10% fetal bovine serum. After three days at 37°C, a second overlay of a 1:1 ratio of 1% Agarose and 2X-MEM supplemented with 10% fetal bovine serum and X-gal (20mg/ml) was applied. Four days after the secondary overlay blue ECTV plaques were counted.

***In vivo* infections:** Unless otherwise stated, ECTV stocks were diluted to 10^6 pfu/ml in 1X PBS prior to intraperitoneal infection in a volume of 100ul, delivering a total of 10^5 pfu per mouse. LCMV Clone 13 stocks were diluted to 10^7 pfu/ml in 1X PBS prior to intravenous infection in a volume of 200ul, delivering a total of $2x10^6$ pfu per mouse. Unless otherwise indicated, co-infected mice received ECTV immediately followed by LCMV inoculation. Mice were monitored daily for clinical disease (hunched posture, ruffled fur, non-motility) and euthanized at the indicated times post infection.

Peptides: Lymphocytic choriomeningitis virus CD8 T cell epitopes GP33 (H-$2D^b$, KAVYNFATC) and NP396 (H-$2D^b$, FQPQNGQFI) were purchased from Genscript (Piscataway, NJ).

Cell surface antibody staining: Single cell suspensions were prepared from splenocytes as previously described(200). Erythrocytes were lysed with ammonium chloride lysis (ACK) buffer purchased from Lonza (Allendale, NJ) and FACS staining was done as previously described(200) in 96 well plates with fluorochrome-labeled monoclonal antibodies: anti-CD8 (clone 53-6.7), anti-CD44 (clone IM7), anti-PD-1 (clone J43), anti-CD4(clone GK1.5) and anti-CD62L (clone MEL-14) or APC labeled GP33-tetramer(238). Samples were then fixed in 1% paraformaldehyde solution and immediately acquired on a BD LSR II Fortessa flow cytometer (San Jose, CA) and analyzed using FlowJo software (Tree-Star, Ashland, OR). All surface monoclonal antibodies were purchased from BD Pharmigen (San Diego, CA) or eBiosciences (San Diego, CA).

Intracellular cytokine staining: For quantitation of LCMV-specific T cell responses, splenocytes were cultured alone or with 1mM LCMV peptide epitopes as previously described(200). After 5 hours of stimulation, cells were permeabilized and intracellular cytokine producing cells detected by staining with anti-IFNγ (clone XMG1.2) and anti-TNF (clone MP6-XT22) antibodies purchased from BD Pharmigen (San Diego, CA) or eBiosciences (San Diego, CA). The samples were acquired and analyzed as described above.

Statistics: Prism software (Graphpad, La Jolla, CA) was used to calculate t-test p values to determine significance or log-rank test to determine survival curve significance (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$).

RESULTS

Persistent variant of LCMV does not rescue mice from lethal pathology during ECTV/LCMV co-infection. The persistent variant of LCMV, LCMV clone-13, induces greater amounts of systemic IFN-I during the first 12-48 hours post infection than LCMV Armstrong(397, 398). We hypothesized that LCMV-clone 13 co-infection would be able reduce ECTV induced mortality and viral loads to the same or greater extent as co-infection with the acute variant of LCMV. 8- week old female mice were infected with ECTV alone, co-infected with ECTV and LCMV Armstrong (ECTV/ARM) or co-infected with ECTV and LCMV Clone-13 (ECTV/Cl13). Mice were evaluated for disease by monitoring physical symptoms, viral loads and survival. As expected, mice infected with ECTV alone exhibited extreme disease symptoms starting at 5 days post infection and required euthanasia by day 6 post-infection.

Mice co-infected with the acute strain of LCMV exhibited a significant delay in morbidity and complete rescue in ~70% of 8-week old mice, consistent with previous experiments. Surprisingly, 8-week old ECTV/LCMV-13 co-infected mice exhibited only a slight delay in disease progression and mortality. 100% of ECTV/LCMV-13 co-infected mice succumbed to disease on day 9 post infection (**Figure 4.1**). Lethal ECTV infection in mice is known to correlate with high viral titers in the liver resulting in in acute hepatic failure(336, 343). Furthermore, the delay in and/or amelioration of ECTV-induced disease in ECTV/ARM co-infected mice was previously found to correlate with suppression of ECTV viral replication in the liver.

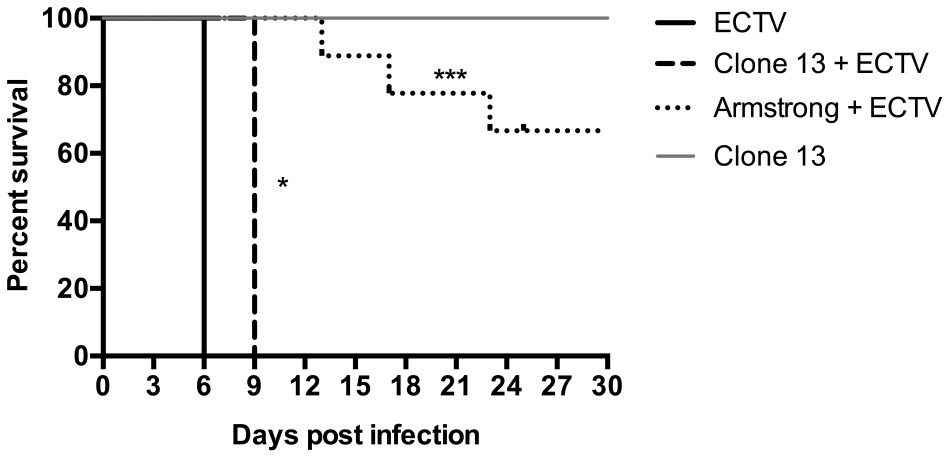

Figure 4.1: Persistent variant of LCMV does not rescue mice from lethal pathology during ECTV/LCMV co-infection. 8-week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with $2x10^6$ pfu LCMV Clone 13 iv or 10^5 pfu LCMV Armstrong ip. All mice were monitored daily for morbidity and mortality. Survival curve of ECTV infected (n=6), LCMV clone 13 infected (n=6), ECTV/ARM (n=7) co-infected, and ECTV/ CL13 co-infected (n=6).

Persistent variant of LCMV does not reduce ECTV viral load in the liver during ECTV/LCMV co-infection. In order to determine whether the mortality seen after ECTV/Cl13 co-infection was due to ECTV replication in the liver, we next measured ECTV viral titers in the liver of ECTV/Cl13 co-infected mice one day prior to evident morbidity requiring euthanasia. Mice infected with ECTV alone exhibited high liver viral titers, on average $5x10^7$ pfu/gram, one day prior to death at day 5 post-infection (**Figure 4.2, black bar**). In comparison, ECTV/Cl13 co-infected mice exhibited high ECTV titer, on average 10^7 pfu/gram, in the liver one day prior to death at day 8 post infection (**Figure 4.2, gray bar**). In contrast, ECTV/ARM co-infected mice with long-term survival had a ~1000 to 100-fold decrease in ECTV liver viral load, measured below 10^5 pfu/gram at day 8 post infection (**Figure 4.2, white bar**). Thus, suggesting that ECTV/Cl13 co-infected mice succumbed to ECTV mediated disease due to uncontrolled ECTV viral replication in the liver.

Prior investigation of ECTV co-infection with LCMV Armstrong demonstrated, in addition to suppression of ECTV replication, a bi-directional effect on LCMV immunity by suppression of LCMV-specific CD8 T cell effector expansion and bias towards $CD62L^{low}$ effector memory cell generation. Therefore, we determined if ECTV immunomodulatory proteins limit primary CD8 T cell effector expansion during ECTV/CL13 co-infection. It is unknown if T cell dysfunction observed during LCMV clone 13 infection due to viral persistence will alter the potential impact of decreased IFN-I on effector expansion.

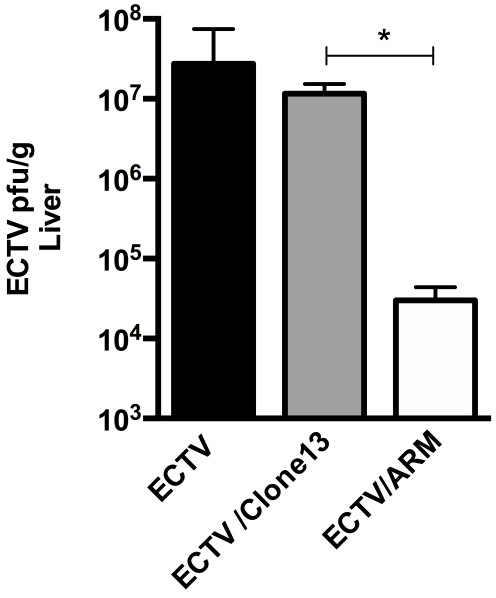

Figure 4.2: Persistent variant of LCMV does not reduce ECTV viral load in the liver during ECTV/LCMV co-infection. 8-week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with $2x10^6$ pfu LCMV Clone 13 iv. Mean ECTV viral load (pfu/gram) in the liver of ECTV infected and ECTV/Cl13 co-infected mice on day 8 indicated post infection (n=3 mice/group, 2 independent experiments).

ECTV limits LCMV-specific CD8 T cell expansion during co-infection with persistent variant of LCMV. In order to determine if ECTV/Cl13 co-infection impaired LCMV-specific CD8 T cell expansion, we compared LCMV effector CD8 T cell generation between co-infected mice and mice infected with LCMV clone 13 alone. Magnitude of the LCMV CD8 T cell response to the immunodominant GP33 epitope(238) on day 8 post Clone 13 infection of mice infected with LCMV alone consisted of on average of 6 $x10^5$ cells in the spleen (**Figure 4.3, black bar**). As expected, ECTV/13 co-infection decreased the LCMV response by over 2-fold, LCMV GP33 epitope-specific CD8 T cells comprised on average $<3x10^5$ cells in the spleen (**Figure 4.3, white bars**). Thus, suggesting that ECTV suppression of Type I IFN during LCMV Clone 13 infection is significant enough to limit LCMV-specific CD8 T cell effector expansion.

However, the significant decrease in effector cell expansion observed after acute LCMV infection did not alter LCMV Armstrong viral control or the protective capacity of immunized mice to limit LCMV clone 13 dissemination after clone 13 challenge. Although, the ability of co-infected mice to protect against LCMV clone 13 challenge may have been due to proliferation of central memory CD8 T cells and/or the presence of LCMV-specific CD4 memory T cells. Therefore, we hypothesize that the significant reduction in the primary LCMV effector response during LCMV clone 13 infection will also result in decreased control of LCMV replication.

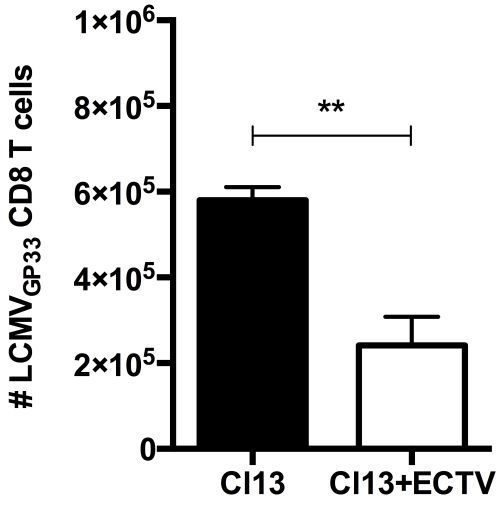

Figure 4.3: ECTV limits LCMV-specific CD8 T cell expansion during co-infection with persistent variant of LCMV. 8-week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with $2x10^6$ pfu LCMV Clone 13 iv. Mean total number of LCMV-specific (GP33 epitope) CD8 T cells in the spleen on day 8 post infection (n=3-5 mice/group).

ECTV co-infection increases LCMV clone 13 viral load. In order to test whether the reduced expansion of LCMV CD8 T cell effectors in ECTV/Cl13 co-infected mice impacted the ability of these mice to limit overwhelming LCMV viral loads, we measured virus load in the serum of co-infected and Clone 13 infected mice. Naive mice infected with LCMV clone-13 are unable to control LCMV replication and present with viral titers around (~10^5 pfu/ml) in the serum at day 8 post-infection (**Figure 4.4, black bar**). As expected, ECTV/CL13 co-infected mice had 10-fold higher LCMV viral titer, greater than 10^6 pfu/ml, in the serum at day 8 post infection (**Figure 4.4, white bar**). Thus indicating, ECTV/Cl13 co-infection impacts not only LCMV- specific CD8 T cell response, but also initial LCMV replication.

IFN-I signaling can induce expression of hundreds of inflammatory genes resulting in pleiotropic effects on multiple cellular processes(37, 40). In some settings of persistent viral infection, IFN-I has sustained production that can result in lymphoid tissue disorganization(398). IFN-I levels in LCMV clone 13 infected mice are consistently higher than in LCMV Armstrong infected mice. LCMV clone 13 has been shown to produce ~4x the amount of IFNβ in the serum(398). Although, IFN-I production during LCMV clone 13/ECTV co-infection did not substantially increase mouse survival or limit ECTV viral replication. However, ECTV/Cl13 co-infection did reduce LCMV immunity, suggesting there is a significant reduction in IFN-I in ECTV/Cl13 co-infected mice. Additionally, blockade of IFN-I signaling with antibodies against IFNAR-/- can result in restoration of splenic architecture and an increase in viral control of Clone 13 infected mice(398). Therefore, we hypothesized that infection of persistently infected LCMV clone 13 mice with ECTV could potentially increase viral control due to production of ECTV immunomodulatory proteins that block IFN-I signaling if sustained IFN-I was great enough to limit overwhelming ECTV replication and disease.

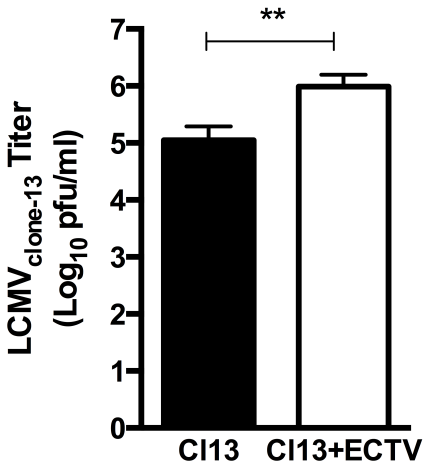

Figure 4.4: ECTV co-infection increases LCMV clone 13 viral load. 8-week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with $2x10^6$ pfu LCMV Clone 13 iv. Mean LCMV viral load (pfu/mL) in the serum of LCMV clone 13 infected and ECTV/Cl13 co-infected mice on day 8 indicated post infection (n=3 mice/group, 2 independent experiments).

Sustained IFN-I production in persistently infected mice does not limit ECTV replication or disease after exposure. In order to test the therapeutic potential of ECTV infection in persistently infected mice, C57Bl/6 mice persistently infected with LCMV clone 13 (30 days prior) were co-infected with 10^2 pfu ECTV ip. All mice were monitored for physical symptoms, viral load and mouse survival. LCMV clone 13 infection in naïve mice results in sustained viral titers up to 60 days post infection, but it does not cause overwhelming pathology due to exhaustion of the responding T cells. Surprisingly, age matched naive mice infected intraperitoneal with 100 pfu ECTV succumbed to ECTV mediated disease at day 8 post ECTV exposure. Thus, highlighting the virulence of ECTV infection given intraperitoneally in C57Bl/6 mice. LCMV clone 13 persistently infected mice also succumbed to disease 8 days post infection ETCV infection. Indicating that the sustained IFN-I produced during clone 13 infection is not great enough to limit ECTV replication and promote survival.

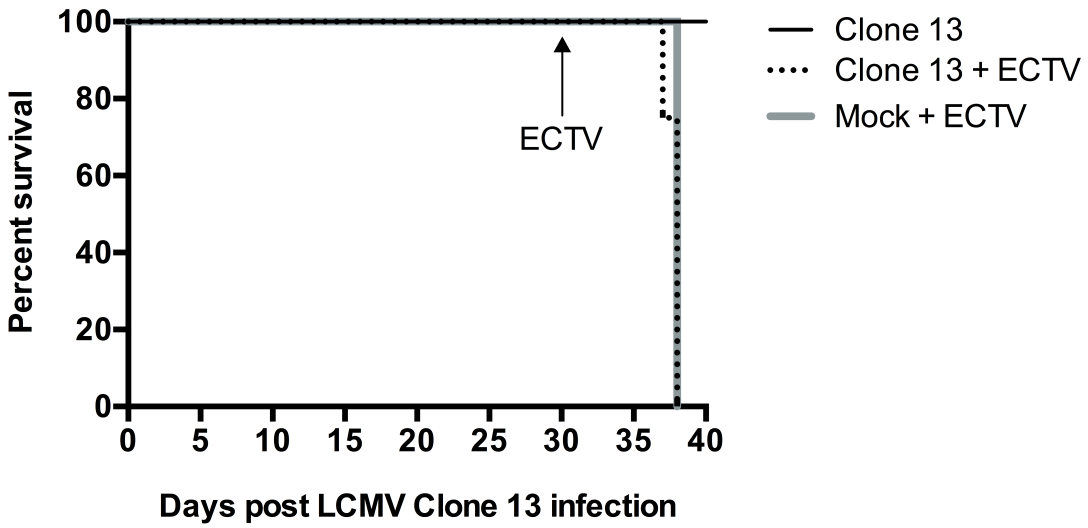

Figure 4.5: Sustained IFN-I production in persistently infected mice does not limit ECTV replication or disease after exposure. C57Bl/6 mice persistently infected with LCMV clone 13 (30 days prior) were co-infected with 10^2 pfu ECTV ip. All mice were monitored for morbidity and mortality. Percent survival of age matched mice infected with 100 pfu ECTV and LCMV clone 13 persistently infected mice co-infected with 10^2 pfu ECTV.

DISCUSSION

Our results highlight the potential consequence of virus-virus co-infection that can result in modulation of T cell immunity and influence disease. However, the ability of the secondary virus to persist in the host dramatically alters the bi-directional affects on immunity and pathology. Mice co-infected with acute LCMV and ECTV demonstrate reduced ECTV replication, resulting in rescue of disease, and enhanced survival. Conversely, mice co-infected with the persistent strain of LCMV and ECTV could not control ECTV replication and perished by 9 days post infection. ECTV viral load in the liver of ECTV/CL13 co-infected mice was of equal magnitude to ECTV viral load in the liver of mice infected with ECTV alone (one day prior to onset of morbidity requiring euthanasia). Maximum viral load was not reached until day nine, resulting in a modest three-day increase in survival prior to fatal disease progression.

One similar outcome of co-infection between acute and persistent strains of LCMV and ECTV was ECTV modulation of Type I IFN production resulting in attenuation of LCMV-specific CD8 T cell effector expansion. The dramatic difference in viral dissemination between Armstrong and Clone 13 due to two amino acid mutations increasing replication rate and viral tropism does not impact the sequence of T cell epitopes presented to virus-specific T cells(276). Therefore, the observed decrease in effector expansion was not unexpected, because LCMV responses are known to be dependent on direct signaling via Type I IFN for sustained proliferation(60, 399). However, the attenuation in the primary response to acute LCMV did not alter initial viral control, nor the protective capacity of LCMV-specific, effector memory biased, CD8 T cells to persistent viral challenge.

The ability of ECTV/ARM co-infected mice to protect against LCMV clone 13 challenge may have been due to proliferation of the small proportion of central memory CD8 T cells and/or the presence of LCMV-specific CD4 memory T cells. On the other hand, attenuation of naïve-originating LCMV-specific CD8 T cell effector expansion by ECTV during persistent LCMV infection results in 10-fold greater LCMV viral load in the serum prior to mice succumbing to liver pathology.

Prior investigation into the mechanism in which LCMV Armstrong influences ECTV replication and disease determined that systemic IFN-I levels changed during co-infection. Serum levels of total IFNα and IFNβ in ECTV infected mice were undetectable, presumably due to the action of ECTV immunomodulatory viral proteins, such as E3L and IFN-I binding protein, that effectively shut down Type I IFN production(336). In contrast, ECTV/ARM co-infection resulted in an increase in serum levels of both IFNα and IFNβ, compared to mice infected with ECTV. However, Serum IFNβ was found to be significantly lower in ECTV/ARM co-infected mice compared to mice infected with LCMV Armstrong alone. LCMV clone 13 has been shown to produce ~4x the amount of IFNβ in the serum(398). Although, the presumed increase in IFN-I during LCMV clone 13 co-infection did not increase survival or limit ECTV viral replication.

However, ECTV/Cl13 co-infection did reduce LCMV immunity, implicating there is a significant reduction in IFN-I in co-infected mice. Additionally, ECTV/Cl13 infected mice had 10-fold greater LCMV viral load in the serum. It is unknown whether the increase in LCMV replication and decrease in viral control was due to lower magnitude T cell responses or if altered IFN-I production between 12-24 hours significantly impacts control of LCMV clone 13, more so than LCMV Armstrong.

Although, the three-day increase in survival in ECTV/Cl13 co-infected mice could be due to the anti-viral effects of Type I interferon during peak induction, between 12-24 hours post infection, initially limiting ECTV replication.

The use of poxvirus vectors to decrease IFN-I signaling in a setting of persistent viral infection that has detrimental sustained IFN-I production is inconclusive. Mice persistently infected with LCMV clone 13 thirty days prior to ECTV exposure succumbed to ECTV mediated disease. Indicating that the sustained IFN-I produced during clone 13 infection was not high enough to overcome lethality of ECTV infection. Mortality due to ECTV viral replication did not allow the determination of the therapeutic potential of ECTV immunomodulatory proteins to limit IFN-I produced during persistent LCMV infection. Future experiments should investigate if less pathogenic ECTV mutants can mediate blockade of IFN-I, without leading to overwhelming pathology in the liver.

Persistent viral infection is characterized by chronic antigen stimulation and persistent inflammation due to continued viral replication over time(234, 237). Under conditions of chronic antigen stimulation, CD8 T cells do not undergo traditional differentiation into functional memory cells(232, 238). There are multiple alterations to the T cell response that occur under situations of chronic antigen stimulation such as T cell localization, functionality, and breadth of response(232, 239). It is unknown at this time how the combination of normal T cell dysfunction, due to increased antigen stimulation, and the attenuation of the CD8 T cell response, due to ECTV alteration of IFN-I production and signaling, is responsible for the decrease in LCMV immunity and viral control during co-infection.

Future studies should investigate if the decreased effector expansion of LCMV specific T cells due to IFN-I modulation affects the level of exhaustion of those cells resulting in decreased viral control. Particularly in settings when the precursor frequency of antigen-specific memory cells can induce increased pathology, due to partial T cell exhaustion(239). The results presented above demonstrate an additional consequence between disease outcomes due to persistent versus acute viral infections.

CHAPTER 5

DISCUSSION

Through the use of mouse-models of acute and persistent virus infection, we have investigated how both virus and immune system variables influence pathogenic versus protective CD8 T cell responses generated during viral infection or after vaccination. In addition, an interdisciplinary collaboration with mathematical modelers has allowed us to provided experimental evidence that validate quantitative mathematical models of viral infection and immune response, which predict the molecular and cellular pathways that mediate T cell protection versus pathology. We have examined the variations in CD8 T cell immunity and disease that may occur during the context of heterologous viral co-infection and we have shown another consequence of viral persistence in the context of viral co-infection. We have shown antigen persistence influences the interplay between two viruses that result in modulation of immune response and disease during co-infection.

Our results have furthered our understanding of potential vaccine-mediated increase in disease, due to variations in antigen-specific T cell precursor frequency that changes the threshold of T cell exhaustion of responding cells. Furthermore, Type I interferon produced during acute viral co-infection can overcome poxvirus immunomodulatory proteins, enhancing survival in co-infected mice. Additionally, viral persistence eliminates the beneficial effects of co-infection between LCMV and ECTV, highlighting the immense difference in T cell responses during persistent versus acute infection.

Immune memory was utilized to decrease disease burden in humans long before the adaptive immune cells responsible for providing the observed protection from subsequent exposure were discovered and meticulously evaluated(1, 2). One of the most relevant and impactful contributions of immunology to human health is the ability to utilize immune memory for the design of vaccines against infectious diseases(7, 10). Expanded vaccination coverage has significantly enhanced our ability to limit or prevent human diseases caused by infectious pathogens(12, 13). Vaccines designed against smallpox, polio and measles are excellent examples of the remarkable impact vaccination can make towards the reduction of the global disease burden(1, 14). Although, the extensive study and characterization of pre-programmed immune cells that respond faster and with greater magnitude to secondary infection to limit disease(7-9), has not facilitated the elimination of all infectious pathogens that cause premature death and disease in humans(11). In 2013, four of the top ten leading global causes of premature death in humans are diseases were due to infectious pathogens(11).

The failure to design a vaccine to prevent HIV/AIDS in humans is leading example of the need to continue to dissect the intricacies of immunity to persistent viral infection. In 2013, 35 million people were estimated to be living with HIV resulting in an estimated 1.5 million deaths due to HIV-related causes(11). Effective antiretroviral therapy has increased the quality and longevity of life in HIV-infected individuals(20). However, in 2013, 2.1 million people worldwide became newly infected with HIV(11), emphasizing the need for a vaccine that prevents infection in order to eliminate HIV/AIDS in humans(21).The traditional approach in order to design a vaccine against HIV has been challenging (17, 21-23).

Therefore, vaccination for T cell responses using recombinant vectors has been proposed as an alternative strategy to elicit immune responses and continues to be the focus of future vaccine candidates(21, 24, 25). However, our data suggest that vaccines designed to only generate T cell responses for persistent viral infections known to induce T cell exhaustion, may result in unintended increases in disease due to partial T exhaustion of responding memory CD8 T cells.

Initial studies have been able to model increased pathology during persistent infection due to changes in antigen-specific CD8 T cell precursor frequency. However, adoptive transfer of naïve transgenic CD8 T cells to study potential vaccine induced pathology is limited by the use of naïve CD8 T cells and transgenic T cells specific for only one epitope of LCMV. The adoptive transfer of naïve T cells is a limitation because vaccination induces the formation of memory CD8 T cells that differ from naïve CD8 T cell subsets. Memory CD8 T cells undergo additional changes in phenotype, function and gene expression during differentiation 1-2 months after infection. This results in the establishment of a pool of antigen-specific T cells that can be maintained without continued antigen stimulation(9).

Memory CD8 T cells can rapidly respond to secondary infection primarily due to a simple increase in precursor frequency of antigen-specific cells and the retained potential to rapidly kill infected cells(223). Memory CD8 T cells can release perforin/granzyme B and produce large amounts of IFNγ and TNF after exposure to an antigen without the activation and proliferation via traditional stimulation from APCs in draining lymph nodes(9, 211, 224). These differences enable memory CD8 T cells to respond more rapidly to subsequent infections.

Previous studies regarding immunopathology during persistent LCMV infection have not compared the ability of naïve and memory CD8 T cells to mediate similar enhancement of pathology during responses to persistent LCMV infection. In addition, the previously developed mathematical model of LCMV infection in mice has generated predictions about which virus and host immune parameters can differentially affect pathology versus protection during persistent viral infection. The model predicts that increased sensitivity to viral infection by memory CD8 T cells should not change the potential for maximum disease, due to an intermediate precursor frequency, because simulations generated from the model are relatively insensitive to the parameter that describes sensitivity. We have tested this prediction and our results indicate that CD8 T cell pathology, due to an intermediate precursor of antigen-specific CD8 T cells, is largely independent of CD8 T cell sensitivity. Morbidity and mortality after adoptive transfer of an intermediate number of transgenic memory antigen-specific or transgenic naïve-antigen specific CD8 T cells was indistinguishable.

However, qualitative differences between the location of pathology indicate the kinetics of the response to virus (influenced by sensitivity) do have a marginal impact. Memory CD8 T cells resulted in pathology contained to the liver, whereas naïve CD8 T cells induced maximum pathology in the lungs. Since memory CD8 T cells respond rapidly upon secondary infection, they may traffic to the liver, an early site of viral replication. Whereas naïve cells may traffic to the lungs, a secondary site of viral replication(273).

Although in either scenario maximum pathology is due to an intermediate number of antigen-specific cells, regardless of antigen-experience. Mice that had high numbers of memory CD8 T cells prior to high dose viral infection did not develop immunopathology because the magnitude of the response was great enough to control viral replication by 8 days post infection. Additionally, pathology did not occur from low precursor frequency of memory LCMV specific T cells prior to LCMV clone 13 infection.

Analysis of T cell persistence and functional capacity support the hypothesis that T cell exhaustion is a mechanism during persistent viral infection to avoid pathology, since responding T cells lose the ability to proliferate and produce inflammatory cytokines such as TNF. Mice that had low numbers of memory CD8 T cells or no memory CD8 T cells prior to high dose infection did not develop immunopathology because the responding CD8 T cells were highly exhausted and unable exert effector function. Whereas mice that had an intermediate number of memory CD8 T cells prior to high dose viral infection developed severe pathology due to partial T cell exhaustion of the response resulting in ongoing high viral titers and pathology presumed due to continued production of TNF.

However, when the initial viral dose decreases by one log, lower numbers of memory CD8 T cells became only partially exhausted, leading to an increase in pathology at a low dose of cells similar to mice receiving intermediate numbers of CD8 T cells after high dose infection. Decreasing the initial amount of stimulation provided to the T cells, by varying the magnitude of viral infection, alters the frequency at which the responding T cells can result in pathogenic responses. These results are consistent with published data indicating that T cell exhaustion may be a mechanism to limit immunopathology of naïve CD8 T cells(275, 384). Increase in pathology can occur due to partial exhaustion of the T cell response, as mediated by initial viral dose.

Our experimental results expand on these findings, indicating there is a fine balance between the magnitude and functionality of the memory CD8 T cell response in addition to initial viral dose (**Figure 5.1**). Although, the use of transgenic memory T cells specific for one LCMV epitope can limit the investigation of T cell pathology to only very narrow T cell responses to viral infection.

Initial studies by Oehen *et al*, demonstrated vaccination with recombinant vaccinia virus vectors expressing LCMV protein antigens resulted in increased pathology during subsequent infection with LCMV, compared to non-vaccinated animals. One main hypothesis from these studies was that T cell mediated immunopathology was a result of the limited breadth of the CD8 T cell response after vaccination using recombinant vectors(303). However, the developed quantitative model predicts that increasing the breadth of the CD8 T cell response should have no impact on resulting immunopathology. Our data showed the increase in immunopathology resulting in mortality, due to transfer of polyclonal LCMV-specific memory cells prior to LCMV clone 13 challenge, was indistinguishable from survival data generated after transfer of an intermediate number of monoclonal-transgenic memory P14 cells. Therefore, TCR diversity does not eliminate the potential for pathology due to partial exhaustion of responding CD8 T cells.

The observed increase in disease from varying precursor frequency of polyclonal or monoclonal memory LCMV specific CD8 T cells validate the mathematical model prediction that the breadth of the T cell response induced by vaccination is independent of the observed pathology. Thus, the breadth of the T cell response has no impact on the dose of cells that cause maximum pathology. Furthermore, generation of LCMV viral variants that evade T cell recognition were not observed after high dose memory transfer (polyclonal or transgenic p14).

Indicating that immune responses generated from memory CD8 T cells are superior in virus control due to the absence of variant escape viruses. Presumably due to the kinetics of the response, indicating memory T cells can control viral loads before selective pressure results in an escape mutant.

Our results indicate a wide range of precursor frequencies could potentially cause pathology depending on viral dose of subsequent exposure. Therefore, the strength of the vaccine response for an individual may or may not be pathogenic depending on the individual's subsequent viral exposure. In order to avoid potential vaccine induced pathology the CD8 T cell response must be above a critical threshold to limit pathology due to early viral control. In addition, vaccines that only elicit one arm of the immune system should be avoided over vaccine candidates that also stimulate antibody production and CD4 T cell help. Since the combination of responding immune cells may influence early viral control decreasing the chance of pathology at a wider range of precursor frequency.

In addition, pathology was observed to be dependent on TNF production by responding CD8 T cells. However, abrogation of TNF signaling did not alter the capacity for effector responses originating after high dose transfer to protect from persistent viral infection. Implicating that blockade of TNF could be a potential treatment for T cell pathology during persistent infection that would not severely impact the ability of T cells to mediate viral control. Our results will provide empirical refinements of the developed mathematical model of LCMV infection and vaccine-induced T cell responses. The refined model may then be used to make predictions about vaccine-induced pathology that might occur during immunization against other pathogens, in particular with vaccines that employ T-cell epitope based approaches.

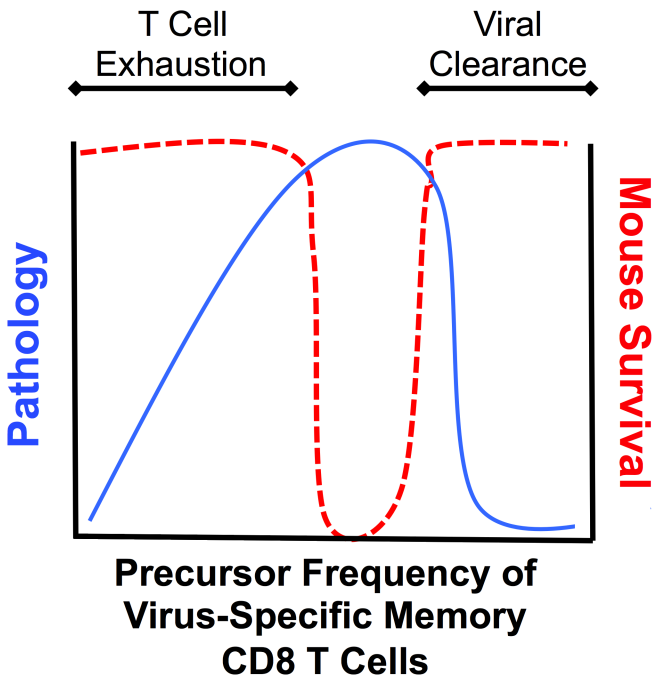

Figure 5.1: Model of vaccine-induced immunopathology due to intermediate CD8 T cell immunity levels prior to persistent viral challenge. Virus-specific memory CD8 T cells primed by vaccination can induce unintended pathology during persistent viral challenge due to high initial viral load and partial exhaustion of CD8 T cells. Thus resulting in greater cytokine production and a corresponding increase in disease compared to naïve infected or low precursor frequency infected individuals that undergo greater T cell exhaustion and loss of effector function. High precursor frequency of virus-specific memory CD8 T cells does not cause disease due to early viral control resulting in contraction of T cell response prior to unintended tissue damage.

The majority of mouse models of persistent viral infection have used a reductionist approach to understand virus-host interactions in which specific cell populations and proteins are classified and dissected to determine their relative impact on the immune response. Therefore, the majority of microbiological research is performed in specific-pathogen-free, clean, and controlled environments that limit potential co-infection by heterologous pathogens. However, most “real-world” infections likely occur in the context of co-infection by heterologous pathogens or host microbiota(346) Viral co-infection can occur by simultaneous exposure of two heterologous viruses that may share the same route of transmission or chronically infected individuals can become co-infected after independent exposure to circulating viral strains. Our results indicating there is a fine balance between pathogenic and protective T cell responses during viral infection, which is dictated by the magnitude and functionality of the memory CD8 T cell response similarly disregarded the potential impact of immune modulation due to heterologous co-infection.

However, HIV co-infection with Hepatitis virus has emerged as a major source of morbidity and mortality in HIV-infected individuals(355). Co-infection with HCV and HIV is a relatively common event occurring in 15-30% of all HIV-infected individuals and 5-10% of all HCV-infected individuals(356, 357). HIV co-infection is associated with weaker HCV adaptive immune responses, increased liver fibrosis, higher HCV RNA levels, reduced response rates to anti-viral treatment, and worse HCV disease progression(356, 358). The differences in immunity and disease in co-infected individuals may be due to alterations in the intrahepatic cytokine milieu as a result of HIV infection(359, 360).

Our results demonstrate interaction between endemic but unrelated viruses during co-infection of mice may limit disease and immunity. Mice co-infected with LCMV and ECTV demonstrated reduced ECTV replication resulting in reduced disease and enhanced survival. Conversely, ECTV co-infection resulted in reduced LCMV-specific CD8 T cell responses. The data suggest that these effects are primarily due to modulation of Type I IFN levels, with primarily lower IFNβ levels in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone. Our results implicate Type I IFN production and/or signaling as the main mechanism by which LCMV suppresses ECTV replication and disease in ECTV/LCMV co-infected mice. LCMV infection post ECTV exposure was likely ineffective as ECTV proteins have effectively shut down Type I IFN signaling(336, 341). Although, treatment with IFN-I prior to 24 hours post ECTV exposure may allow for prophylactic IFN-I induction by LCMV.

Our results also show that ECTV modulation of Type I IFN production during ECTV/LCMV co-infection attenuates LCMV-specific CD8 T cell responses that are dependent on direct signaling via Type I IFN for sustained proliferation. Although we found minimal differences in IFNα in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone, we did observe significantly and consistently lower levels of systemic IFNβ in co-infected mice. Therefore, our data suggests that IFNβ, rather than IFNα, plays a larger role in supporting sustained CD8 T cell proliferation during LCMV infection. We also show that ECTV partial suppression of Type I IFN during LCMV co-infection results in decreased memory CD8 T cell functionality and biasing towards an effector-memory phenotype (**Figure 5.2**).

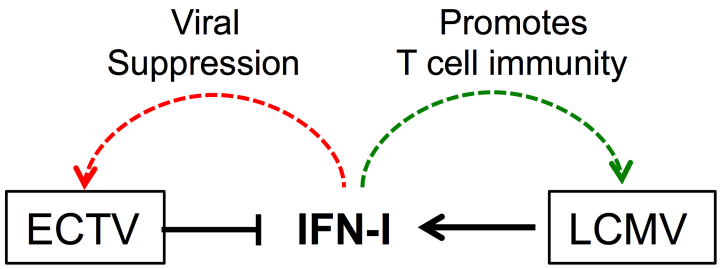

Figure 5.2: Bi-directional effects of viral co-infection on disease and immunity. Mice co-infected with LCMV and ECTV demonstrated reduced ECTV replication resulting in reduced disease and enhanced survival. Conversely, ECTV co-infection resulted in reduced LCMV-specific CD8 T cell responses. The data suggest that these effects are primarily due to modulation of Type I IFN levels, with primarily lower IFNβ levels in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone. Our results implicate Type I IFN production and/or signaling as the main mechanism by which LCMV suppresses ECTV replication and disease in ECTV/LCMV co-infected mice.

While we observed no difference in the ability of memory cells in either group to control subsequent LCMV infection, it has been shown that central-memory are better able to control LCMV infection compared to effector-memory cells(9). Taken together with the decreased TNF production by these cells, our results suggest that ECTV co-infection results in a slight impairment of LCMV-specific immunity.

The critical finding of these studies is that ECTV/LCMV co-infection alters the magnitude, function, and phenotype of CD8 T cell responses. One implication of this observation is that heterogeneity in T cell responses during vaccination and/or viral infection(415) may be in part explained by the context of other infections. This is particularly relevant for vaccination studies using recombinant orthopoxviruses as vaccine vectors for other pathogens(25), as individuals with other infections (or other vaccinations) may attenuate the effectiveness of such strategies. Furthermore, extension of this model to compare the effect of viral immunomodulatory proteins altering the cytokine milieu during co-infection with persistent strains rather than acute strains highlight additional consequences of viral persistence on immunity.

Our results demonstrate that ECTV/CL13 co-infection of mice results in differential outcomes in T cell immunity and disease. Persistent Clone 13 co-infection with ECTV induced only a marginal increase in survival, as compared to complete rescue in 70% of older Armstrong co-infected mice. Indicating there may be a lower threshold for ECTV-replication mediated liver pathology. This may be due to persistent LCMV Clone 13 in the liver also driving liver pathology, due to responding LCMV-specific CD8 T cells. We also show that ECTV co-infection with persistent LCMV results in diminished LCMV-specific CD8 T cell effector expansion. However, the attenuation in the primary response to acute LCMV did not alter initial viral control, nor the protective capacity of LCMV-specific, effector memory biased, CD8 T cells to persistent viral challenge.

Although, the ability of ECTV/ARM co-infected mice to protect against LCMV clone 13 challenge may have been due to proliferation of the small proportion of central memory CD8 T cells and/or the presence of LCMV-specific CD4 memory T cells.
On the other hand, attenuation of naïve-originating LCMV-specific CD8 T cell effector expansion by ECTV during persistent LCMV infection results in 10-fold greater LCMV viral load in the serum prior to mice succumbing to liver pathology.

Together our data provide experimental evidence for differential effects of acute versus persistent viruses during virus infection and viral co-infection that modulate disease and immunity. Intermediate precursor frequency of virus-specific memory CD8 T cells prior to LCMV infection resulted in maximum T cell mediated pathology and the threshold for exhaustion of responding CD8 T cells ultimately influenced the precursor frequency that causes enhanced disease. Furthermore, we also show that ECTV co-infection with persistent LCMV results in diminished LCMV-specific CD8 T cell effector expansion. Thus, suggesting viral co-infection may influence pathogenic and protective T cell responses that are dictated by the magnitude and functionality of the memory CD8 T cell response. Furthermore, co-infection could either abrogate pathology, via reduction of the magnitude of an intermediate response, or induce pathology, via reduction of the magnitude of a high precursor response of antigen-specific memory CD8 T cells.

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