Immunological basis of age-related vulnerability to viral infection

by

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Abbreviations

APC- Antigen presenting cell

B6- C57BL/6

BCR- B cell receptor

C'- Complement

CNS- Central Nervous system

DC- Dendritic cell

dLN- Draining lymph node

DV- Dengue virus

FCM- Flow Cytometry

FFU- Focus forming units

GrB- Granzyme B

GST- glutathione transferase tag

ICCS- Intracellular cytokine staining

IFN- Interferon

IFNα– Interferon alpha

INV- Inactivated virus vaccine

IP- Intraperitoneal injection

IRF3- Interferon regulatory factor 3

JEV- Japanese Encephalitis virus

LAV- Live attenuated virus

MOI- Multiplicity of infection

MST- Mean survival time

NS#- Non-structural protein

PBMC- Peripheral blood mononuclear cells

PFU- Plaque forming units

PIV- Pseudo infectious virus

pMHC- Peptide-Major Histocompatibility Complex

RAG- recombination activating gene

sem- Standard error of the mean

sq- Subcutaneous injection

SVP- Sub-viral particle

TCM- Central memory T cells

TCR- T cell receptor

TEM- Effector memory T cells

Tet- Tetramer

TYT-1- N'-(1,1-dioxido-2-phenyl-1,4,2-dithiazolidin-3-ylidene)-N,N-diphenylthiourea

VACV- Vaccinia virus

WNE- West nile virus encephalitis

WNV- West Nile virus

YF- Yellow Fever virus

Abstract

    West Nile virus is an arthropod borne virus that can cause severe central nervous
system diseases including meningitis and encephalitis. This emerging infectious disease
was introduced into North America in 1999 and has since spread throughout the
Americas causing thousands of cases of disease. Severe West Nile virus (WNV) disease
disproportionately affects the elderly population. This dissertation describes a novel
model of WNV infection in aged mice that was developed to study the age-related factors
involved in the increased susceptibility of the elderly to WNV. Using this model we have
been able to show that aged mice are more susceptible to WNV infection and that
deficiencies in the antigen-specific T cell response play a role in this susceptibility. We
have characterized the impact of two molecular defects within the antigen-specific T cell
compartment that lead to the increased susceptibility of old mice to WNV encephalitis.
We have identified defects in production of both Interferon gamma and Granzyme B
within WNV-specific T cells. We have also compared and contrasted our model of
immune senescence in old mice infected with WNV with the effects of immune
senescence on old mice infected with Vaccinia virus.

    During the development of this model we were able to identify WNV-encoded
MHC class I and class II-restricted CD8 and CD4 T cell epitopes and characterize the
functional epitope-specific T cell response that is elicited in WNV infected C57BL/6
mice (H-2^b haplotype). These newly described epitopes are currently being used to
characterize the development and maintenance of memory T cells in old and adult mice
as well as evaluate novel vaccine vectors and treatments designed to reverse the
susceptibility of old mice to serious West Nile virus infection.

The experimental results described herein are divided into seven chapters, whose main points are outlined below:

Chapter One: Introduction and Background

Chapter Two: Protection of immunocompetent mice from WNV disease requires naive CD8 T cell lymphocytes. Additionally, antigen-specific CD8 T cells can protect mice from the development of severe WNV disease.

Chapter Three: CD4 T cells protect against West Nile virus infection by direct anti-viral cytotoxicity and cytokine secretion.

Chapter Four: The discovery of functional defects in antigen-specific T-cells in old mice leading to age-related susceptibility to severe WNV disease.

Chapter Five: Contrasting the impact of immune senescence on old mice infected with either WNV or VV

Chapter Six: T cell memory develops after the acute response to WNV infection and is maintained for the life of the animal.

Chapter Seven: Analysis of protective T cell responses in adult and old mice generated by several vaccination approaches.

Thesis Summary

A protective immune response relies on a tightly controlled set of events which include pathogen recognition, activation of appropriate cellular immune responses, removal or control of the infectious agent, and silencing of the immune system. This response relies upon what is traditionally called the two arms of the immune system, the innate and adaptive, with each arm of the immune system having different responsibilities but still work together in an orchestrated manner. During the aging process both arms of the immune system begin to deteriorate, leading to a breakdown in the protective capacity of the immune system. The cumulative effects of aging on the immune system are referred to as immune senescence. Immune senescence results in increased frequency and severity of infectious diseases in the elderly.

Immune senescence consists of changes at the cellular and molecular level. As we age, there are increased serum levels of inflammatory cytokines such as IL-6 and TNFα that alter the environment in which immune responses occur. At the cellular level there is a decrease in proportion of naïve lymphocytes that results in a narrowing of the T cell receptor repertoire. The remaining antigen-specific memory T cell population also undergoes changes at the molecular level, exhibiting reduced secretion of effector cytokines such as IL-2 and IFN-γ. Lastly, the absolute landmarks of T cell aging are defects in proximal TCR signaling and IL-2 production by naïve T cells. However, many of these defects have only been documented using *in vitro* systems or transgenic mouse models, contributing to the large number of conflicting reports. Very few studies have used live pathogens or have been able to correct specific defects in vivo to show the relative contribution of the immune defects to immune senescence.

One pathogen that disproportionately impacts the elderly population is WNV. WNV was recently introduced into the Western hemisphere and since then has rapidly spread across North and, subsequently Central and South America, becoming a major public health problem. WNV can cause two types of severe disease, West Nile virus fever and West Nile virus encephalitis (WNE), both of which primarily occur in the elderly population. This thesis describes the development of a novel and biologically relevant mouse model, which can be used to study both WNV pathogenesis and the impact of immune senescence. Several features distinguish this model. First, this model uses primary WNV isolates derived from infected birds found within the United States and the isolated viruses were shown to cause human disease. Second, the use of large numbers genetically identical old and adult mice allow for a directed study of specific mechanisms of immune senescence without the confounder of genetic diversity. Lastly, WNV infection of mice can lead to a self-limiting disease or to a lethal encephalitis depending on the initial dose given. Altering the dose allows us to specifically focus on either the development of a protective immune response occurring with a low dose infection or on the resulting pathology and the inadequate immune response seen in a high dose infection.

We started our studies by investigating the role of naive CD8 T cells in controlling disease caused by the lineage I strains of WNV. Indeed, numerous mechanisms have been implicated in *in vivo* protection against WNV (type I interferons, complement, antibodies, etc.), but the relative importance of each one of them remains unclear. In Chapter 2, we addressed this issue by showing that the transfer of highly enriched naïve CD8 T cells protects the majority of alymphoid mice against lethal WNV

infection. To substantiate and expand this finding we have defined the peptide-specificity of the CD8 response in H-2^b mice. We then used a panel of identified peptides to map one dominant (NS4b$_{2248-2256}$) and several subdominant epitopes. Hierarchy of these epitopes was stably maintained in the memory responses and during re-infection. Importantly, CTL lines directed against these peptides conferred protection against lethal WNV infection in direct proportion to the epitope immunodominance. These results provided a platform for future characterization of T cell responses against WNV and demonstrated for the first time that CD8 T cells can single-handedly protect from this disease.

We next investigated the role of naive CD4 T cells during primary infection. In Chapter 3, we show that the transfer of highly enriched naive CD4 T cells protects the majority of alymphoid mice against lethal WNV infection. To define the functional requirements of the responding CD4 T cells, we have defined the peptide specificity of the CD4 response in H-2^b mice using a panel of peptides to map two dominant epitopes (ENV$_{641}$ and NS3$_{1616}$) and several subdominant epitopes covering approximately sixty percent of the total CD4 T cell antigen-specific response. We used these epitopes to map the functionality of the CD4 antigen-specific population in vivo. The CD4 T cells respond in a strong Th1 fashion and we clearly show that in addition to producing IL-2, and IFN-γ, these cells can also be cytolytic *in vivo*. These same epitopes conferred protection from a lethal WNV challenge of immunocompetent mice after peptide vaccination, further confirming our epitope mapping findings. These results show the importance of naive CD4 T cells in preventing severe WNV disease *in vivo*.

In Chapter 4, we describe the underlying immunological basis of increased susceptibility of old mice to severe WNV. In a mouse model of age-related vulnerability to WNV we demonstrate that viral spread and titers were significantly higher in old compared to adult mice, indicating that a loss of protective immune mechanisms required for virus control occurs during aging. This loss of viral control is due defects in both the quantity and the quality of the CD4 and CD8 T-cell response against dominant WNV epitopes exhibited by decreases in the frequency and amount of cytokine and lytic granule production. Finally, while adult CD4 or CD8 T cells readily protected immunodeficient host mice upon adoptive transfer, old T-cells of either subset were unable to protect against WNV. These findings identify key defects as well as potential targets for immunomodulation and treatment to combat lethal WNV infection in the elderly.

In Chapter 5, we describe an additional mouse model that uses a prototype poxvirus, Vaccinia virus (VV) to examine the impact of immune senescence. Poxviruses produce some of the most severe human infections and are known to disproportionally affect the elderly. This chapter describes the initial work in old and adult C56BL/6 mice to begin to address the causes of the age-related immune vulnerability to poxvirus infection and the inefficacy of poxvirus vaccines. Here we demonstrate that old C57BL/6 mice are more susceptible to VV infection than adult mice. The diminished adaptive immune response of the old mice is one potential cause for the increase in disease severity, however the age-related defects seen in flavivirus and poxvirus infections are only partially overlapping.

Chapter 6 addresses the development and maintenance of the CD4 and CD8 T cell memory populations after WNV infection. The evaluation of quality and quantity of the memory responses from animals that survived primary infection demonstrates a direct correlation between the fitness of the memory response and the ability to survive WNV re-infection. These experimental findings can be applied to rational vaccine design that specifically targets highly vulnerable populations such as the elderly.

Chapter 7 describes the potential protective effect of a novel therapeutic anti-viral compound and a novel WNV vaccine approach, referred to as a pseudo-infectious virus (PIV). We show that treatment of mice with a Sultam thiourea compound reduces WNV induced mortality, making these drugs strong candidates for anti-WNV therapy. We also evaluated the antigen-specific T cell response to the PIV vaccine in old and adult mice as well as in several target gene-knockout mice, which are highly susceptible to severe WNV disease. Our results show that vaccination with PIV can generate a robust T cell population in C57BL/6 mice as well as B cell deficient mice (μMT-/-). Although PIV vaccination of μMT-/- mice generated a memory T cell response, this response was insufficient to protect the mice from a high dose viral challenge, indicating that a memory T cell response is not sufficient to protect mice. PIV vaccination of old mice does induce a T cell response, and unlike the vaccination of μMT-/- mice, PIV vaccinated old mice are protected from a high dose virus challenge.

Taken together, this work further clarifies the role of T cells in the control of infection. These findings include: 1) the first description of a mouse model of age-related susceptibility to a viral infection. 2) Demonstration that increased mortality from WNV infection is linked to a poor adaptive immune response. 3) The observation that either

CD4 or CD8 T cells are sufficient to protect against WNV infection. 4) The

identification of a correlation between the fitness of the immune response and the ability

to survive WNV re-infection.   5) The use of tools developed during this thesis period to

evaluate current vaccines strategies. Through this work we have advanced both the

understanding of WNV immunity and of the mechanisms involved in immune

senescence. Future vaccine studies will need to incorporate what we have learned about

immune senescence to design affective vaccines for one of the most susceptible

populations, the elderly.

Chapter One

Background and Introduction

The topic of this thesis is the elucidation of immune deficiencies that develop during aging and lead to a generalized increase in the severity of viral infections in the elderly. We have developed an animal model of West Nile virus (WNV) infection that mirrors human disease and have used it to determine the age-related immune deficiencies most relevant to anti-viral defense. We will begin by providing an overview of the immune response, focusing specifically on the immune response to viral infections and on the changes in immune response in the elderly population. This will be followed by a discussion of WNV infection and the immune response generated against WNV. The chapter will conclude with a brief discussion of the immune responses to other viral infections and an overview of the outstanding questions addressed within this dissertation.

Overview of Immunity

The immune system has evolved over tens of millions of years to protect us from infectious agents (including viruses) (reviewed in (1)). In order to achieve this task the immune system has evolved two arms, the innate and the adaptive immune systems. The innate immune system is driven by the recognition of pathogen associated molecular patterns (PAMPs) (2), whereas the adaptive system is guided by the recognition of specific antigens by clonotypic receptors expressed on the surface of lymphocytes (reviewed in (3-5)). These two arms are linked together by a series of complex interactions that consist of both positive and negative feedback loops that activate and/or silence the immune response as necessary to control an infection and limit immune-mediated pathology (reviewed in (6-9)).

Upon infection the innate immune system has three main goals; the first is to limit pathogen spread; the second is to alert the adaptive immune system and the third is to direct the initial steps of the adaptive response. The innate immune response is driven and guided by germ-line encoded receptors and soluble molecules (reviewed in (10)). The innate immune system deploys both general mechanisms such as complement, cytokine release or intracellular defenses as well as cell-specific mechanisms such as NK cells and macrophage responses to infections. However, upon a repeat challenge by a pathogen, the innate immune response lacks a characteristically rapid and improved responsiveness that is the hallmark of immunological memory (10, 11).

To alert the adaptive immune system the innate immune system uses professional antigen presenting cells (APCs). APCs play an essential role as mediators of the innate immune response by releasing cytokines and directly removing pathogens and are also critically involved in priming and stimulating the adaptive immune response (reviewed in (10, 12, 13)). APCs continuously survey their environment by engulfing self and nonself debris, and process and present it to T cells as a mechanism for monitoring the environment. Following infection there is an activation of APCs by PAMPs or by cytokines released during the early stages of the innate immune response. APC activation causes several phenotypic and functional changes including the cessation of environmental sampling, the increased expression of costimulatory molecules such as CD80 and CD86, and migration to the draining lymph node (reviewed in (14)). Upon arrival in the draining lymph node APCs upregulate expression of major histocompatibility complex (MHC) class I and II molecules, which are absolutely required for the priming of the adaptive immune response.

The adaptive immune system has two key characteristics that distinguish it from the innate immune system. First, T and B cell receptors used for antigen recognition are generated through somatic mutation and recombination, generating a tremendous diversity allowing for the identification of antigens unique to each specific pathogen. This is in contrast to the innate immune system that more broadly recognizes molecular structures of pathogens and products of their infection. The second major defining characteristic of the adaptive immune system is the development of immunological memory that manifests itself as increased functionality and increased number of responding cells upon re-exposure to the same antigen. Lymphocytes undergo an education/selection process independent of foreign antigen to learn to distinguish self from non-self, foreign. The education occurs either in the bone marrow for B cells or within the thymus for CD4 or CD8 $\alpha\beta$ T cells, where strongly self-reactive lymphocytes are removed. This education process must occur before naive lymphocytes are released into the periphery to limit the probability of developing an autoreactive immune response and to focus the lymphocyte response to the recognition of non-self foreign antigens.

Naive B cells use their receptor (B cell receptor, BCR) to recognize structural antigenic determinants that are either expressed on the cell surface or on soluble molecules (reviewed in (8, 15)). Once properly stimulated, antigen-specific B cells divide, start to mature and have several potential fates depending on the structure of the antigen, T cell help and the cytokine milieu. A fraction of B cells will become short-term plasma cells and secrete high levels of IgM antibody. Other antigen-specific B cells will enter a germinal center within a draining lymph node (dLN), and undergo somatic hypermutation. Somatic hypermutation is a critically important process where mutations

are randomly introduced into the BCR complementarity-determining (CDR) region #3, the portion of the BCR that is responsible for recognizing antigen. This leads to generation of a BCR that may exhibit better or worse antigen recognition. B cells bearing the latter BCR will be eliminated, whereas those bearing the improved, "affinity-matured" BCR will be selected, leading to the improved affinity of the B-cell response against the cognate antigen.   Antigen-specific B cells that survive somatic hypermutation can then undergo isotype class switching, where the Fc domain of an antibody can be changed from IgM to IgG or IgA etc. This process alters the biological function of antibodies, by allowing it to be recognized differently by different subsets of receptors. Eventually these cells are destined to become non-Ig secreting memory cells or long lived plasmablasts that secrete high levels of antibody (reviewed in (8, 16)).

T cell receptor (TCR) $\alpha\beta$ T cells consist of two main subsets, CD4 T cells, which are also called helper T cells, and CD8 T cells, which are often called cytotoxic T lymphocytes. Naïve $\alpha\beta$ T cells undergo priming after the recognition of their cognate antigen within the context of an MHC class I or II molecule on a professional antigen presenting cell. The priming-process is essential because the T cell not only receives signals through the TCR (signal 1), which provides specificity to the response, but also receives co-stimulatory signals (signal 2), which play a role in fate determination. Finally cytokine mediated signals (signal 3) such as IL-12 augment the functional heterogeneity of the T cell and is involved in optimal functional maturation of the T cell (reviewed in (17)). The cumulative effect of these three signals is massive expansion and acquisition of a wide range of effector functions (18-21) (reviewed in (22)).

CD4 T cells have evolved to monitor extracellular spaces by recognizing fragments of extracellular proteins that are processed and presented in the context of MHC class II molecules (3, 23). Upon antigen recognition CD4 T cells have the potential to secrete a broad range of cytokines. CD4 T cells traditionally have been classified according to the their cytokine secretion pattern as either Th1, helping cellular immunity, or Th2, helping antibody responses. However this initial classification scheme is rapidly becoming an oversimplification. The recent description of regulatory T cells and Th17 cells (24, 25) as well as the description of CD4 T cells that are capable of direct effector function such as cytotoxicity or cytokine release (26) requires a more flexible system of classifying CD4 T cell subsets. Each subset of CD4 T cells is most precisely classified based on their effector function during an immune response as wells as the expression of specific markers (27)(reviewed in (28, 29)). Different CD4 T cell subsets are primed by MHC class II bearing APCs and can be driven into distinct differentiation pathways depending upon specific receptor-ligand interactions with an APC, and upon the cytokine environment (reviewed in (30)).

CD8 T cells have evolved to monitor for and protect against intracellular pathogens by scanning for the presence of their cognate antigen complexed to MHC class I molecules (31). MHC class I molecules present on the surface of the cell can present both self-and non-self peptides. CD8 T cells survey the health of cells by distinguishing between the presentation of self and non-self-peptides on the surface of cells. MHC class I molecules are assembled and loaded with both self and non-self peptides imported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). The peptides brought into the ER by TAP are derived from a pool of peptides

present within the cytosol. This peptide pool mainly consists of proteasome-cleaved

cytosolic proteins, which can arise from either cellular proteins or proteins produced by

an intracellular pathogen (reviewed in (32)).

The anti-viral immune response (**Figure. 1**)

The aforementioned systems work in concert to respond to a viral infection.

Immediately upon viral entry, the innate immune system responds by releasing type I

interferons (IFN-α and IFN-β) (reviewed in (33, 34)). Type I interferon (IFN) is induced

by Toll-like receptor (TLR3, 4, 7/8 or 9) or other intracellular molecules that then signal

through several of the IFN-regulatory factor family members (IRF1, 3, 7) (35-37). In

turn, type I IFNs reduce viral replication within the infected cell by activating intra-

cellular anti-viral proteins such as interferon-inducible 2'-5' oligoadenylate synthetase 1b

protein (OAS1b) (38). Type I IFN also alerts the host of an invading virus by

upregulating antigen processing and presentation to T cells via MHC molecules and by

initiating the natural killer cell response (39, 40). In this way the innate immune response

maintains partial control of virus replication while allowing the adaptive immune

response to develop (reviewed in (11, 34, 41)).

The adaptive response is initiated in the draining lymph node (dLN) by the arrival

of professional APCs, chiefly dendritic cells (DCs) that have captured viral antigen to

present to naïve lymphocyte (reviewed in (14)). Antigen presentation in acute infections

begins within hours of an infection and lasts approximately 1-2 days (42-44). During this

first 48 hours of infection CD4 and CD8 T cells as well as B cells traffic to the dLN and

respond to foreign antigen, captured and presented by APCs (45, 46). Antigen-specific T
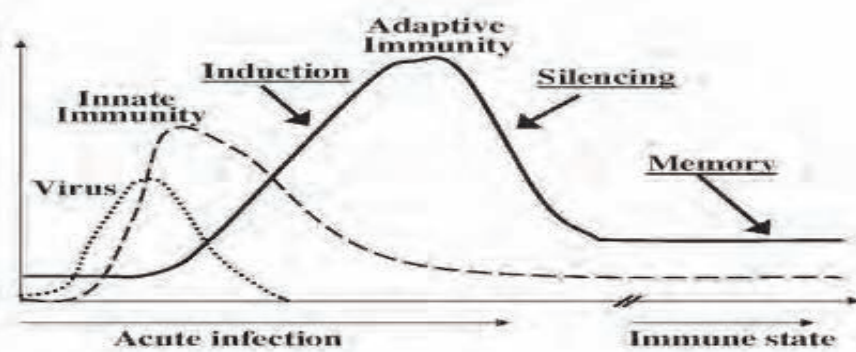
Figure 1. A representative time line of an immune response during an acute viral infection.

cells proliferate and differentiate into effector cells as a result of three signals: 1) Antigen-specific receptor (TCR) pMHC interaction, 2) the interaction of co-stimulatory receptors on the lymphocytes with ligands on the APC, and 3) stimulation of T cell activation by the cytokine milieu (47-49).

A successful adaptive immune response eliminates virus reservoirs in three ways. First, neutralizing antibody produced by B cells can function directly by blocking viral infectivity. Antibody produced by B cells can also undergo isotype switching allowing the new isotype of antibody to activate C' or trigger opsonization by phagocytic cells (reviewed in (50)) to improve viral clearance. Second, cytokine secretion by antigen-specific T or B cells can either have a direct anti-viral effect or aid in recruitment of other cells that clear the virus. The third mechanism of viral clearance by the adaptive immune response is direct cell killing (or cytolysis) by CD8 or CD4 T cells (26, 51, 52).

Once the virus is eliminated, the adaptive immune response undergoes a reduction in antigen-specific lymphocyte numbers, called the contraction phase (reviewed in (53)). The contraction phase is an essential homeostatic mechanism that is required for the reduction of effector cells following the resolution of infection. Most cells die by apoptosis during the contraction phase, while a small number of antigen-specific T cells survive and are maintained for the life of the animal as memory lymphocytes (17, 54, 55).

Memory T cells have several functional characteristics and based on those characteristics they are divided into two groups: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) (56). T_{EM} cells patrol peripheral tissues prepared to eliminate an invading pathogen and exhibit immediate effector function, but they proliferate poorly when stimulated and undergo slow homeostatic cycling. T_{CM} cells do

not rapidly develop effector functions but do migrate through secondary lymphoid organs and undergo self-renewal by homeostatic cycling. T_{CM} cell can become effector cells upon secondary stimulation (reviewed in (28)). However, whether these two groups represent two distinct cell lineages in development is still controversial.

Memory T cell populations play an essential role in preventing the development of disease upon reinfection because of their ability to respond rapidly. There are rare instances when memory T cells are not essential to prevent reinfection. This occurs when antibody titers are of the correct specificity, and are at a high enough concentration that sterilizing immunity upon re-infection is possible. These two tenets are taken into account when developing a vaccine, to prevent infection or disease. Newly designed vaccines normally target both the B cell and T cell response. Targeting both the B cell and T cell response is important because there is a potential for viral variants to emerge with mutations that allow a virus to evade the antibody or T cell response (antigenic shift). The goal of a vaccine is to generate an antigen-specific memory response. Specifically, a vaccine that can generate a memory T cell response may have a greater efficacy because of the CD8 T cells' ability to scan cells for intracellular infection, and the essential role of CD4 T cells that can simultaneously support the development of rapidly responding CD8 T cells and a strong antibody response. The relative contributions and functional requirements for each lymphocyte subset will most likely vary based upon the pathogen in question. There is a second reason why most vaccines target multiple arms of the immune system and that is the potential that vulnerable populations, like the elderly, will develop an insufficient response to a vaccine that only triggers a single component immunity(57).

Immune senescence

People, specifically in the developed world, are living much longer then previous generations (58). There are currently thirty-seven million people over the age of 65 in the United States and this number is expected to rise to over seventy million by 2030 (58). In addition to the increase in the number of aged individuals, there is also an increase in life expectancy. More specifically, in year 1900 the life expectancy was forty-seven years whereas the current life expectancy is seventy-eight years (58). With this increased life expectancy a greater percentage of the elderly are living with chronic diseases such as arthritis, hypertension, heart disease and diabetes (59). All of these factors increase the severity of disease caused by infection. In fact, infectious diseases are consistently the 4-5th leading cause of increased morbidity and mortality in the elderly. This increased susceptibility is believed to be due to a diminished immune function, generally referred to as immune senescence.

Immune senescence is the combination of diverse cellular and molecular changes that occur as the organism ages and results in a diminished protective immune response (reviewed in Nikolich-JNZ in press, (60-64). The most significant defects that contribute to immune senescence are those associated with the adaptive arm of the immune system, specifically T cells (65, 66). However, there are additional deficiencies that impair innate immunity including the Type I interferon response (67-69), NK cell reactivity (70, 71), and macrophage activation (72).

Specific changes in T cell function seen during aging include: (i) impaired signaling including IL-2- IL-2R (73) and the T cell receptor signaling (74) and effector responses (75, 76); (ii) an increase in circulating memory and a corresponding decrease

in naïve T cell populations (61, 77); (iii) a decrease in T cell diversity which is exacerbated by T cell and B cell clonal expansions (78-80). An acute viral infection causes a spectrum of symptoms, such as fever and malaise, as well as multiple alterations in the levels of circulating cytokines and chemokines (81-84). As stated above, it has been shown that chronic diseases seen in the elderly, including diabetes and heart disease (85), exacerbate these symptoms. In addition the stress of an inflammatory response following acute viral infection can lead an even greater number of adverse events such as myopericarditis (83, 85-91), the progression of atherosclerosis (92) and progression of Alzheimer's disease (93, 94).

The changes described above contribute to greater morbidity and mortality from infectious disease within the elderly population. The elderly show increased morbidity and mortality following infection with several bacterial pathogens such as *S. pneumoniae*, *H. influenzae*, *C. difficile* (95, 96) and viral pathogens, such as Influenza, Severe Acute Respiratory Syndrome-causing corona virus (SARS-CoV), Respiratory Syncytial virus (RSV) and West Nile virus (97-103). Affecting an even larger proportion of the elderly population is a decrease in the quality of life, and pronounced sickness, or morbidity, caused by the same infections described above, as well as by viral reactivation of viruses like VZV (104, 105).

As a result of the age-related defects in adaptive immunity, the elderly also respond poorly to vaccination against the most frequent and severe pathogens affecting them: influenza virus, pneumococcus and VZV (106-108). Age-related immunological changes have the greatest clinical impact on influenza vaccination and infection. The rate of serious illness and mortality from influenza is highest among persons 65 years old or

older (109-111). On average 142,000 aged individuals are hospitalized each year because of an influenza infection (112). Within the elderly population, influenza can cause mortality both directly as well as by allowing development of a secondary infection or exacerbating a pre-existing chronic disease (113, 114). Approximately 36,000 influenza-related deaths occur annually in people above the age of 65 in the United States alone (114) and this number is predicted to increase because of the increased size of the population above 85 years old (115). Vaccination does improve the outcome of infection in both the adult and elderly populations. However the efficacy is quite different considering that vaccination prevents symptoms in 70%-90% of adults but only in 30%-40% of the elderly (116-118). Furthermore, elderly patients are at a greater risk for complications from live vaccines (108, 119). As a result very few vaccines have been tested or optimized for the elderly. Thus, older populations are not only at an increased risk of infection, but cannot be adequately protected by vaccination, which can moreover pose a risk, specifically for those that already suffer from chronic illnesses (88).

Specific age-related defects in innate and adaptive immunity relevant West Nile virus-infected humans

As mentioned, specific age-related defects are seen in both arms of the immune system, although the significance of changes in the innate immune system remains unclear at the present. One potential factor that has led to the controversy is the differential use of inclusion and exclusion criteria for human studies. For example, one common selection method, called the SENIEUR protocol, sets guidelines for the "healthy

aging", which is based on the exclusion of disease (120), where up to 90% of subjects can be excluded (121).

<u>Innate immunity</u>

Although controversial, it is necessary to describe the most commonly held beliefs about the changes that occur to the innate immune response during aging because several of those may play a direct role in immune protection or increased pathogenesis during WNV infection. During aging there are changes that occur with both baseline cytokine levels as well as changes at the cellular level. Within the aged human population there is an increase in NK cell numbers and diminished cytokine production by NK cells (reviewed in (122)), but whether their cytotoxic potential is altered is not clear (70, 123, 124). Similar to the consensus about NK cell numbers, it is widely accepted that circulating levels of pro-inflammatory cytokines such as TNF-α and IL-6 are increased (125-127). But it is believed that type I IFN's decrease as one ages, despite the fact that there have been very few studies looking at this question (69, 128). The actual mechanism of this decrease is still debated, although there is evidence that there is a decrease in the number of plasmacytoid DCs (pDC's), a DC population that can rapidly respond to infection by producing large amounts of IFNα, as one ages (129, 130), and that several cell types show diminished responsiveness to type I IFNs (131, 132). Unfortunately, most recent publications have questioned the results of these studies by publishing data that directly contradict the above results (129, 130, 132).

Adaptive immunity

The changes that occur in the T cell percentages within the periphery during aging can be quite dramatic. As people age the ratio of phenotypically naïve lymphocytes to memory lymphocytes decreases. The alteration in the naïve:memory T cell ratio, a result of immune senescence, is thought to be one potential explanation for the increased susceptibility to severe disease in the elderly (reviewed in (60, 61, 133, 134)). Several factors affect the decrease in naive lymphocytes; 1) diminished bone marrow potential (135), 2) thymic involution (atrophy) (135), 3) compensatory homeostatic proliferation driving memory cell formation (136), 4) repeated antigenic stimulation of memory lymphocytes during reactivation of latent viral infections (103, 137). These factors do not function independently, but cooperatively, and a discussion of how these factors interact in regard to peripheral T cells is contained below.

Peripheral homeostatic mechanisms strive to maintain constant numbers of T lymphocytes within the periphery (138). As one ages, fewer new naïve T cells enter the peripheral T cell pool, because there is diminished seeding of the thymus by the bone marrow, and there are fewer T cells that are released by the thymus into the periphery (reviewed in (135, 139)). Lymphocytes in the periphery replicate more often due to a surplus of cytokines that regulate survival and proliferation, these lymphocytes take up space normally occupied by newly arrived naïve T cells (reviewed in (135, 139)). In conjunction with increased peripheral T cell proliferation, over time latent herpes virus infections, specifically human cytomegalovirus (HCMV), periodically reactivate, reintroducing antigen into the periphery and requiring T cells to respond in order to prevent viral spread (103, 140). This causes an incremental increase in the number of

memory cells that respond to HCMV, which in turn adds additional homeostatic pressure reducing both number of naïve lymphocytes capable of responding to a novel pathogen, such as West Nile virus, or reinfection with an infrequent pathogen such as Influenza.

Models of Immune Senescence

Currently there are very few experimental animal models that are used to determine why the severity of infectious diseases increases with age (141-143). Mice and rats are the most common models used for immunological aging experiments (reviewed in (142, 144-146)), although the rhesus macaque model has been developed as a model to study vaccination and primary viral infection in the old age (review in (143)).

Inbred laboratory mouse strains are good models to study aging because the inbred nature of the population eliminates genetic variability as a confounding factor. In addition, only a model system can be used to properly determine if the increased severity of disease within the elderly population is a result of increased viral load versus increased pathology. This is accomplished by simultaneously determining viral load, monitoring pathology and immune response strength and kinetics. Influenza, RSV, Lymphocytic Choriomeningitis virus (LCMV), Herpes Simplex virus type I (HSV-1), SARS and E55 murine leukemia virus (E55+MuLV) infection are examples of mouse models of viral infection used to study the impact of aging.

Of the models described above the most common model is the Influenza mouse model, that takes advantage of several well characterized strains of virus such as A/PR8/34, to infect or vaccinate old and adult mice (reviewed in (147, 148)). Infection of BALB/c, C57BL/6 or CBA mice with sublethal doses of Influenza virus results in a

delayed clearance of infectious virus from the lungs of old mice during days 7 to 10 post infection, compared to adult animals (149-151). This delayed viral clearance is directly related to a diminished CD8 T cell response (149, 151). This is characterized by a delayed and diminished proliferation of antigen-specific cells, from both effector sites such as the lung (150, 152) and central sites such as the spleen (153, 154). In addition, cells isolated from the spleen or the lung exhibit diminished IFNγ production and cytolytic activity (149, 150, 153), believed to be caused mostly by a decrease in the number of antigen-specific cells (150).

A diminished CD4 antigen-specific response develops as well, which is responsible for decreased concentrations of IL-2 (153, 155), IFN-γ (153, 155) and cytotoxicity (155). In addition, this depressed CD4 T cell response leads to diminished antibody production (156-158). These antigen-specific responses were measured using bulk T cell populations, often stimulated with the whole virus, rather than with specific epitopes. Therefore, the full extent of the existing defects may not have been completely elucidated.

Old mice have equal or greater probability of surviving a high viral challenge with Influenza virus than adult mice (152, 159), thus showing a fundamentally different outcome to that seen in elderly humans. However, this does not hold true after vaccination. Once old mice are vaccinated with either live or formalin inactivated Influenza A virus, they remain susceptible to severe disease, whereas adult mice become resistant (158, 160).

RSV, SARS and E55+MuLV have also been studied to determine the effects of virus infection on old mice and have yielded similar results to infection with Influenza

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virus. Old mice have a delayed antigen-specific immune response, which even at the peak contain fewer antigen-specific CD4 and CD8 T cells (161-164). The CD8 T cell population exhibits lower cytotoxicity in old mice than in the corresponding adult populations (161, 163, 164). During this delayed immune response viruses continue to persist at an equal viral load to what is found in adult mice although the immune response is measurably diminished (161-164).

LCMV is a non-cytopathic virus that is rapidly cleared from the periphery by antigen-specific CD8 T cells (reviewed in (165)). When old and adult mice are infected intraperitoneally with LCMV or with Influenza, old mice exhibit diminished antigen-specific CD8 and CD4 T cell responses, as shown by diminished cytotoxicity (166, 167) or tetramer staining (167). This diminished responsiveness leads to increased viral titer and a protracted time line for viral clearance within the spleen, liver and blood (167). Upon eventual clearance of LCMV, both adult and old mice develop a stable memory pool with equivalent numbers of memory cells (167). These memory cells are able to control reinfection as well as the memory cells from adult mice, although there are fewer cells in old mice that respond to reinfection than in adult mice (167). If LCMV is given intracranially, it causes a rapid immune-mediated encephalitis caused by the cytotoxic attack on virally infected neurons by activated CD8 T cells (reviewed in (168)). In this model system, old mice do succumb to LCMV infection but with delayed viral kinetics, as one would expect from diminished CD8 T cell responsiveness (166).

West Nile virus Virology and Ecology

WNV is a member of the *Flavivirus* genus of the *flaviviridae* family (169, 170). Of the 75 viruses in the *Flavivirus* genus, approximately 40 cause human disease (170). The *Flavivirus* genus is further broken down into 12 antigenic serogroups, with the WNV being a member of the Japanese Encephalitis virus serogroup (171). WNV isolates are further sub-divided based on nucleotide sequence homology into two lineages I and II, with the Kunjin virus classified as a third sub-group (170, 172). Lineage I strains of WNV are present in North America, Europe and the Middle East and have been responsible for most if not all outbreaks of human disease (173-179).

WNV is a small, enveloped virus, 50nm in diameter that contains a single-stranded, positive sense RNA genome (169) (**Figure. 2**(180)). Infection starts with the viral envelope protein binding to an unidentified receptor and several potential co-receptors, including CD209L (181) and αvβ3 integrin (182) (reviewed in (183)). After receptor binding, the virion enters the cell via receptor-mediated endocytosis followed by fusion with the endocytic vesicle mediated by a low-pH event, where the capsid is released into the cytoplasm (184). Once in the cytoplasm, the 11kb single stranded positive sense RNA genome is accessible and translated into a single polyprotein (185). The polyprotein is cleaved by various cellular proteases and by a virally encoded protease (consisting of the NS2b and NS3 proteins, described below), into 10 mature viral proteins (186, 187). Of the ten proteins three are structural [capsid (C), membrane (prM/M), and envelope (Env)], while seven are nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5), and as with most viral proteins (188), each protein has multiple functions (reviewed in (183)). Upon cleavage of the viral polyprotein, NS5, the viral RNA-

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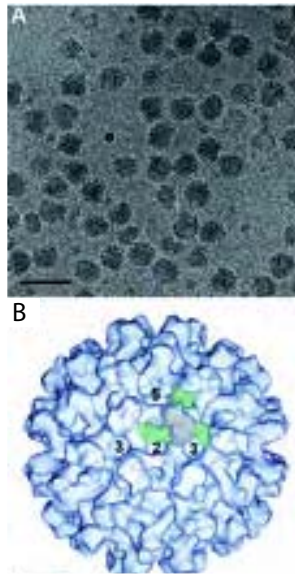
Image taken from: Structure of Immature West Nile Virus: Journal of Virology 2007
(179)

Figure. 2 Two images of West Nile virus virions. A. Cryo-EM micrograph of immature West Nile virus viron. The scale bar represents 1,000 Angstroms. **B.** Surface representation of a mature WNV particle.

dependent RNA polymerase/ methyltransferase, uses the positive stranded genome as a template to generate negative strand RNA which are used for the synthesis of new genomic RNA's (185, 189, 190). Replication and virus assembly occur in close proximity of the endoplasmic reticulum (190, 191). During this process, NS2a, NS2b, NS4a and NS4b facilitate the assembly of the replication complex as well as virus assembly (185, 189-191). The complex of NS2b + NS3 acts as a serine protease, where NS3 is the serine protease and NS2b is an essential co-factor (187). NS1 acts as a co-factor during replication and is thought to be immunomodulatory during mammalian infection (192-194). As virions accumulate and associate with the ER (195), they are transported through the Golgi, where the membrane protein is cleaved by furin (196). Mature virions are released from infected cells by exocytosis (197).

The transmission cycle of WNV is maintained by infection in an arthropod vector -wild bird enzoonotic cycle (reviewed in (198, 199)). Many species of birds develop long-term high titer viremia, which is sufficient to infect a mosquito vector (200-202). The number of infected mosquitoes and birds increases over time, starting in early spring and peaking during the middle of the summer (200, 203-205). At this point in time, the increased number of infected mosquitoes increases the possibility that an incidental host, mouse or human, will be bitten for a blood meal and become infected (206). Most mammals can become productively infected with WNV (206), but current evidence indicates that birds are the only animals that develop a sufficient viremia to continue the viral life cycle (198).

WNV: viral spread and pathogenesis

Once a mosquito takes a blood meal, virus that is contained within the saliva is transported to the skin as well as to the blood stream. Virus has the potential to replicate with in the Langerhans' cells within the skin (207, 208). Infected Langerhans cells are believed to deliver the virus to the draining lymph node where it continues to undergo additional rounds of replication leading to a brief viremia and spread to the visceral organs (209, 210). The initial stages of this route of infection are still debated and there is compelling evidence that during mosquito feeding that WNV is delivered directly into the blood stream and spreads to the parenchymal organs (211) (Mason, PW and Tesh, RB pers comm). A cautionary note is that in most animal models mosquitoes are not used to initiate infection, and it has been shown that several of the components of mosquito saliva increase the severity of West Nile virus disease (205, 212).

Once viral replication begins after sub-cutaneous infection of C57BL/6 mice, virus replication occurs in the draining lymph node from 24 hours to 8 days post infection (213-215). During that same time viremia occurs in mice for approximately six days where viral titers peak on days 3-4 in the blood and day 4-5 in the spleen; in some experiments virus can be isolated from the kidney and liver as well (213, 216, 217). Although very limited data is currently available, *in vitro* experiments indicate that several cell types, including monocytes, DCs, and B cells, can be infected by WNV. However during in vivo infection only monocytes and B cells have been found to support a full round of replication, indicated by the presence of both + and – strand WNV genomes by real-time PCR (214, 218). In C57BL/6 mice, West Nile virus enters both the spinal cord and the brain between day 4 and 6 depending on the experimental design

(219, 220) and infects neurons (221, 222). The method of entry into the CNS is still hotly debated. WNV may cross the blood-brain barrier by a hematogenous or Trojan horse route (216, 223, 224), by active replication in endothelial cells (169, 225), retrograde axonal transport, or even by passive transport (226, 227). In a recent report, it was suggested that stimulation of the TLR-3 pathway within the periphery causes an upregulation of TNFα, which induces transient permeability in the blood-brain barrier and allows neuroinvasion (220).

WNV: Epidemiology and Clinical Symptoms

WNV was introduced into the United States in 1999 and has since become endemic. WNV had spread to forty-two states in less than three years (228, 229) and was responsible for thirty-seven hundred documented human infections leading to two hundred and fourteen deaths within the US in 2002 alone (228, 229). The median age of the decedents is 78 years and the range of decedents is between 24-99 years (99, 230). Within the past few years' outbreaks of WNV have lead to a greater mortality rate within birds (231), the natural host for the virus (177, 178), as well as horses (incidental hosts) (232) suggesting there may have been a change in the virulence of WNV. Birds that succumb to WNV experience substantial infection of the heart, subsequent heart failure and death (91, 200, 233, 234).

Since the mid-1990's there has been an increase in the frequency and clinical severity of WNV infection within the human population (179, 235-237). Approximately 1 in 150 WNV infections results in meningitis or encephalitis, with advanced age being the most significant risk factor for severe neurological disease (99, 230, 238). The

incidence of infection is fairly uniform with age (230) however, persons aged 50-59 years of age have a 10x higher incidence of severe disease while persons aged 80 years or greater have a 43x higher incidence of severe disease (100). Many patients that suffer from WNV encephalitis require greater than one year to fully recover physically, functionally, or cognitively (239). Encephalitis and febrile illness are the most critical signs of a severe infection. A severe infection results from infection of the central nervous system, as well as the heart, liver and kidney (240). Viral spread within an infected person is thought to be biphasic (241). Initial virus replication occurs within the skin and local lymph nodes, this virus replication seeds the reticuloendothelial system, which may in turn spread to the central nervous system (241). It is thought that the length and level of viremia affect clinical manifestations and disease outcome (241, 242); this correlates well with animal studies (221).

West Nile Virus: Immune response

A great deal of effort has recently focused on studying the immune response to flaviviruses. Studies including those using mice deficient for various components of the immune system have demonstrated the importance of multiple components of the adaptive immune response in controlling infection. The following paragraphs give a brief overview of the immune responses to flavivirus infections.

Innate Immunity: Intracellular Responses, Interferon, Complement

Type I interferon (IFNα/β) are essential components of the anti-viral innate immune response (reviewed in (243)) to any virus infection. IFNα/β is produced by

almost every cell type, and has both direct and indirect modes of action. IFNα/β has been shown to be absolutely essential for effective control of WNV infection in mice. All IFNα/βRI-/- mice rapidly succumb to lethal WNV encephalitis 6 days post infection (244). Upon infection by Type I clade a strains of WNV, the induction of interferon regulatory factor 3 (IRF3), a transcription factor critical for IFNβ responses, is delayed, therefore allowing the progression of WNV replication and viral protein translation (245, 246). The delay in IRF3 induction also causes a delay in IFNβ secretion by infected cells, preventing neighboring cells from preparing for potential infection. This is important because WNV replication is strongly inhibited by IFNα/β. If fibroblasts, neurons, or even mice are pretreated with type I IFN, WNV replication is reduced by several logs (244, 247). Conversely, when IFNα/βRI-/- mice are infected with WNV there is an increased titer within the visceral organs and increased number of neurons infected within the CNS (244).

Almost every cell within the body has internal anti-viral effector mechanisms triggered by pattern recognition receptors (PRR) and type I IFN. Upon ligation of IFNα/βR and PRRs, such as retinoic acid-inducible gene-I (RIG-I) or TLR3, intracellular effector molecules such as RNase L, 2'-5' oligoadenylate synthetase (OAS), and double-stranded-RNA-activated protein kinase (PKR) are activated and limit WNV replication. Signaling by RIG-I, a PRR, is delayed but not completely ablated during WNV infection, leading to a delay in the anti-viral response that allows for increased virus production from infected cells (245). Once PRR's become activated they trigger intracellular RNAse effector molecules such as RNase L and PKR, which then cleave WNV RNA lowering viral titers both in vitro (248), and *in vivo* (214).

The complement system, a series of extracellular effector proteins, is also used to control initial stages of WNV infection. All three complement pathways, classical, alternative and lectin, function together to prevent severe disease in mice during the course of WNV infection, as indicated by the increased mortality of mice that lack any one of the several components of the complement pathway, including C3, C4, complement receptor 1 or 2, and factors b or d (244, 247). Depending on the specific defect, either WNV entered the CNS earlier in infection as in C4-/- mice, or there was a defect in the antigen-specific response in mice deficient in the classical or lectin pathway leading to increased mortality (218). In total, the alternative pathway restricts virus replication within the periphery, while the other pathways are essential in priming the adaptive immune response (218).

West Nile virus: Cellular Innate response

Macrophages, dendritic cells, γδ T cells and natural killer cells are all essential components of the cellular innate immune response. During WNV infection, it has been shown that depletion of macrophages leads to extended and increased viremia, as well as decreased survival time and increased mortality (249, 250). This clearly shows the direct role of macrophages controlling viral spread, although it does not rule out potential pathogenic effects of macrophages. Dendritic cells also play an essential role during WNV infection. It is likely that pDCs are responsible for secreting the large quantities of IFN-α seen early in infection (209) reviewed in (14). Although NK cells are activated during infection (251), both antibody depletion studies and the use of Ly49A-/- mice indicate that NK cells do not directly control infection (252-254).

γδ T cells have been described to be essential for early control of WNV infection. The survival of γδ T cell-/- mice is decreased compared to C57BL/6 controls when infected with high dose of WNV, and increased mortality upon low dose infection (255, 256). During infection γδ T cell-/- mice have increased viremia and increased viral titers in the spleen, spinal cord and brain (256). This increased viral titer correlates with the ability of γδ T cells to produce IFN-γ, as shown by the rescue of IFNγ-/- mice by the adoptive transfer of γδ T cells from IFNγ competent mice (256) as well as by a series of experiments using mixed bone marrow chimera experiments with similar results (257). IFN-γ is also required later during infection, indicating that IFN-γ is produced by additional subsets of cells including γδ T cells (257).

West Nile virus: Adaptive Cellular response

B cell response

West Nile virus induces a strong antigen-specific response during primary infection. Currently it is believed that antigen-specific CD4 and CD8 T cells as well as antigen-specific B cells are all required to protect an immunocompetent mouse (reviewed in (258, 259))

Humoral immunity was shown to be essential for protection from severe WNV disease, (213, 216, 260, 261). This was shown by an elegant series of experiments completed by Diamond et al. First, μMT-/- mice, deficient in B cells, were shown to be uniformly susceptible to mortality from WNV infection; but these mice can be protected temporarily by WNV specific IgM (216) and fully protected by the transfer of IgG (213). In conclusion the presence of early IgM on day 4 and IgG from day 8 on, reduced the

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viral load early during infection, leading to a decrease in viral loads within the spinal cord and the brain late in infection (216). Although it is clear that passive transfer of anti-WNV IgG antibodies can protect in animal models (261), and can play a role as a potential therapy (262), it is unknown what role IgG may play during primary infection since IgG develops after virus has already entered the CNS.

Most WNV neutralizing antibodies bind the envelope protein, although some bind the membrane protein (216, 217, 263-268). The most potent neutralizing antibodies generated in mice bind to the DIII region of the envelope protein (269-271). Antibody responses to most of the non-structural proteins also develop, and in the case of NS1 these antibodies can be protective (193, 260). During infection of a mammalian host NS1 is found on the cell surface of infected cells, but not located within the virion, and the ability of antibody to bind to NS1 on the surface of the cell triggering a C' cascade, may be one reason why antibodies to NS1 are protective (192, 272).

WNV T cell responses

In vivo T cell responses to flavivirus infection have been an area of interest for over 40 years (273, 274). T cells play an essential role in viral clearance in both the periphery and in the CNS (253, 275-278), and limiting disease severity upon potential re-infection (278, 279). It has been shown that both naive CD4 and CD8 T cells are required for control and clearance of West Nile virus (219, 275, 280), but the role of each cell population and the mechanisms used by these cell populations both within the periphery and the CNS have not yet been fully elucidated.

Although CD4 T cells are required for survival from WNV infection (277), no direct effector function has been assigned to this cell subset. It has been shown that CD4 T cells are most likely responsible for aiding in the survival and proliferation of CD8 T cells and the priming of B cells (277); whether this occurs via a cytokine mediated mechanism or by cell-to-cell contact is unknown. The requirement for CD4 T cells has been shown with other Flavivirus infections, such as the Japanese Encephalitis virus (JEV) (281) and Yellow Fever virus (YFV) (282), but once again no specific mechanism was shown. CD4 T cells may play a direct role in viral clearance from the periphery and the CNS, using either cytokines such as IFNγ or direct cytotoxicity as seen in other viral infections. During Influenza infection CD4 T cells use perforin mediated cytotoxicity to clear virus from the periphery (283) and, during measles virus infection, CD4 T cells use IFNγ to control virus within the CNS (284, 285).

The requirement for CD4 T cells during WNV infection was shown using CD4-antibody depletions and CD4 or MHC class II deficient animals (219). Antibody-depleted mice have high WNV viral titers for over 50 days within the CNS, which eventually leads to death (219). The mice exhibit similar splenic viral titers compared to controls indicating that virus control in the periphery is predominantly CD4 T cell-independent (219). After infection with a high dose of WNV, CD4 T cells have an indirect effect on controlling WNV infection by maintaining both CD8 and B cell responses (219), potentially through CD40-CD40L interactions (280). However lower dose infection leads to a strong CD4 T cell responsive population that can produce IFN-γ and proliferate in vitro in response to WNV antigens (286, 287).

In the mouse model of WNV infection several laboratories have shown that CD8 T cells are essential for controlling virus within the spleen and elimination of virus from the central nervous system, in order to prevent encephalitis (253, 261, 275, 288-291). This is consistent with the increased severity of disease in individuals with impaired T cells that have an increased risk of WNV CNS disease (99, 292). Interestingly the first immunological difference between the control of lineage I and II virus strains involves the use of granzymes and perforin. During infection with lineage II strains of WNV Granzyme A and B are essential and perforin is not required (293), whereas infection with lineage I strains of virus requires perforin (253) and the requirements for granzymes is unknown. Other CD8 T cell effector mechanisms are also thought to be involved in controlling WNV infection however detailed studies have yet to be performed.

Lymphocyte trafficking plays an essential role in protection from WNV infection, since an influx of CD4 and CD8 T cells into the CNS is required to decrease viral loads and prevent encephalitis (276, 280, 294). Expression of CD40, CCR5 and CXCL10 alters recruitment of T cells into the CNS and results in increased mortality (276, 280, 294). WNV infected neurons secrete CXCL10, and in this process recruit both CD4 and CD8 T lymphocytes, and in CXCL10-/- mice there is a significant reduction in CD8 T cells that are recruited into the CNS, along with increased mortality (276). WNV infection also induces the expression of CCR5 on lymphocytes, and infection of CCR5-/- mice results in diminished trafficking of lymphocytes into the CNS and100% mortality. In addition, humans with CCR5 deficiency (CCR5Delta32) have been found to have a higher risk of severe WNV disease (295). Adoptive transfer of CCR5+/+ lymphocytes into CCR5-/- mice increased trafficking into the CNS and diminished mortality (294).

Similar to CCR5 and CXCL10, the loss of CD40 prevented recruitment of CD4 and CD8

T cells into the CNS and increased mortality of WNV infected mice (280).

Very little is known about memory T cell responses to lineage I strains of WNV.

What is known is that flavivirus infections, such as WNV, Banzi, YFV and JEV, generate

memory T cells that upon adoptive transfer can provide protection to naive animals

exposed to lethal challenge (255, 281, 293, 296). Experimental vaccines that are

designed to induce a memory response mostly contained components aimed to induce

both an antibody responses and a T cell response. Animals vaccinated in this fashion

have been protected against lethal challenge (297-300). But as of so far very few groups

have tried to separate the impact of the antibody response from that of the T cell

response. In one JEV vaccine T cells provided little additional protection above that

provided by antibody alone (301).

Poxviruses- Epidemiology and Immunology

Many virus infections, such as WNV and Influenza described above, as well as

many live attenuated vaccines adversely affect the elderly population. Currently there are

very few animal models that closely mimic the morbidity and mortality as well as

pathology of these human infections, increasing the difficulty of studying immune

senesce. Historical data on poxviruses and the vaccination to prevent poxviruses,

document that, similar to WNV infection, both situations adversely affect the elderly

population (302, 303).

Vaccinia virus (VACV) is a live vaccine for variola virus (smallpox). Vaccinia

virus is a large DNA virus that replicates within the cytoplasm of infected cells and is a

member of the genus orthopoxvirus of the family *poxviridae* (169). Use of live attenuated VACV results in a cross-reactive and cross-protective immunity (304). The use of live attenuated vaccine has lead to a higher rate of complications than most other currently used vaccines, including myopericarditis, generalized Vaccinia, and progressive Vaccinia (87, 302, 305, 306 , 2002 #738, 307).

VACV and Ectromelia virus (mousepox) have been used extensively in animal studies for decades. The correlates of protection have been well documented in many strains of adult mice (308-311). This type of study has not been completed in aged mice.

In animal models heat inactivated or formalin fixed poxviruses provide inadequate protection from challenge in most animal models (312, 313), implying that T cell mediated immunity is required for clearance of primary infection. In addition, people with defects in cell-mediated immunity usually succumb to Vaccinia vaccination (314). It has been shown that both neutralizing antibody (310, 311) and/or a T cell response (308, 309) can be protective against lethal VACV infection.

Aim of the thesis

The aim of this thesis is to determine why the elderly are at a greater risk of morbidity and mortality from some infectious diseases. We begin to answer this question by characterizing two small animal models of immune senescence, where old mice disproportionally suffer severe disease from a viral infection.

Chapter Two

Protective capacity and epitope specificity of CD8^{+} T cells responding to lethal West Nile virus infection

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Abstract

West Nile virus (WNV) is a small, positive-strand RNA virus belonging to the *Flaviviridae* genus, which causes lethal encephalitis in a subset of infected birds and mammals. In humans, WNV exhibits pronounced age-related morbidity and mortality, but the basis of this effect is unclear, and the molecular and cellular parameters of the host-WNV infection are just beginning to be elucidated. Indeed, numerous mechanisms were implicated in *in vivo* protection against WNV (IFN-I and IFNγ, Ab, C', CD8 and CD4 T cells), but the individual importance of each one of them remains unclear. Here, we show that the transfer of highly enriched naïve $CD8^+$ T cells protects the majority of RAG1-/- mice against lethal WNV infection. To substantiate and expand this finding, we defined the peptide specificity of the CD8 response in H-2b mice and used a panel of identified peptides to map one dominant ($NS4b_{2248-2256}$) and several subdominant epitopes. Hierarchy of these epitopes was stably maintained in the memory responses. Most importantly, CTL lines directed against these peptides conferred protection against lethal WNV infection in direct proportion to the epitope immunodominance. These results provide a springboard for future characterization of T cell responses against WNV and demonstrate, for the first time, that CD8 T cells can single-handedly protect from this disease.

West Nile virus is a small, enveloped virus that contains a single, positive-sense ~11 kb RNA genome encoding a polyprotein that is post-translationally cleaved into three structural (envelope–E, pre/membrane–prM/M and capsid–C) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. These ten, multifunctional proteins play a role in invasion, entry, viral replication, assembly and modulation of host cell functions, including the immune response (rev. in (183, 315)).

Since its appearance at the Eastern seaboard of the United States (1999), WNV-NY strain 385-99 (173, 231) and its immediate descendents have spread through all 48 continental states, infecting more than 10,000 people and killing 264 in 2003 alone (316, 317). The incidence of death is disproportionately frequent in the elderly (median age at death= 75 yr) (100). WNV leads to systemic disease in approximately 20% percentage of individuals, with the most severe disease being due to neuroinvasion and the consequent meningitis and encephalitis (177, 318). While about 1 in 150 infections results in meningitis or encephalitis, advanced age and impaired immunity are the most significant risk factors for severe neurological disease (230); persons 50-59 yr of age have a 10-fold, and those >80 a 43-fold higher incidence of severe disease compared to adults between 20 and 40 years of age (100).

In order to understand the immunological basis of this age-related susceptibility to WNV, we have developed the model of WNV infection and vulnerability in old C57BL/6 mice (J. B. and J. N-Z. unpublished data). Our preliminary results suggest the existence of age-related defects in T cell responses, whereas data from literature implicated CD8 T cells in anti-WNV resistance (275, 291). Therefore, we focused on the impact of naïve

and memory CD8 T cells in anti-WNV responses and showed that naïve T cells can protect Rag1-/- mice from lethal WNV infection. We then defined H-2^b restricted CD8$^+$ T cell epitopes from multiple WNV protein segments and have demonstrated that these epitopes can protect against lethal WNV infection in direct proportion to their immunodominance, showing that CD8$^+$ T cells can be sufficient for protection against WNV.

Results

Transfer of naïve CD8+ T cells provides significant protection against WNV in RAG1-/-
mice

CD8+ T cells were implicated in anti-WNV resistance using knockout animals.
However, it was not clear whether these cells, by themselves, can protect against the
disease. To test whether naïve, unprimed CD8+ T cells can provide protection against
lethal WNV, splenic CD8+ T cells, purified to 90-95% purity (<2% CD4+ and <3% B cell
contamination), were transferred into RAG-KO animals, and recipients were infected
with a lethal dose of WNV. While all control recipients, receiving saline died, transfer of
CD8+ T cells provided protection in up to 75% of recipient animals (**Figure. 1**). This
suggests that naïve CD8+ T cells can provide significant level of protection against WNV,
but does not formally rule out other elements of adaptive immunity. We therefore set to
investigate the epitope specificity of the CD8+ T cell response and test whether such
epitopes are important determinants of anti-WNV protection.

WNV protein overload provides means to identify protein targets for CD8 cells

Previously, it was shown that *in vitro* incubation of antigen-presenting cells
(APC) with massive amounts of proteins (mg range) can result in processing and
presentation of the MHC class I epitopes of the protein (319, 320). This "protein
overload" could theoretically lead to uptake of some of the protein and its leaking into
MHC class I biosynthetic/Ag processing pathway; or to the extracellular cleavage of just
enough of the correct peptide from imperfectly synthesized or even fully synthesized
proteins to produce MHC targeting (where only a few peptides, and even a single one
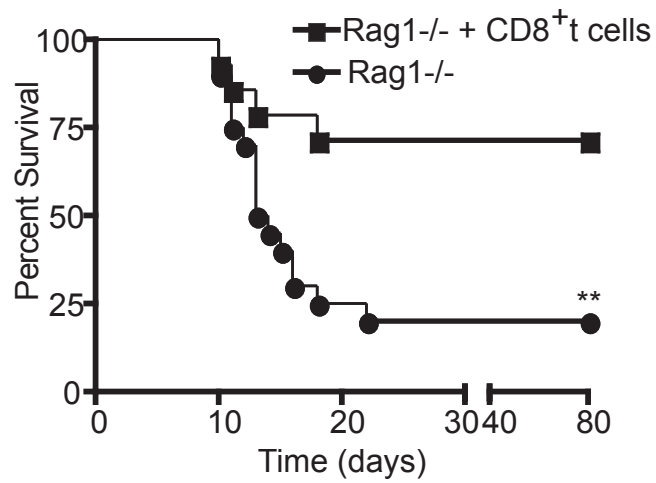
Figure 1. Protective effect of Naive CD8 T cells Rag1-/-. Splenic CD8^{+} T cells (2-5x10^{6}) from naive C57BL/6 mice were isolated by positive selection (90-95% purity) and transferred to C57BL/6 Rag1^{-/-} mice. 24 hours after transfer, mice were challenged with 300pfu WNV sub-cutaneously. Significant difference according to the log-rank test ** p<0.005.

(321, 322) would suffice to detect biological activity); or by other, unknown mechanisms. Regardless of the exact mechanism(s), we reasoned that this strategy could allow us to screen for those WNV segments that contain the H-2^b-restricted CD8$^+$ epitopes. To that effect, we expressed each of the WNV protein segments as glutathione-transferase fusion proteins in E. coli, as described in Methods, and illustrated in **Figure 2** for representative proteins, (arrows pointing to the molecular weight of indicated GST proteins before and after induction with IPTG). Each of the proteins was incubated at 0.1 mg/well with H-2^b splenocytes from infected mice for 12h and production of IFNγ by splenic CD8$^+$ T cells measured by the ICCS assay as described in Methods. A dominant response was detected against NS4b, a prominent response against E and a borderline response to M and NS5 segments (**Table 1**). The combined magnitude of the response against all proteins amounted to 50-120% of the IFNγ response obtained with polyclonal stimulation with anti-CD3 mAb (not shown), and was typically higher than the response to virally infected cells (not shown, but see also **Figure 4**, values shown for "total"). Importantly, reactivity against each of the segments was confirmed using pools of overlapping peptides (**Table 1**), consistent with the idea that epitopes indeed existed within these proteins.

Definition of dominant and subdominant WNV epitopes that elicit CD8+ T cell responses in vivo

To identify optimal epitopes within the above WNV proteins, we screened smaller peptide pools and finally individual 15-mer peptides. From the identified 15-mer, we aligned the amino acid sequence based on known H-2K^b and H-2D^b peptide binding motifs (323), and synthesized the optimal predicted peptides, as well as their potential N-
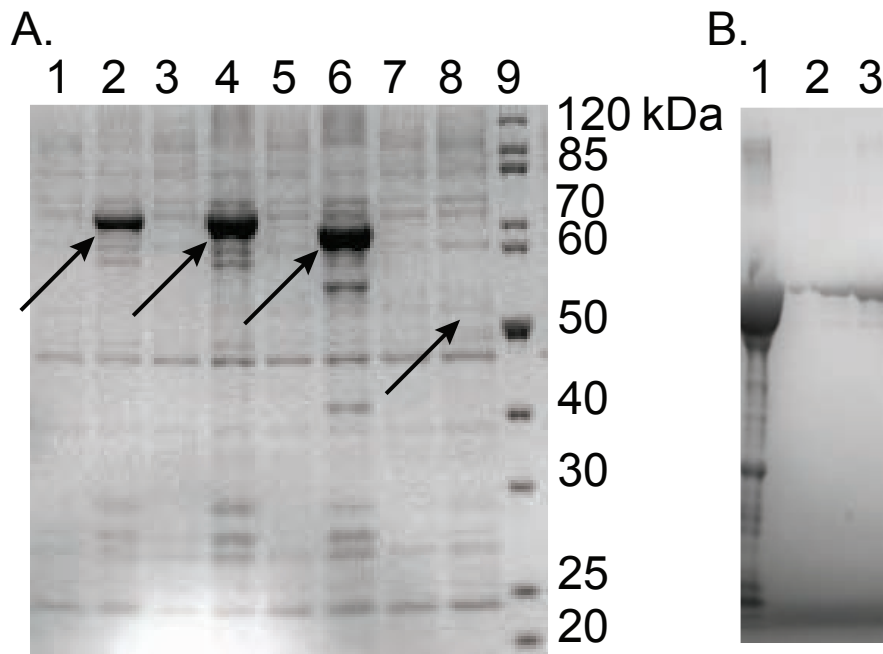
Figure 2. Production of GST-WNV orf proteins. Plasmid pGEX 4T-1 was used to express each of the 10 functional proteins from WNV. Transformed BL21 (DE3) cells were grown in Luria broth and induced with IPTG as described in Methods, lysed, purified over a GST column and resolved on a 10-15% gradient SDS-PAGE. **A.** Lanes show uninduced and induced whole cell protein lysates, with arrows indicating the position of E, NS1 and NS4b. **B.** Lane 1- whole lysate after refolding of GST-envelope, Lane 2,3- elution of GST-envelop from GST column.

GST-protein	Percent Response	Peptide Pool (15mers)	Exp. 1 - % Response	Exp. 2 - % Response	Exp. 3 - % Response
Capsid	1.8	Capsid	0.47	1.45	0.87
pre-membrane + membrane	7.8	pre-membrane + membrane	7.45	5.06	1.09
Envelope	31.9	Envelope	26.36	13.74	10.67
NS1	0.6	NS1	0.71	0.00	0.52
NS2	0.5				
NS2a	ND	NS2a	0.59	0.54	0.25
NS2b	ND	NS2b	1.06	0.18	0.93
NS3	0.4	NS3	0.35	0.36	0.02
NS4a	0.8	NS4a	0.35	0.36	0.59
NS4b	55.8	NS4b	60.40	72.51	83.15
NS5	ND	NS5	2.25	5.79	2.79

TABLE 1. CD8 T-cell response to WNV proteins and peptide pools. Immune spleen cells were treated by protein overload approach (second column) or with overlapping peptide pools (last three columns) as described in Methods and were then scored for production of IFN by CD8 T-cells. Percent response was calculated by adding the response to all protein segments or all peptide pools, respectively, and using that value as 100%; responses to individual proteins/peptide pools were calculated from those values.

and C-terminal extensions, to identify optimal peptides that represent major targets for

CD8$^+$ T cells. These peptides were then used in T cell functional assays to narrow down

and confirm the optimal epitopes. Representative examples of T cell reactivity by IFNγ

secretion against 15-mers and optimal peptides are shown in **Figure 3**, and the summary

of the results for all epitopes are shown in **Table 2**, which lists all identified peptides and

their restriction elements. All peptides described are cited with their inclusive amino acid

numbers the first time in the text, as well as in **Table 2**. Subsequently, abbreviated

nomenclature was used, based upon designation of the protein component from which the

peptide is derived, e.g. E (envelope), etc., followed by the initial amino acid at which the

peptide begins, counting from the beginning of the polyprotein; therefore, the envelope

peptide 347-354 is designated E_{347} etc.

In agreement with the reactivity to whole proteins, the strongest reactivity was

observed against one of the NS4b 15-mers, amounting to 50-70% of the reactivity to the

whole virus. From that 15-mer, we identified the NS4b nonamer peptide 2488-2496,

SSVWNATTA (**Table 2**), which was shown to bind in a quantitative manner to H-2D^b

(**Figure 4**), to cause robust IFNγ secretion (**Figure 5A**) and to readily sensitize target

cells for CTL lysis (**Figure 5B**). In the same manner, we have identified other WNV

epitopes – the H-2K^b binder $E_{347-354,}$ RSYCYLAT, which is responsible for 17-30% of the

whole response, the H-2D^b binder $E_{521-529}$ TVWRNRETL, the H-2D^b binder $E_{293-301}$

LGMSNRDFL, the H-2K^b-restricted NS4b$_{2286-2295}$ ISSLFGQRI, and the H-2D^b-restricted

NS5$_{2863-2871}$, DTITNVTTM peptide (**Figure 4 & 5**, **Table 2** and not shown), with smaller

and more variable contributions to the response. Of the peptides tested, the NS4b$_{2488}$

peptide bound strongly to D^b, whereas the two next peptides on the immunodominance
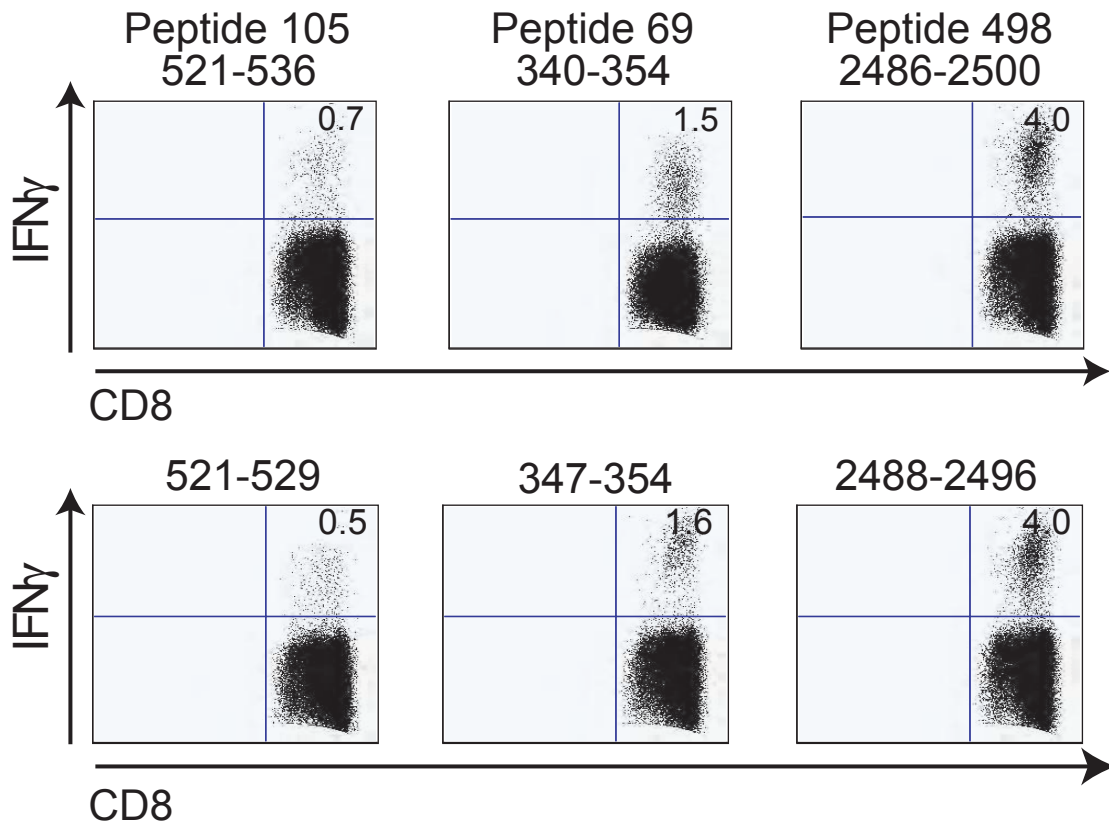
Figure 3. Narrowing down of epitopes from 15-mers to optimal epitopes. Three examples of the IFNγ response to 15-mer peptides from E and NS4b (top panels), and the response to optimal peptides contained within these 15-mers, identified by deduction using predicted MHC binding motifs (bottom panels). Percentages denote the representation of CD8$^+$ cells secreting IFNγ in response to indicated peptides among the total CD8$^+$ population on day 7 after infection. ICCS and FCM were done as described in Methods, using T-cells from a WNV-immune donor. A summary of the results for all epitopes identified in this manner is shown in Table 2.

15-mer peptide	Origin	sequence	Optimal epitope /stimulus	peptide sequence/ restriction	Exp. 1 %CD8^{+} IFN^{+} cells	Exp. 2 %CD8^{+} IFN^{+} cells	Exp. 3 %CD8^{+} IFN^{+} cells	Exp. 4 %CD8^{+} IFN^{+} cells
			2c11		17.66	4.80	15.12	5.13
pep 59 (294 - 303)	ENV	FNC **LGMSNR DFL** EGV	**ENV 294 -302**	**LGMSNR DFL (Db)**	ND	ND	0.77	0.52
pep 69 (347 - 354)	ENV	ANLAEV **RSYCYL ATV**	ENV 345 -353	EVRSY CYLA	0.23	0.48	0.25	ND
pep 70	ENV	V **RSYCYL ATV** SDLST	**ENV 347 -354**	**RSYCYL AT (Kb)**	2.17	0.81	3.22	1.84
			ENV 347 -355	RSYCY LATV	0.35	0.07	0.18	ND
pep 104 (521 - 529)	ENV	SSAG **STVWR NRET L** M	ENV 518 -528	AGSTVWRNR ET	0.19	0.54	0.26	ND
pep 105	ENV	**TVW RNRE TLM** EFEEP	**ENV 521 -529**	**TVW RNRE TL (Db)**	0.59	0.62	0.46	0.26
pep 155 (771 - 778)	ENV	**IALTFL AV** GG VLLFL	**ENV 771 -778**	**IALTFL AV (Kb)**	ND	ND	2.28	0.64
pep 498 (2488 - 2496)	NS4b	GA **SSVWNATTA** IGLC	**NS4b 2488 - 2496**	**SSVWNATTA (Db)**	5.49	2.90	6.00	3.14
	NS4b	GA **SSVWNATTA** IGLC	NS4b 2488 - 2497	SSVWN ATTAI	5.02	ND	5.17	3.0
pep 573 (2863 - 2872)	NS5	PW **DTI TNVTTM** AMTD	**NS5 2863 - 2872**	**DTITN VTTM (Db)**	ND	ND	0.27	0.21
pep 635 (3171 - 3181)	NS5	GKGPKV RTW LFEN GE	NS5 3171 - 3181	GKGPKV RTWL	ND	ND	0.16	ND
			NS5 3172 - 3181	KGPKV RTWL	ND	ND	0.17	ND
			NS5 3177 - 3184	**RTW LFENG (Kb)**	ND	ND	0.15	0.21
pep 643 (3172 - 3181)	NS5	LHFLN AMSKVRKDIQ	NS5 3216 - 3224	AMSKVRKDI	ND	ND	0.16	0.18

TABLE 2. **Identification of optimal CD8 T-cell epitopes using overlapping WNV peptides** a)Experiments were performed as in Tab. 1, except that individual peptides were used, and that results are not represented relative to total reactivity, but rather as % of total splenic CD8^{+} cells secreting IFN . Boldfaced are the sequences and the peptide designations for those peptides that represent optimal epitopes, which produced unambiguous T-cell reactivity. ND – not determined.
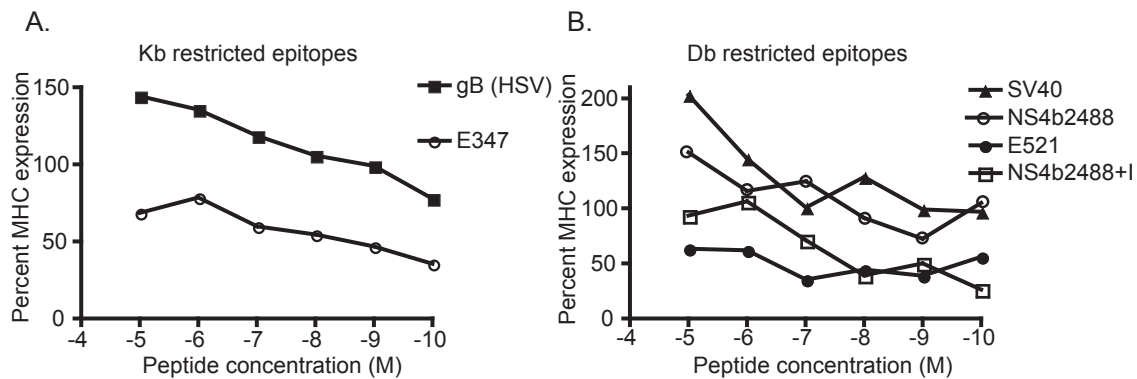
Figure 4. Epitope binding kinetics of selected peptides to H-2K^b and H-2D^b as estimated by the RMA-S MHC stabilization assay. Optimal peptides, used at indicated concentration, were tested for their ability to stabilize empty class I MHC molecules on RMA-S cells. RMA-S stabilization assay was performed exactly as described in Methods, and stable expression of H-2K^b (left panel) and H-2D^b (right panel) detected using FCM and mAb AF6 and B22-249, respectively. Results are plotted as % maximal class I expression, calculated as mean relative fluorescence intensity compared to that of class I molecules expressed by RMA-S cells at 29^{0}C, which was taken as 100%. Given that optimal peptides often induce higher expression of class I molecules than that achieved with empty molecules at 29^{0}C, results in this assay can (and do) exceed 100%. Peptides known to bind to K^b and D^b (HSV gB$_{498-505}$; and SV40 large T$_{404-412}$, respectively) were used as positive controls. Representative results for one out of three experiments are shown.

A.

IFNγ →

CD8 →

| Irrelavant peptide 0.11 | 294-302 0.51 | 347-354 1.87 | 521-529 0.23 | 771-778 0.98 |
| 2488-2496 2.97 | 2488-2497 2.88 | 2863-2871 0.23 | 3177-3184 0.17 | 3216-3224 0.16 |

B.

Percent Specific Lysis vs Effector:Target Ratio

- □ 2488-2496
- ▲ 347-354
- ○ 771-778
- ■ 294-303
- △ 521-529
- ● el-4

C.

NS4b 2488 tetramer →

CD43 →

Naive: 1.07 | 0.03 | 96 | 2.86 — CD8+ IFNγ+=0.13

Day 7: 2.46 | 3.76 | 87 | 6.75 — CD8+ IFNγ+=3.81

Day 50: 1.52 | 0.18 | 96.2 | 2.09 — CD8+ IFNγ+=1.36
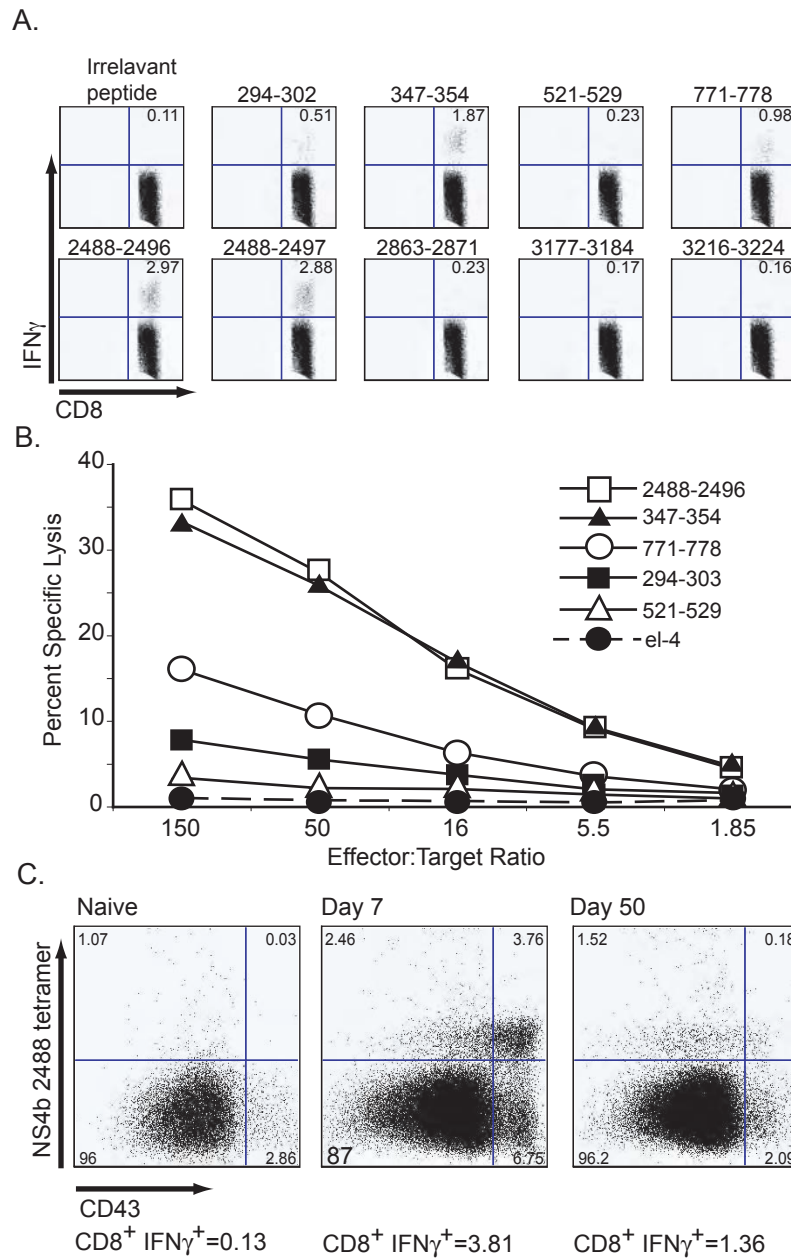
Figure 5. CD8 T cell response to the WNV epitope. B6 mice were infected with WNV, their splenic cells isolated 7 days later and tested by three functional assays. **A.** Peptide-stimulated cytokine secretion of IFNγ was determined following 6h stimulation by ICCS. **B.** Cytotoxicity was tested by a direct ex-vivo ^{51}Cr-release assay. **C.** NS4b$_{2488}$:D^b pMHC tetramer staining of immune CD8$^+$ T cells on days 7 and 50 post infection as gated on CD8$^+$ T cells. Numbers under the panels denote percentage of CD8$^+$ cells secreting IFN in samples corresponding to the above tetramer staining. All experiments were performed exactly as in methods. Results are representative of a minimum of 3 experiments shown in each figure.

scale, E_{347} and, in particular, E_{521}, exhibited weaker binding (**Figure 4**), possibly accounting for the weaker responses. Of interest, direct ex vivo CTL activity directly correlated to the observed immunodominance by ICCS (**Figure 3B**). We also identified several other candidate peptides from NS5, with less prominent or borderline contribution to the overall response (sum of these peptides accounts for >3% of total reactivity, not shown), which were not tested extensively (**Table 2** and not shown).

We next confirmed the above results using pMHC tetramers against the dominant NS4b$_{2488}$ peptide. This analysis independently confirmed that the response to identified epitopes occurs in vivo and is a direct consequence of viral infection (**Figure 5C**). Phenotypic analysis of epitope-responding CD8 T cells (**Figure 6**) further confirmed their correct identification as WNV-specific cells. Not surprisingly, on day 7 post infection, the cells responding to the epitopes in vivo exhibited the expected phenotypic characteristics of effector cells during the acute phase of the response CD11a^{hi}CD62L^{lo}CD44^{hi}, secreting IFNγ, with a minor population secreting IL-2 as well (**Figure 6**). However, these cells did not downregulate CD127 at this time point (**Figure 6**), and also remained CD27^{hi}. A thorough kinetic analysis of these and other activation markers will be needed to resolve whether CD127 downregulation indeed fails to occur in WNV infection, or whether the kinetics of T cell marker expression in response to WNV may differ from that published for other viral infections (324).

At this point in the course of our study, we became aware that the group at the Washington University was performing a search for WNV epitopes using computer-assisted epitope prediction. To compare methods and validate each other's results, we swapped the identified sequences, and have realized that we identified largely
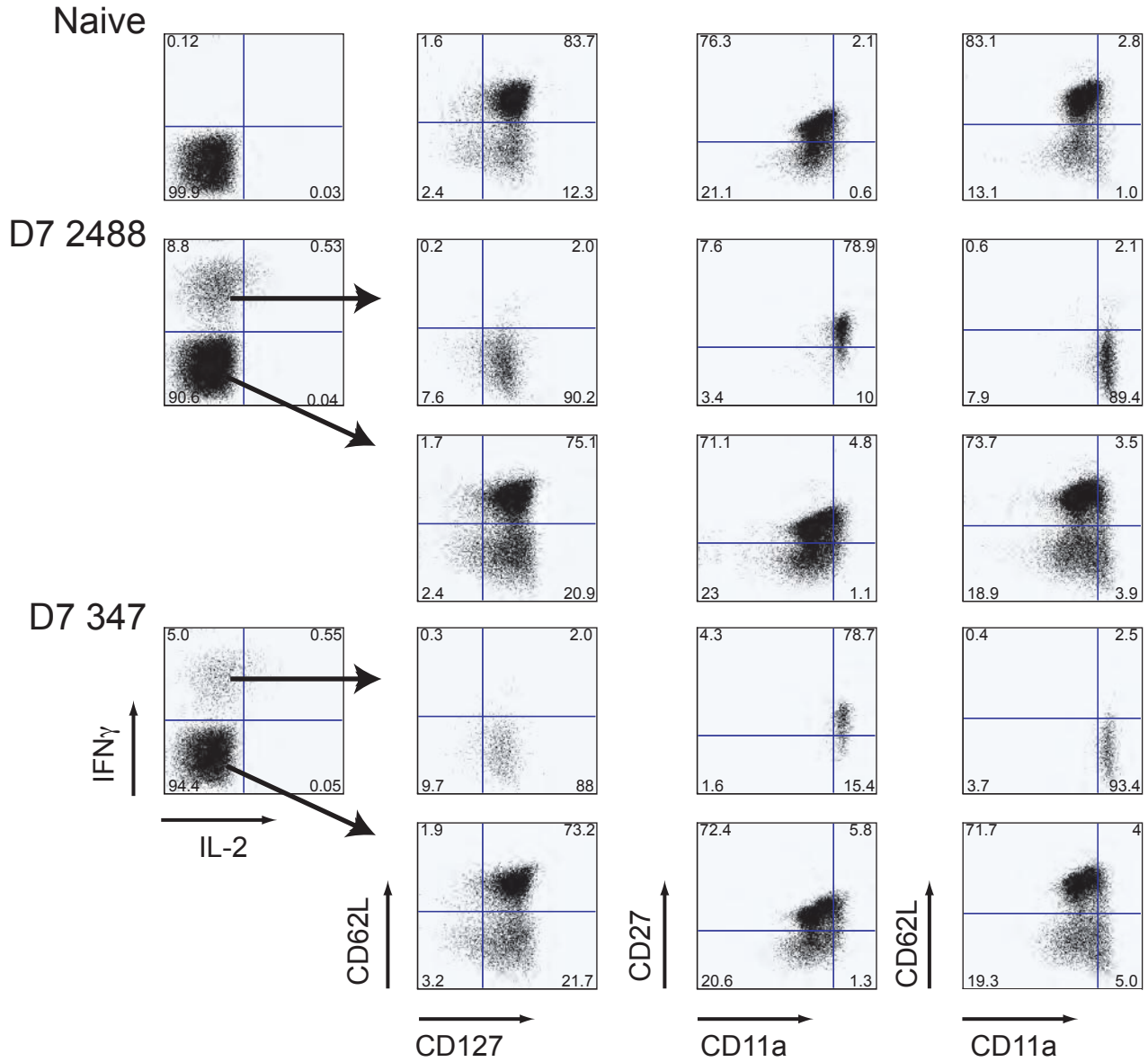
Figure 6. Phenotype of CD8 T-cells responding to NS4b$_{2488}$ and E$_{347}$ epitopes in the acute infection. Phenotypes of CD8$^+$ cells responding to NS4b$_{2488}$ and E$_{347}$ epitopes. Progressive gating is based upon the response measured by IFNγ and is indicated by arrows. For comparison, IFNγ-negative cells are shown in parallel panels. Results are representative of three experiments.

overlapping, but not identical, epitope sets. Thus, our approach had failed to identify one important epitope the H-2K^b binder E$_{771-778}$ IALTFLAV, (making up about 12-20% of the response). This was probably due to the problems surrounding synthesis of the 15-mer peptide, which was subsequently showed not to be stable in functional assays. Regardless, we synthesized this epitope and used it in subsequent studies to examine epitope hierarchy.

Primary and memory response of CD8 T cells to WNV epitopes

We next used the identified epitopes to examine and compare effector and memory stages of the CD8$^+$ response to WNV. ICCS was performed using either peptide pools (not shown) or optimal peptides (**Figure 7** and **Table 3**) on day 7 (effector phase) and day 50 (memory phase) post infection. Frequencies of the responding cells dropped from the peak of 5-8% of responding cells/total CD8+ T cells in the primary to ~1-1.5% of the cells in the memory phase, consistent with the expected expansion and contraction of the primary response (**Figure 7** and **Table 3**). In this experiment, E$_{347}$ yielded a very strong response, however, it still ranked behind NS4b$_{2488}$ in dominance. Otherwise, the hierarchy was similar to that noted in other experiments at the peak of acute infection (day 7) (**Figure 7**), and showed little change at 50 days after infection, in the memory phase of the response (**Figure 7** and **Table 3**), regardless whether we used peptide pools (not shown) or individual peptides (**Figure 7**). If anything, the immunodominance was more pronounced in favor of the NS4b and the two major E epitopes in the memory phase. Thus, whereas seven of the peptides tested gave signals above the background in the primary response, accounting for >90% of the response to the whole virus (**Figure 7**
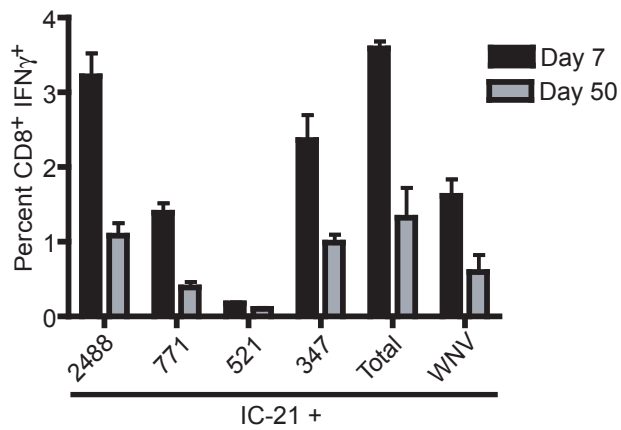
Figure 7. Primary and memory response to dominant and subdominant epitopes.
ICCS was performed at indicated times post infection using peptide pulse or WNV
infection of the H-2[b] macrophage cell line, IC-21. Results represent 4 animals/group (x ±
SEM) from one out of three experiments with comparable results.

15-mer peptide	Origin	sequence	Optimal epitope /stimulus	peptide sequence/ restriction	Exp. 3 %CD8$^+$ IFN$^+$ cells – d. 50	Exp. 4 %CD8$^+$ IFN$^+$ cells – d. 50
			HSV gB-8p	SSIEFARL (Kb)	0.06	0.12
			2c11		0.52	1.14
pep 59 (294-303)	ENV	FNC**LGMSNRDFL**EGV	**ENV 294-302**	**LGMSNRDFL(Db)**	0.05	0.36
pep 69 (347-354)	ENV	ANLAEV**RSYCYLAT**V	ENV 345-353	EVRSYCYLA	ND	ND
pep 70	ENV	V**RSYCYLAT**VSDLST	**ENV 347-354**	**RSYCYLAT (Kb)**	0.16	0.44
pep 104 (521-529)	ENV	SSAGS**TVWRNRETL**M	ENV 518-528	AGSTVWRNRET	0.01	ND
Pep 105	ENV	**TVWRNRETL**MEFEEP	**ENV 521-529**	**TVWRNRETL(Db)**	0.02	0.18
pep 155 (771-778)	ENV	**IALTFLAV**GGVLLFL	**ENV 771-778**	**IALTFLAV(Kb)**	0.05	0.27
pep 498 (2488-2496)	NS4b	GA**SSVWNATTA**IGLC	**NS4b 2488-2496**	**SSVWNATTA(Db)**	0.29	1.36
pep 498 (2488-2496)	NS4b	GA**SSVWNATTA**IGLC	NS4b 2488-2497	SSVWNATTAI	0.3	1.05
pep 573 (2863-2872)	NS5	PW**DTITNVTTM**AMTD	**NS5 2863-2872**	**DTITNVTTM(Db)**	0.04	0.14
pep 635 (3171-3181)	NS5	GKGPKV**RTWLFENG**E	NS5 3177-3184	**RTWLFENG (Kb)**	ND	0.14
pep 643 (3172-3181)	NS5	LHFLNAMSKVRKDIQ	NS5 3216-3224	AMSKVRKDI	ND	ND

Table 3. Memory response to optimal epitopes: Experiments were performed as described within the methods. Experiment three and four are the same experiments completed within table 2 of the main manuscript. Boldfaced are the sequences and the peptide designations for those peptides that represent optimal epitopes which produced unambiguous T-cell reactivity. ND – not determined.

and **Table 3**), the response to $NS4b_{2866}$ and E_{521} epitopes in the memory phase was no longer reliably detectable, consistent with further focusing of the immune response (**Figure 7**). Moreover, the response to individual peptides was always at least equal, and often significantly larger than the response to WNV-infected cells (**Figure 7** and not shown), probably due to peptide competition, unequal temporal expression of all epitopes or a combination of these and other, unknown, factors. Regardless of the exact reason, this suggested that we have indeed identified the vast majority of WNV epitopes in this MHC haplotype.

In vivo relevance of anti-WNV epitope CD8 T cell responses

The above epitopes were all identified using $CD8^+$ T cells from infected animals, and therefore would be expected to represent physiological targets of the cellular immune system. As the choice of the epitopes is dictated by both host and pathogen factors, and because factors such as precursor frequencies, p:MHC affinities, pMHC:TCR affinities and T cell avidities and viral immune evasion all influence the relative protective value of a response to a given epitope, we sought to investigate whether responses to individual epitopes can influence resistance to WNV infection. To that effect, we sought to test whether peptide-specific CTL lines can confer protection against the lethal WNV infection to $RAG-1^{-/-}$ mice. Splenocytes from infected animals were explanted on day 7 p.i. and were restimulated in vitro with indicated peptides. Seven days later, we obtained cell lines that were further purified by immunomagnetic sorting to contain <0.5% CD4 T cells and B-cells. These CTL lines were transferred into $RAG-1^{-/-}$ recipients, engraftment confirmed and animals infected 24 h later. In the absence of transferred cells, recipients

exhibited high mortality typical of animals lacking adaptive immune system (J.B. and

J.N-Z., unpublished results; (213)), and transfer of an irrelevant CTL line (specific for the

HSV-1 gB-8p epitope) did not improve their survival (**Figure 8**). By contrast, cell lines

directed against the two major WNV epitopes ($NS4b_{2488}$ and E_{347}) conferred a high

degree of protection (>75%); the CTL line directed against a minor epitope E_{521} provided

clear, but less strong, protection, which did not reach statistical significance. Therefore,

we conclude that CD8+ CTL lines, directed against major WNV epitopes identified in

this study, can provide significant protection against lethal WNV in vivo, in the absence

of other components of the adaptive immune system. This protection appeared to

correlate to immunodominance for the peptides tested.
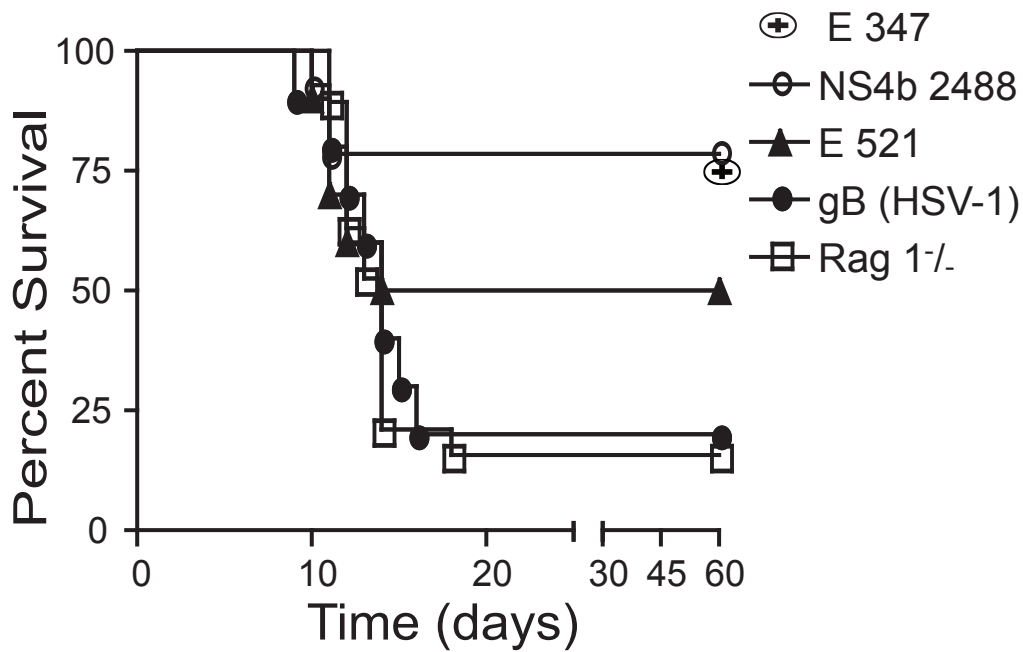
Figure 8. Antigen-specific CD8$^+$ T cells are sufficient for protection against lethal WNV encephalitis. CTL cell lines specific for indicated WNV or control epitopes were obtained as described in Methods, and were transferred into RAG-1$^{-/-}$ recipient, one day prior to challenge with a lethal dose (300 pfu) of WNV. Both NS4b$_{2488}$ (open circles) and E$_{347}$ (open triangles) significantly increased the resistance of RAG-1$^{-/-}$ mice to WNV challenge in comparison to RAG-1$^{-/-}$ mice that received gB specific CD8 T cells (log-rank test p<0.01), whereas CTL lines directed against E$_{521}$ (closed triangles) provided some protection, that did not reach statistical significance. This is the compilation of 2 experiments with each group containing a total of 10-12 mice.

<u>Discussion</u>

In this study, we report on the ability of CD8$^+$ T cells to protect alymphoid mice from lethal infection, and on the identity of the molecular targets, which are the focus of attack by these CD8$^+$ T cells. Highly enriched naïve CD8 T cells transferred into RAG-KO mice protected 75% of the mice from WNV encephalitis, suggesting that in a large number of cases, CD8 T cells can be sufficient for protection against this flavivirus. This finding is consistent with previous observations of Shresta et al. (275) and Wang et al. (325), which implicated CD8+ T cells as necessary in the recovery from primary WNV infection. Further experiments will be needed to determine whether naïve CD8 T cells can confer complete protection, and what other elements of the immune system, previously implicated in WNV resistance, may be necessary and sufficient to confer full protection.

We next investigated the molecular complexity of the anti-WNV CD8 T cell response at the target epitope level. Six H-2^b WNV epitopes recognized by the CD8$^+$ T cells were identified, using a combination of protein overload (suitable for rapid identification of proteins that contain T cell epitopes (319)) and overlapping peptide library approaches. Of these six, the strongly immunodominant peptide NS4b$_{2488,}$ was responsible for 50-70% of the total reactivity to peptide pools. Next in the hierarchy was E$_{347}$, which consistently carried 17-25% of the reactivity, followed, in order, by E$_{521,}$ E$_{293}$, NS4b$_{2286}$ and NS5$_{2863}$, which gave smaller and more variable responses.

As mentioned above, concurrently with our studies, a group from the Washington University had used a computer-based epitope prediction algorithm to identify epitopes from this same pathogen, also in the H-2^b haplotype (companion paper – Purtha et

al.)(278). Our results were largely overlapping but not identical. Of importance, both approaches identified the same immunodominant $NS4b_{2488}$ epitope as the main CD8 target albeit the computer algorithm approach identified the longer version, SSVWNATTAI, which, in our hands, tended to stimulate slightly smaller functional responses compared to the nonamer SSVWNATTA (Table 2). Of more interest were the differences between the two approaches. Thus, while some of the peptides listed in Tables 1& 2 were not identified by the computer algorithm approach, we also realized that our approach had failed to identify one epitope, E_{771}, (making up about 12-20% of the response). This was probably due to the problems surrounding synthesis of the 15-mer peptide, which was subsequently shown not to be stable in functional assays. This peptide was subsequently used in functional assays.

Using all of the above peptides, we have assigned the immunodominance hierarchy to the identified epitopes in the order $NS4b_{2488} > E_{347} > E_{771} >$ all other epitopes. The three most dominant epitopes above could readily account for >80% of the total response. Therefore, they provide means to easily track the $CD8^+$ T cell response to WNV in B6 mice in a single ICCS sample.   An interesting finding was that in the primary response, there was no downregulation of CD127 in response to any of the epitopes tested (Figure 6). It is unclear whether this is due to the nature of the viral infection or due to a possible difference in kinetics of activation marker expression. Nevertheless, immunological memory was clearly formed against the key epitopes. In fact, in the course of the memory responses, the $CD8^+$ T cell response to WNV further focused: while the response to these three epitopes dominated even more, the responses to other epitopes diminished and were often not detectable.

Most importantly, when a few identified peptides were tested for their ability to elicit CTL lines which can protect animals against lethal WNV, those cell lines showed protective activity in vivo, and peptide immunodominance appeared to correlate with protective activity. We conclude that the identified epitopes present not only targets of CD8 attack, but that they have the capacity to elicit in vivo protective CD8$^+$ T cells. These epitopes should therefore help unravel the kinetics of CD8 T cell response to WNV, and help us provide insight into the presence or the absence of specific defects in WNV adaptive immunity in old mice.

Protection against WNV in mice has received considerable attention in the last several years, and it is clear that both innate and adaptive immune systems play a role (33, 259, 326). This includes natural and acquired early antibodies (213), complement (265), type I IFN and IFNγ (33, 216, 327) and T cells (325), including CD8 T cells (275, 288, 328) and perforin (253). What is less clear is which of these mechanisms are absolutely required and are both necessary and sufficient for protection against WNV. With regard to this last point, we show (Figure 8) that virus-specific CD8$^+$ T cells are not only necessary, but can be sufficient to mediate protection against WNV, in that they can protect the majority of adoptive hosts in the absence of other components of adaptive immunity. Therefore, a vaccine that would include CD8$^+$ epitopes may be a viable strategy to prevent severe WNV disease.

We clearly show the protective capability of CD8 T cells during WNV infection, however, we have not addressed the potential requirement for CD4 T cells during WNV infection. In the next chapter we address both the requirement for the CD4 T cell

response to WNV infection, and the protective capacity of antigen-specific CD4 T cells

during WNV infection.

Material and Methods

Cloning and production of GST-WNV proteins. WNV RNA was isolated using Tri-reagent (MRC), and was converted into cDNA using SuperScript III (Invitrogen, Carlsbad, CA) and random hexamers. cDNA was used as template and PCR products were generated using Accuprime PFX enzyme (Invitrogen). PCR products were enzyme digested and ligated into the pGEX 4T-1 vector (GE Healthcare Life Sciences, Piscataway, NJ) to produce 10 individual plasmids encoding a GST-fusion protein of each of the 10 WNV protein subunits. Individual GST-WNV proteins were produced in BL-21 (DE3) cells (Stratagene, La Jolla, CA) as inclusion bodies after induction with IPTG. Inclusion bodies were solubilized with urea, and the GST proteins refolded during dialysis in the presence of protease inhibitors. Following refolding, proteins were purified using glutathione-Sepharose 4B beads (Pharmacia, Piscataway, NJ). Purified proteins were dialyzed overnight in 1xPBS, concentrated using a Centricon membrane (Millipore, Freehold, NJ) and protein concentration determined using a BCA kit (Biorad, Hercules, CA). A small aliquot was run on a SDS-PAGE gel transferred to a PVDF membrane (BioRad) and probed with anti-WNV serum from immune mice. Remaining portions of protein were frozen at -80°C until further use.

Mice. Adult (2-6 months old) male C57BL/6 (B6) mice were purchased from the National Cancer Institute Breeding Program (Frederick, MD). B6.Rag1$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and bred at the VGTI vivarium (Oregon Health & Science University); they were used at 2-4 months of age. All animals were housed and bred under specific pathogen-free conditions at the Oregon

Health & Science University. All WNV experiments were completed within a United

States Department of Agriculture (USDA, Frederick, MD) approved Biosafety Level

(BSL) 3 facility, and were approved by the Institutional Animal Care and Use

Committee, and the Institutional Biosafety Committee in accordance with the applicable

federal, state, and local regulations.

Virus, Peptides, and Cell Lines. West Nile virus strains NY99-crow, 31A and 385-99

were used and all virus strains yielded similar results. West Nile virus strains: NY99 and

385-99 were kind gifts of Drs. W. Ian Lipkin (Columbia University, New York, NY) and

Robert Tesh (University of Texas Medical Branch, Galveston, TX), respectively; strain

31A was provided by the USDA reagent program (Ames, IA). An overlapping peptide

library covering the entire length of the viral polyprotein (15-mers overlapping by 10 aa)

was obtained from Sigma Aldrich (St. Louis, MO). Additional synthetic peptides were

purchased at >95% purity from Sigma Aldrich and 21st Century Biochemicals (Marlboro,

MA), diluted in 10% H_2O/90% DMSO, stored at -80^0 C and subsequently used at

indicated concentrations. Virus was grown in mycoplasma-negative Vero cells, cultured

under aseptic conditions as described previously; mycoplasma-negative EL-4, IC-21 and

MC57g cells (all H-2^b) were used in target-cell and stimulation assays. Cells were

infected using variable multiplicity of infection (MOI) as indicated, however, for

intracellular cytokine staining and for ^{51}Cr assays or for any experiments using MC57g

cells, the cells were infected at the MOI of 10 for 30 hours prior to use.

Intracellular cytokine and surface flow cytofluorometric (FCM) staining. Cytokine-producing T cells were detected using the Cytofix-Cytoperm Kit (BD PharMingen, San Diego, CA), as described. Single-cell splenocyte suspension was depleted of red blood cells and was incubated with 1μM peptide or infected with WNV in the presence of 5μg/ml Brefeldin A (Sigma Aldrich) for 6 h at 37 °C. After six hours the cells were washed and blocked with Fc block (anti–mouse FcγRI/III; BD PharMingen) and incubated overnight in the presence of a saturating dose of surface antibodies against CD8, CD3, CD4, CD11a, CD43 (Clone 1B11), CD44 and CD62L (BD-PharMingen). After washing, the cells were fixed, permeabilized and intracellular antibodies (anti-IFN-γ, or anti- IL-2; BD-PharMingen) added for 30 minutes. The samples were then washed and analyzed using either a FACSCalibur or LSR II cytometer (Becton Dickinson Immunocytometry Systems, Sunnyvalle, CA) instrument. Where indicated, pMHC tetramers, conjugated to APC (NIH Tetramer Facility, Atlanta, GA) were added to unstimulated cells in conjunction with other markers for surface staining. FCM analysis was performed by collecting a minimum of 5×10^4 events and gates set on lymphocyte population based on forward and orthogonal light scatter, followed by marker positioning to denote fluorescence greater than that of control stained or unstained cells.

pMHC stabilization assay. This assay was performed using the RMA-S cell line, exactly as previously described (329, 330), using mAbs Y3 and B22/249, followed by secondary, allotype-specific antibodies conjugated to PE (Southern Biotech, Huntsville, AL).

<u>Infection, immunization, CTL line generation and CTL assays</u>. Mice were immunized subcutaneously (s.c.) between the shoulder blades with 20-600 pfu WNV, or with 1μg of indicated WNV peptides emulsified in the adjuvant TiterMax (CytRx, Norcross, GA), exactly as previously described (331). Seven days later, splenocytes were isolated and subjected to FCM, ICCS or CTL assay analysis as described above, or were restimulated in vitro to generate CTL lines for adoptive transfer experiments below. Briefly, splenocytes were co-cultured with irradiated (30Gy), peptide-coated (0.1 μg/ml) syngeneic spleen cells for 7 days, cells harvested, purified using anti-CD8-coated magnetic beads (Milteny Biotech, Santa Cruz, CA) and transferred as described below. Infection was performed s.c. using pre-titrated virus dose lethal for B6.Rag-1$^{-/-}$ (300 pfu).

Direct ex vivo CTL activity was determined using peptide-coated and control EL-4 thymoma cells as targets. Radioactivity was measured using TopCount Packard δ/γ radioactivity reader (Packard Co., Detroit, MI), using a standard ^{51}Cr-release assay, exactly as previously described (331).

<u>Adoptive transfer and virus challenge experiments</u>. For naïve CD8^{+} T cell transfer, spleens from 4-wk old B6 mice, containing <u>less than </u>5% CD44^{hi} (memory) splenic T cells, were coated with anti-CD8-coated beads, and CD8^{+} cells isolated at 90-95% purity. These cells were transferred i.v. (5-10 x 10^{6} cells/recipient), transfers monitored and challenge performed as below.

Peptide-specific CTL lines were generated by in vitro restimulation of WNV-primed spleen cells, as above, were purified to deplete CD4+ and B220+ cells at <1%, and were injected (2-5 x 10^{6} cells/recipient) i.v. into RAG1-/- recipients and

engraftment success was evaluated by FCM 24 h later. Animals were challenged 24 h

after cell transfer as described above. Survival was scored on a daily basis. Death

occurred between days 10 and 18, and all animals surviving this period remained disease

free for 60-90 days at which point the experiment was terminated. Data is shown as

percent survival at the termination of the experiment, with the statistical significance

determined using Fisher's exact test.

Chapter Three

CD4 T cells protect against West Nile virus infection by direct anti-viral
cytotoxicity and cytokine secretion.

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Abstract

        CD4 T cells have been shown to be necessary for prevention of encephalitis during West Nile virus infection. However, the mechanisms used by antigen-specific CD4 T cells to protect mice from West Nile virus encephalitis has not been fully elucidated. It is currently believed that CD4 T cells are protective because they maintain the CD8 T cell response and improved antibody production. To unambiguously test whether CD4 T cells may also exhibit direct anti-viral activity, we have adoptively transferred naïve CD4 T cells into RAG1-/- mice, which were then infected with an otherwise lethal West Nile virus dose. To our surprise, transfer of highly enriched <u>naive CD4 T cells</u> protected the majority of RAG1-/- mice against lethal West Nile virus infection. To more clearly define the functional mechanisms of protection employed by the responding naïve CD4 T cells, we defined the peptide specificities of the CD4 T cells responding to West Nile virus infection within C57BL/6 (H-2^b) mice, and used these peptides to functionally characterize the CD4 T cell response to West Nile virus. WNV-specific CD4 T cells produced IL-2 and IFN-γ, but also exhibited *in vivo* cytotoxicity. Furthermore, peptide vaccination using CD4 epitopes conferred protection against lethal West Nile virus infection in immunocompetent mice. These results demonstrate the role of antigen-specific CD4 T cell direct effector function in preventing severe West Nile virus disease.

<u>Introduction</u>

West Nile virus (WNV) is a small-enveloped arbovirus of the Flaviviradae family that persists in an enzootic cycle between mosquitoes and birds, with humans and many other animals as incidental hosts. Since WNV appeared at the Eastern seaboard of the United States in 1999 (173, 231), it has spread through all 48 continental states, infecting more than 10,000 people and killing nearly 300 people in 2003 alone (316, 317). WNV leads to systemic disease in approximately 20% of individuals, with the most severe disease caused by viral neuroinvasion resulting in meningitis and encephalitis (177, 318) in about 5% of the infected people. T cells play an essential role in preventing meningitis and encephalitis upon primary infection, and limiting disease severity upon potential re-infection. It has been shown that both CD4 and CD8 T cells are required for the control and clearance of West Nile virus (219, 275, 280). Still, the relative importance of each cell population at different stages of infection and the critical anti-viral mechanisms employed by each cell population in controlling systemic and CNS infection remain to be fully elucidated.

Although CD4 T cells were shown to be required for survival following WNV infection in antibody-depleted and the CD4 T cell deficient mouse strains (CD4-/- and MHC class II-/-), (277), their direct effector function was not fully investigated. CD4 T cell deficient mice, generated by continued antibody depletion, exhibited high viral titers for over 50 days within the CNS, eventually leading to death (219). In these same mice, viral titers in the spleen were not altered, suggesting that systemic, but not CNS, virus control may be predominantly CD4 T cell-independent (219). Moreover, these same experiments suggested that CD4 T cells are responsible for aiding in the survival and

proliferation of CD8 T cells and the priming of B cells (277) but the underlying mechanisms remain unknown.

Similarly, prior work has identified a requirement for CD4 T cells in controlling other Flavivirus infections, including those with the Japanese Encephalitis virus (JEV) (281) and Yellow Fever virus (YFV) (282), but no direct role for CD4 T cell effector function was investigated. CD4 T cells, in either infection, may play a direct role in viral clearance from the periphery and the CNS using either cytokines such as IFNγ or direct cytotoxicity. For example, during influenza infection CD4 T cells use perforin-mediated cytotoxicity to clear virus from the periphery, (283) whereas measles-specific CD4 T cells use IFNγ to control virus within the CNS (284, 285).

In this study we show that CD4 T cells are sufficient for the control of WNV infection in RAG1-/- mice. Using newly defined T cell epitopes, we show that CD4 T cells are capable of both secreting cytokines and being cytolytic during an infection. Whereas eliminating antigen-specific CD4 T cells in C57BL/6 mice increased WNV mortality, vaccinating mice with CD4 epitopes increased protection. This clearly identifies a direct effector role of CD4 T cells that contributes to protection during primary WNV infection.

Results

Naïve CD4 T cells protect mice from WNV severe disease

To confirm previous results on the CD4 T cell requirement for control of WNV infection, we depleted CD4 T cells in B6 mice just prior to WNV infection. The CD4 T cells were depleted using the monoclonal antibody GK1.5 and depletion was confirmed by flow cytofluorometric (FCM) analysis of peripheral blood mononuclear cells, where there were fewer than 0.05% CD4 T cells remaining (data not shown). Antibody depletion of CD4 T cells in C57BL/6 mice during WNV infection resulted in a significant increase in the mortality rate (p<0.005), but not in the mean survival time (MST) (**Figure 1A**). When mice were infected with 400 plaque forming units (pfu) of WNV, 66% of the C57BL/6 mice survived compared to only 20% of the CD4 T cell depleted mice. From this result we concluded that within an immunocompetent animal, CD4 T cells are necessary for protection against severe WNV disease, confirming results previously published by Sitati et al. (277).

After confirming the necessity of CD4 T cells during WNV infection we sought to determine whether naïve CD4 T cells were sufficient for direct control of WNV disease in the absence of CD8 T cells or B cells. We and others have previously shown that recombination activating gene 1-/- mice (RAG1-/-), which contain no T and B cells, are extremely sensitive to WNV infection (216, 279). Therefore RAG1 -/- mice can serve as an excellent host to determine the relative contribution of different lymphocyte subsets in protecting from disease upon adoptive transfer. For our protection experiment, CD4 T cells were purified by negative selection using magnetic beads. The resulting populations were 85-95% CD4$^+$, but never contained more than 0.5% contaminating
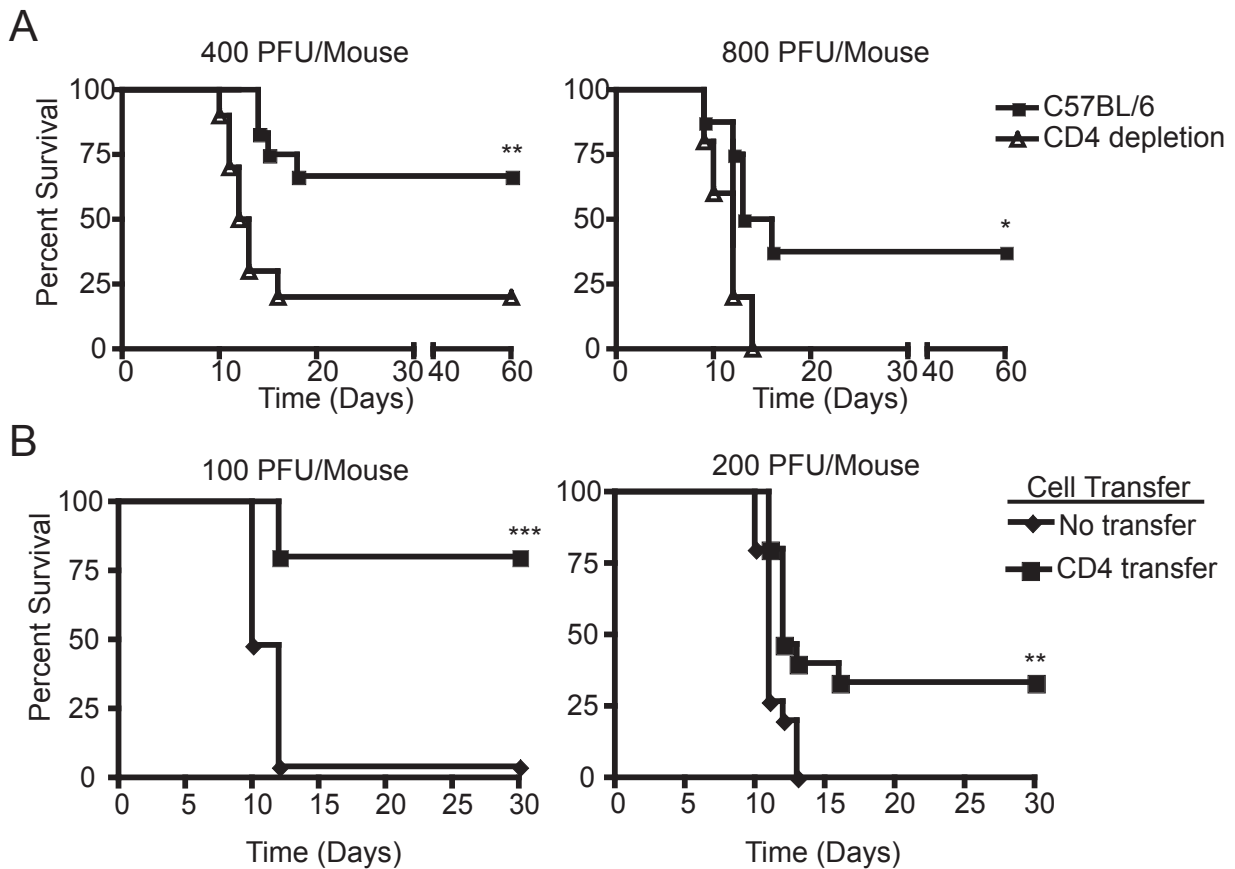
Figure 1. Protective effect of Naïve CD4 T cells. **A**. Antibody depletion of CD4 T cells renders C57BL/6 mice significantly (**p<0.005, *p<0.04) more susceptible to WNV induced mortality. CD4 T cells were depleted using two doses of GK1.5 antibody on day -3 and day 0, then infected with either 400pfu/mouse (left) or 800pfu/mouse (right). N=10 mice per group. One representative experiment of two. **B**. Adoptive transfer of naïve CD4 T cells provided protection to Rag1-/- mice against lethal WNV infection. Splenic $CD4^+$ T cells ($5-10x10^6$) from naive C57BL/6 mice were isolated by negative selection (80-95% purity) and transferred to C57BL/6 $Rag1^{-/-}$ mice. 24 hours after transfer, mice were challenged with 100 pfu or 200 pfu WNV sc. Significant difference according to the log-rank test (*** p<0.0005, left) (**p<0.002, right). Two experiments are shown, representative of a total of 4.

CD8$^+$ or CD19$^+$ cells. We transferred 5x10^6-1x10^7 CD4 T cells from naïve C57BL/6 mice into RAG1-/- deficient mice and challenged these mice with WNV. Adoptively transferred naïve CD4 T cells significantly protected (p<0.0005) the RAG1-/- mice from mortality following subcutaneous WNV challenge with 100pfu or 200pfu (**Figure 1B**). Indeed, 80% of the RAG1-/- mice that received naïve CD4 T cells survived versus only 4% of the RAG1-/- controls when challenged with 100pfu/mse of WNV. Transferred CD4 T cells also significantly protected (p<0.0001) the RAG1-/- mice following intra-peritoneal WNV challenge (data not shown). Moreover 60 days post infection, we could not detect infectious WNV in the brains of Rag-/- mice that had received CD4 T cells by either plaque assay or co-culture. Altogether, our data indicates that naïve CD4 T cells are both necessary and sufficient to protect mice from a lethal WNV challenge to the point of sterilizing immunity.

In order to confirm that CD8 T cell or B cell contamination did not play a role in protection, we bled mice 7 days post infection (8 days post transfer) and stained cells to enumerate CD8 and B cell contaminants by FCM (data not shown). If a contaminating population was playing a role in protection, the cell population would have expanded by 7 days post infection, making it easy to identify. Such an expansion was detected in only 1 mouse out of 30 and that animal was eliminated from the study. Based upon our adoptive transfer results we conclude that protection from WNV challenge was a result of direct CD4 T cell control of WNV.

Direct *ex vivo* CD4 T cell function

To evaluate the direct ex vivo effector function(s) of the CD4 T cells upon WNV infection, we chose to monitor Granzyme B (GrB) levels. This marker is expressed on effector T cells with lytic potential and its expression does not require *in vitro* stimulation, limiting any potential misinterpretation of data due to *in vitro* bias (332). FCM analysis revealed that a significant percentage of CD4 T cells upregulated GrB content on days 4 ($p<0.02$) and 7 ($p<0.02$) post WNV infection compared to naïve controls (**Figure 2A and 2C**). By day 10 post infection we began to see a decrease in GrB content and by day 50 post infection the percentage of CD4 T cells that contained GrB returned to baseline levels (**Figure 2A and 2C**). The kinetics of IFNγ production by CD4 T cells was similar to what we observed for GrB during the course of WNV infection, except that the detection required brief in vitro stimulation of CD4 T cells with anti-CD3e *in vitro* (**Figure 2B**). The similar kinetics of expression of GrB and IFNγ illustrates the strengths of using Granzyme B expression to monitor an anti-viral response without biasing the outcome with an *in vitro* stimulation. From the results shown in Figure 2 we concluded that antigen-specific CD4 T cells can protect mice against lethal WNV challenge and that these cells express molecules involved in direct effector T-cell function, including lytic granule release and cytokine production.

Functional characteristics of WNV antigen-specific CD4 T cells

To gain deeper understanding of the mechanism by which CD4 T cells protect WNV infected mice, we needed tools to track and study WNV-specific T-cells. To that effect, we identified multiple CD4 T cell epitopes encoded by WNV using pools of
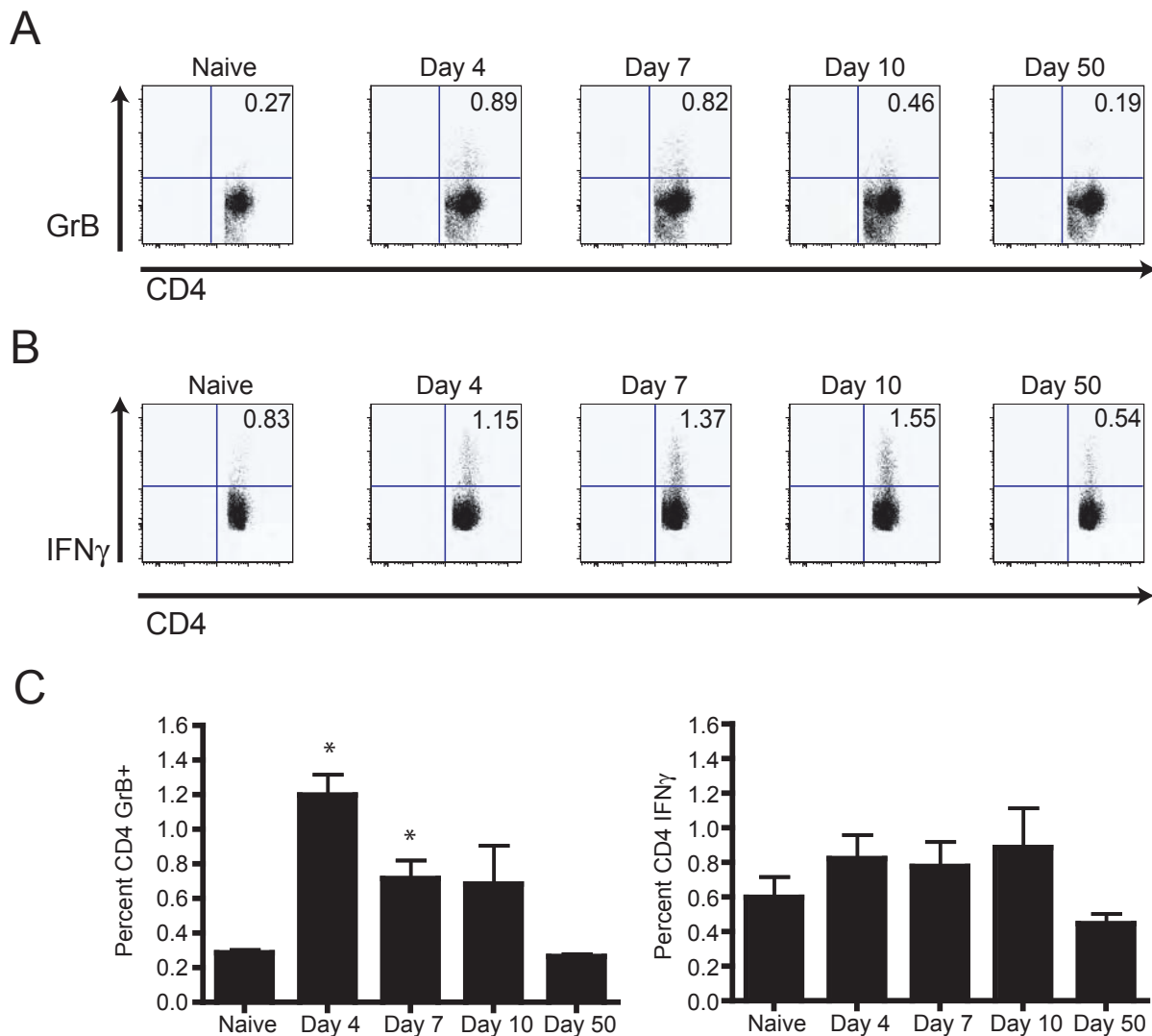
Figure 2. CD4 T cell response during West Nile virus infection. **A**. Representative example of GrB expression in CD4 T cells after WNV infection measured by direct ex-vivo intracellular FCM, without in-vitro stimulation. A naïve animal shown as a control. One mouse out of four from each time point is shown. One experiment of two shown. **B**. Representative example of IFN expression in CD4 T cells after WNV infection measured by ICCS, stimulated with 0.5μg/ml anti-CD3e (clone 2c11), with a naïve animal shown as a control. One mouse out of four from each time point is shown. One experiment of two shown. **C**. Left panel-Enumeration of CD4 T cell GrB expression shown in panel A. Percentage of CD4 GrB$^+$ T cells for the time points given above. There is a significant induction of GrB in CD4 T cells on day 4 (p<0.02) and day 7 (p<0.02), but not on day 10 (p>0.05) .   Average of four mice per time point. X+/-sem One representative experiment of two. Right panel-Enumeration of CD4 T cell IFN expression shown in panel B. Percentage of CD4 IFNγ$^+$ T cells for the time points given above. Average of four mice per time point. X+/-sem One representative experiment of two.

overlapping peptides that cover the entire WNV polyprotein. To identify optimal epitopes within the above WNV proteins, we screened smaller peptide pools and finally individual 15-mer peptides (**Table 1**). For the strongest three epitopes several peptide truncations were synthesized in order to determine the optimal epitope (**Table 2**). All peptides described are cited with their inclusive amino acid numbers the first time in the text, as well as in Table 1. Subsequently, abbreviated nomenclature is used based upon designation of the protein component from which the peptide is derived, e.g. NS3 (nonstructural protein 3), followed by the initial amino acid at which the peptide begins counting from the beginning of the polyprotein. Therefore, the NS3 peptide 2066-2080 is designated $NS3_{2066}$. A summary of the results for all identified CD4 T cell epitopes is listed in Table 1. Through this process we identified three dominant epitopes, $Env_{641-645}$, $NS3_{2066-2080}$, and $NS3_{1616-1630}$ (**Figure 3A**) and three sub-dominant epitopes (**Table 1 and Figure 4**). Epitopes Env_{641} and $NS3_{2066}$ are each responsible for 30% of the total response, $NS3_{1616}$ is responsible for 20% of the total response and $Env_{431-445}$, $NS3_{2081-2095}$, and $NS3_{1866-1880}$ are each responsible for less than 8% of the total response. The response to Env_{641}, $NS3_{2066}$, and $NS3_{1616}$ peptide pool was always at least equal to, and often significantly larger than, the response to WNV-infected cells (**Figure 4B**), probably due to peptide competition, unequal temporal expression of all epitopes or a combination of these and other, unknown, factors. Regardless of the exact reason, this suggested that we have indeed identified the vast majority of WNV epitopes in this MHC haplotype.

We also demonstrated that the hierarchy and the immunodominance of the three identified CD4 T cell epitopes are maintained into memory (**Figure 3B**). However, we

Day Post Infection		Day 7	Day 7	Day 7	Day 7	Day 7
# of mice		N=4	N=3	N=4	N=5	N=2
Anti-CD3		1.16	1.136	0.52	ND	0.911
431-445	IFVHGPTTVESHGNY	0.105	0.179	0.03	0.14	0.152
641-655	PVGRLVTVNPFVSVA	0.505	0.326	0.1	0.41	0.273
1616-1630	TKPGVFKTPEGEIGA	0.317	0.499	ND	0.30	ND
1866-1880	WFVPSVKMGNEIALC	0.082	0.089	0.01	0.13	ND
2066-2080	RRWCFDGPRTNTILE	0.527	0.406	0.487	0.49	0.176
2081-2095	DNNEVEVITKLGERK	0.096	0.029	0.01	0.14	ND

Table 1. List of all identified CD4 T cell epitopes. This table lists the aa numbers and sequence of the CD4 T cell epitopes we have found cytokine response to by intracellular cytokine staining. ND-not determined

Day Post Infection	Day 7	Day 7	Day 7	Day 7	Day 7
# of mice	N=4	N=3	N=4	N=5	N=2
Anti-CD3	1.16	1.14	0.52	1.33	0.91
646-660	ND	0.32	ND	0.29	0.16
641-655	0.51	0.33	0.10	0.41	0.27
1616-1630	0.32	0.50	0.15	0.30	0.14
1617-1627	ND	0.11	ND	ND	0.08
1618-1628	ND	0.10	ND	0.17	0.08
1619-1629	ND	0.15	ND	0.28	0.37
1620-1630	ND	0.16	ND	ND	ND
2066-2080	0.53	0.41	0.41	0.49	0.43
2068-2078	ND	0.25	ND	0.24	ND
2070-2080	ND	0.20	0.01	0.34	0.08

Table 2. **List of truncated CD4 T cell peptides**. This table lists the truncated CD4 T cell peptides used to determine the optimal epitope. The optimal epitope is shown in bold. ND-not determined
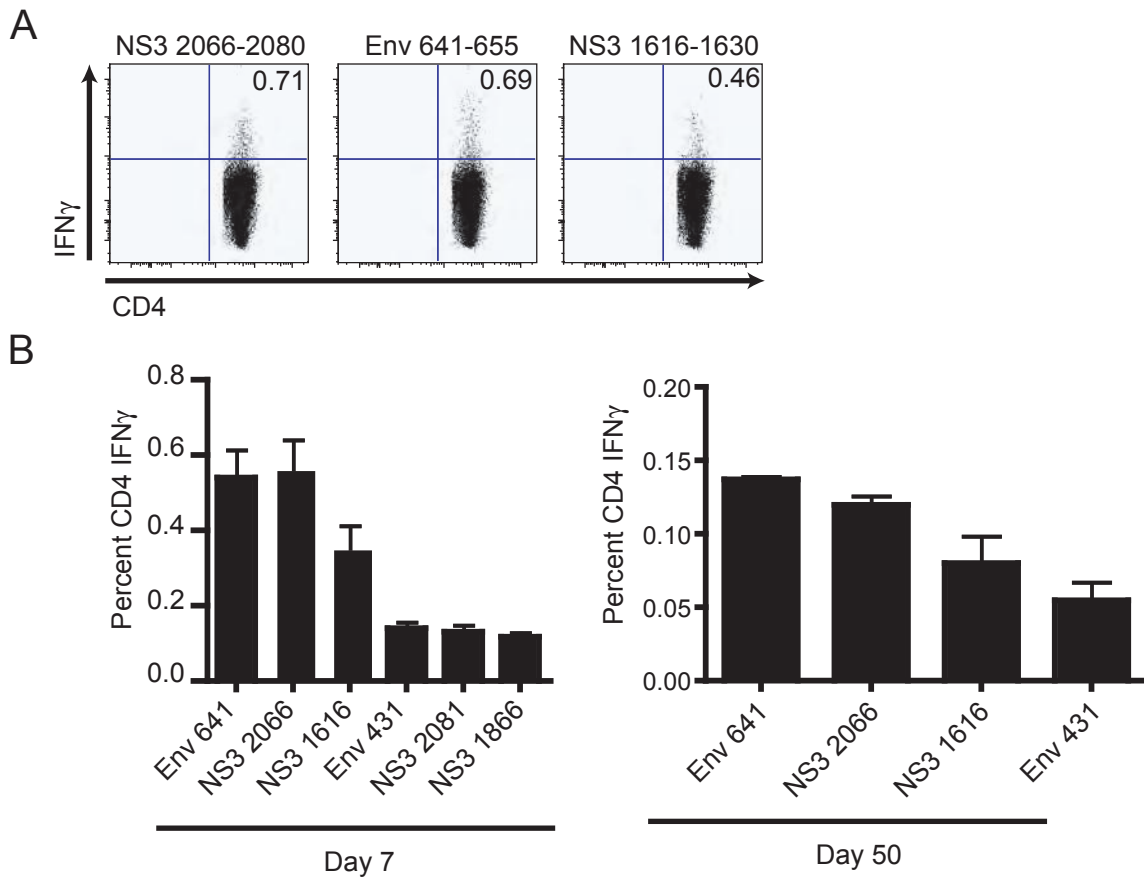
Figure 3. Antigen specific CD4 T cell IFN response. **A**. Representative example of CD4 T cell IFNγ ICCS response to the three immunodominant CD4 T cell epitopes as measured by 6 hour ICCS. CD4 T cells were from mice 7 days post infection and stimulated with each peptide (10^{-6}M) as indicated above each plot. One mouse of 5 is shown. **B**. Left panel- Quantification of the CD4 T cell IFNγ 7 days post infection. CD4 T cell IFNγ ICCS data shown in panel A. Average of 5 mice for each peptide, x+/-sem. One representative experiment of three. Right panel- Quantification of day 50 CD4 T cell IFNγ ICCS. CD4 T cells were stimulated for 6 hours with each peptide (10^{-6}M) as indicated above each plot. Average of 4 mice for each peptide, x+/-sem. One representative experiment of three.

A.

B.
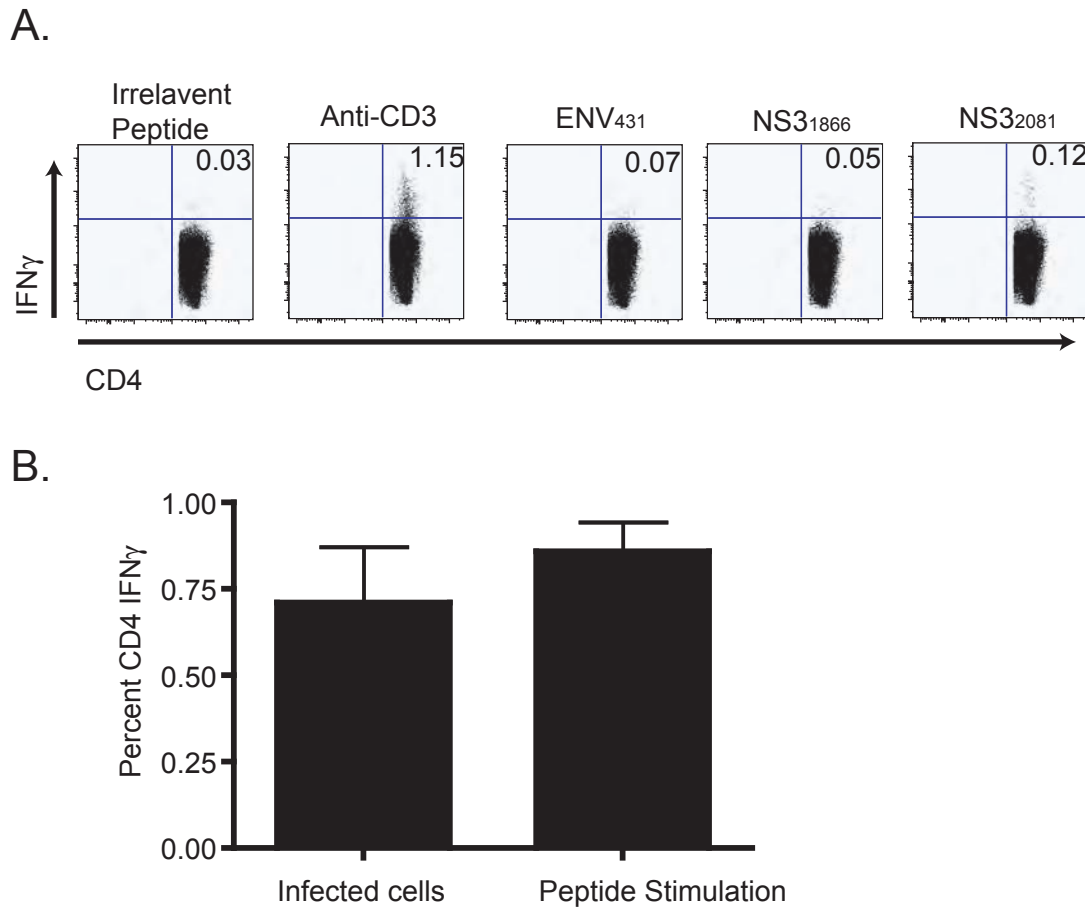
Figure 4. ICCS of CD4 T cell epitopes. **A**. Representative example of CD4 T cell IFNγ ICCS response to the three sub-immunodominant CD4 T cell epitopes as measured by 6 hour ICCS. CD4 T cells were from mice 7 days post infection and stimulated with, media, 2c11 (0.5μg/ml) or peptide (10^{-6}M) as indicated above each plot. One mouse of 5 is shown from one representative experiment of three. **B**. Quantification of day 7 CD4 T cell IFNγ ICCS response to either WNV-infected IC-21s (MOI:40) or peptide pulsed IC-21s 10^{-6}M (Env$_{641}$, NS3$_{1616+2066}$) peptide. CD4 T cells were stimulated for 6 hours in the presence of BFA. Average of 4 mice per stimulation, x+/-sem. One representative experiment of two.

noted that upon secondary challenge the top two epitopes maintain their immodominance but Env_{431} becomes more dominant than $NS3_{1616}$ (data not shown).

Antigen-specific CD4 T cell cytokine bias

Using these newly identified epitopes we wanted to determine the functional capabilities of the WNV-specific CD4 T cells. As shown in Figure 3, the peptide stimulation of splenocytes isolated from mice 7 days after infection resulted in a robust IFNγ response. Notably, we observed spontaneous IL-4 production by CD4 T cells from day 7 infected animals (infected mice CD4 $IL-4^+$= 0.91%, naïve mice CD4 $IL-4^+$ 0.20%, n=4 mice per group), but did not see an increase in IL-4 following stimulation with anti-CD3ε, or with any of the newly defined epitopes (**Figure 5A**). During stimulation of antigen-specific cells with $NS3_{1616+2066}$ we did not see any IFNγ +IL-4+ double positive cells (naïve mice $IL-4^+IFNγ^+$=0.005, day 7 mice $IL-4^+IFNγ^+$=0.0005; n=4 /group) indicating that the IL-4 production is most likely not part of the antigen-specific response and may represent a bystander response. The results of the cytokine profile produced by the WNV-specific CD4 T cells directly *ex vivo* indicates that the WNV CD4 T cell response shows a strong Th1 bias.

We next used these epitopes to track the percentage of the antigen-specific CD4 T cells within the spleen during the course of an infection (**Figure 5B**), and at range of infectious doses (**Figure 5C**). Using ICCS, we were able to define the time course of the systemic antigen-specific CD4 T cell responses (**Figure 2A**). We could start to detect the antigen-specific response by day 4 and the peak of the response in the spleen occurred at
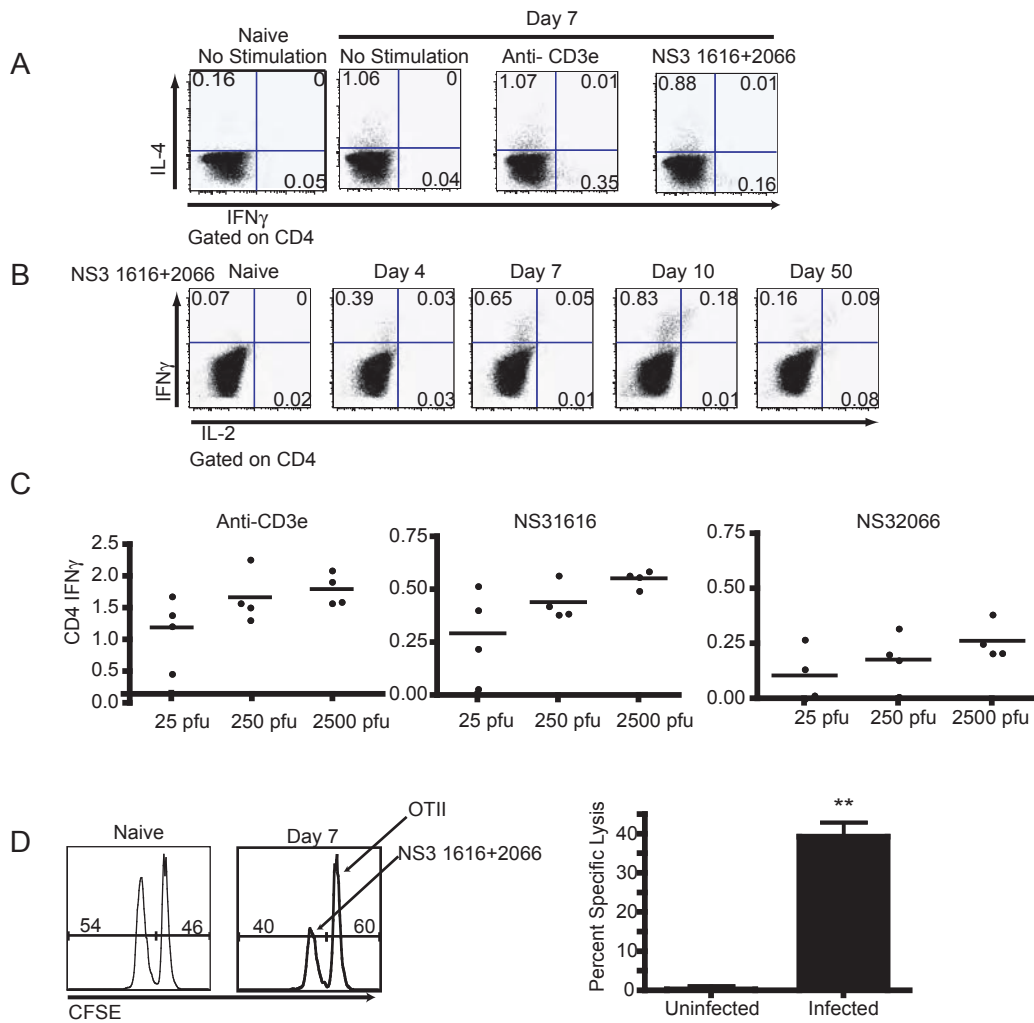
Figure 5. Functional potential of antigen specific CD4 T cells. **A**. Representative example of a CD4 T cell ICCS 7 days post infection. Gated on CD4, IFNγ and IL-4 are measured after 6 hr stimulation in Monensin. Cells were stimulated with either: media, 2c11 (0.5 μg/ml) or $NS3_{1616+2066}$ (10^{-6}M), one naïve mouse shown as a control. One representative mouse is shown of four from one representative experiment of two. **B**. Representative example of CD4 T cell ICCS during the course of infection, as indicated above each plot. Gated on CD4, IFNγ and IL-2 are measured after 6 hr stimulation with $NS3_{1616+2066}$ (10^{-6}M) in BFA, one naïve mouse shown as a control. Results show one representative mouse of four per time point from one representative experiment of two independent experiments. **C.** Quantification of percent CD4 IFNγ⁺ 7 days post infection, from mice infected sc with increasing doses of WNV. Cells were stimulated for 6 hours with either: 2c11 (0.5 μg/ml) or $NS3_{1616}$ (10^{-6}M), $NS3_{2066}$ (10^{-6}M). Percent CD4 IFNγ⁺ for 4 mice shown per infection dose from one experiment of two, bar=mean. **D**. *In vivo* CD4 T cell CFSE cytotoxicity assay. Left panel-Representative histogram of splenocytes 12 hours after adoptive transfer of target cells into naïve (left histogram) and infected (right histogram) mice. Right panel- Quantification of *in vivo* CD4 T cell CFSE based cytotoxicity assay. CD4 T cells are cytolytic *in vivo* on day 7 post WNV infection (p<0.008). Results represent the average of 5 mice per group, and are representative of three independent experiments.

day 10 (**Figure 5B**). Upon peptide stimulation of memory CD4 T cells they were capable of immediately producing both IFNγ and IL-2 (**Figure 5B**).

In vivo CD4 T cell cytotoxicity

Since we saw a strong induction of GrB in the CD4 T cell population during infection we next wanted to determine whether the CD4 T cells were capable of *in vivo* cytotoxicity (**Figure 5D**). We chose to use a CFSE- based *in vivo* cytotoxicity assay as initially described by Jellison et al. (26). In these experiments we adoptively transferred peptide-pulsed splenocytes from C57BL/6 Ly-5.1 congenic mice (target cells), into day 6 WNV infected C57BL/6 mice, where the congenic marker was used to identify transferred cells and two concentrations of carboxy fluoroscein succinimidyl ester (CFSE) labeling used to differentiate specific targets from the control ones. CFSE^{hi} splenocytes were peptide pulsed with ovalbumin epitope 323-339, Ova$_{323}$ (OTII; control) and CFSE^{low} cells were pulsed with NS3$_{1616+2066}$ (experimental peptide). The *in vivo* CTL assays showed the cytotoxic capacity of the antigen-specific CD4 T cells, where 39% of the MHC class II^{+} targets were killed within a 12-hour time period (p<0.008). Direct ex vivo CD4 ^{51}Cr release CTL assays were also completed using a peptide pulsed IC-21 cells, a macrophage cell line, where we observed peptide specific lysis (data not shown). This data shows that during the course of WNV infection CD4 T cells differentiate into in vivo cytotoxic effectors capable of killing infected cells and presumably clearing them from the periphery.

In vivo relevance of antigen-specific CD4 T cell response

Based upon adoptive transfer of naive CD4 T cells and the direct *ex vivo* functions of antigen-specific T cells, we proposed that antigen-specific CD4 T cells use both their cytolytic and cytokine capacity to control WNV infection, preventing the development of encephalitis/meningitis. In order to test this hypothesis we completed a peptide tolerization experiment as well as a series of peptide vaccination experiments to directly determine the *in vivo* role of the antigen-specific CD4 T cell population in animal survival.

In order to diminish the development of antigen-specific CD4 T cells in naïve mice during WNV infection, C57BL/6 mice were given high doses of soluble peptide ENV_{641} and $NS3_{2066}$, every other day for 15 days to induce peripheral tolerance as described previously (333, 334). This treatment leads to deletion or functional inactivation of peptide-specific cells. Mice were tolerized to either a control peptide, $(OVA_{323-339,}$ OTII) (335) or to the two dominant CD4 epitopes, then infected with WNV. Mice that were tolerized to the WNV CD4 epitopes exhibited a 40% survival rate, whereas mice tolerized to ovalbumin had a 60% survival. Although these results failed to reach statistical significance ($p<0.07$), they suggested that reducing the antigen-specific CD4 T cell populations by half may increase WNV mortality (**Figure 6A**). This trend is suggestive of the protective role of epitope-specific CD4 T-cells during primary WNV infection.

To look at the direct protective capacity of antigen-specific CD4 T cells we used a peptide vaccination approach. C57BL/6 mice were vaccinated two times with an emulsion of $NS3_{1616}$ and $NS3_{2066}$ peptide in Titermax Gold (336). Twenty days after the
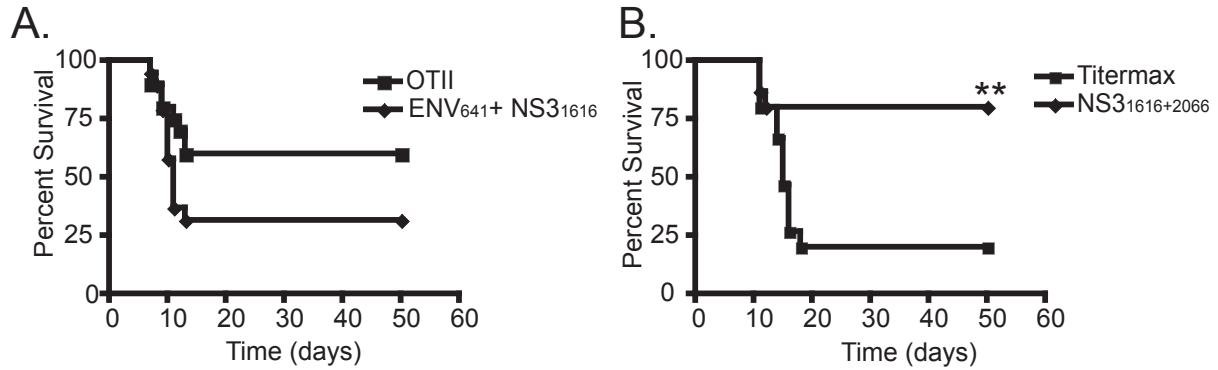
Figure 6. Antigen-specific CD4 T cell responses are essential for protection against WNV. **A**. Peptide induced CD4 T cell peripheral tolerance increases the susceptibility of B6 mice to WNV. Peripheral tolerance was induced by injecting 100ug of soluable ENV_{641} + $NS3_{1616}$ i.p. for 15 days. After 7 days mice were challenged with 400pfu of WNV and survival monitored. **B**. CD4 T cell peptide vaccination leads to a significant ($p<0.03$) increase in protection of WNV infected mice. Mice were vaccination using 20μg of $NS3_{1616+2066}$ peptide and titermax emulsion. Mice were challenged with 1200 pfu of WNV. One representative experiment of three.

last vaccination mice were challenged with a high dose of WNV (800pfu). The mice were observed for 60 days, and mice that received the vaccination with WNV epitopes NS3$_{1616+2066}$ were protected significantly better then the mice vaccinated with OVA$_{323-339}$ ($p<0.03$) (**Figure 6B**). This data suggest that the loss of antigen-specific CD4 T cell precursors prior to infection may lead to more severe disease and demonstrates that the dominant peptide epitopes recognized by CD4 T cells can be used as a vaccine to protect mice given a lethal dose of WNV.

In this study, we show that naïve CD4 T cells differentiate into primary effector cells and protect RAG1-/- mice from lethal WNV infection. This indicates that not only do CD4 T cells have the ability to directly control WNV infection, but that they are sufficient to do so in a RAG1-/- environment. In this report we also defined in the H-2^b haplotype the majority of CD4 T cell epitopes that develop during the course of a WNV infection. Most importantly, we used these CD4 T cell epitopes as a vaccine to show that the generation of memory CD4 T cells response can be protective.

Previous reports studying anti-viral immune responses to Flaviviruses, and WNV specifically, have indicated that T cells are required to prevent the development of encephalitis/meningitis (281, 282, 337). More recent work indicates a requirement for CD8 T cells in protection from lineage I strains of WNV (275, 279, 338). While CD4 T cells are not required during primary Dengue virus infection of mice (339), there is an absolute requirement for CD4 T cells during primary WNV infection (data above and (219)). Our studies indicate that not only is the CD4 antigen-specific response responsible for the protection originally described by Sitati et al. (219), but that these cells protect *in vivo* at least in part by the direct effector function, and that this is sufficient to confer anti-WNV protection to adoptive hosts.

There has been a long-standing interest in the CD4 T cell functional response to many of the flaviviruses that infect humans. It has been shown, predominantly with T cell lines, that CD4 T cells responding to Dengue virus (DV), JEV, YF and WNV can proliferate, produce IFNγ and IL-2 and are cytotoxic in response to viral antigens (286, 287, 337, 340, 341). It has been recently shown that during resolution of JEV infection

the presence of a strong Th1 T cells response, including IFNγ production, results in a reduction of neurological sequalae (342). Little work has been done to monitor the CD4 T cell response to lineage I WNV infection in real time. We provide what we believe is the first description of a CD4 T cell antigen-specific response to WNV and a list of WNV determinants recognized by CD4 T cells. Our data indicates that the CD4 T cells respond in a strong Th1 fashion, including pronounced IFNγ, but not IL-2, production,

The one report that examined the direct effector role of CD4 T cells during a lineage I WNV infection did observe a protective effect upon the transfer of CD4 T cells, but did not observe a difference in the protective capacity of CD4 T cells that lacked either IFNγ, perforin or Fas-FasL (219). The authors interpreted this as evidence for the lack of direct effector function. Here we clearly show that CD4 T cells can lyse peptide-coated targets *in vivo*. Although we have not determined whether this occurs through a perforin or Fas-FasL mediated pathway, our GrB expression data implies that a direct Grb/perforin mediated response is certainly plausible. We believe that WNV-specific CD4 effector T cells use all three effector mechanisms, IFNγ, perforin and Fas-FasL, with a level of redundancy built into the antigen-specific response. Simply denying CD4 T cells the use of one effector function may not be sufficient to reduce their antiviral activity and to observe a difference in survival in an adoptive transfer model. This could explain why Sitati et al. (219) saw a similar increase in survival in mice receiving CD4 T-cells, regardless of whether they were wt, IFNγ−/−, perforin-/- or FasL-/-.

Several studies have identified proper T cell trafficking as critical for protection from WNV neurological disease. CXCL10 and CD40 deficient mice have impaired trafficking of CD4 T cells into the CNS (276, 338). In B6 mice, WNV infection of the

CNS begins approximately 3 to 4 days post-infection, which is the same time period when we begin to detect antigen-specific, GrB positive CD4 T cells that are prepared to secrete anti-viral cytokines. The early, day 4, antigen-specific CD4 T cells during WNV infection may not only play a direct role in controlling early infection within periphery, but trafficking of antigen-specific CD4 T cells to the CNS may prevent viral spread within the CNS. Control of viral spread within the CNS requires the use of non-cytolytic mechanisms of viral clearance for host survival (343).   The secretion of IFNγ by lymphocytes is required for the clearance of neurotropic viral infections, such as Sindbis virus (344) and yellow fever (282), in order to keep neurons intact. Although neurons are not known to express MHC class II in situ, they do constitutively express IFNγR (345), and upon exposure to IFNγ secreted by WNV-specific CD4 T cells can up regulate essential anti-viral molecules such as RNaseL (214).

In summary our experiments show that CD4 T cells are sufficient for controlling and clearing WNV from RAG1-/- mice. Antigen-specific CD4 T cells rapidly respond to infection within the periphery by secreting a multitude of cytokines. In addition to cytokine production, our data shows that CD4 T cells have in vivo cytotoxic capabilities. These antigen-specific CD4 T cells most likely use both these mechanisms to prevent WNV encephalitis/meningitis during viral challenge.

Materials and Methods

Mice. Adult (2-6 months old) male C57BL/6 (B6) mice were purchased from the National Cancer Institute Breeding Program (Frederick, MD). B6.Rag1$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and bred at the VGTI vivarium (Oregon Health & Science University); they were used at 2-4 months of age. B6.SJL-Ptprc^a Pepc^b/BoyJ, commonly referred to as B6. Ly-5.1 congenic mice were purchased from the National Cancer Institute and were used at 2-4 months of age. All animals were housed and bred under specific pathogen-free conditions at the Oregon Health & Science University. All WNV experiments were completed within a United States Department of Agriculture (USDA, Frederick, MD) approved Biosafety Level (BSL) 3 facility, and were approved by the Institutional Animal Care and Use Committee, and the Institutional Biosafety Committee in accordance with the applicable federal, state, and local regulations.

Virus, Peptides, and Cell Lines. West Nile virus strains 31A and 385-99 were used and both virus strains yielded similar results. West Nile virus strain 385-99 was a kind gift of Dr. Robert Tesh (University of Texas Medical Branch, Galveston, TX); strain 31A was provided by the USDA reagent program (Ames, IA). An overlapping peptide library covering the entire length of the viral polyprotein (15-mers overlapping by 10 aa) was obtained from Sigma Aldrich (St. Louis, MO). Additional synthetic peptides were purchased at >95% purity from Sigma Aldrich and 21st Century Biochemicals (Marlboro, MA), diluted in 10% H$_2$O/90% DMSO, stored at -80^0 C and subsequently used at indicated concentrations. Virus was grown in mycoplasma-negative Vero cells, cultured

under aseptic conditions as described previously (279); mycoplasma-negative IC-21 cells were used in stimulation assays. Cells were infected using variable multiplicity of infection (MOI) as indicated.

Determination of viral titer. Viral titer was determined by plaque assay where a virus sample was serially diluted onto Vero cells. After co-culture of the virus with the cells for two hours, agarose overlay was added. Two days after the initial overlay, cells were overlayed with additional agarose-containing Neutral Red (0.2%). Plaques where then counted to determine viral load.

To evaluate a potential chronic virus infection, brains were homogenized, as described above but not centrifuged. The cellular suspension was seeded onto a monolayer of Vero cells, in triplicate, and cultured for 7 days, while monitored for cytopathic effect (CPE). CPE was monitored visually and defined as the rounding up of cells and loss of a monolayer. At the end of the culture period, cells and supernatant were transferred to a second monolayer of Vero's and left for 48 hours, which were then trypsinized, fixed and permeabilized and stained intracellular for WNV envelope protein using an E16-alexa647 (346) conjugated antibody.

Intracellular cytokine and surface flow cytofluorometric (FCM) staining. Cytokine-producing T cells were detected using the Cytofix-Cytoperm Kit (BD PharMingen, San Diego, CA), as described. Single-cell splenocyte suspension was depleted of red blood cells and was incubated with 1μM peptide or infected with WNV in the presence of 5μg/ml Brefeldin A (Sigma Aldrich) for 6 h at 37 °C, except when looking at production

of IL-4, where monensin was substituted for Brefeldin A. After six hours the cells were washed and blocked with Fc block (anti–mouse FcγRI/III; BD PharMingen) and incubated overnight in the presence of a saturating dose of surface antibodies against CD8, CD3, CD4, CD11a, CD43 (Clone 1B11), CD44 and CD62L (BD-PharMingen). After washing, the cells were fixed, permeabilized and intracellular antibodies (anti-IFN-γ, anti-TNFα or anti- IL-2; BD-PharMingen) were added for 30 minutes. For detection of Granzyme B, splenocytes were isolated and kept on ice, surface stained, fixed and permeabilized as described above without stimulation. Cells were stained with Granzyme B Alexa647 (clone: gb11, BD PharMingen). The samples were then washed and analyzed using either a FACSCalibur or LSR II cytometer (Becton Dickinson Immunocytometry Systems, Sunnyvalle, CA) instrument. FCM analysis was performed by collecting a minimum of 5×10^4 events and gates set on lymphocyte population based on forward and orthogonal light scatter, followed by marker positioning to denote fluorescence greater than that of control stained or unstained cells.

Infection, immunization, and CTL assays. Mice were immunized subcutaneously (s.c.) between the shoulder blades with 20-600 pfu WNV. Four, seven, ten or fifty days later, splenocytes were isolated and subjected to FCM, ICCS or CTL assay analysis as described below.

Direct ex vivo CTL activity was determined using peptide-coated or virally infected and control IC21 cells as targets. Radioactivity was measured using TopCount Packard δ/γ radioactivity reader (Packard Co., Detroit, MI), using a standard ^{51}Cr-release assay, exactly as previously described (347).

In vivo CTL assays were performed by isolating splenocytes from B6. Ly-5.2 mice, and labeling them to produce a CFSE^{hi} (1uM) and a CFSE^{low} (10nM) population using standard CFSE labeling protocol (Molecular probes, Eugene OR). After CFSE labeling, cells were peptide pulsed for 1hr at 37 degrees with 1uM NS3$_{1616+2066}$ or 1uM OTII peptide. Cells were counted and equal numbers were mixed then injected i.v. into 5 infected mice and 5 naïve mice. After 24 hours mice were sacrificed and cells were gated on LY5.2 (clone A20) MHC class II double positive cells. The percent killing was calculated as follows: (1 – (ratio immune/ratio naive)) x 100. Ratio = number of events NS3$_{1616+2066}$ peptide-coated target/number of events reference target (26).

Peptide tolerance was induced by solubilizing NS3$_{1616+2066}$ peptides in sterile 1xPBS. Mice were given 100ug of each peptide ip every other day for 15 days, then rested for 7 days and challenged with WNV. For peptide vaccinations 20ug at 1mg/ml of each peptide (NS3$_{1616+2066}$) were mixed in equal volume of the adjuvant TiterMax (Sigma-Aldrich), by vortexing in an eppendorf tube for 30 minutes. Mice were immunized 3 times, 1 time every 21 days, at the base of the tail.

Adoptive transfer and virus challenge experiments. For naïve CD4$^+$ T cell transfer, spleens from 4-wk old B6 mice, containing less than 5% CD44^{hi} (memory) splenic T cells, were coated with anti-CD8, B220, NK1.1-coated beads, and CD4$^+$ cells isolated at 80-95% purity. These cells were transferred i.v. (at 5-10 x 10^6 cells/recipient), transfers monitored and challenge performed as below. Virus specific CD4 T cell lines were generated by *in vitro* restimulation of WNV-primed spleen cells, as above, were purified to deplete CD8+ and B220+ cells at <1%, and were injected (2-5 x 10^5 cells/recipient)

i.v. into RAG-1$^{-/-}$ recipients and engraftment success was evaluated by FCM 24 h later. Animals were challenged 24 h after cell transfer as described above. Survival was scored on a daily basis. Death occurred between days 10 and 18, and all animals surviving this period remained disease free for 60-90 days at which point the experiment was terminated. Data is shown as percent survival at the termination of the experiment, with the statistical significance determined using Log-Rank test. All calculations were done using the Prism (GraphPad, San Diego, CA) software.

Chapter Four
Key role of T cell defects in age-related vulnerability to West Nile virus

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Abstract

West Nile virus (WNV) infection causes a life-threatening meningoencephalitis 40-50 times more often in people over the age of 70, compared to adults under the age of 40. In a mouse model of age-related vulnerability to WNV we demonstrate that viral spread and titers were significantly higher in old compared to adult mice, indicating that a loss of virus control occurs with age. This was due to age-related defects in both the quantity and the quality of the aged CD4 and CD8 T cell response against dominant WNV epitopes at the level of cytokine and lytic granule production. Finally, while the adult CD4 or CD8 T-cells readily protected immunodeficient host mice upon adoptive transfer, old T-cells were unable to afford any protection against WNV. These findings identify potential targets for immunomodulation and treatment to combat lethal WNV infection in the elderly.

Introduction

West Nile virus (WNV) is a small, enveloped, single-stranded, positive sense RNA-containing virus with a genome of approximately 11Kb that belongs to the family *flaviviridae* (348). WNV is a typical arbovirus that cycles between its natural hosts, mosquitoes and birds (177). Mosquitoes transmit WNV to a wide range of other species (including humans), which serve as incidental, dead-end hosts. In the US, this virus has been responsible for over 23,000 clinically registered human infections, leading to nearly 1,000 deaths. Annual outbreaks in the United States were registered in every year since 2000, and were marked by increasing mortality rate in infected birds (231) and horses (232), and by an increase in the frequency and clinical severity of WNV infection within the human population (349). These observations are consistent with findings elsewhere in the world since the mid 1990's (179, 350, 351) and suggest that the US strains of WNV may exhibit an increase in virulence versus historical strains of WNV.

The incidence of WNV infection is fairly uniform with age (230) and in most immunocompetent humans the disease is asymptomatic (239, 349). However, severe WNV disease, which includes the involvement of the nervous system (meningitis and encephalitis) is primarily a disease of old age with a lethality of 10% and a mean age at death of 78 years (99, 230). Persons between 50-59 years of age have a 10 times higher incidence of severe disease while persons aged 80 years or greater have a 43 times higher incidence of severe disease compared to the adults between 20-40 years of age (99, 100). Moreover, many elderly patients that suffer from WNV encephalitis require several years to fully recover and the overall mortality in the first year post infection is significantly increased compared to age-matched controls (99).

Aging leads to a widespread but poorly understood state of immunodeficiency, which is associated with an increased incidence, and severity of infectious disease in the elderly (352). Many facets of innate and adaptive immunity have been shown to be altered by aging (62, 135, 353, 354), but it is unclear at this point, which of these defects are critical to impaired immune defense. Moreover, it is possible, and in fact likely, that the critical defects will vary depending on the biology of the encountered pathogen. Of note, T-cells have been shown to exhibit some of the most pronounced age-related defects (61, 143), and reversion of T cell defects has been associated with improved immune function in old animals (355-358), although this improvement never been tested in a model of infectious disease. It was therefore of interest to examine the immunological basis of the age-related increase in susceptibility to WNV.

Age related susceptibility to West Nile virus

We first established a novel and robust mouse model of the age-related susceptibility to WNV. In our model old mice exhibited increased susceptibility to WNV regardless of the route (intraperitoneal, ip; or subcutaneous, sc) or the isolate of WNV Ia (NY-99, 31A or 382-99, **Table 1,Figures 1-5** and not shown), or strain (C57BL/6, **Figures 1-5** or BALB/c, **Figure 2**) used, although, as expected, intraperitoneal (ip) infection produced lethal effects at a lower dose (1-20 plaque-forming units, pfu) than the more physiological sub-cutaneous (sc) infection (50-1,000 pfu). Overall, old mice were at least 6 times more susceptible to WNV as measured by survival rates over many viral concentrations (**Figure 1A** and not shown). To examine whether this susceptibility was caused by loss of viral control, infectious viral titer was determined in different organs of adult and old mice. Following infection with a viral dose that kills most old but not adult mice, we found significantly higher viral titers in the brains of old mice compared to the adult counterparts (**Figure 1B**), whereas differences in other organs were far less pronounced (not shown). Importantly, viral titers within the brain directly correlated to mortality as indicated by moribund mice regardless of age having equivalent brain WNV titers (**Figure 1B**) as well as at higher infection doses of WNV, that were lethal to both adult and old mice, the difference in brain viral titers was lost (**Figure 3**). Therefore, in all subsequent experiments where we sought to dissect the immunological basis of vulnerability to WNV, we used the viral doses at which most old animals died and the majority of adult mice survived.

Mouse group	Virus strain	Virus dose(route)	Live/Total	MST (Days)
Adult	NY99	20 pfu (IP)	0/5	14.5
Old	NY99	20 pfu (IP)	0/5	13.5
Adult	NY99	10 pfu (IP)	0/3	14
Old	NY99	10 pfu (IP)	0/3	13
Adult	NY99	6pfu (IP)	9/20	18.5
Old	NY99	6pfu (IP)	4/11	14.5
Adult	NY99	3pfu (IP)	19/20	U
Old	NY99	3pfu (IP)	5/21	14.5
Adult	NY99	1pfu (IP)	20/21	U
Old	NY99	1pfu (IP)	5/21	15
Adult	NY99	0.5pfu (IP)	21/22	U
Old	NY99	0.5pfu (IP)	13/20	15
Total of 3 experiments				U= Undefined

Mouse group	Virus strain	Virus dose(route)	Live/Total	MST (Days)	Fisher exact test p value	Chi Squared test p value
Adult	NY99	80 pfu (S.c.)	10/10	U	0.0006	0.00039
Old	NY99	80 pfu (S.c.)	1/7	12		
Adult	NY99	40 pfu (S.c.)	8/10	U	0.023	0.0176
Old	NY99	40 pfu (S.c.)	2/9	12		
Adult	NY99	20 pfu (S.c.)	10/10	U	0.0031	0.004
Old	NY99	20 pfu (S.c.)	3/9	12		
Total of 2 experiments						

Table 1: Summary of old and adult mortality experiments
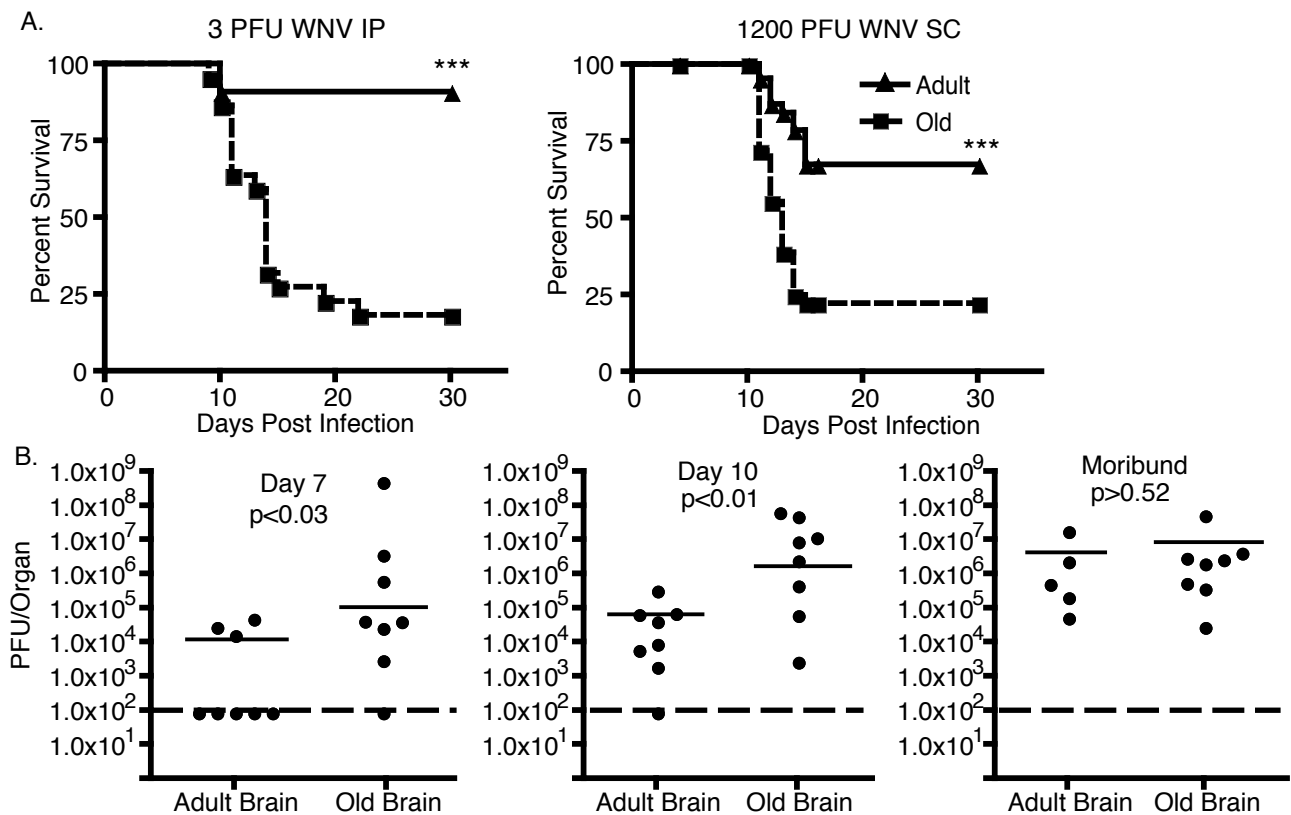
Figure 1. Age-related susceptibility to West Nile virus disease in old mice. **A**. Survival of adult (4-6 months old) and old mice (18-22 months old) following the challenge with WNV-NY99 i.p. or WNV 385-99 s.c. Old mice are 5- and 6 times more susceptible to West Nile virus upon i.p. (mean survival time - MST - 13 days) and s.c. (MST 14 days) infection, respectively, compared to adult controls. Statistical significance was evaluated by the Log rank test (***p<0.0005). Results are representative of at least 4 experiments with similar results. **B**. Viral titers within the brains of adult and old mice after s.c. infection (1200pfu/mouse) were determined by plaque assay. The brains of old mice contained significantly more virus on both day 7 and day 10 post infection. By contrast, there was no difference between brain viral levels of old and adult moribund mice on day 12-16.
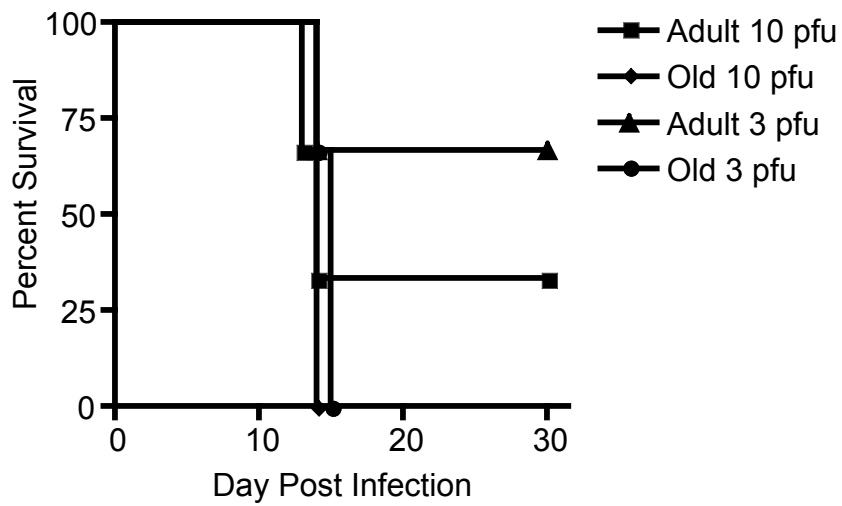
Figure 2. Survival of Adult and Old BALB/c mice infected with WNV. Adult and old BALB/c mice were infected with strain NY99 ip. with 10pfu or 3pfu. At both doses adult mice survived at a higher frequency then their old counterparts. (n=3).
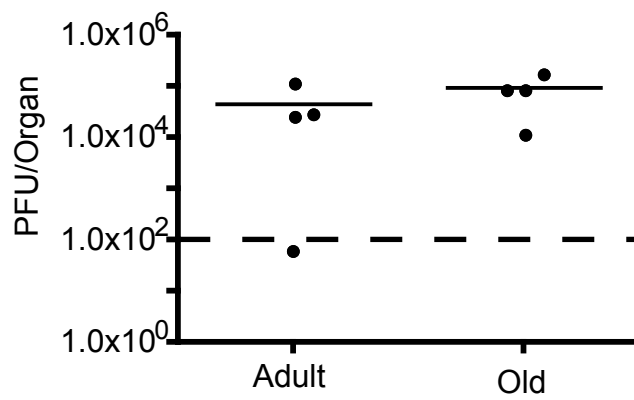
Figure 3. Viral titer in the brains of adult and old mice 10 days after a lethal dose of WNV. Adult and Old mice were given a lethal dose of WNV s.c. and sacrificed 10 days post infection and viral titer in their brains was determined to be equivalent. (n=4)

We next addressed whether the above differences could be explained by age-related defects in innate or adaptive immunity. We did this by comparing mortality rates and mean survival times (MST) in mice that lacked key elements of either the innate or adaptive immune system to those in adult and old wild-type mice. Interferon α/β receptor-deficient mice (IFNAR-/-) (359) are unable to respond to type I interferons (IFN-I), and are known to be highly susceptible to numerous infections (reviewed in (243)), including WNV (244). Conversely, Rag-1-/- mice (360) lack the recombinase essential for generation of T and B cell receptors and therefore have no adaptive immune system (no T or B cells); these animals are also susceptible to many infections, including WNV (217). Our results confirmed that both of these strains are highly susceptible to WNV (**Figure 4A**), but also highlighted a difference in the MST (**Figure 4A**). Thus, IFNAR-/- mice died rapidly post infection (MST 5 days), consistent with the lack of innate defensive mechanisms, whereas the RAG1-/- mice died within the same temporal window as old and adult mice (MST 13 days). Since the MST of old mice is 13-14 days and they are more sensitive to WNV than adult mice, similar to RAG1-/- mice, we concluded that any putative innate immune defects, if present in old mice, likely do not account for their increased WNV susceptibility. In order to confirm this we have also looked at functional levels of IFN-I within the serum and see no difference (**Figure 4C**). To address whether the main defect in old mice lies within the adaptive immune system, we performed adoptive transfers of old and adult spleen cells (**Figure 4B,** left panel) or T cells (CD4+8) (**Figure 4B**, right panel) into RAG1-/- mice. In this series of experiments, we found that adult spleen cells as well as the adult T cells conferred significant
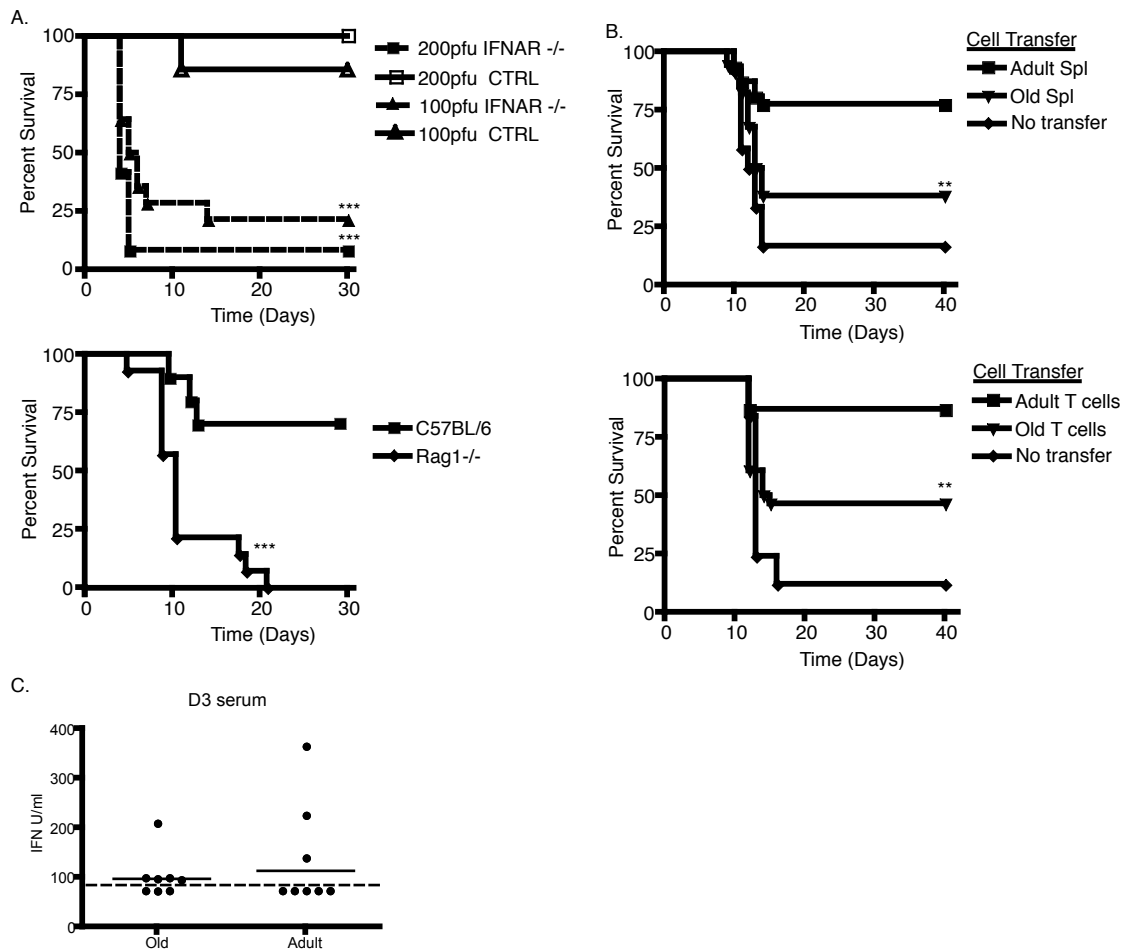
Figure 4. Relative roles of innate and adaptive immunity and the importance of the age of T-cells in resistance to WNV infection. A. IFNAR-/-, RAG1-/- and wt mice were infected with indicated WNV doses s.c. and MST and % survival scored. WNV caused rapid mortality (200 pfu MST:4.5 days) with a very low rate of survival in IFNAR^{-/-} mice compared to wt controls (p<0.0001 at 200 pfu). West Nile virus infection also caused significant mortality in RAG-1^{-/-} mice compared to wt controls (p<0.0001) but MST was similar to that of wt mice (200 pfu MST=13 days). Data were compiled from two independent experiments. **B**. Adoptive transfer of lymphocytes from old and adult mice reveals age-related defects in adaptive immunity. Splenocytes (10^7/animal) from adult mice protect RAG-1^{-/-} significantly better than splenocytes from old mice (Top panel; 100pfu MST=14.5; p<0.0025). Transfer of purified T cells (CD4 and CD8 T cells; 5 x 10^6/mouse) reveals defects in old T cells, which were inferior at protecting RAG1^{-/-} mice compared to the purified T cells from adult mice (Bottom panel; 100pfu p<0.03). Compared to RAG1-/- mice with no transfer, old splenocytes conferred no significant protection, whereas old T cells showed some protection (p<0.04). Significance: * - p=0.05-0.01; ** - p=0.01-0.001; *** - p<0.001. **C**. Type I Interferon activity in the serum of old and adult mice. Old and adult mice were infected s.c. with WNV at a dose that caused decrease survival of old mice compared to adult animals. In this experiment the levels of type I IFN were equivalent in old and adult mice. These mice were bleed 3 days post infection, blood allowed to clot then spun down and serum kept at -80. A VSV based bioassay for murine IFN-I was completed using this serum, as explained in the methods. This experiment has been completed once.

protection to RAG1-/- mice. By contrast, mice that received old cells were afforded less protection, which was not significant in the case of splenocytes. The protection afforded by the transfer of old T cells ($p<0.04$) compared to RAG1-/- mice with no transfer was significant, but even that was significantly worse than the protection afforded by adult T cells ($p<0.003$, **Figure 4B**). Because antigen presentation and priming is not affected by the targeted deletion in the adult RAG1-/- mice, these results collectively indicate that the process of aging impairs resistance to WNV at the level of generation of the adaptive immune response.

Antigen-specific T cell responses

IgM and the virus-specific CD8 and CD4 T cells have all been implicated in affording protection against WNV in adult mice (213, 219, 275). Given the published accounts on the decline of T cell immunity with aging, and the above results with T cell transfers, we initiated experiments to test possible defects in the T cell pool. We first examined signs of T cell activation using multicolor flow cytofluorometric analysis. On day 7, at the peak of the adaptive T cell response, old CD8^{+} T cells exhibited signs of quantitative as well as qualitative hypoactivation. CD43, a molecule involved in T cell activation, costimulation and effector function (361) was found to be expressed on a much smaller fraction of old CD8^{+} T cells (**Figure 5**), but the same was not true for old CD4^{+} T cells (not shown). In particular, the fraction of CD43^{+}GzmB^{+} cells was 10-20-fold reduced in old animals (**Figure 5**). Moreover, at the same time point, many fewer old CD8^{+} T cells exhibited intracellular granzyme B, and the amount of granzyme B per cell was also significantly reduced compared to adult counterparts. While these signs
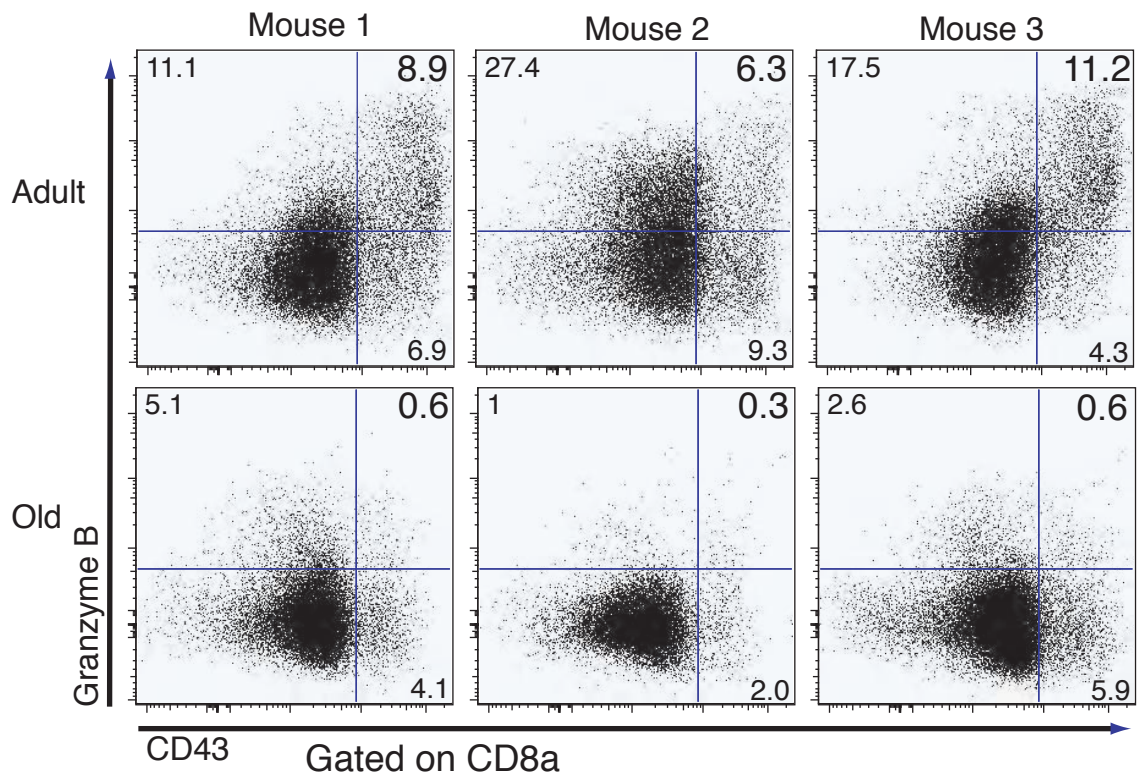
Figure 5. Functional and phenotypic defects in CD8 T cell activation in response to West Nile virus infection. CD8$^+$ T cells derived from spleens of old and adult mice were harvested on day 7 and analyzed for the expression of CD43 and granzyme B. Old cells exhibit quantitative and qualitative defects in activation as measured by fraction of cells expressing CD43 and gra nzyme B and by the amount of each molecule per cell. Results are shown for individual mice and are representative of 4 experiments.

were highly suggestive, they were observed by analyzing total T cell populations, rather than the virus-specific T cells. To circumvent this caveat, we used recently identified immunodominant WNV peptide epitopes that stimulate $CD8^+$ and $CD4^+$ T-cells [(279) Brien, J. et al, in preparation]. Adult and old mice were infected with WNV and day 7 ex vivo T cells analyzed for the response to the immunodominant $CD8^+$ T cell peptide $NS4b_{2488}$ and the two immunodominant $CD4^+$ T cell epitopes ($NS3_{1616+2066}$) by measuring intracellular content of IFNγ. This analysis revealed that significantly fewer $CD8^+$ T cells made this cytokine in old mice, and those that did produced less cytokine per cell (**Figure 6A**). A similar situation was seen with old $CD4^+$ T cells, which showed a trend towards lower percentage of IFNγ producers and a significant reduction of the amount of IFNγ produced per cell (**Figure 6B**). We also used the dominant CD8 T cell epitope to complete the analysis of CD8 T cell cytotoxic potential and to evaluate whether the differences in Granzyme B expression correlated to functional cytotoxicity in adult and old CD8 T cells. Direct ex vivo cytotoxicity correlated to Granzyme B expression as both were decreased in CD8 T cells from old mice (**Figure 7A**). This difference in the cytotoxic potential was abrogated upon one round of *in vitro* stimulation with the dominant WNV CD8 peptide (**Figure 7B**) indicating that CD8 T cells capable of functioning equally well as the adult T cells can be expanded from the old mice.

T cell mechanisms used for protection against WNV disease

We next wanted to determine whether the observed reduction in IFNγ secretion and/or Granzyme B expression were relevant for protection against WNV in vivo. To that effect, we performed two types of adoptive transfer experiments using RAG1-/- mice as
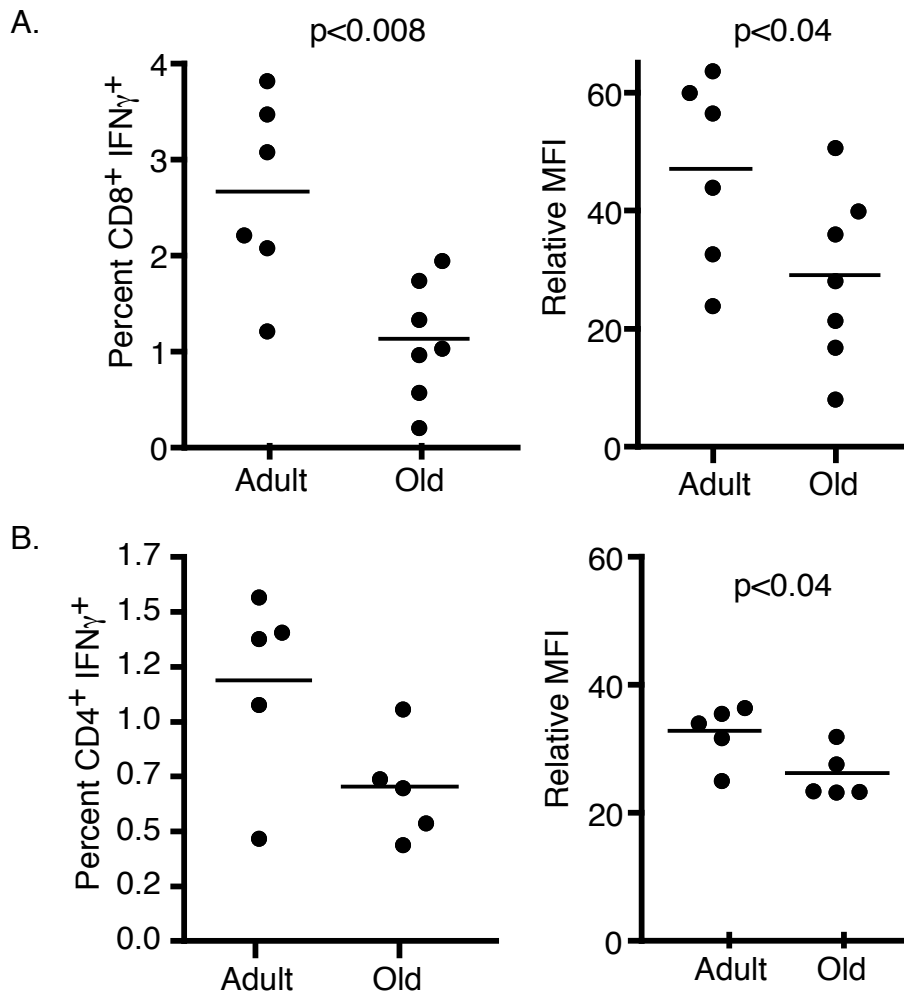
Figure 6. Quantitative and qualitative age-related defects in antigen-specific T cell IFN response to West Nile virus infection and the importance of IFN and perforin in anti-WNV resistance. Old and adult wt or mutant mice were infected with WNV and survival and/or production of IFNγ measured in response to immunodominant CD8 and CD4 T cell epitopes by ICCS at the peak of the immune response (day 7). **A.** Old mice mobilized significantly lower number of IFNγ-producing WNV-specific CD8^{+} T cells than their adult counterparts (p<0.008) (left panel) and produced less IFNγ per cell than old mice (p<0.04). **B.** Old mice showed a trend towards mobilizing fewer antigen specific CD4^{+} T cells then adult mice (p<0.09) and their CD4 T-cells produced significantly less IFNγ per cell than CD4 T cells from adult mice (p<0.04). Results were compiled from three independent experiments.
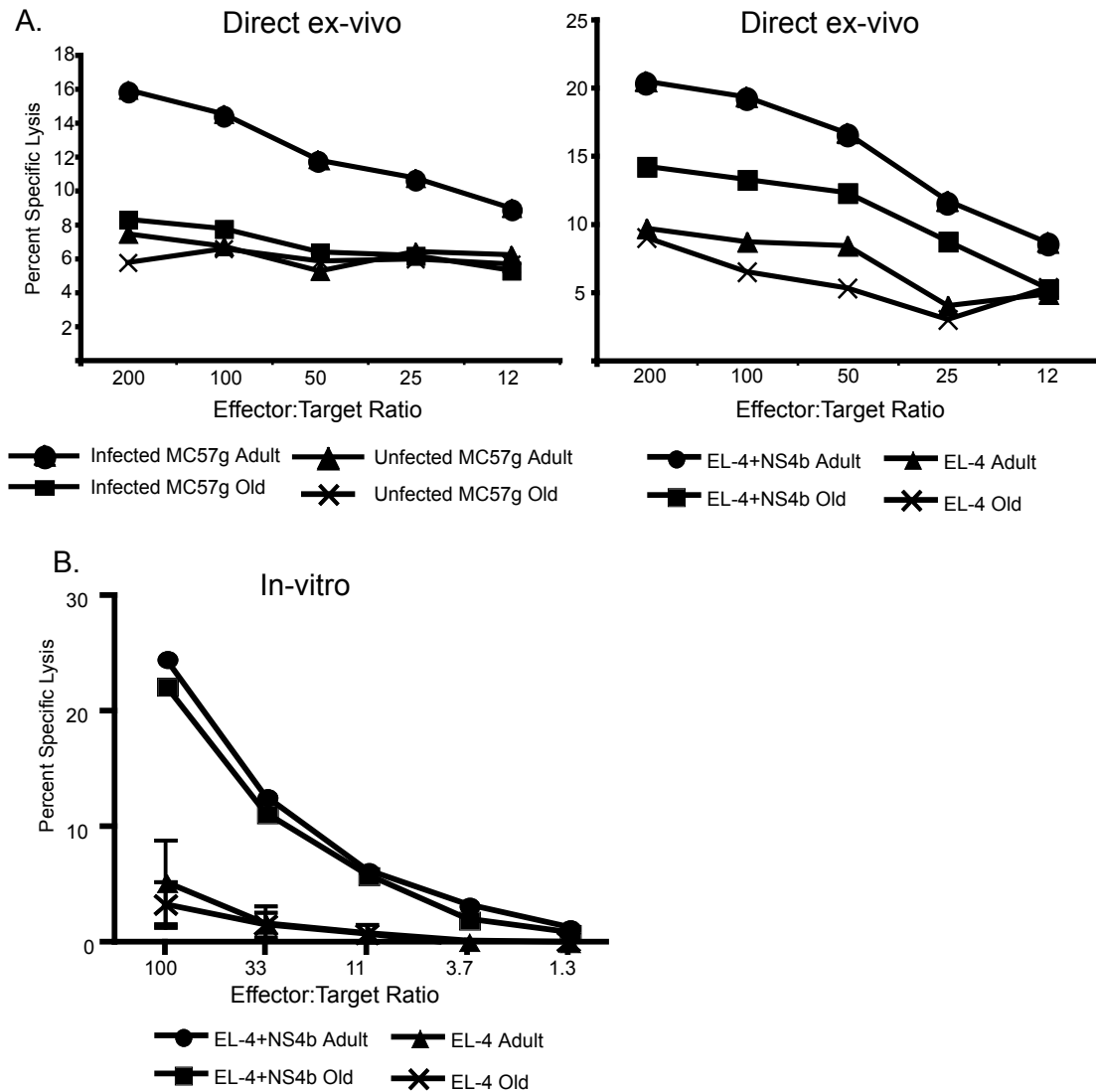
Figure 7. Cytotoxic potential of Adult and Old CD8$^+$ T cells in a ^{51}Cr assay. Adult and old mice were infected with s.c. with WNV at a dose that caused decreased survival of old mice versus adult mice. **A**. CD8$^+$ T cells from adult mice have a greater cytotoxic potential than T cells from old mice, directly ex-vivo 7 days post-infection, whether killing is measured against peptide pulsed targets(right) or virally infected cells (left) (avg. of 4 mice, representative example of three experiments). **B**. Adult and old CD8$^+$ T cells have similar cytotoxic potential against peptide coated targets after 6 days of in-vitro stimulation with the dominant CD8$^+$ T cell epitope (NS4b$_{2488}$).

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recipients. First, we transferred total T-cells from wild type, IFNγ-/-, or Perforin-/- donors

into RAG1-/- recipients, and show that T-cells defective in IFNγ or perforin provide

negligible, if any, protection as compared to animals, which received no cells at all

(**Figure 8A**). This confirms and extends prior results on the importance of these

molecules in anti-WNV protection (217, 291). Finally, we separately transferred highly

purified (< 0.5% cross-contamination) CD4 and CD8 T cells from old or adult naïve

donors into adult RAG1-/- recipients. These results convincingly showed that either adult

CD4 or adult CD8 T cells were sufficient to confer significant protection to RAG1-/-

mice against primary WNV infection in the absence of other components of the adaptive

immune system (**Figure 8B**). More importantly, neither the CD4$^+$ nor the CD8$^+$ T cells

from the old mice were able to confer any protection upon RAG1-/- mice over the level

seen in the absence of transfer (**Figure 8B**), although the combination of the two old T

cell subsets did show some synergy, affording low level of protection (**Figure 4B**, bottom

panel).
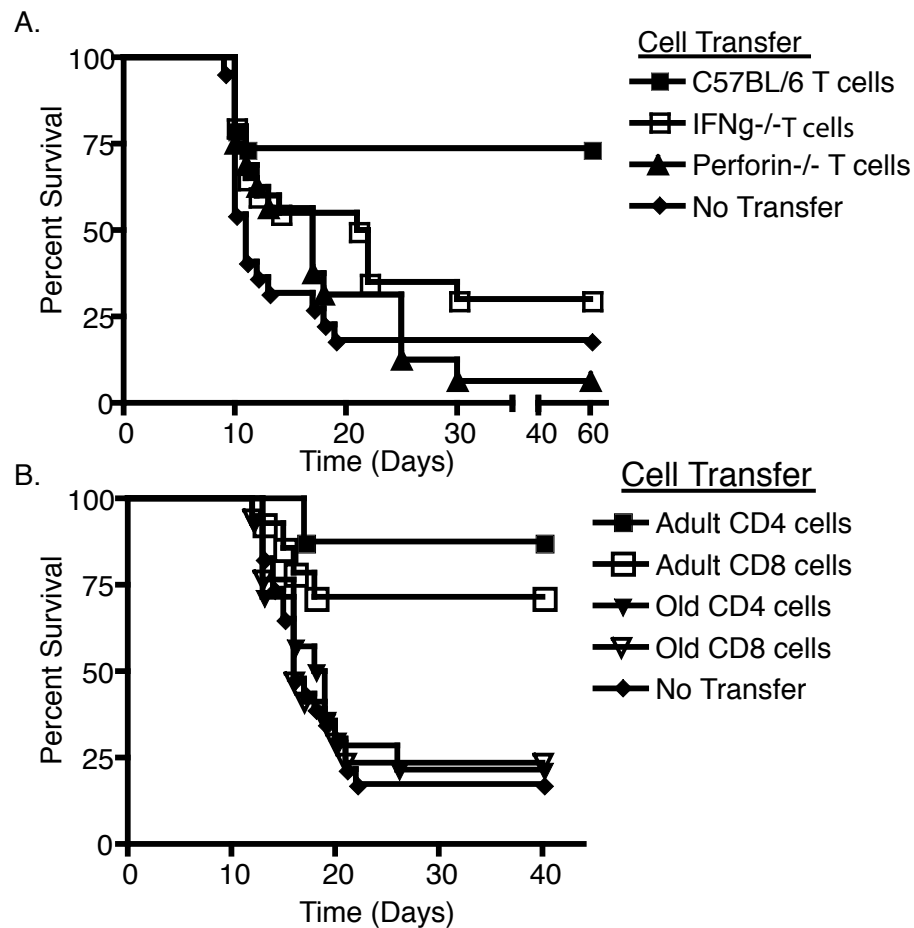
Figure 8. Inability of aged CD4 and CD8 T cells to protect RAG-1$^{-/-}$ mice against lethal WNV infection. **A.** Purified CD8 and CD4 T cells from C57BL/6, IFNγ$^{-/-}$ or Perforin$^{-/-}$ mice were transferred into RAG-1$^{-/-}$ mice, which were then infected with 200 pfu WNV and scored for survival. Wild-type T-cells exhibited significantly enhanced protection when compared to RAG-1$^{-/-}$ mice with no transfer (p<0.0006) or RAG-1$^{-/-}$ mice receiving Perforin$^{-/-}$ (p<0.003) or IFNγ$^{-/-}$ T cells (p<0.01). **B.** Young RAG-1$^{-/-}$ mice received highly purified adult or old CD4 or CD8 T cells (5 x 10^6 cells/mouse). Engraftment was verified after 24 h, animals infected with 200 pfu WNV and survival scored thereafter. Adoptive transfer of old CD4 or CD8 T-cells failed to confer any protection to RAG-1-deficient hosts, whereas transfer of adult CD4 (p<0.0001) or CD8 (p<0.01) T cells afforded a high degree of protection.

Discussion

The above results show that specific age-related defects in T cell immunity, affecting both CD8 and CD4 T cells, underlie the susceptibility of old mice to WNV. This is in contrast to some of the other viral infections, such as Vaccinia, where no defects in granzyme B expression directly ex-vivo can be detected (J. B. and J. N-Z., in preparation), or influenza, where there is no age related decrease in survival in mice (152, 159), and where the loss of CD8 T-cells was not accompanied by impaired viral resistance (362), but is similar to impaired lytic function of CD8 T-cell s against HSV-1 in old mice (363). Numerous innate (IFN-I, complement, innate IgM antibody) and adaptive (B cells, CD4 & CD8 T-cells) were implicated in anti-WNV resistance in adult mice (364) but their relative importance and primary roles still remain incompletely mapped. Our results show that both T cell subsets play important and independent anti-viral roles in adult mice, although it is likely that they could also synergize (e.g. Figure 4). Adult CD4 and CD8 T-cells were both able to secrete IFNγ (results above) and to kill infected target cells (279, 338), and (Brien et al., in preparation), which are critically important anti-WNV effector mechanisms (219, 257), (Figure 6 & 8A), and both functions were impaired with aging (Figure 6). Of major importance are the results from adoptive transfers into RAG1-/- mice, where adult, but not old, CD4 or CD8 T cells could confer significant anti-WNV protection. Since these animals possess functional (and young) innate and reticuloendothelial system components, and only lack B, T and NK-T cells, our results reveal that the defects in transferred aged T cells are cell-autonomous in nature, and not precipitated by the aging of accessory and/or antigen presenting cells.

This would suggest that the use of T cell rejuvenation therapies to remedy the above condition might offer the best chance for therapeutic intervention.

However, it will also be important to understand other, perhaps more subtle, potential defects in innate and adaptive immunity. Indeed, since correction of T cell defects may be achieved by providing enhanced activation of innate immunity, by cytokine manipulation (357, 365) and/or antigen presentation, that approach may be more straightforward for therapeutic manipulation. Overall, given the multitude of defects that affect an aging immune system, it seems prudent to elucidate specific and key defects affecting resistance to each pathogen, and then rationally target the identified defects for intervention.

Materials and Methods

Mice: Old (18-22 month) and adult (4-6 months old) C57BL/6 (B6) mice were purchased from the National Institute of Aging breeding colony (Harlan). C57BL/6 RAG1$^{-/-}$, 129, C57BL/6 Perforin$^{-/-}$ and C57BL/6 IFN$\gamma^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and bred at the Oregon Health & Science University West Campus vivarium. IFNα/β receptor-/- mice were a kind gift of Dr. Ann Hill (OHSU). All animals were housed and bred under specific pathogen-free conditions at the OHSU and experiments conducted under the Institutional Animal Care and Use Committee, and the Institutional Biosafety Committee approvals in accordance with all applicable federal, state, and local regulations. All West Nile virus experiments were completed within a United States Department of Agriculture (USDA) inspected Biosafety Level three facility.

Virus, Peptides, and Cell Lines: West Nile virus strains NY99, 31A and 385-99 were used and all virus strains yielded similar results. West Nile virus strains: NY99 and 385-99 were kind gifts of Robert Tesh, MD, (University of Texas Medical Branch), strain 31A was provided by the USDA. Peptides NS3 $_{1616}$, NS3 $_{2066}$, NS4b $_{2488-2496}$ were purchased from 21st Century Biochemicals, diluted in 10% H_2O, 90% DMSO and stored at -80deg C. Vero, MC57g and EL-4 cell lines were mycoplasma negative, and were cultured under aseptic conditions DMEM (VERO and MC57g) or RPMI (EL-4) supplemented with antibiotics and 5% fetal calf serum. MC57g's were infected using an MOI of 10 for 30 hours prior to use for ^{51}Cr assays. MC57g's were infected using an MOI of 10 for 30 hours prior to use for ^{51}Cr assays.

Flow cytofluorometric (FCM) analysis and Intracellular cytokine staining (ICS):

Cytokine-producing T cells were detected using the Cytofix-Cytoperm Kit (BD

PharMingen, San Diego, CA), as described. Single-cell splenocyte suspension was

depleted of red blood cells and was incubated with 1μM peptide or infected with WNV in

the presence of 5μg/ml Brefeldin A (Sigma Aldrich) for 6 h at 37 °C. After six hours the

cells were washed and blocked with Fc block (anti–mouse FcγRI/III; BD PharMingen)

and incubated overnight in the presence of a saturating dose of surface antibodies against

CD8, CD3, CD4, CD11a, CD43 (Clone 1B11), CD44 and CD62L (BD-PharMingen,

Ebioscience, Biolegend). After washing, the cells were fixed, permeabilized and

intracellular antibodies (anti-IFN-γ, or anti- IL-2; Ebioscience) added for 30 minutes. The

samples were then washed and analyzed using either a FACSCalibur or LSR II cytometer

(Becton Dickinson Immunocytometry Systems) instrument.

Granzyme B (Caltag) intracellular staining was completed directly ex-vivo with

no stimulation.

Infection and CTL Analysis: Mice were infected intraperitoneally (i.p.) with 0.16-20

PFU of WNV virus, or subcutaneously (s.c.) with 20-1200pfu per mouse, as denoted in

the figure legends. Seven days after infection, lymphocytes were isolated and used for

direct flow cytometry (FCM) analysis, for direct ex-vivo restimulation for cytokine

production, and direct ex-vivo ^{51}Cr assay. Percent specific lysis was calculated as [(E -

S)/(M - S)] times 100, where E equals the counts per minute released from targets

incubated with lymphocytes, S equals the counts per minute released from target cells

incubated with no lymphocytes and M equals the counts per minute released from cells after lysis with 1% Nonidet P40 (USB, Cleveland, OH).

Lymphocyte purification and adoptive transfer: Old and adult T cells from corresponding mouse populations were enriched by positive selection of CD4 and/or CD8 T cells using MACS separation (Miltenyi Biotec) in accordance with manufacturer's protocol. Purity of obtained cells was 90-95% CD8+/CD4+ as determined by FCM, and the opposite subset and B cells were present at <0.5% in all transferred populations. 2-10 x 10^6 cells were injected i.v. and engraftment success was evaluated by FCM 24 h later, at the time of infection.

Survival experiments: Animals were challenged with no other manipulations or 24hrs after cell transfer with either 3pfu i.p. or 100 pfu WNV s.c.. Survival was scored on a daily basis. Death occurred between days 10 and 18, and all animals surviving this period remained disease free for >60 days, at which point the experiment was discontinued. Data is shown as percent survival at the termination of the experiment.

Determination of viral burden: Animals were sacrificed and liver, spleen, brain, kidney were removed and homogenized in RPMI using a beadbeater-96 (Biospec). Samples were spun at 2000rpm for 10 min at 4^oC and aliquots of each sample were stored at -80^oC. Viral titer was determined by plaque assay by serially diluting sample onto Vero cells. After co-culture of the virus with the cells for two hours, agarose overlay was

added. Two days after the initial overlay, cells were overlayed with agarose containing

Neutral Red (0.2%). Plaques where then counted to determine viral load.

Type I IFN Bioassay: Serum levels of IFN a/b were measured using a common bioassay.

Type I IFN standards (NIAID international standard) and mouse serum is serially diluted

2-fold down a flat bottom 96-well tissue culture treated plate in complete media, 10%

FBS, Pen/Strep, DMEM. Interferon responsive L929 cells are trypsinized and plated

with equal number of cells per well (5×10^4 cells/well). Cells are incubated overnight

with the serum. Following day (day2) media is aspirated and media containing 5 pfu

VSV-Indiana is added to each well, except for control wells. On day 3 media is aspirated

and the plate is washed 2 times with 1xPBS. The monolayer is than fixed with 5%

formaldehyde, incubate for 10 minutes, and then stain with 0.05% crystal violet for 10

minutes. Wash monolayers with water and allow to dry. Once dry, 100% methanol is

used to elute the dye and absorbance is measured at 595nm on molecular probes ELISA

plate reader. Experimental samples are compared to the standard curve to determine

value, to determine endpoint values.

Statistical analyses: Fisher's exact test and Log Rank test was used to analyze results

from survival experiments. Statistical significance of viral titer observed between groups

was analyzed using Mann Whitney test. All calculations were done using the Prism

(GraphPad, San Diego, CA) software.

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Chapter Five

A mouse model for age-related vulnerability to Poxvirus-induced disease

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Abstract

The elderly human population suffers greater consequences from infectious disease then the adult population. In the face of an increasing number of aged individuals as well as an increase in the spread of emerging and re-emerging virus infections there is a need to develop better animal models to explore the mechanisms of immune senescence and it's relation to the increased severity of disease in the elderly. Specific causes behind these deficiencies in generating safe and protective immune responses remain incompletely understood and suggest that vaccines and treatments may need to be tailored for the elderly population. Poxviruses cause some of the most severe human infections and are known to disproportionally affect the elderly. Here we demonstrate that old C57BL/6 mice are more susceptible to Vaccinia virus infection then adult mice. During acute infection of old and adult mice, old mice develop an inferior CD8 T cell response, as shown by diminished antigen-specific cytokine production and limited cytotoxicity. Although a similar outcome in terms of animal survival is seen in our West Nile virus model, the molecular defects identified in each model are different.

<u>Introduction</u>

Vaccinia virus (VACV) is a member of the genus orthopoxvirus of the family *poxviridae* (169). Vaccinia is best known for its role as a live vaccine for variola virus (smallpox). Immunization with live VACV results in a cross-reactive and cross-protective immunity (366) against the variola (VARV) virus. Although most of our knowledge of immunity to poxviruses is derived from the use of ectromelia virus (ECMV) in mice, some of the essential protective mechanisms against VACV infection in vivo have been defined. Previous studies have shown that both a strong humoral immune response (consisting of IgM and IgG) and a robust cellular immune response (including CD4 or CD8 T cells) are critical for protection from severe VACV disease (367-369). Interestingly, CD8 or CD4 T cells subsets individually are dispensable during primary VACV infection of mice, but survival is dependent upon one of the two subsets being present during primary infection, indicating that they overlap in their essential functions (367, 368, 370). While some T cell protective functions are not required for survival (perforin, Fas) during VACV infection, IFNγ production is absolutely critical (371-373).

These immunological requirements for protection in mice are mirrored during mass vaccinations of humans with VACV, since people with defects in either humoral or cellular immunity responded poorly to VACV given during immunization (374). The increased incidence of vaccination-associated disease, seen during the use of a live attenuated virus (LAV) vaccine highlights the risks that these types of approaches have when thinking about protecting the immunocompromised or the elderly (88, 375, 376). This is of particular concern, in considering how best to protect vulnerable populations from bioattack. Recently, it was shown that the use of VACV as a vaccine did not

prevent infection by a heterologous poxvirus, Monkeypox virus (MPV), but did prevent severe disease (377). If MPV was used as a potential bioweapon, this limited level of viral control by the current vaccine could cause a greatest loss of life in the elderly population due to the waning of immunity.

Immune senescence is a state of immunodeficiency that occurs during the aging process and is believed to be responsible for the increased severity of disease within the elderly population. The impact of immune senescence has an ever-increasing importance due to the increasing size of the elderly population. Overall, we are far from identifying the primary and the most important age-related defects in immunity and how these defects can be ameliorated and this is in part due to the lack of suitable animal models. Moreover, the importance of different defects is likely to vary with each pathogen, and our knowledge of interactions between specific pathogens and the aging immune system is even more limited.

In this present study, we determined the susceptibility of old and adult mice to VACV infection. We did this in order to be able to develop additional models to identify T cell defects that develop during immune senescence and which may lead to increased disease severity. We show that old mice are more susceptible to intraperitoneal (ip) VACV challenge than adult mice. Old mice have fewer antigen-specific T cells capable of producing IFNγ after a short *in vitro* stimulation. Although GrB expression levels are similar in old and adult mice during VACV infection, cytotoxic T lymphocytes (CTL) generated in response to VACV in old mice have diminished cytotoxic potential. These studies provide critical insight into defects in the antigen-specific T cell population that can develop during aging.

Increased susceptibility of old mice to VACV infection.

Few small animal models exist to study the increased susceptibility of old mice to infection. Old and adult mice were challenged with three incremental doses of VACV. Based on this experiment we have determined that old mice are 15 fold more susceptible to lethal VACV infection than adult mice (**Figure 1**). At 5×10^8 plaque forming units (pfu)/mouse, all of the old mice succumb to infection, while only 33% of adult mice succumb to infection. At 1.25×10^8 pfu, all adult and no old mice survived showing significant differences in susceptibility ($p < 0.04$) more susceptible than adult mice to VACV infection. Finally, at 3×10^7 pfu, all adult mice and 66% of old mice survived. This model was therefore suitable to investigate the root causes for the increased susceptibility of severe disease.

Characterization of the VACV-induced T cell responses in old and adult mice.

Although perforin is not absolutely required for the control of VACV during the infection of adult B6 mice, we chose to evaluate the cytolytic pathway in old mice because of the defect in Granzyme B (GrB) production we see during WNV infection of old mice, as well as the pronounced T cell defects that develop as an overall part of immune senescence. Using direct ex-vivo immunophenotyping, we looked at the expression of GrB in CD8 T cells without any *in vitro* stimulation (**Figure 2A**). There was no difference in either the number of cells that express GrB or the amount of GrB expressed per CD8 T cell with age. We next chose to look at IFNγ production by antigen-specific CD8 T cells. We chose to use B8R, the dominant CD8 T cell epitope, to stimulate IFNγ production for 6 hours in the presence of BFA. After peptide stimulation
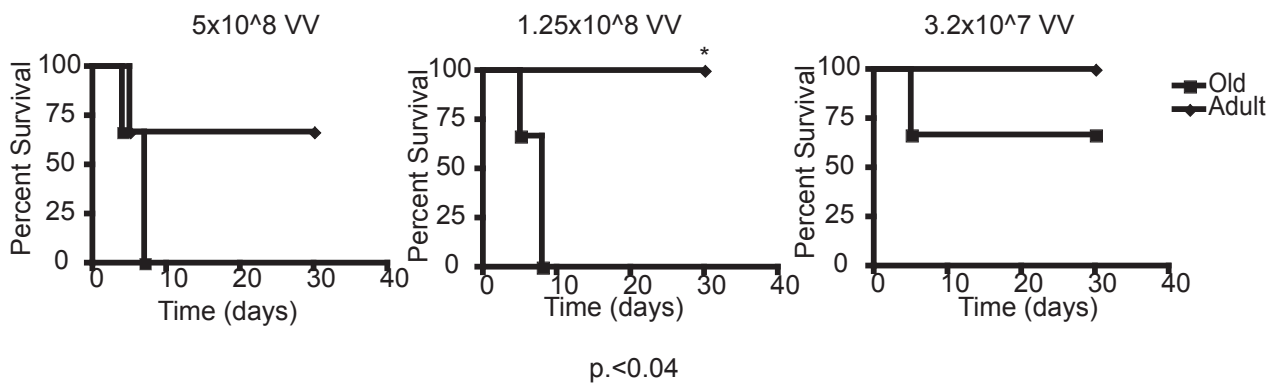
Figure 1. Increased age-related mortality C57BL/6 mice in response to VACV infection. Old mice were significantly more susceptible (p<0.04) than adult mice at 1.25x10^7 pfu VACV. Adult (4 mo old) and old (20 mo) C57BL/6 mice were infected i.p. with indicated doses of VACV strain WR, and mortality scored over 60 days. Three animals per group were analyzed, and results are representative of three similar experiments.

A.

B.
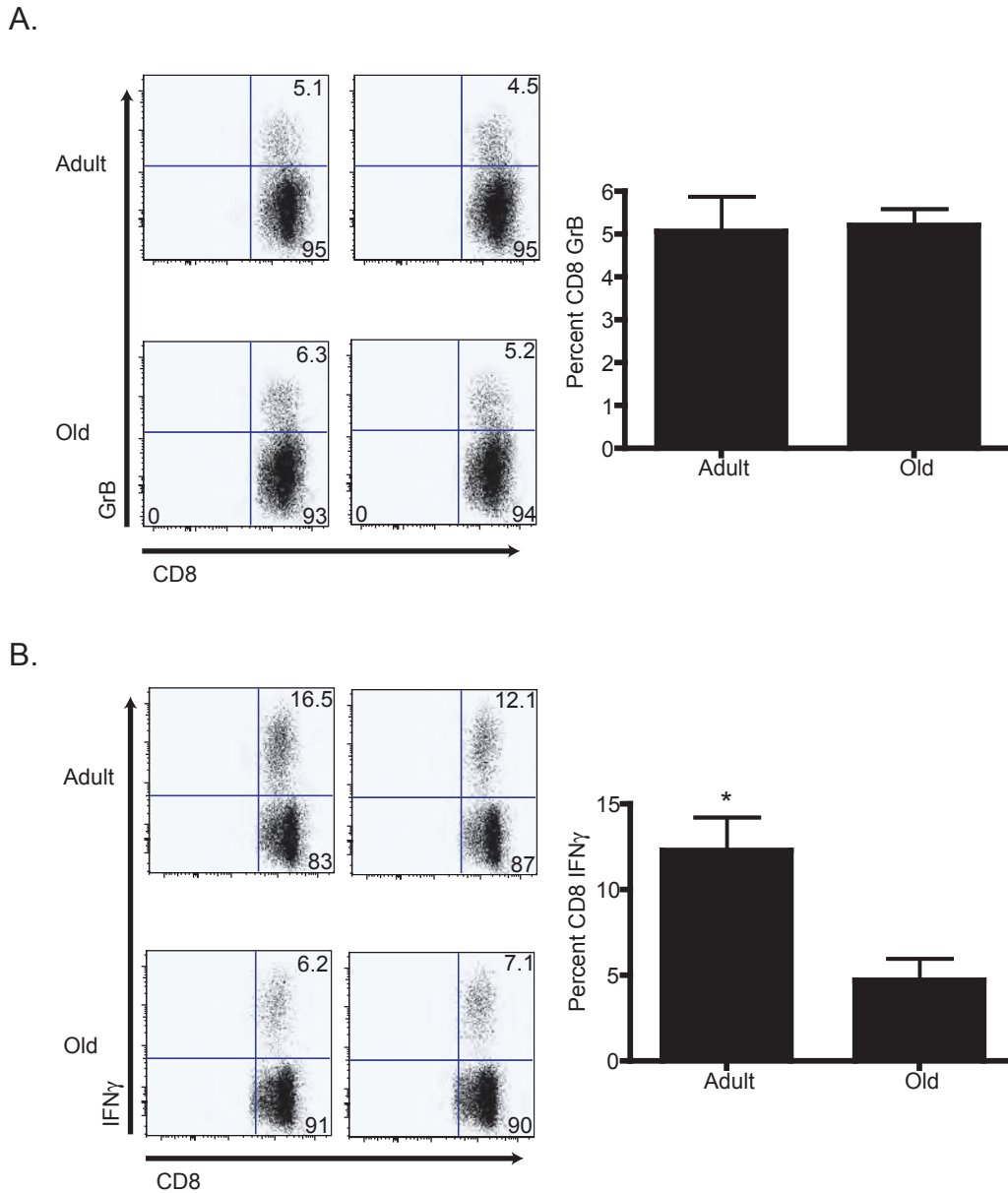
Figure 2. Poor response of old splenic CD8 T cells following systemic infection.
Animals were infected with 1×10^7 VACV. **A.** Direct ex-vivo GrB staining of CD8 T
cells from old and adult mice. **B.** Day 7 direct ex-vivo ICCS performed using 6 h
stimulation with the B8R peptide in the presence of BFA. N=5 mice per group. Peptide
was used at 10-7M. Data are representative of two experiments.

12% of adult CD8 T cells produced IFNγ, whereas only 5 % of old CD8 T cells produced IFNγ (**Figure 2B**). Significantly (p<0.02) more adult CD8 T cells responded to the B8R peptide than CD8 T cells from the old mice. This clearly shows that adult mice have a more robust antigen-specific response to VACV infection than old mice.

Direct ex-vivo cytotoxic potential of CD8 T cells in old and adult mice.

In a direct ex-vivo ^{51}CR-release assay CD8 T cells from old mice were unable to recognize virally infected target cells (**Figure 3A**), but were still capable of cytolytic activity as indicated by their ability to kill targets in a re-direct killing assay (**Figure 3B**). If target cells were peptide pulsed ($1x10^{-7}$M) with the immunodominant CD8 peptide B8R, splenocytes from old mice still killed fewer peptide-pulsed targets cells than adult cells, indicating there was still a T cell defect in killing, although the greatest defect may be in target recognition (**Figure 3C**).

Interestingly, there was a reduction in total lymphocyte numbers at the peak of the immune response (day 7) in the spleens of old mice, although the percentage of CD8$^+$ T cells was equivalent (data not shown). In addition, when virally activated lymphocytes were stimulated by virally infected cells, to estimate the total number of VACV-specific T cells within the CTL assay, the reduction in cytolytic function did not correlate with a decrease in measured cytokine production (data not shown). Based upon these results, we propose that the decrease in the quality, specifically the reduction in cytokine production, of responding CD8$^+$ T cells in old animals may be responsible for the increased risk for severe viral disease.
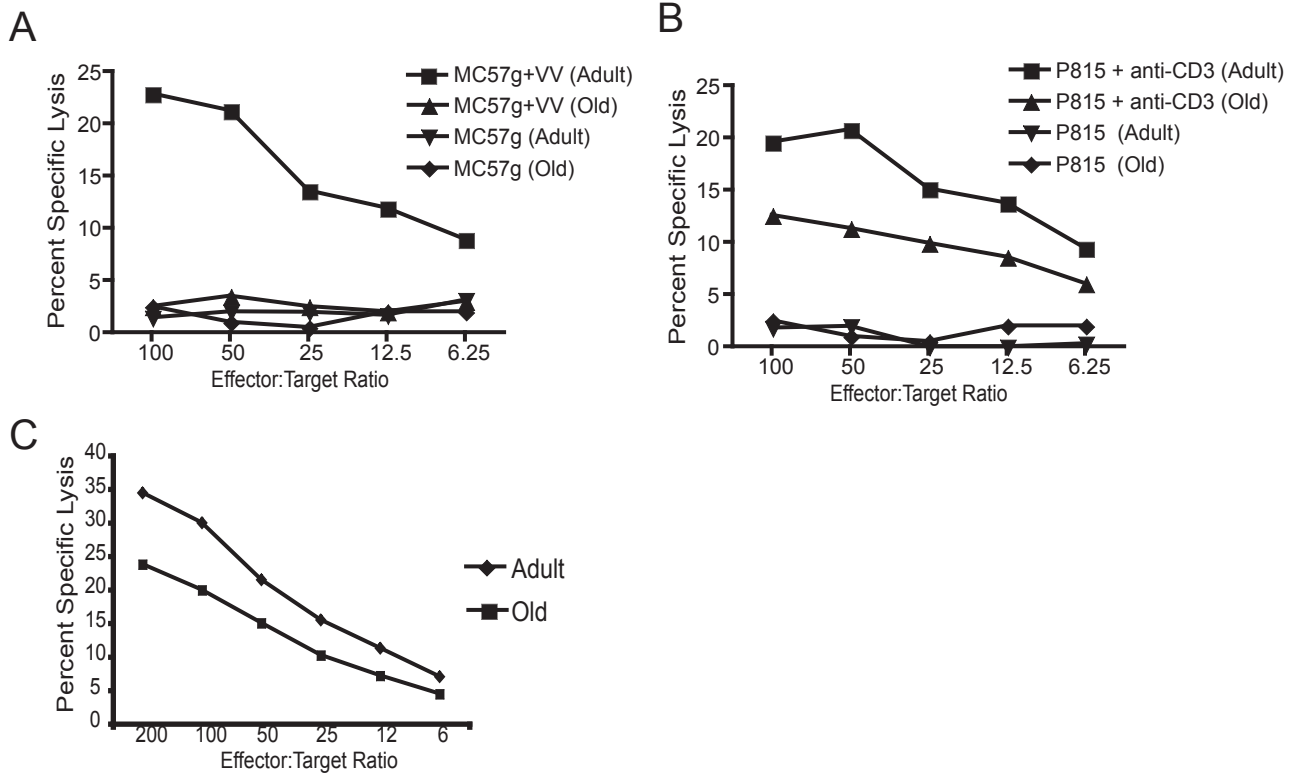
Figure 3. Direct ex-vivo cytotoxicity of splenocytes from old and adult mice. A. Old mice were unable to recognize and kill virally infected target cells directly ex-vivo. 6 hour ^{51}Cr-release assay was performed using splenocytes from B6 mice 7 days post infection with VACV. MC57g cells were infected (2 h, m.o.i. 10) or used uninfected. Cytotoxicity of splenocytes of virally infected splenocytes. **B.** Re-directed lysis assay using identical splenocytes as used in panel A. Here the cytolytic capacity of adult splenocytes is greater than 8x that of old splenocytes cells. Representative experiment of 3. N=4 mice per group. **C.** Cytotoxicity of B8R peptide pulsed MC57g target cells, 1×10^{-7}M peptide. In this experiment the cytolytic capacity of adult splenocytes is greater than 3x that of old splenocytes. This is one representative experiment of two.

We present the initial description of another viral infection model where increased age leads to increased severity of disease. We note that during the primary immune response, CD8 T cells from both old and adult mice upregulate GrB expression, but do not have equal number of antigen-specific CD8 T cells that are able to produce IFNγ. We also show that CD8 T cells from old mice are unable to recognize and kill virally infected targets, but this deficiency can be partially restored by the use of peptide-coated targets. This indicates that antigen-specific CD8 T cells, which develop during VACV infection, may not only have a defect in delivering a cytotoxic signal, but also have a defect in target recognition.

VACV has long been used as a human vaccine, and over the past decade has been adapted to be used as a vaccine vector in a broad number of studies. At the time when VACV vaccination was commonly used to prevent variola, little was known about the T cell response and therefore little work was done to determine the efficacy of T cells during vaccination. With the use of VACV as a vaccine vector, once again little work was completed looking at the immune response to the vector backbone. It has not been until the recent concerns over bioterrorism that basic questions about protection using VACV been discussed. In addition, since VACV vaccination ended within the United States in 1972, there has been a tremendous cultural change in the publics understanding of medicine and health. The general public no longer would accept the complication rate seen with VACV vaccination. There has also been an increase in the potential complication rate with VACV vaccination due to changes in population, where there are

many more people who are immunosuppressed for a number of reasons as well as a tremendous increase in the number of elderly.

In light of the current climate of potential bioterrorism and an ever-increasing elderly population, a pox model of increased severity of disease in the elderly is essential. We believe this model can be used to assess two specific questions that affect vulnerable populations. First, what are the correlates of protection during a primary immune response to a live vaccine? Second, what are the correlates of protection during either a homologous or heterologous virus challenge? Both questions are of the utmost importance.

In VACV infection, antibody response plays a key factor in protection (367, 370), although in the absence of B cells, CD4 and CD8 T cells have been sufficient to provide protection during primary VACV infection (367, 368). Therefore based on previous reports where knockout mice or antibody depletion studies were used to map correlates of protection, there is probably a cumulative series of defects in both the antibody response as well as the T cell response, that leads to the increased severity of disease in old mice. We have here demonstrated the existence of antigen-specific CD8 T cell defects, and these defect alone or in combination with potential defects in the CD4 and antibody response are likely to result in the increased severity of disease in the old mice.

Materials and Methods

Mice. Adult (2-6 months old) male C57BL/6 (B6) mice were purchased from the National Cancer Institute Breeding Program (Frederick, MD). Old (18-22 months old) mice were purchased from the National Institute of Aging. All animals were housed under specific pathogen-free conditions at the Oregon Health & Science University. All VACV-wr experiments were completed within an approved Biosafety Level (BSL) 2 facility, and were approved by the Institutional Animal Care and Use Committee, and the Institutional Biosafety Committee in accordance with the applicable federal, state, and local regulations.

Virus, Peptides, and Cell Lines. Vaccinia virus strain Western Reserve (WR) was a kind gift of Dr. Jon Yewdell (National Institute of Health). Synthetic peptide B8R (TSYKFESV) was purchased from 21st Century Biochemicals (Marlboro, MA), diluted in 10% H_2O/90% DMSO, stored at -80^0 C and subsequently used at indicated concentrations. Virus was grown in mycoplasma-negative Vero cells, cultured under aseptic conditions as described previously. Virus was purified by pelleting cells and dounce homogenizing the cell pellet in 10mM Tris pH 8.0. Cell homogenate was spun at 3,000rpm for 10 minutes at 4 degrees C. Supernatant was taken and layered over a 36% sucrose cushion made in   10mM Tris pH 8.0 and spun in an SW41 ultracentrifuge at 18,000 xg for 80 minutes. Virus pellet was resuspended in 2mls of 10mM Tris pH 8.0 and dounce homogenized again then aliquoted and stored at -80 for use in future experiments.

<u>Determination of viral titer</u>. Viral titer was determined by plaque assay by serially diluting sample onto Vero cells. After co-culture of the virus with the cells for two hours, agarose overlay was added. Two days after the initial overlay, cells were overlayed with additional agarose containing Neutral Red (0.4%). Plaques where then counted to determine viral load.

<u>Intracellular cytokine and surface flow cytofluorometric (FCM) staining.</u> Cytokine-producing T cells were detected using the Cytofix-Cytoperm Kit (BD PharMingen, San Diego, CA), as described. Single-cell splenocyte suspension was depleted of red blood cells and was incubated with 1μM peptide or infected with WNV in the presence of 5μg/ml Brefeldin A (Sigma Aldrich) for 6 h at 37 °C. After six hours the cells were washed and blocked with Fc block (anti–mouse FcγRI/III; BD PharMingen) and incubated overnight in the presence of a saturating dose of surface antibodies against CD8, CD3, CD4, CD11a, CD43 (Clone 1B11), CD44 and CD62L (BD-PharMingen). After washing, the cells were fixed, permeabilized and intracellular antibodies (anti-IFN-γ, BD-PharMingen) were added for 30 minutes. For detection of Granzyme B, splenocytes were isolated and kept on ice, surface stained, fixed and permeabilized as described above without stimulation. Cells were stained with Granzyme B PE (clone: gb12, Invitrogen), The samples were then washed and analyzed using either a FACSCalibur or LSR II cytometer (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA) instrument. FCM analysis was performed by collecting a minimum of 5 x 10^4 events and gates set on lymphocyte population based on forward and orthogonal

light scatter, followed by marker positioning to denote fluorescence greater than that of control stained or unstained cells.

Infection and CTL assays. Mice were immunized intraperitoneally (i.p.) $5x10^8$-$1x10^7$ pfu VACV-wr. Direct ex vivo CTL activity was determined using a standard ^{51}Cr-release assay, with peptide-coated or virally infected and control MC57g cells as targets. MC57g's were infected at an MOI of 10 for two hours prior to the experiment. Radioactivity was measured using TopCount Packard δ/γ radioactivity reader (Packard Co., Detroit, MI).

Statistical analysis. All calculations were done using the Prism (GraphPad, San Diego, CA) software. All p values are given as exact values.

Chapter Six

T cell memory after primary West Nile virus infection

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Abstract

Memory T cells are an essential component of the adaptive immune response, in charge of protection against re-infection. The development of memory T cells is also essential for effective vaccination. We provide here a detailed phenotypic and functional characterization of antigen-specific memory T cells that develop in C57BL/6 mice after primary West Nile virus infection. A strong memory T cell response develops after West Nile virus infection, which declines slowly overtime, but is still functionally responsive. These results are discussed in light of designing immunotherapeutic strategies to prevent West Nile virus disease. The description of the antigen-specific memory T cells can have important implications for designing immunotherapeutic strategies to prevent West Nile virus disease.

The characterization of memory T cell populations has drastically changed with the development of the central and effector memory model (378). Although the process of cell fate commitment is incompletely understood, the existence of different populations of memory T cells as defined by phenotypic markers and functional capabilities is well accepted (17, 56, 379). Initial reports indicate that the generation of central memory T cells may be the best target for long-term protection by a vaccine (380).

Memory T cell development is a defining component of the adaptive immune response. A viral infection or vaccination triggers the innate immune system to release a series of chemokines and cytokines recruiting immature dendritic cells (DCs) to the site of inflammation. When DCs are in an immature state they are able to uptake antigen. Once exposed to cytokines such as type I IFN or IL-12, DCs begin to mature and stop taking up antigen. DC maturation is marked by upregulation of major histocompatibility (MHC) Type I and II molecules, as well as co-stimulatory molecules including CD80, CD86. Mature DCs then stimulate naïve T cells to differentiate into antigen-specific effector cells. The naïve T cell requires 3 signal 1) direct signaling through the T cell receptor, provided by a DC; 2) co-stimulatory receptor-ligand interaction on the DC providing signal #1 3) an environmental cytokine cue such as Type I IFN or IL-12, generated by the innate immune system in response to an infection. With these three signals a naive T cell will rapidly proliferate, acquire effector functions such as cytokine production and/or cytotoxicity, and upregulate anti-apoptotic intracellular molecules such as BCL-2. It is the upregulation of these anti-apoptotic signals that is absolutely required

for the development of memory T cells (reviewed in (17, 28). However, the question of whether memory cells are derived from this initial population of naïve T cells or some of the effector cells that survive the acute phase of infection has yet to be resolved.

Memory T cells are divided into two dominant subsets, central memory and effector memory (28). Central memory T cells are long term memory cells that are capable of vigorous proliferation, but have limited immediate effector function. Effector memory cells are able to rapidly deliver a variety of effector function such as cytokine release or cytolytic activity, but have limited proliferation potential. Maintenance of the T cell memory pool requires expression of cytokine receptors and anti-apoptotic molecules by memory T cells (reviewed in (381)). Homeostatic control or maintenance of memory cells involves several signals, of which IL-7 and IL-15 are the most important. In general, IL-15 mediates proliferation, while IL-7 promotes survival. CD4 memory T cells may also require signals through the TCR for survival (382).

Only a limited number of studies have looked at memory T cell response to WNV (275, 279, 287, 383, 384), and no studies at this time have looked at memory T cell responses in an antigen-specific fashion for either the CD4 or CD8 T cell population. Many vaccination studies have shown that mice can be vaccinated and protected against West Nile virus encephalitis (WNE) by a variety of approaches (297, 299, 300, 385-387), however, there is limited information as to which subsets of lymphocytes are playing the most important role in that protection. In one study Pan et al used a JEV DNA-vaccine in mice, and showed that a combination of neutralizing antibody response and T cells were required for protection (341).

Our data indicates that WNV infection induces a strong CD4 and CD8 T cell response that develops into a robust memory T cell population that lasts well over a year and a half after primary infection. Although the T cell memory population does decline over time, the remaining cells are still functionally responsive.

Results

Phenotypic characteristics of Tetramer positive CD8 T cells after WNV infection

In order to evaluate the generation of effector and central memory populations we used the CD8 NS4b$_{2488}$ tetramer to compare and contrast the antigen-specific CD8 T cell populations during the course of a primary and memory response by flow cytometry (FCM) (**Figure 1**). C57BL/6 mice were infected sub-cutaneously (sc) with 200-800 pfu of WNV strain 385-99 and sacrificed either 7 or 60 days post-infection. During acute infection, antigen-specific CD8 T cells clearly down regulate CD127 (IL-7Rα) and CD62L, while upregulating CD44, CD43 (1b11) and Ly6c. By contrast, memory cells exhibited high expression of CD127, CD44 and Ly6c, with an intermediate level of CD43 (1b11) expression. Memory cells also showed a broad distribution of CD62L expression, giving the impression that both an effector-memory and central-memory population exist without either one dominating the memory pool at two month post-infection.

Cytokine responsiveness of effector and memory WNV specific CD8 T cells

To evaluate the protective potential of these memory T-cells, we next evaluated their ability to respond to peptide stimulation by cytokine secretion. Splenocytes from C57BL/6 mice infected with WNV strain 385-99, were stimulated with $1x10^{-6}$M NS4b$_{2488}$ peptide for 6 hours in the presence of BFA (**Figure 2**). We observed that memory T cells are able to respond to antigen by producing cytokine, and that the percentage of antigen-specific lymphocytes was lower in memory mice than acutely infected mice. We then gated on IFNγ$^+$ and IFNγ$^-$ CD8 T cells and completed a four-way
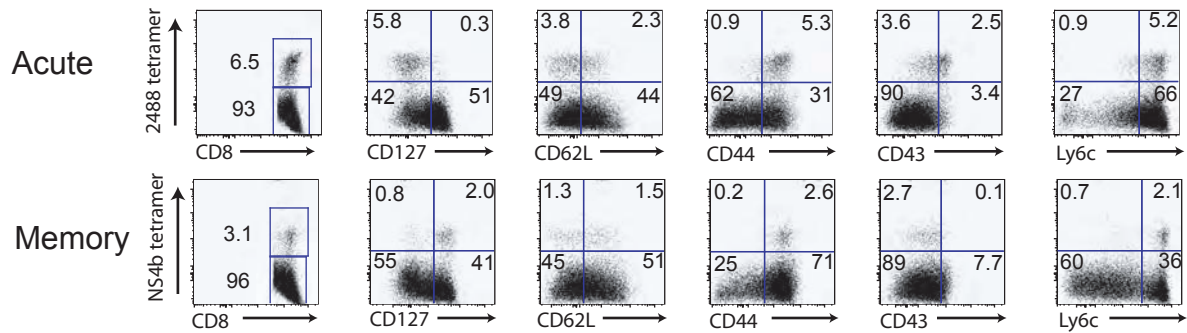
Figure 1. Immunophenotyping of effector (day 7) and memory (day 60) C57BL/6 mice infected with WNV strain 385-99. Top panel- CD8 NS4b2488 tetramer$^+$ effector cells show the CD127^{low}, CD62L^{low}, CD44^{hi}, CD43^{hi}, Ly6c^{hi} phenotype. Bottom panel-CD8 NS4b2488 tetramer$^+$ memory cells, which are CD127$^+$, CD44^{hi}, CD43^{low}, Ly6c^{hi}. Direct ex-vivo staining of splenocytes with NS4b2488 tetramer and various other cell surface markers, see methods. The cells are gated on CD8 T cells (data not shown). Panels depict the analysis of T cells from one animal of four analyzed in this experiment, and are prepresentative of a total of three experiments and 16 mice.

Figure 2. **Intracellular Cytokine staining of acute (day 7) and memory (day 50) CD8 T cells.** Phenotype of antigen-specific effector and memory CD8 T cells. Top panel-CD8 IFNɣ+ effector T cells show the CD27+, CD127-, CD11a^hi, CD62L^low phenotype. Bottom panel- CD8+ IFNɣ+ memoryT cells which are CD27-, CD127-, CD11a^hi, CD62Llow^/hi, CD8 IFNɣ+ T Splenocytes from C57BL/6 mice were stimulated with 1x10^-6M NS4b_{24 88} peptide for 6 hours in the presence of BFA and stained for various other cell surface markers, see methods, then stained intracellulary for IFNɣ. Progressive gating is based upon the response measured by IFNɣ and is indicated by arrows. For comparison, IFNɣ negative cells are shown in parallel panels. Panels depict the analysis of T cells from one animal of four analyzed in this experiment, and are representative of a total of three experiments and 16 mice).

comparison of cell phenotype between the responding and non-responding populations of both effector and memory T cells. Antigen-specific, cytokine responding $CD8^+$ $IFN\gamma^+$ T cells from acute mice were $CD11a^{hi}$, $CD127^-$, $CD62L^{low}$, $CD27^+$, while cytokine responding memory cells were $CD11a^{hi}$, $CD127^-$, $CD62L^{low}$, $CD27^-$. $IFN\gamma^-$ cells from both acute and memory mice exhibited heterogeneous phenotype. The dominant $IFN\gamma^-$ population from both acutely infected mice and memory mice were comparable, they were $CD27^+$, $CD127^+$, $CD62L^{hi}$, $CD11a^{low}$. When interpreting the phenotype of CD8 $IFN\gamma^+$ cells from acute mice we can clearly see the homogenous response of a primary effector cell population, with the downregulation of CD127 correlating with cell survival. While the CD8 $IFN\gamma^+$ memory cells after stimulation the cells once again downregulate CD127 (IL-7Rα), but CD27 has been downregulated.

Enumeration of WNV antigen-specific memory T cells

We next evaluated the percentage of memory T cells in individual mice that had been infected sc with WNV strain 385-99 (**Figure 3**). Enumeration of $CD8^+$ $NS4b_{2488}Tet^+$ cells was completed through a progressive gating strategy as described above. NS4b tetramer positive cells from the spleen were gated and enumerated from naïve and acutely infected mice. Background tetramer staining ranged from 0.1-0.8% and was subtracted from the positive signal. Over time we saw a decrease in the number of memory T cells (**Figure 3A**). However, even at 17 months post-infection 8 out of 8 mice still exhibited tetramer staining above background. Using the same mice we evaluated the frequencies of $IFN\gamma$-secreting cells by stimulating splenocytes with 1×10^{-6}M $NS4b_{2488}$ peptide for 6 hours in the presence of BFA. Background levels of staining
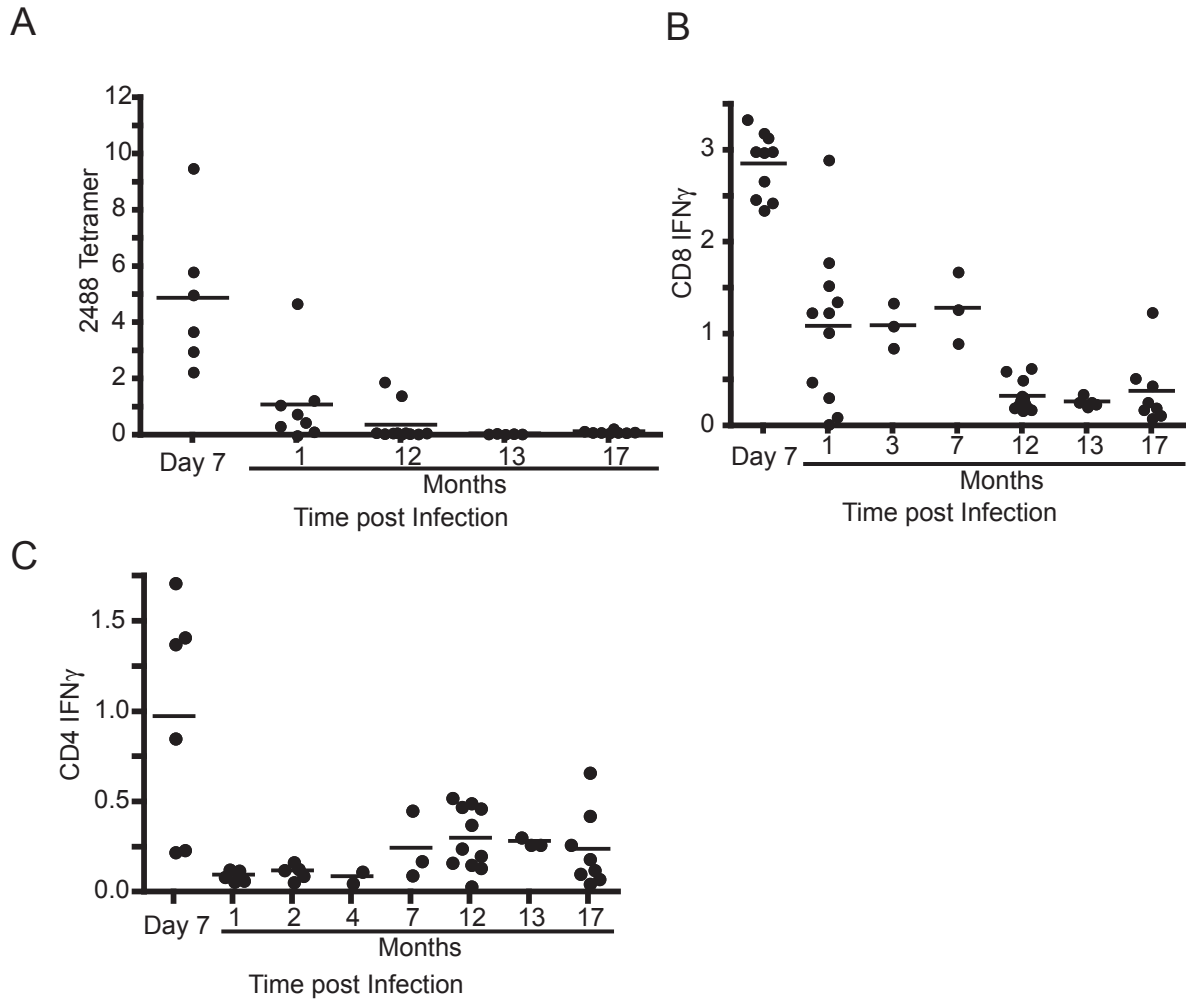
Figure 3. **Quantification of Memory T cells over time in response to WNV**.
Enumeration of the percentage of memory T cells found in mice infected sc with WNV
strain 385-99. **A.**) Enumeration of CD8 NS4b$_{2488}$ tetramer^{+} from the spleen by FCM.
Background tetramer staining ranged from 0.1-0.8% and was subtracted from the positive
signal. **B.**) Enumeration of CD8 IFNγ^{+} T cells from the spleen by ICCS. Splenocytes
were stimulated with $1x10^{-6}$M NS4b$_{2488}$ peptide for 6 hours in the presence of BFA and
stained for various surface markers, see methods, then stained intracellularly for IFN .
Background levels of staining were based on percentage of CD8 IFNγ^{+} cells from non-
stimulated samples for each mouse and was subtracted from the positive signal. **C.**)
Enumeration of CD4 IFNγ^{+} T cells from the spleen by ICCS. Splenocytes were
stimulated and stained as in panel B, except splenocytes were stimulated with 3 peptides
ENV$_{641}$, NS3$_{1616+2066}$ $1x10^{-6}$M peptides. Background levels of staining were based on
percentage of CD4 IFNγ^{+} cells from non-stimulated samples for each mouse and was
subtracted from the positive signal. Panels depict the analysis of T cells from 3-12 mice
per time point, and this data represents the total of 4 experiments and 46 mice.

were based on percentage of CD8$^+$ IFNγ^+ cells from non-stimulated mice, otherwise the analysis was similar to what was described above. We observed a decline in the percentage of memory CD8 T cells that respond to the dominant CD8 T cell epitope (**Figure 3B**). All mice except for one showed an antigen-specific response; that animal did not have any NS4b tetramer specific cells. Based on our experience, we believe this mouse never received an infectious dose of WNV and therefore was not primed. Using similar methods described in Figure 3B, with the same mice we evaluated the antigen-specific response to the three dominant CD4 T cell epitopes ENV$_{641}$, NS3$_{1616+2066}$. Similar to CD8 T cells we saw a decline in the percentage of CD4 memory T cells over time, except for the one mouse described above. We observed an increase in CD4 memory frequency starting at 7 months, but we believe that this increase is due to a new batch of peptide. Additional mice from the majority of the time points shown will be analyzed to determine if the CD4 T cell population is actually increasing, or if the variation is due to a new batch of peptide.

The data presented here clearly shows that memory T cells persist at least for 17 months after infection. Both CD4 and CD8 T cells are able to respond to peptide stimulation and produce IFNγ.

Discussion

    We present here what we believe is the first report describing the phenotype and functionality of WNV-specific memory T cells in an antigen-specific fashion. The data we present here clearly shows that primary WNV infection generates a robust memory T cell population that can persist for at least 17 months post infection (Figure 3). Memory CD8 T cells can produce cytokines and bind tetramers. Memory CD4 T cells are also able to produce cytokines upon antigen stimulation. This life long presence of memory T cell populations could potentially play a role in protection from re-infection with WNV and, perhaps, other Flaviviruses (388, 389).

    It has been recently shown that a robust memory T cell response is required for protection upon rechallenge of mice, since mice with inferior memory CD8 T cells due to priming in the absence of $\gamma\delta$ T cells, were more susceptible to re-challenge (255). In addition, several groups have shown that the adoptive transfer of memory CD8 T cells to naive mice improve survival over controls groups (278, 293), demonstrating the ability of at least memory CD8 T cells to play a role protection upon rechallenge.

    Knowledge on the functional and phenotypic characteristics of memory T cells after primary WNV infection has been limited. This data, taken in light of current vaccination studies, will allow us to start to determine the relative contribution of antibody versus T cell memory in control of WNV spread and pathogenesis upon re-infection.

Methods

Mice. Adult (2-6 months old) male C57BL/6 (B6) mice were purchased from the
National Cancer Institute Breeding Program (Frederick, MD). All animals were housed
and bred under specific pathogen-free conditions at the Oregon Health & Science
University. All WNV experiments were completed within a United States Department of
Agriculture (USDA, Frederick, MD) approved Biosafety Level (BSL) 3 facility, and were
approved by the Institutional Animal Care and Use Committee, and the Institutional
Biosafety Committee in accordance with the applicable federal, state, and local
regulations.

Virus, Peptides, and Cell Lines. West Nile virus strain 385-99 was a kind gift of Robert
Tesh (University of Texas Medical Branch, Galveston, TX). Synthetic peptides were
purchased at >95% purity from 21st Century Biochemicals (Marlboro, MA), diluted in
10% H_2O/90% DMSO, stored at -80^0 C and subsequently used at indicated
concentrations. Virus was grown in mycoplasma-negative Vero cells, cultured under
aseptic conditions as described previously.

Intracellular cytokine and surface flow cytofluorometric (FCM) staining. Cytokine-
producing T cells were detected using the Cytofix-Cytoperm Kit (BD PharMingen, San
Diego, CA), as described. Single-cell splenocyte suspension was depleted of red blood
cells and was incubated with 1μM peptide or infected with WNV in the presence of
5μg/ml Brefeldin A (Sigma Aldrich) for 6 h at 37 °C. After six hours the cells were
washed and blocked with Fc block (anti–mouse FcγRI/III; BD PharMingen) and

incubated overnight in the presence of a saturating dose of surface antibodies against CD8, CD3, CD4, CD11a, CD43 (Clone 1B11), CD44, CD27, Ly6c, and CD62L (BD-PharMingen). After washing, the cells were fixed, permeabilized and intracellular antibodies (anti-IFN-γ; BD-PharMingen) added for 30 minutes. The samples were then washed and analyzed using a LSR II cytometer (Becton Dickinson Immunocytometry Systems, Sunnyvalle, CA) instrument. Where indicated, pMHC tetramers, conjugated to APC (NIH Tetramer Facility, Atlanta, GA) were added to unstimulated cells in conjunction with other markers for surface staining. FCM analysis was performed by collecting a minimum of 5×10^4 events and gates set on lymphocyte population based on forward and orthogonal light scatter, followed by marker positioning to denote fluorescence greater than that of control stained or unstained cells.

Infection. Mice were immunized subcutaneously (s.c.) between the shoulder blades with 200-800 pfu WNV. Seven days or as indicated in the text, splenocytes were isolated and subjected to FCM, or ICCS assay analysis as described above.

Chapter Seven

Prevention and therapy of West Nile virus disease in vulnerable populations

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Abstract

    The potential for the spread of emerging infectious diseases became apparent
following the introduction of West Nile virus into the United States during the summer of
1999. West Nile virus has served as a global warning sign of the potential dangers of
more severe viral infections spreading outside of there current geographic locations and
of the need for treatments for these diseases. Currently there is no WNV therapy or
vaccine approved for use in humans. Here we describe the initial testing of a novel
vaccine platform, called a pseudo-infectious virus (PIV), and a novel therapeutic
treatment, consisting of the *in vivo* use of a small molecule inhibitor of WNV. Both the
vaccine and the small molecular inhibitor are designed to limit the development of West
Nile virus disease. We show here that vaccination of mice with a pseudo-infectious virus
induces a robust and long standing T cell response, similar to primary WNV infection.
The vaccine also induces a robust T cell response in B cell deficient mice (μMT-/-). We
also show that the small molecule inhibitor from a cyclic sulfonamide family, TYT-1, can
limit the development of severe West Nile virus disease *in vivo*. One benefit of these
approaches is the high level of safety and the strong potential for efficacy in vulnerable
populations, suggesting that these two could be used in tandem in the most vulnerable
populations.

West Nile virus (WNV) was introduced into the United States during the summer of 1999. By 2005 the virus had spread to every state of the US except Alaska and Hawaii, going from an isolated outbreak to a countrywide epidemic, and documenting the potential for the rapid spread of an emerging infectious diseases. As WNV rapidly spread, vulnerable subsets of the human population were identified, including the elderly, and immunocompromised. Developing safe and effective vaccines to protect these susceptible groups has become a major priority in WNV research.

There is currently no approved human vaccine or therapeutic designed specifically to protect against WNV disease, which is urgently needed to protect vulnerable populations. WNV is a member of the *Flaviviradae* family, within the *Flavivirus* genus, which contains approximately 40 viruses that are capable of causing human disease. To date, vaccines are available for only three flaviviruses, Yellow Fever virus (YF), Japanese Encephalitis virus (JEV) and Tick-borne Encephalitis virus (TBE). The TBE and JEV vaccines are inactivated virus vaccines (INV), while the YF vaccine is a live attenuated vaccine (LAV). Both the TBE and JEV vaccines have a high incidence of mild side effects, such as headache or rash, but a low incidence of potentially life threatening side effects (390-392). However, the LAV for YF does have the potential to cause disease in the immunosuppressed (393) or the elderly (394). Unlike many other commonly used INVs, the TBE and JEV vaccines have limited potency and require three vaccinations and repeated boosters to be effective at preventing disease (395). By contrast, the LAV for YF only requires one immunization to prevent disease (396).

The incidence of WNV infection is fairly uniform with age (230) and in most immunocompetent humans the disease is asymptomatic (239, 349). However severe WNV disease, which includes the involvement of the nervous system (meningitis and encephalitis) disproportionally afflicts the elderly with a lethality of 10% and a mean age at death of 78 years (99, 230). Persons between 50-59 years of age have a 10 times higher incidence of severe disease while persons aged 80 years or greater have a 43 times higher incidence of severe disease compared to the adults between 20-40 years of age (99, 100). Moreover there is an increase in morbidity within the elderly population with many elderly patients suffering from WNV encephalitis requiring several years to fully recover. The overall mortality in the first year post infection is also significantly increased compared to age-matched controls (99).

WNV contains a single positive-sense ~11 kb RNA genome that is translated into a polyprotein that is post-translationally cleaved into three structural envelope (E), pre/membrane (prM) and capsid (C)] and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. These ten multifunctional proteins play a role in invasion, entry, viral replication, assembly, and modulation of host cell functions, including the immune response (rev. in (183, 315)). The WNV virion consists of three structural proteins, C, E and M, which package a single RNA copy of the WNV genome. Many of the antigenic determinants that induce the immune response to WNV are found within E and are the focus for the development of many potential vaccines.

The ability to intelligently design vaccines relies on the development of reverse genetics systems and on increased understanding of the correlates of protection. One vaccine that takes advantage of these developments is the pseudo-infectious virus (PIV)

(397), based upon the reverse genetic system to develop an infectious particle and our understanding of protective immune responses to flaviviruses to promote the expression of antigenic determinants that drive a protective immune response. The WNV PIV consisting of a nucleocapsid wrapped WNV RNA genome that lacks the coding region for the capsid protein, surrounded by a lipid layer (397).

Upon exposure of a permissive cell to a PIV particle, viral binding and entry occur just like for the wt virus, and the viral genome is then translated, generating 9 out of the 10 WNV proteins. While the lack of the capsid protein prevents production of infectious virus, the presence of the E and M proteins affords generation and secretion of sub-viral particles (SVP) (398: Mason, 2006 #555). These particles are smaller but antigenically indistinguishable from viral particles and therefore stimulate a similar antibody response to what is seen during WNV infection (397). The WNV PIV's have been shown to be effective vaccines in outbred swiss Webster mice, but the relative contribution of the humoral and cellular immune response to vaccine-mediated protection has not yet been determined (397).

In the present work we examined the effector and memory T cell response to a WNV PIV in C57BL/6 mice. A specific goal of these studies was to test whether the cardinal characteristics of this potential WNV vaccine – safety due to the inability to replicate, and the expression of multiple WNV antigens that should drive a strong protective immune response (397) – are sufficient to allow its use and to afford the efficacy in immunocompromised, vulnerable segments of the population.

We documented the ability of a PIV to safely induce a T cell response in two models of vulnerable populations, B-cell deficient mice, and old mice. Despite this

immunogenicity, PIV was unable to protect B–cell deficient mice from high dose viral challenge, indicating protection against high dose viral challenge requires participation of Ab. However, WNV PIV was able to protect another susceptible animal population, the old mice (J.D.Brien & J.N-Z., submitted), from high dose viral challenge. We conclude that PIV generates a strongly protective immune response in some, but not all, vulnerable populations, and that protective effects of a given vaccine need to be tested in a variety of populations with suboptimal immunity in order to devise optimal vaccination strategies for each of them.

In addition to the development of new vaccines to prevent severe WNV disease, the ability to use a small molecule inhibitor against WNV would also be a benefit, for two reasons. Currently there is no vaccine present to protect against WNV infection and therefore there is no clear strategy for developing an effective vaccine. The cost and the ability to rapidly increase production of small molecule inhibitors make them an attractive alternative to viral base vaccines. Several small molecular inhibitors have been shown to reduce the viral titer of WNV and other flavivirus *in vitro* (399, 400), but to our knowledge this is the first indication that these types of molecules work against WNV *in vivo,* and would therefore be a viable alternative to virus-based vaccines.

In the present work we describe the WNV-specific effector and memory T cell response to a WNV PIV. We documented the ability of a PIV to safely induce a T cell response, in two models of vulnerable populations, B cell deficient mice (μMT) and old mice. We also demonstrated the ability of TYT-1, a cyclic sulfonamide, to protect WNV infected C57BL/6 mice. This work highlights the importance of developing and testing multiple approaches to target at risk populations. Vaccines that stimulate a potent and

protective immune response in one group may be ineffective or deleterious in another

group. By testing multiple approaches we can target the vaccine to protect different

groups within the population, which may be poorly served by a general vaccine, aimed at

fully immunocompetent subjects.

We first chose to evaluate the quantity and quality of the T cell response to PIV in comparison to WNV infection. Second, we wanted to assess the safety of PIV vaccines in vulnerable populations and measure the WNV-specific T cell response within these vulnerable populations. Third, we wanted to determine contribution of protection derived from the humoral response versus the cellular response in adult B6 mice. Lastly we wanted to determine if there was a change in the correlates of protection within a vulnerable population. These studies are still ongoing and the data presented here will address only the first two goals of this continuing study.

Generation of antigen-specific T cells during PIV vaccination

We first sought to determine whether a WNV PIV vaccine could generate a T cell response. There were two reasons why PIV should generate a strong T cell response: (i) PIV has the ability to generate large quantities of viral proteins within a cell (209, 397), providing a substrate for generation of antigenic determinants; and (ii) the introduction of viral RNA into the cell stimulates cellular anti-viral defenses (209), which should increase antigen processing and presentation. We immunized mice with decreasing doses of WNV PIV ($1x10^5$-$1x10^2$ focus forming units -ffu) intraperitoneally (i.p.) and compared the induction of T cell responses to those induced by 50pfu of WNV i.p. on day 7 post immunization (peak of T-cell responses). To compare the antigen-specific CD8 T cell responses we measured the percentage of antigen-specific CD8 T cells responding to the immunodominant NS4b WNV epitope in B6 mice, using the $NS4b_{2488:}D^b$ tetramers, (278, 279). $1x10^5$ ffu PIV induced T-cell response comparable to

the one induced by 50 pfu WNV in percentage and number of antigen-specific CD8 T

cells 7 days post immunization (**Figure 1A and data not shown**).   Moreover, the

percentage of NS4b$_{2488}$-specific CD8 T cells was proportional to the dose of injected PIV.

We next evaluated the percentage of responding CD8 and CD4 T cells 7 days post

immunization by measuring IFNγ production in an intracellular cytokine staining assay

(ICCS) (**Figure 1B**). Here we saw that the ICCS gave similar results to the tetramer

staining, where immunization with $1x10^5$ffu of PIV generated similar percentages of

NS4b$_{2488}$ specific CD8 T cells compared to WNV infection, and that decreasing doses of

PIV generated decreasing percentages of antigen-specific CD8 T cells. We obtained

similar results with CD4 T cells, except that larger percentages and numbers of CD4 T

cells apparently were induced by $1x10^5$ffu PIV compared to WNV infection (Figure 1B).

However, that trend failed to reach statistical significance. PIV vaccination also

generated CD8 T cells that respond to several other viral determinants, including Env$_{347}$

and all other previously identified CD8 and CD4 T cell epitopes (data not shown) ((401)

and manuscript in preparation).   This illustrates the ability of the PIV vaccine to generate

a broadly specific T cell response.

We next evaluated the maintenance of this antigen-specific response during the

memory phase (**Figure 1C**) in C57BL/6 mice. We used ICCS to determine the

percentage of CD8 and CD4 T cells that would recognize viral determinants and produce

IFNγ when stimulated with peptide antigen 50 days post immunization. Both antigen-

specific CD8 and CD4 T cells were present at similar percentages as compared to the

WNV infection at a comparable time point (data not shown).
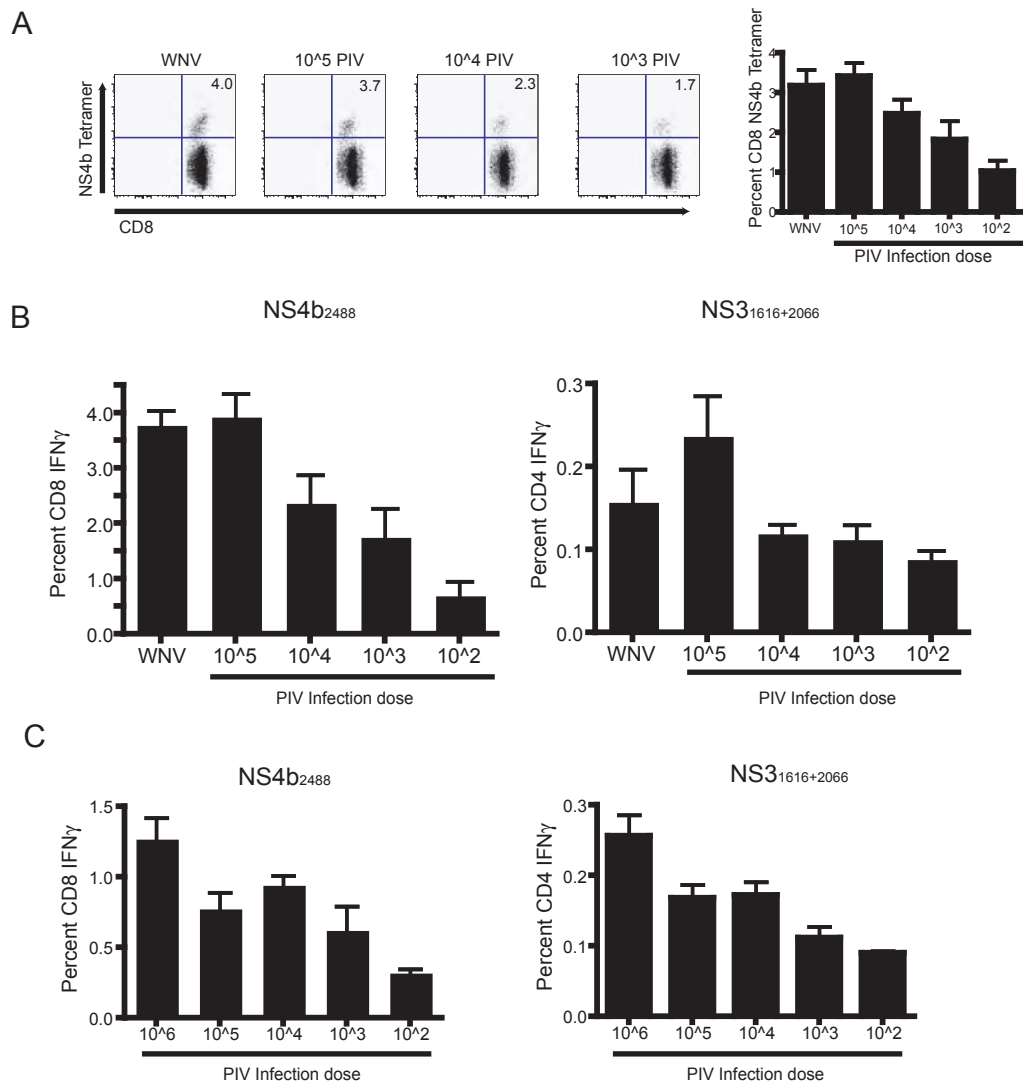
Figure 1. Enumeration of antigen-specific T cells in response to PIV vaccination and WNV infection. **A.** CD8 NS4b$_{2488}$ tetramer^{+} T cells 7 days post infection from mice given decreasing doses of PIV. Left panel- Representative example of CD8 NS4b$_{2488}$ tetramer[+] T cell staining 7 days post infection, completed by FCM. Panels depict CD8 and tetramer staining in a representative mouse from one out of two experiments, where a total of 36 animals were analyzed with comparable results. Right panel- Enumeration of CD8 NS4b$_{2488}$ tetramer[+] staining showing decreasing doses of PIV mice. CD8 Tetramer staining was collected by FCM, by gating on CD8. Results represent 4 mice per dose, and are expressed as x+/- SEM, from one out of two comparable experiments. **B.** Left panel-Enumeration of day 7 CD8 T cells by IFNγ ICCS, showing decreasing doses of PIV infected mice. CD8 T cell ICCS stimulated with $1x10^{-6}$M NS4b$_{2488}$ peptide for 6 hours in BFA. N=4 mice per dose, x+/- SEM. Right panel- Enumeration of day 7 CD4 T cells by IFNγ ICCS, showing decreasing doses of PIV infected mice. CD4 T cell ICCS stimulated with $1x10^{-6}$M NS3$_{1616+2066}$ peptide for 6 hours in BFA. N=4 mice per dose, x+/- SEM. One representative example of two experiments. **C.** Left panel-Enumeration of day 50 CD8 T cells by IFNγ ICCS, showing decreasing doses of PIV infected mice. CD8 T cell ICCS was performed as in B. N=4 mice per dose, x+/- SEM. Right panel-Enumeration of day 50 CD4 T cells by IFN ICCS, showing decreasing doses of PIV infected mice. CD4 T cell ICCS was done as above, on 4 mice per dose. Results are shown as x+/- SEM, and are from one experiment of two with similar results.

The data presented here indicates that PIV vaccine can generate a robust T cell response, quantitatively similar to that seen during a virus infection, and that these T cells survive to become memory T cells. This data also indicates that PIV vaccination allows the processing and presentation of both structural and non-structural proteins by both major histocompatibility complex-encoded (MHC) class I and II molecules, unlike an inactivated vaccine or a Virus like particle, which cannot express non-structural proteins.

Functional Capabilities of PIV generated antigen-specific CD8 T cells

We next compared the functional ability to respond to decreasing concentrations of peptide in CD8 T cells generated by PIV immunization or WNV infection (**Figure 2A, left panel**). In order to look at the functional avidity of antigen-specific T cells 7 days post infection, we expressed the results as a percentage of maximum of CD8 T cell IFNγ production obtained in the presence of saturating concentrations of NS4b$_{2488}$ (10^{-6}M). Results obtained indicate that PIV vaccination generates CD8 T cells of equal functional avidity to that generated within the context of a primary WNV infection (**Figure 2A, right panel**). This was observed across a wide range of PIV doses, suggesting that the effect is largely PIV-independent.

This prompted us to examine the phenotype of the responding CD8 T cell population by flow cytometry (FCM). Using this technique we saw that both WNV infection and PIV vaccination generated Granzyme b (GrB)$^{+}$, CD11a^{hi} CD127^{low} CD8 T cells (**Figure 2B**), the phenotype that corresponds to fully mature effector CD8 T-cells (324, 379). Due to the robust direct ex-vivo GrB expression, we next evaluated the cytotoxic potential of PIV-generated CD8 T lymphocytes 7 days post infection in a direct

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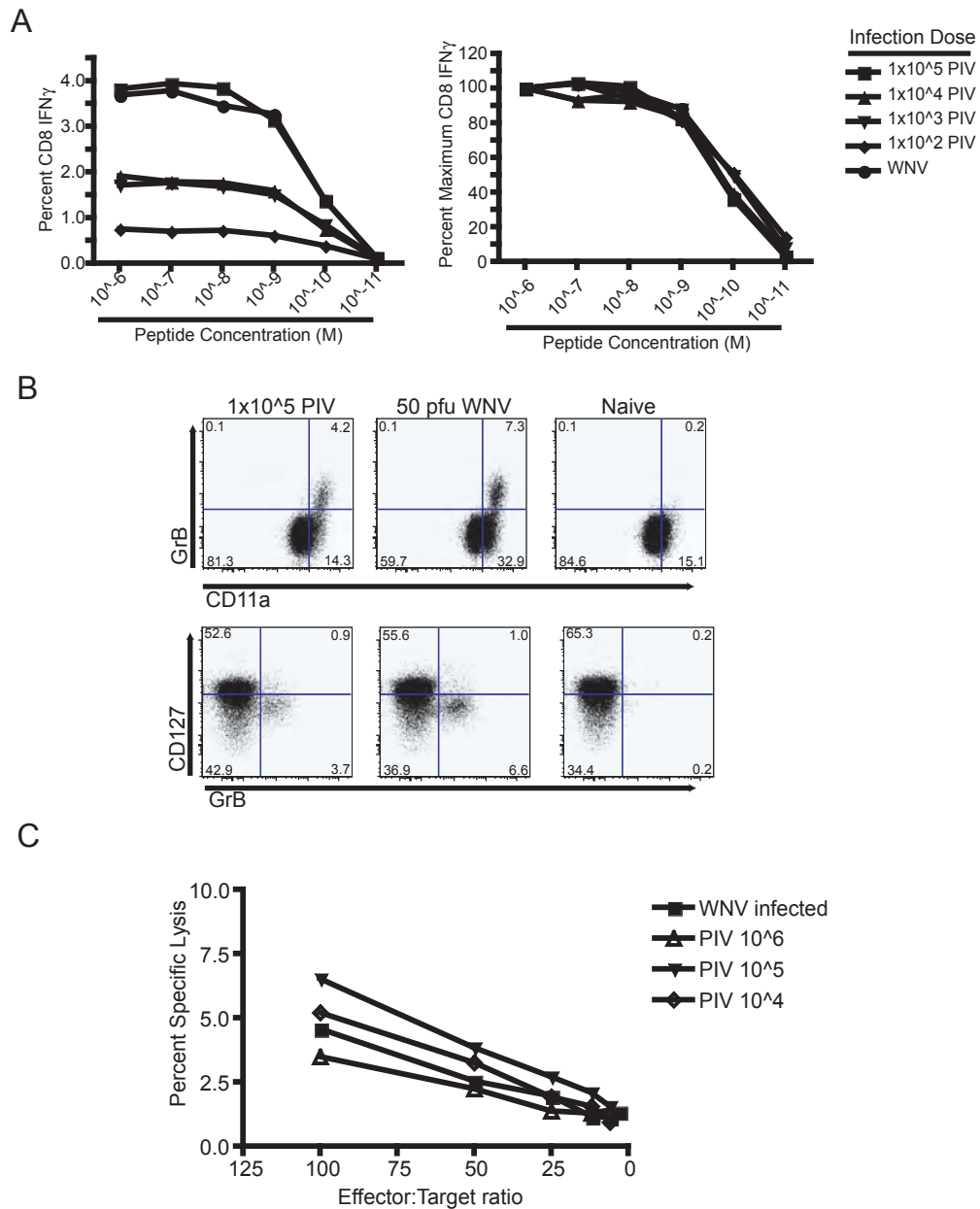
Figure 2. Comparison of T cell effector (day 7) function from WNV infected and PIV vaccinated mice. A. Right panel-Enumeration of day 7 CD8 IFNγ^+ T cells by ICCS, showing decreasing doses of NS4b$_{2488}$ peptide. CD8 T cell ICCS stimulated with $1 \times 10^{-6--11}$ M NS4b$_{2488}$ peptide for 6 hours in BFA. N=4 mice per dose of PIV. Left panel- CD8 T cell avidity curve with results expressed as a percentage of maximum of CD8 T cell IFNγ production with saturating peptide concentrations of NS4b$_{2488}$ 1×10^{-6}M peptide N=4 mice per dose of PIV. One representative example of two experiments. **B.** Representative example of direct ex-vivo immunophenotyping of day 7 splenocytes, from PIV vaccinated, WNV infected or naïve mice. Dot plots are gated CD8. Staining was completed as described within the methods. One representative mouse of four. One representative experiment of two. **C.** Day 7 CD8 T cell direct ex-vivo 6-hour ^{51}Cr-release assay with NS4b peptide coated EL-4 T cells and control cells. N=4,5 mice per group. One representative example of two experiments.

ex-vivo ^{51}Cr release assay. Again, PIV vaccination was largely indistinguishable from WNV infection in that it generated CD8 T cells that were capable of recognizing and lysing peptide coated EL-4 target cells with similar efficiency (**Figure 2C**).

<u>CD8 and CD4 antigen-specific T cell response in PIV vaccinated μMT mice.</u>

To determine whether the T cell response in isolation is sufficient to provide protection against high dose WNV challenge after PIV vaccination we immunized B cell deficient mice (μMT-/-) with $1x10^5$ffu ip PIV vaccine or with 50pfu of WNV ip. Using ICCS we evaluated the CD8 and CD4 T cell IFNγ response 7 days post immunization (**Figure 3A**). Both PIV and WNV generate equivalent percentages of CD8 T cells specific for NS4b$_{2488}$ epitope in both strains of mice. Interestingly, the percentages of antigen-specific CD4 T cells were the same in both μMT-/- mice and B6 controls. As was seen in Figure 1B, PIV induced a significantly (p<0.02) higher percentage of antigen-specific CD4 T cells in B6 mice cells than virus infection (**Figure 3A right panel**).   An increase was seen in μMT-/- mice given PIV versus WNV, however the difference was not significant.

The vaccination of μMT-/- mice also allowed us to further evaluate the safety of PIV vaccination, since it is known that μMT-/- mice are exquisitely sensitive to WNV infection (217, 402). If we infect μMT-/- with 10pfu of WNV, all of these mice succumb to infection, whereas 100% of the mice vaccinated with PIV survive (**Figure 3B**). This further demonstrates the safety of PIV vaccination initially shown by Mason et al (397). This data shows that PIV vaccination can generate a robust CD8 and CD4 T cell effector response in B cell deficient mice early after vaccination.

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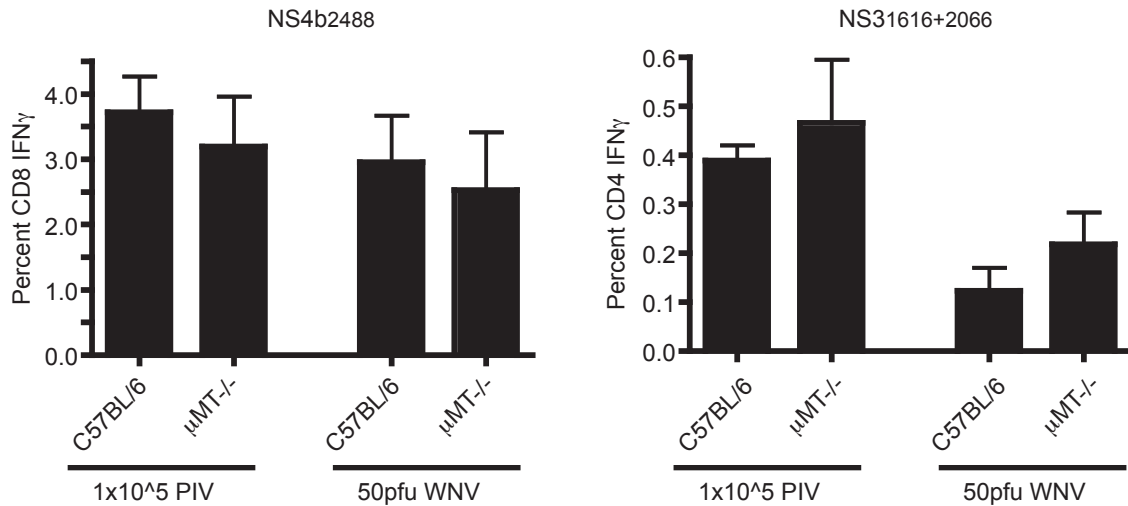
B
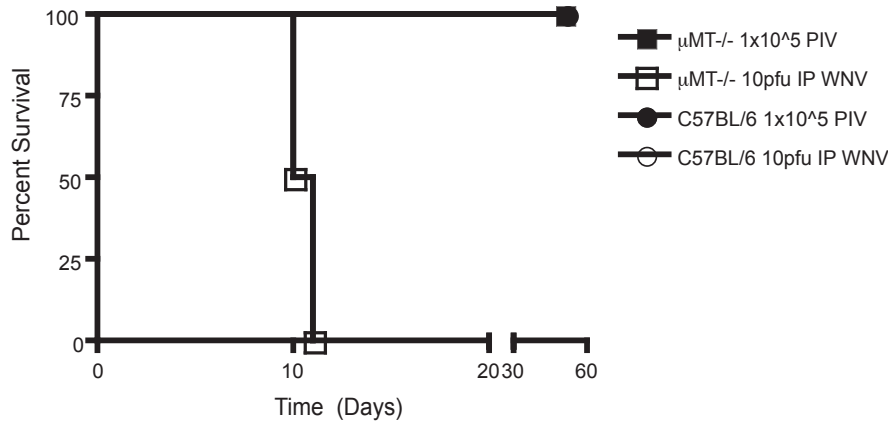
Figure 3. Antigen-specific T cell response to PIV and WNV in μMT mice. A. Left panel- Enumeration of day 7 CD8 T cells from μMT and B6 mice immunized with either PIV or WNV by IFNγ ICCS. CD8 T cell ICCS stimulated with $1x10^{-6}$M NS4b$_{2488}$ peptide for 6 hours in BFA. N=5 mice per dose, x+/- SEM. Right panel- Enumeration of day 7 CD4 T cells from μMT and B6 mice immunized with either PIV or WNV by IFNγ ICCS. CD4 T cell ICCS stimulated with $1x10^{-6}$M NS3$_{1616+2066}$ peptide for 6 hours in BFA. N=5 mice per dose, x+/- SEM. One representative example of two experiments. **B.** PIV vaccination ($1x10^{5}$ffu) and WNV infection(10pfu ip) of μMT-/- and control C57BL/6 mice. μMT-/- are highly sensitive to WNV infection (μMT-/- PIV vs μMT-/- WNV p<0.008).

Phenotype and antigen-specific T cell response in old and adult mice 7 days post PIV vaccination.

We have previously shown that old mice are more susceptible to severe WNV disease than adult mice (manuscript in preparation). We therefore initiated experiments to test PIV vaccine efficacy in this vulnerable population. In order to evaluate if PIV vaccination can generate an antigen-specific response in aged mice, we used FCM to evaluate the phenotype of CD8 T cells 7 days after vaccination with $1x10^5$ ffu PIV vaccine (**Figure 4A**). We found that PIV vaccination generated a larger CD8 CD62L^{low} GrB$^+$ population in adult animals than in old animals. One potential reason for this reduced responsiveness is highlighted by the differences seen in CD44 and CD62L expression levels on CD8 T cells. Old mice clearly have a larger pre-existing memory T cell compartment (CD44^{low} CD62L^{low}). This increased memory population and corresponding decrease in the naïve precursor pool thus may explain diminished immune responsiveness to vaccination seen within old mice.

To further characterize this response of old animals to PIV vaccine, we measured the percentage of IFNγ antigen-specific CD8 and CD4 T cells generated in old mice (**Figure 4B**). Similar to the GrB expression data we saw a significant decrease in the percentage of CD8 T cells ($p<0.01$) and CD4 T cells ($p<0.01$) in old mice that are able to produce IFNγ after a 6-hour peptide stimulation in the presence of BFA. Vaccination of old mice with PIV did not produce a robust antigen-specific T cell response during the acute phase of infection in old mice (**Figure 4A/B**) as compared to adult counterpart. This is reminiscent of profound age-related defects in T-cell immunity seen in other

A.
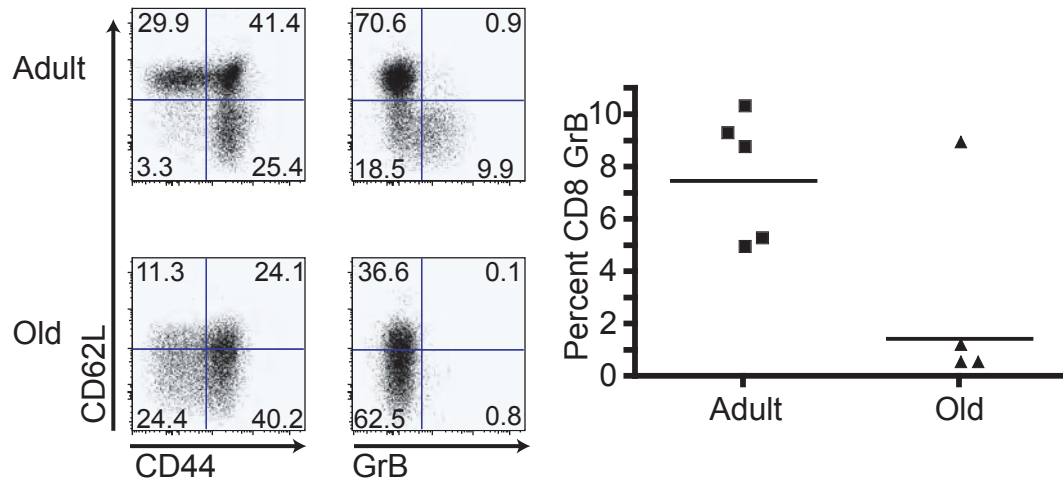
B.
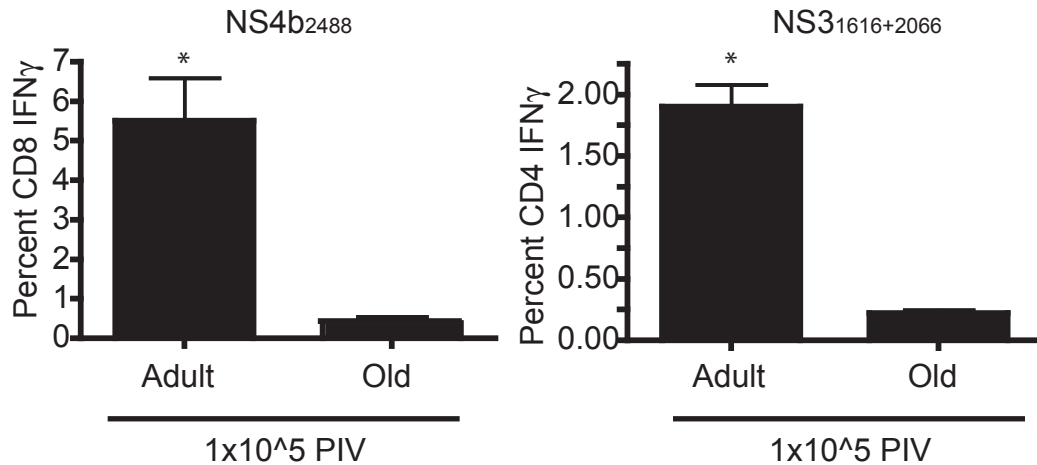
Figure 4. Phenotype and antigen-specific T cell response in old and adult mice 7 days post PIV vaccination. A. Left panel- Representative example of day 7 direct ex-vivo immunophenotyping of old and adult mice vaccinated with $1x10^5$ ffu of PIV. Gated on CD8. Right panel- Enumeration of day 7 direct ex-vivo GrB staining of CD8 T cells of old and adult mice vaccinated with $1x10^5$ ffu of PIV. N=4,5, bar=mean, One representative example of two experiments. **B.** Left panel-Enumeration of day 7 CD8 T cells from old and adult mice immunized with $1x10^5$ ffu of PIV by IFNγ ICCS. CD8 T cell ICCS stimulated with $1x10^{-6}$M NS4b$_{2488}$ peptide for 6 hours in BFA. N=5 mice per dose, x+/- SEM. Right panel-Enumeration of day 7 CD4 T cells from old and adult mice immunized with $1x10^5$ ffu of PIV by IFNγ ICCS. CD4 T cell ICCS stimulated with $1x10^{-6}$M NS3$_{1616+2066}$ peptide for 6 hours in BFA. N=5 mice per dose, x+/- SEM One representative example of two experiments.

models of infectious diseases (Messaoudi et al., JEM 2004) including those seen during primary infection with WNV (Brien et al., in preparation).

<u>Protective Capacity of PIV vaccine</u>

As a corollary to the above phenotypic and functional studies, we tested whether PIV vaccination and challenge of µMT-/- mice would generate a strong and protective recall response. µMT-/- and C57BL/6 mice were sacrificed 6 days post challenge with 2000 pfu per mouse of WNV and CD8 and CD4 T cell cytokine production was monitored by ICCS. PIV vaccination resulted in >3x increase in antigen-specific CD8 T cell frequencies compared to controls, indicative of a recall response (**Figure 5A and data not shown**). Although both µMT-/- and C57BL/6 mice exhibited recall responses, only C57BL/6 mice survived this high-dose infection (**Figure 5B**). These experiments indicate that PIV vaccination of µMT-/- mice does generate a memory T cell response, which is able to respond to a viral infection, but that T cells by themselves can only protect against low dose **(Fig. 3b)** and not high dose **(Fig. 5b)** viral challenge.

PIV vaccination was also tested for the ability to protect old mice against WNV challenge. Although vaccination of old mice did not generate a robust T cell response we wondered if the immunity provided by the vaccination was sufficient to protect old mice from a high dose WNV challenge. Old and adult, vaccinated and control C57BL/6 mice were sacrificed 6 days post challenge with 2000 pfu per mouse of WNV and CD8 and CD4 T cell cytokine production was monitored by ICCS. CD8 T cell cytokine production indicated a recall response in PIV-vaccinated the old mice, but there was no increased T cell response within the PIV vaccinated adult mice (**Figure 6A and data not**
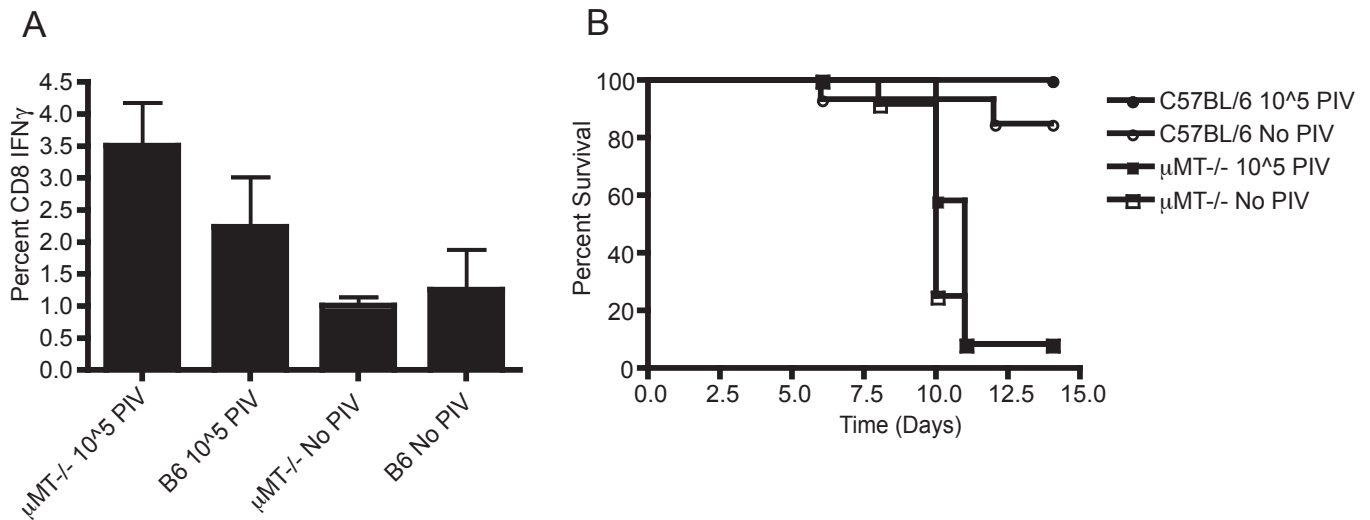
Figure 5. CD8 T cell response and animal survival after high dose WNV challenge of μ MT-/- mice. A. Enumeration of antigen specific CD8 T cells by IFNγ ICCS, 6 days post viral challenge, showing increased T cell responses in PIV vaccinated mice. CD8 T cells were stimulated with $1x10^{-6}$M NS4b$_{2488}$ peptide for 6 hours in BFA. N=3 mice per dose, x+/- SEM. **B.** Survival of PIV vaccinated and control μ MT-/- and C57BL/6 mice following challenge with 2000pfu of WNV 385-99 i.p.. μ MT-/- mice were not protected by PIV vaccination. C57BL/6+PIV mice survived significantly (p <0.0001) better than μ MT-/-+PIV. One experiment out of one experiment is shown.
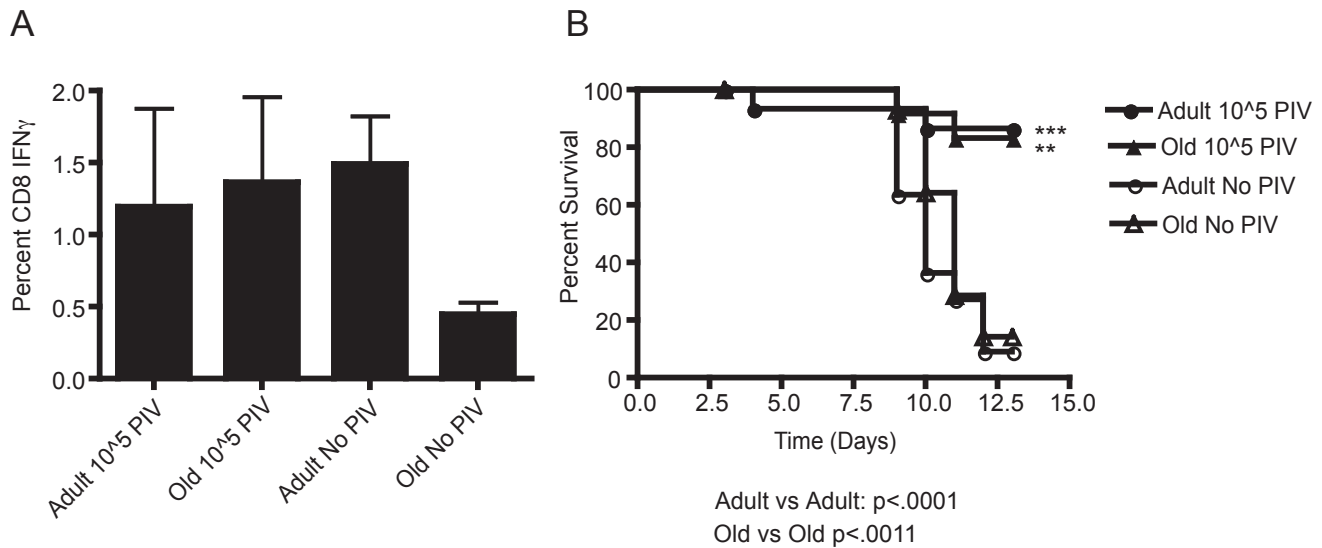
Figure 6. CD8 T cell response and animal survival after high dose WNV challenge of old and adult C57BL/6 mice. A. Enumeration of antigen specific CD8 T cells by IFNγ ICCS, 6 days post viral challenge, showing increased T cell responses in PIV vaccinated mice. CD8 T cells were stimulated with 1×10^{-6}M NS4b$_{248\ 8}$ peptide for 6 hours in BFA. N=3 mice per dose, x+/- SEM. **B.** Survival of PIV vaccinated old and adult mice following challenge with 2000pfu of WNV 385-99 i.p.. PIV vaccinated old and adult mice were protected from high dose WNV challenge. C57BL/6+PIV mice survived significantly (adult PIV vs ctrl p <0.0001, old PIV vs ctrl p <0.0011) better than unvaccinated mice. One experiment out of one experiment is shown.

shown). It is possible that a strong antibody response limited secondary infection in primed adult mice, leading to diminished T cell activation during challenge. Most importantly, PIV vaccination significantly (adult PIV vs ctrl p<0.0001, old PIV vs ctrl p<0.0011) protect both old and adult mice from a high dose infection with WNV (**Figure 6B**), highlighting potential utility of this vaccine in protecting this vulnerable population.

Results-Treatments

Protective capacity of TYT-1, a Sultam thiourea compound, during WNV infection

  Since vaccine development to protect against WNV disease is in its infancy, small molecule inhibitors could provide a welcome alternative. Several laboratories have identified small molecular inhibitors of WNV *in vitro*, including the identification of TYT-1 by Barklis et al. (403). Here we present data showing the use of this compound to protect mice from a lethal dose of WNV (**Figure 7A**). In this experiment mice were infected with 300 pfu of WNV subcutaneously, and given 25 μl of TYT-1 (1mg/ml) or 25 μl of DMSO ip daily for the next 7 days. Seven days post infection mice were bled and percentage of antigen-specific CD8 T cells were determined using NS4b$_{2488}$ tetramer. Mice were then monitored for 60 days for survival. Mice that received TYT-1 had a significantly (p<0.05) greater level of survival than DMSO control (**Figure 7A**). The TYT-1 treated and untreated mice do develop an antigen-specific T cell response, where the DMSO treated group does not. With only one time point and one experiment completed it is difficult to determine the validity of this trend.
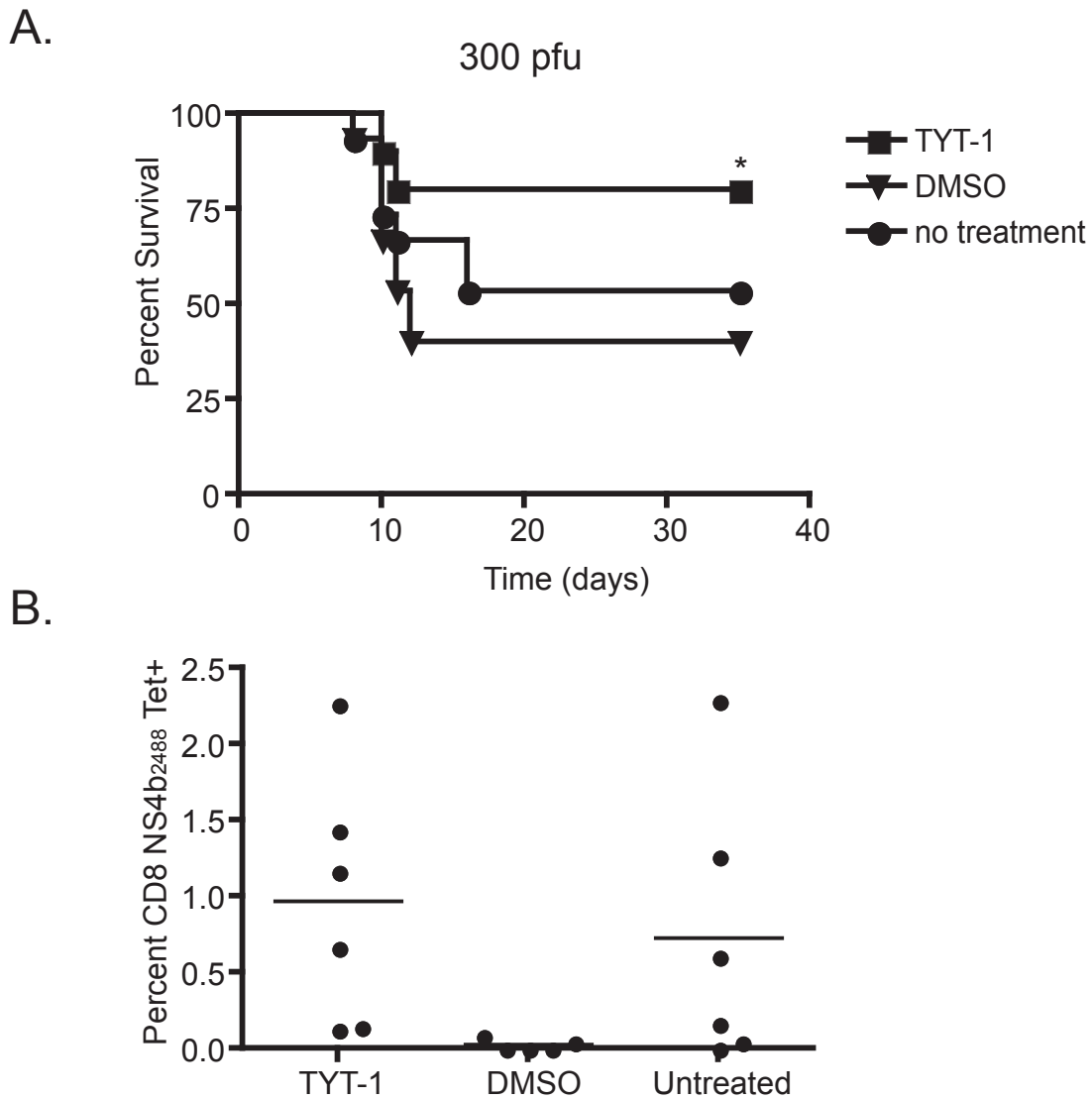
Figure 7. Treatment of C57BL/6 mice with TYT-1, a Sultam thiourea compound. A. Significant (p< 0.05) survival of C57BL/6 mice treated with TYT-1 vs DMSO carrier control when mice were infected with 300pfu of WNV. Mice were treated everyday for 7 days with 25μl of TYT-1 (1mg/ml) or DMSO intraperitoneally. One representative experiment of 2, N=10-15 mice per group, statistical significance was determined using the log-rank test. **B.** Enumeration of day 7 CD8 $NS4b_{2488}$ tetramer[+] from PBMCs. CD8 Tetramer staining was collected by FCM, by gating on CD8. N=6 mice per dose, x+/- mean. Only experiment completed is shown.

Discussion

This chapter discusses the initial testing of a vaccine and a therapeutic treatment against severe WNV disease, including the first steps in implementing a novel vaccine platform, that has so far proven to be safe, easy to generate and effective in vivo (397, 404). In this study, we initially compared the quantity and quality of the acute (day 7) and the memory (day 50) T cell response of the vaccine in B6 mice to the T cell responses that develop during WNV infection. When mice are given $1x10^5$-$1x10^4$ffu, they develop a robust CD8 CTL response capable of cytotoxicity and cytokine production. At the same time those mice also develop a CD4 T cell response that is capable of producing IFNγ, indicating that PIV- and WNV-generated T cells exhibit the same level of effector functions. The phenotypic characteristics of PIV and WNV generated effector cells are identical. Given that both PIV and WNV elicit similar T cell responses we next sought to evaluate this vaccine platform in B cell deficient mice (μMT) and in old C57BL/6 mice, which are more susceptible to WNE.

Many laboratories have designed vaccines that have been successful in protecting immunocompetent mice (297, 299, 300, 385-387). However no study to date has looked at which immune mechanisms are necessary and sufficient to protect during a WNV challenge after vaccination. One group mutated a JEV DNA vaccine candidate to include or exclude a T cell epitope to determine whether protection was best provided by a combination of neutralizing antibody and T cells (341). It is now clear that antibody, specifically immune serum, is not sufficient to provide protection against WNE, since transfer of immune serum to Rag1-/- mice still ends in 100% lethality (216, 217). If immune serum is transferred to μMT-/- mice, prior to primary infection, they do not

develop WNV disease, indicating that preexisting neutralizing antibody in conjunction with primary T cell response is sufficient to provide protection (216). Finally our laboratory has shown that previously primed CD8 T cells are capable of protecting Rag1-/- mice independent of antibody, but this challenge was completed at a low viral dose (279). Understanding what immune mediated mechanisms are necessary for protection will be crucial for effective vaccine design.

It will be important to determine if a memory T cell response is maintained in the μMT-/- mice and if they are capable of with standing a viral challenge. The same is true for the old mice that were PIV-vaccinated. Although the old mice had a diminished response, we do not currently know whether that response may be sufficient to provide protection. In the event that PIV-based vaccines are unable to fully protect vulnerable populations, there is still the option to use small molecular inhibitors of WNV, such as TYT-1, to alleviate or eliminate WNE in a vaccinated but vulnerable population.

Materials and Methods

Mice: Old (18-22 month) and adult (4-6 months old) C57BL/6 (B6) mice were purchased from the National Institute of Aging breeding colony (Harlan). C57BL/6, C57BL/6 μMt-/- mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and bred at the Oregon Health & Science University West Campus vivarium. IFNα/β receptor-/- mice were a kind gift of Dr. Ann Hill (OHSU). All animals were housed and bred under specific pathogen-free conditions at the OHSU and experiments conducted under the Institutional Animal Care and Use Committee, and the Institutional Biosafety Committee approvals in accordance with all applicable federal, state, and local regulations. All West Nile virus experiments were completed within a United States Department of Agriculture (USDA) inspected Biosafety Level three facility.

Virus, Peptides, and Cell Lines: West Nile virus strain 385-99 was used. West Nile virus strains 385-99 was a kind gift of Robert Tesh, MD, (University of Texas Medical Branch). Peptides NS3 $_{1616}$, NS3 $_{2066}$, NS4b $_{2488-2496}$ were purchased from 21st Century Biochemicals, diluted in 10% H_2O, 90% DMSO and stored at -80deg C. Vero, MC57g and lines were mycoplasma negative, and were cultured under aseptic conditions DMEM (VERO and MC57g) or RPMI (EL-4) supplemented with antibiotics and 5% fetal calf serum. MC57g's were infected using an MOI of 10 for 30 hours prior to use for ^{51}Cr assays.

Flow cytofluorometric (FCM) analysis and Intracellular cytokine staining (ICS): Cytokine-producing T cells were detected using the Cytofix-Cytoperm Kit (BD

PharMingen, San Diego, CA), as described. Single-cell splenocyte suspension was depleted of red blood cells and was incubated with 1μM peptide or infected with WNV in the presence of 5μg/ml Brefeldin A (Sigma Aldrich) for 6 h at 37 °C. After six hours the cells were washed and blocked with Fc block (anti–mouse FcγRI/III; BD PharMingen) and incubated overnight in the presence of a saturating dose of surface antibodies against CD8, CD3, CD4, CD11a, CD43 (Clone 1B11), CD44 and CD62L (BD-PharMingen, Ebioscience, Biolegend). After washing, the cells were fixed, permeabilized and intracellular antibodies (anti-IFN-γ, or anti- IL-2; Ebioscience) added for 30 minutes. The samples were then washed and analyzed using either a FACSCalibur or LSR II cytometer (Becton Dickinson Immunocytometry Systems) instrument.

Granzyme B (Caltag) intracellular staining was completed directly ex-vivo with no stimulation.

Infection and CTL Analysis: Mice were infected intraperitoneally (i.p.) with 0.16-20 PFU of WNV virus per mouse, as denoted in the figure legends. Mice were vaccinated intraperitoneally (i.p.) with $1x10^2$-$1x10^6$ ffu of PIV per mouse, as denoted in the figure legends Seven days after infection, lymphocytes were isolated and used for direct flow cytometry (FCM) analysis, for direct ex-vivo restimulation for cytokine production, and direct ex-vivo ^{51}Cr assay. Percent specific lysis was calculated as [(E - S)/(M - S)] times 100, where E equals the counts per minute released from targets incubated with lymphocytes, S equals the counts per minute released from target cells incubated with no lymphocytes and M equals the counts per minute released from cells after lysis with 1% Nonidet P40 (USB, Cleveland, OH).

Statistical analyses: Fisher's exact test and Log Rank test was used to analyze results

from survival experiments. Statistical significance of viral titer observed between groups

was analyzed using Mann Whitney test. All calculations were done using the Prism

(GraphPad, San Diego, CA) software.

Chapter 8:

Discussion

West Nile virus (WNV) is an emerging infectious disease that has the potential to cause devastating human disease. The elderly are at the greatest risk of developing a severe WNV infection (99). As the "baby boomer" generation begins to age a significant proportion of the United States (US) population is at risk for developing severe WNV disease. This, combined with the recent threat of a biological terrorist attack has resulted in close attention paid to the WNV epidemic. After WNV was introduced into the US in 1999 it has continued to spread (405, 406). This atmosphere drove one of the largest recent health care campaigns within the US. The health care campaign focused on documenting cases of WNV infection to determine the number of people infected in a region and the dynamic of virus spread each year (407). Based on data complied from this campaign it is now known that WNV has spread throughout the US and there have been documented cases of human infection in every state except Alaska and Hawaii. This data has also confirmed the observation that the elderly are at the highest risk for developing severe WNV disease (408).

This thesis focused on the impact of aging on the immune system, and the defects that develop within the adaptive immune system during aging which contribute to the increased susceptibility of old mice to severe disease. In Chapters 2-4 we used an adoptive transfer system to define correlates of infection that lead to the increased susceptibility of old mice to WNE. We then chose to use a VACV animal model (Chapter 5) to compare and contrast age-related defects seen in WNV- and VACV- antigen-specific CD8 T cell populations. The results provided within this dissertation thus enhance our knowledge that should aid in the development of specialized vaccines designed to achieve protective immunity within the elderly population.

<u>Protective capacity of naive T cells in adult C57BL/6 mice</u>

In the second and third chapter of this thesis, we describe the individual roles of antigen-specific CD8 and CD4 T cells during WNV infection. These results demonstrated that protection from the most severe disease requires both of these T cell subsets. Our observations are consistent with experiments completed by several different laboratories, where B6 mice were depleted of either CD4 or CD8 T cells (219, 253, 275, 278). However, it is important to discuss potential explanations as to why these cell subsets may be both necessary and sufficient, as a function of the experimental model system. We have shown that both CD4 and CD8 T cells subsets are able to protect RAG1-/- mice during low dose viral challenge and we believe that this protection is due to the functional heterogeneity of the T cells ((279) and manuscript in preparation). We define functional heterogeneity as the ability of antigen-specific CD4 and CD8 T cells to both produce IFNγ and become cytotoxic. Both CD4 and CD8 T cells can produce cytokines (Chapter 2, Figure 3, and Chapter 3, Figure 2) as well as be cytolytic (Chapter 2, Figure 3, and Chapter 3 Figure 4) after the recognition of antigenic peptides within the context of MHC II or I molecules, respectively. This functional heterogeneity allows either subset in isolation not only to control but clear WNV within RAG-/- mice, when a low dose challenge is applied. Similar mechanisms of clearance are used by each respective T cell subset for Measles virus (284, 285), YF virus (282), Influenza virus (283, 409, 410) and WNV (253, 275-280, 328, 411).

Moreover, Sparks-Thissen et al. showed that the adoptive transfer of a murine gammaherpesvirus 68 (MHV-68) specific CD4 T cell clone was able to control viral replication in RAG-/- mice (412) through the secretion of IFNγ. This result highlights the

ability of CD4 T cells to clear a viral infection by a direct cytokine effector response as opposed to providing help to other populations of responding lymphocytes. However in our system we were able to demonstrate that both the responding CD4s and CD8s were able to release lytic granules as well as cytokines in response to viral infection. This functional heterogeneity of the antigen-specific CD4 and CD8 T cells may have been sufficient to prevent WNE. Alternatively, each T cell population may use a separate approach to control viral spread, whereby the CD4s could control viral infection through the release of cytokines and CD8 control WNV infection via cytolytic activity.

To confirm the hypothesis about functional heterogeneity further experiment using the established adoptive transfer model should be completed to determine the impact of the individual effector functions of either CD4 or CD8 T cells. Purified CD8 or CD4 T cells derived from B6, IFNγ-/- and Perforin-/- mice would be adoptively transferred into the RAG1-/- recipients followed by WNV challenge. By transferring in either CD4 or CD8 T cell subsets deficient in the individual effector mechanisms we could determine whether both cell subsets take advantage of the same or different effector mechanisms to prevent WNE. If the multifunctionality is important, we would predict that loss of either effector function, IFNγ or lytic granule release, would lead to a reduction in the protection of the RAG1-/- recipient mice.

Other laboratories have taken a different approach to defining the role of these T cell populations during WNV infection. Shrestha et al. had shown that WNV-immune CD8 memory T cells clear virus from infected primary neuronal cells in tissue culture (253). This does indicate that CD8 T cells are capable of controlling WNV infection of neurons in vitro. However, because these experiments were performed in vitro in a single

cell culture system it is unclear whether memory CD8 T cells alone can clear WNV from the CNS of an infected mouse. Again, based on our observation that antigen-specific CD8 T cells can control WNV infection in RAG1-/- we would predict that adoptively transferred WNV-specific CD8 T cells could control WNV replication in the CNS. However, cytokine control of viral infection may be better suited to control viral infection in the CNS due to the potential detrimental effects of lytic granule release on neuronal cells.

Our result indicate that both CD4 and CD8 T cells independently are sufficient to provide protection to RAG1-/- mice when given a low dose WNV infection contradicts our antibody depletion findings, where in B6 mice antibody depletion of either of these subsets renders mice susceptible to WNE. We believe that the difference in results obtained between the adoptive transfer model and the antibody depletion model is related to a difference in the dose of WNV given to the mice in the separate systems. We would predict that if the RAG1-/- recipients were given a high viral dose (which was given to wt Ab-depleted mice; 10xLD100 of RAG-/- mice) neither the CD4 nor CD8 T cell subsets alone would be able to protect RAG1-/- mice. This finding is consistent with our previous observations as well as those of Shresta et al. (275), Wang et al. (325), and Sitati et al. (219) where antibody depletion of either CD8 or CD4 T cell subset rendered the B6 mice highly susceptible. In that regard, it is now known that WNV-infected mosquitoes deliver a high WNV dose (1x104-6pfu) in the course of a blood meal (413), making it highly likely that the in vivo relevant immune defense has to deal with a high viral dose and that no single lymphocyte T cell population would be sufficient to protect under these conditions.

Protective capacity of antigen-specific CD8 and CD4 memory T cells

In order to determine the protective capacity of antigen-specific CD8 T cells, using newly identified WNV encoded CD8 T cell epitopes (Chapter 2 Table 2), we transferred *in vivo* primed, *in vitro* restimulated T cells specific for individual WNV epitopes into RAG1-/- mice (Chapter 2, Figure 5). Survival of the RAG1-/- mice, which received CD8 T cells, matched the immunodominance of the T cell population they received. In similar experiments, Purtha et al conducted adoptive transfer experiments where *in vitro* expanded NS4b$_{2488}$ specific and OT-1control, CD8 T cells were transferred into B6, and found that the WNV-specific CD8 T cells protected the mice significantly better than controls (278). This group did not test other peptides and therefore could not address the role of immunodominance. Since in our experiments immunodominance hierarchy was predictive of the level of protection provided by the antigen-specific CD8 T cells, this would suggest that future vaccination experiments should be designed to elicit an immune response directed against the immunodominant epitopes.

Our finding that immunodominance matches the ability of WNV antigen-specific T cells to protect mice is however not unique to WNV and has been documented in other viral systems (414).Yet, there have also been instances where the transfer of subdominant T cells did not provide protection (415) or provided equal protection to the dominant epitope (416). There are many factors which affect immunodominance, such as antigen processing and transport, competition for antigen-presenting cells, availability of the CD8 T cell repertoire and peptide affinity for the MHC (Chapter 2 Figure 2) (reviewed in (417)). In that regard, in our experiments the affinity of the peptide for the MHC class I molecule correlated with immunodominance (Chapter 2 Figure 2)

Moreover we have shown the protective capacity of antigen-specific memory CD4 T cells, by both adoptive transfer of antigen-specific CD4 T cells or peptide immunization of B6 mice (data not shown and Chapter 3, Figure 5). Several labs have documented that *in vitro* grown WNV-specific CD4 T cells can proliferate (286, 384, 418), produce cytokine (286, 384, 418) and are cytotoxic (287), but very few groups have looked at the role of CD4 T cells *in vivo*. Sitati et al. has shown that removal of CD4 T cells by antibody depletion prior to primary infection decreases the survival of mice infected with WNV (219), illustrating the requirement for CD4 T cells during primary infection of WNV as discussed above. Our results represent the first *in vivo* documentation of antigen-specific CD4 T cells by themselves sufficient to protect adult mice from a lethal WNV challenge.

Increased susceptibility of old C57BL/6 mice to WNE

It has been observed that WNV causes increased disease severity in the elderly (99, 230). Our laboratory has had a long-standing interest in the impact of aging on the T lymphocyte response and we were interested if we could establish a WNV mouse model that would correlate with the impact of WNV infection on elderly humans. We have shown that old mice are more susceptible to WNE than adult mice (Chapter 4, Figure1/Table 1), due to increased viral titers within the CNS (Chapter 4, Figure 1).

Unlike WNV infection of mice, RSV and Influenza infection do not lead to increased mortality of old mice but old mice do take longer to clear virus from infected organs (149-151, 161). Differences in viral clearance with these other virus infections have been linked to poor T cell responses (150, 161). However, because the models of

Influenza and RSV only show a minor delay in viral clearance it is difficult to ascertain the correlates of protection within the old mice. The age-related difference in survival during WNV infection has allowed us to be in a unique position to carefully examine the causes of enhanced susceptibility of old mice to an infectious disease.

Role of type I IFN in old and adult mice infected with WNV

Old mice could be more susceptible to WNV due to defects in innate or adaptive immunity. For example, type I IFN pathway is essential for controlling WNV infection (244, 419), and an age-related defect in this innate immunity pathway could have an impact on the increased susceptibility of old mice to WNE. By comparing the mean survival times of type I interferon α/β receptor-/- mice (IFNAR-/-) and RAG1-/-, we concluded that type I IFN is required early in infection. Any potential age-related deficiency in this pathway would lead to significantly shorter survival time of old mice, which we do not observe (Chapter 4 Figure 1). We confirmed that there was no substantial defect in type I responses by bioassay (Chapter 4 Figure 3). Old mice may have subtle defects in the overall type I IFN response, or may have a defect in type I IFN responses in a specific microenvironment, like lymph nodes, which we did not sample in our studies. In addition, there may also be other aspects of the innate immune response that have an impact on WNV infection of old mice, but based upon our experimental data we believe that neither the type I IFN response nor other early-acting antiviral innate mechanisms are affected by aging, and are therefore not the root causes of the age-related increased WNV susceptibility. We have therefore focused on identifying potential defects in the adaptive immune response.

Role of T cells in the increased susceptibility of old mice to WNE

We identified defects in the adaptive T cell response of old mice using two different approaches. One approach was the adoptive transfer of naïve T cells into RAG1-/- mice and monitoring of survival. The second approach was to monitor WNV-specific T cells within old and adult mice during the course of infection using FCM (Chapter 4 Figure 2,4-8).

We chose to use an adoptive transfer system because this allowed us to compare old and adult lymphocytes within the same (otherwise adult) immunological environment. RAG1-/- recipients provide equal innate immune responsiveness (type I IFN) and antigen processing and presentation abilities. In addition, young adult RAG1-/- mice do not exhibit the increased levels of IL-6, altered NK cell responsiveness, and many other potential defects found within old mice. An additional benefit of using adult RAG1-/- mice is that they allow us to compare the adaptive immune response of an adult mouse and an old mouse, within a young organism where physiology of other critical organs and components, including the blood brain barrier, cardiovascular system, etc., would be controlled and equal.

We targeted the T cell populations for two reasons; first, it is the diminished CD8 T cells responses that lead to a delay in viral clearance during RSV and Influenza virus infection of old mice (150, 161). Second, the most pronounced defects that occur in immunological aging affect the T cell population (reviewed in (61, 62)). Using the RAG1-/- adoptive transfer model, we demonstrated that there is a defect in the T cell responsiveness of old T cells to WNV leading to increased susceptibility of WNE in RAG1-/- mice (Chapter 4 Figure 4). We also showed that the combination of old CD4

and CD8 T cells does provide some protection against WNE, although the protective capacity of T cells from old mice is completely lost when these populations are transferred separately (Chapter 4 Figure 8). This indicates that the T cells from old mice are able to recognize WNV infection and mount a response within the adoptive transfer model but that the response is simply not sufficient to prevent the development of WNE. Since these results occurred in our adoptive transfer system, they certainly indicate that the defect is cell intrinsic, since adult RAG1-/- recipients provide the necessary innate immune pathways, including natural killer cells, type I IFN and C', etc.

Many other experimental systems have been used to show substantial defects in CD8 T cells from old mice. Defects in proliferation (420), IL-2 production and signaling (73, 153, 161, 421), IFNγ production (161, 421) and cytotoxicity (152, 161, 422) have all been shown to occur both *in vitro* and *in vivo* with CD8 T cells from old mice. There are equivalent defects in CD4 T cells in old mice. CD4 T cells from old mice have been shown to have diminished IL-2 production and signaling (164, 357, 423), IFNγ production (164) and proliferation (164, 357, 423). Several of these defects in either the CD8 or CD4 T cell population of old mice have been linked to a delay in viral clearance *in vivo* (152, 153, 161, 421). However, our data is the first description of diminished T cell responsiveness that is directly linked to increased mortality.

Defects in T cell effector mechanisms within old mice that results in the increased spread of WNV

By adoptively transferring T cells from either wild type, Perforin-/- or IFNγ-/- mice, we show that T cells require both IFNγ and perforin to effectively control WNV

infection and prevent the development of WNE. We could now use these results to pinpoint specific defects in CD4 and CD8 T cell responses in old mice during WNV infection.

Several other laboratories have shown the importance of both perforin and IFNγ production in the prevention of WNE. Shrestha et al. has shown that Perforin-/- mice have higher viral titers within the CNS then control mice, and that the adoptive transfer of pre-primed wt CD8 T cells into Perforin-/- mice leads to decreased viral titer within the CNS (253). Although perforin has also been shown to be essential in the control of the lineage II strains of WNV, perforin can also lead to increased disease during infection with Murray Valley encephalitis virus, a closely related flavivirus (411).

Several groups have shown the importance of IFNγ during infection with lineage I strains of WNV (256, 257). Dr. Erol Fikrig's laboratory has shown that γδ T cells use IFNγ to control WNV replication early during infection (256, 257). Although this data indicates that IFNγ is important during infection with a lineage I strain of WNV; IFNγ does not seem to be important for protection during infection with lineage II strains of WNV or for Kunjin virus as well as several other flaviviruses (282, 293).

Our adoptive transfer experiments are the first to directly address the protective role of IFNγ production by αβ CD4 and CD8 T cells. By evaluating the protective capacity of IFNγ-/- and IFNγ+/+ T cells we were able to demonstrate that αβ T cells also require IFNγ to protect mice from WNE. Mixed-bone marrow chimera experiments completed to formally demonstrate that γδ T cells use IFNγ to limit early WNV growth, did not address the question whether additional T cell subsets used IFNγ later in infection (257).

<u>Age related defects in antigen-specific CD8 and CD4 T cell response to WNV</u>

We have clearly demonstrated three key points; first, old mice are more susceptible to WNE than adult mice (Chapter 4 Figure 1), second, T cells from old mice do not protect RAG1-/- from WNE as well as T cells from adult mice (Chapter 4 Figure 2 and Figure 5), third, adult T cells require both perforin and IFNγ to protect mice from WNE (Chapter 4 Figure 5).

To extend these discoveries, we have identified two molecular defects in the antigen-specific response within old mice to WNV. We showed that CD8 T cells from old mice not only mobilize fewer antigen-specific CD8 T cells but that these CD8 T cells expressed less IFNγ per cell then adult CD8 T cells (Chapter 4 Figure 4). Furthermore, old mice also mobilized fewer CD4 T cells, but in this case CD4 T cells from old and adult mice produce equal quantities of IFNγ (Chapter 4 Figure 4). We observed a similar defect in IFNγ production by antigen-specific CD8 T cells during VACV infection of old mice (Chapter 5 Figure 3). The second molecular defect involves Granzyme B (GrB). We found that old mice mobilize fewer CD8 T cells that produce GrB and that the CD8 T cells from old mice contain less GrB than adult CD8 T cells (Chapter 4 Figure 3), these cells are also less cytotoxic. Interestingly CD8 T cells from old mice infected with VACV did not exhibit a reduction in GrB content but showed a defect in cytotoxic potential (Chapter 2 Figure 3,4). These results are currently being pursued in a separate series of experiments that are not a topic of this thesis.

In order to formally prove that the molecular defects within the WNV-specific T cell population are the cause of the increased viral load within old mice, and therefore the increased probability of developing WNE, the molecular defects would need to be

reversed. We describe two potential experimental schemes to achieve this goal. One approach would be to adoptively transfer adult T cells into old mice then challenge with WNV. If these T cells were capable of protecting old mice, and the adult T cells within an old host still had improved IFNγ and GrB expression, we could infer that the improved T cell function was able to control virus replication and prevent the development of WNE. As a control similar transfers would be completed so that the old T cells are transferred in old mice and challenged, and these mice did not survive, we could definitively say it was due to improved T cell functionality not due to an increased T cell numbers.

A second approach would be to try to ameliorate the T cell defect in old mice, perhaps by treatment with IL-2+antibody immune-complex. It has been recently shown that IL-2 regulates the expression of perforin and GrB (424), it is also known that old mice have defects in both IL-2 production and signaling ((153) reviewed in (60, 154, 425)). *In vivo* treatment of mice with IL-2 combined with S4B6 antibody (anti-IL-2 antibody) increases both the cytolytic and cytokine potential of T cells *in vivo* (426-428). By increasing the level of available IL-2 within old mice we may be able to increase both cytotoxic and cytokine potential of the antigen-specific T cells in old mice.

Unfortunately, there are several caveats to this type of treatment. First, IL-2 concentrations also control regulatory T cell populations (reviewed in (429)), so regulatory T cells would need to be monitored. Second, an optimal dose of IL-2:antibody immune-complex would need to be determined, because the initial high dose treatments were described to bypass the requirement for TCR stimulation, allowing most naïve T cells to become memory cells, and to even protect against a lethal bacterial challenge in a

non-specific manner (430). By potentially shortening the treatment period and lowering the treatment dose, it may be possible to limit bystander activation and target only activated T cells.

We have tried both of these approaches more than one time, but each time the experiments have yielded uninterruptible results due to technical problems. In order to determine if the T cell defects we see in old mice are specific to WNV infection or aging we chose to switch to a different model and attempt correction of defects by vaccination.

Induction of T cell responses by a pseudo-infectious virus particles

In chapter seven, we describe the T cell responses to a novel vaccine approach referred to as a pseudo-infectious virus (PIV) particle. This is a virus particle that carries a defective genome, so that upon infection of a cell, the virus cannot produce infectious progeny (397). One interesting aspect of this approach is the ability of these vaccines to induce a robust type I IFN response (397). Our goal was to determine if the PIV vaccine was capable of inducing a strong immune response in old mice that was sufficient to protect them from a lethal WNV challenge.

When adult B6 mice were vaccinated with PIVs we measured a strong T cell response that developed into a memory T cell population and was protective (Chapter 7 Figure 1-3 and data not shown). The PIV vaccine safely induced T cell responses in both μMT-/- as well as in old mice.   However, old mice but not μMT-/- mice were protected against high dose viral challenge (Chapter 7 Figure 5,6). These results indicate that an antibody response is necessary to protect against a high dose challenge.

Attempts at vaccinating mice against other Flaviviruses has given us a model to compare to the PIV vaccine. A successful vaccination against a high dose viral challenge with JEV requires both an antibody response as well as a T cell response. This was clearly demonstrated in μMT-/- that develop a memory T cell response following JEV DNA vaccination, but the memory T cells were not protective against JEV challenge (341), similar to what is seen with PIV vaccination of μMT-/- (Chapter 7 Figure 5). Vaccination of beta$_2$microglobulin (B$_2$M)-/- mice, which lack CD8 T cells, also were unable to protect mice from a lethal JEV challenge (341). These results are supported by several reports using WNV, which indicated that transfer of immune serum to RAG1-/- or transfer of immune serum to μMT-/- after 4 days post infection did not provide protection (213, 216, 217).

One potential reason why old mice did not respond as well as adult mice to PIV vaccination is the pre-existence of a large memory pool specific for other antigens in the old mice (Chapter 7 Figure 5). As described in the introduction, old mice develop of a large pool of memory T cells overtime (136), and the existence of homeostatic pressure to keep a stable number of T cells (138) causes a reduction in the naïve T cell precursor pool in old mice (reviewed in (135, 139)). In adult mice one mechanism that is responsible for creating space during the acute phase of an infection is the type I IFN response (431). It has been shown that the induction of type I IFN in old mice does not lead to the apoptosis of T cells which allows a new effector population to expand (432). The PIV vaccine also induces a strong type I response in adult mice (209, 397), which may lead to the induction of apoptosis in the T cell population. One potential approach to improve the PIV vaccination in the old mice would be to immunize the mice with a

combination of PIV and Poly I:C to induce a stronger type I IFN response, allowing for a temporary reduction in the T cell pool to allow the responding effector T cells to expand. This increased type I IFN may also improve the functionality of the effector T cells by generating an improved signal 3 during T cell priming.

Summary

The elderly make up an increasing portion of the population within the United States as well as in other developed countries (58). The increased age of the human population has both social and economic impacts. The elderly are highly susceptible to infectious diseases and the treatments needed to combat infection within the elderly are different than the treatments given to a younger population (107). As a large percentage of the population ages the need for specialized treatments designed for the elderly population has increased. More attention is now being paid to why the elderly are at greater risk of morbidity and mortality from some infections, including WNV. This dissertation addresses this question by establishing and characterizing a mouse model of age-related susceptibility to WNV disease, and by identifying several T cell defects that lead to this susceptibility. The results described within this dissertation will aid in the development of specialized vaccines designed to achieve protective immunity within the elderly population.

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