VIRUS-SPECIFIC CD8+ T CELL IMMUNITY AND AGING

RELATIONSHIP BETWEEN CD8+ T CELL IMMUNITY AND AGING USING WEST NILE VIRUS INFECTION AS A MODEL

By

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— ABSTRACT —

The incidence and severity of infectious diseases increases in elderly people (>60 years of age). It is believe that the age-associated changes in the immune system, a phenomenon referred to as immunosenescence, lead to diminished effectiveness of the immune system leaving the aged susceptible to infectious pathogens and associated diseases. The limited efficacy of the currently available vaccines in elderly populations contribute immensely to the frequency of infectious diseases in the globally growing aging population. As such, the demographic shift warrants the development of effective prophylactic vaccines for the elderly to reduce the disease burden and the burden on the global health economy.

West Nile virus (WNV) became endemic in North America in 1999, and although it infects people of all ages, the incidence of severe neuroinvasive disease is more prevalent in the elderly. I hypothesized that the susceptibility of the elderly towards severe WNV disease is a consequence of aberrant immune function, and specifically lack of functional virus-specific CD8+ T cells that mediate clearance of virus-infected cells. The work presented in this thesis utilized WNV infection as a tool to study the development of primary CD8+ T cell responses in a cohort of WNV naturally infected people and compare these responses with respect to age.

Virus-specific CD8+ T cell epitopes were identified using three different epitope mapping techniques including the use of libraries of synthetic peptides that span the WNV proteome, mass spectroscopy sequencing of peptides isolated from HLA molecules of

infected cells, and predictive computer algorithms. Several immunoreactive epitopes were successfully identified displaying a hierarchy of reactivities. Furthermore, a restricted set of epitopes exhibited dominance across the patient population. Direct stimulation of PBMCs from WNV-immune subjects with synthetic peptides proved to be the most effective method of WNV epitope discovery. This method identified the greatest number of highly immunogenic virus-specific CD8+ T cell epitopes with diverse HLA restrictions.

Contrary to my hypothesis, I found that the magnitude, breadth and functionality of WNV-specific CD8+ T cells were not different between the aged and young members of our cohort. These results argue that advanced age does not limit the development of functional CD8+ T cell responses following primary infections with an acute virus. Furthermore, the aged members of our cohort displayed functional CMV- and EBV-specific CD8+ T cell immunity, thus showing that CD8+ T cell functionality is maintained with age, even in the case of persistent viruses.

Collectively, my results demonstrate that elderly individuals can mount effective CD8+ T cell responses. Thus, development of vaccines designed to elicit CD8+ T cell immunity rather than humoral immunity may be warranted for elderly individuals. Alternatively, given the robust immune response produced by WNV in the elderly, perhaps live vectors should be considered for this population.

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— ABBREVIATIONS —

Abs	antibodies
AIDS	acquired immunodeficiency syndrome
Ags	antigens
APC	antigen presenting cell
AATC	American type culture collection
BBB	blood-brain-barrier
BCR	B-cell receptor
C	capsid
CCL #	C-C chemokine ligand
CCR #	C-C chemokine receptor
CD#	cluster of differentiation
CD#L	cluster of differentiation ligand
CEF	cytomegalovirus, Epstein-Barr virus, influenza
CMV	cytomegalovirus
CNS	central nervous system
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic lymphocyte antigen 4
DC	dendritic cell
DENV	Dengue virus
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
E	envelope
EBV	Epstein-Barr virus
EC	elite controllers
EDTA	ethylenediamine tetra-acetic acid

ELISPOT	enzyme-linked immuno spot
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FasL	Fas ligand
FBS	fetal bovine serum
FISH	flourescence *in situ* hybridization
FLOCK	flow cytometry without K
FMOC	9-flourenylmethyloxycarbonyl
GrB	granzyme B
HBV	hepatitis B virus
HCV	hepatitis C virus
HEV	high endothelial venule
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HHV #	human herpesvirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
HSV #	herpes simplex virus
ICAM#	intracellular adhesion molecule
ICS	intracellular cytokine staining
IFN-γ	interferon gamma
IgG	immunoglobulin G
IgM	immunoglobulin M
IL#	interleukin
IRP	immune risk phenotype
JEV	Japanese encephalitis virus
KSHV	Kaposi sarcoma-associated herpesvirus
K_D	dissociation constant
KS	Kolmogorov-Smirnov

LAMP1	lysosomal-associated membrane protein
LCMV	lymphocytic choriomeningitis virus
LFA-1	lymphocyte function-related antigen 1
LPS	lipopolysaccharide
LTNP	long-term non-progressors
MDA5	melanoma differentiation associated gene 5
MDR-TB	multi-drug-resistance tuberculosis
MHC	major histocompatibility complex
MIP-1β	macrophage inflammatory protein 1 beta
MPEC	memory precursor effector cell
MS	mass spectroscopy
Mtb	*Mycobacterium tuberculosis*
MVA	modified vaccinia Ankara
NK cells	natural killer cells
NS#	non-structural protein
PCA	principal component analysis
PCs	principal components
PCR	polymerase chain reaction
PRNT	plaque reduction neutralization test
PBMC	peripheral blood mononuclear cell
prM/M	pre-/membrane
PRR	patter recognition receptor
RAG1/2	recombinase-activating genes
RhCMV	rhesus cytomegalovirus
RIG-I	retinoic acid-inducible gene I
RNA	ribonucleic acid
RP-HPLC	reverse-phase high-performace liquid chromatography
RPMI	Roswell Park Memorial Institute medium
RSV	respiratory syncytial virus

RT-PCR	real time polymerase chain reaction
SARS	severe acute respiratory syndrome
SEM	standard error of the mean
SFC	spot forming cells
SFU	spot forming units
sHLA	secreted class I human leukocyte antigen
ssRNA	single stranded ribonucleic acid
TAP	transporter associated with antigen processing
TB	tuberculosis
T_{CM}	central memory T cell
TCR	T-cell receptor
T_{EFF}	effector T cell
T_{EM}	effector memory T cell
T_{EMRA}	terminally differentiated memory T cell
TLR#	Toll-like receptor
TNF-α	tumor necrosis factor alpha
T_{SCM}	stem-cell memory T cell
V/D/J	variable/ diversity/ joining
VP	vesicle packets
VV	vaccinia virus
VZV	varicella zoster virus
WNV	West Nile virus
YFV	yellow fever virus

— CHAPTER 1 —

INTRODUCTION

Prologue: Infection and immunity

Infectious diseases present a continuous global health problem. According to a 2004-report on global burden of disease, viral, bacterial and parasitic infections were the second leading cause of mortality accounting for an estimated 16.2% of global deaths [1]. The most prevalent infectious diseases that are largely responsible for global mortality rates included human immunodeficiency virus (HIV) infections, tuberculosis (TB), and malaria. In spite of all the advancements in the development of drug treatments for various infectious diseases, their prevalence continues to rise. This has been attributed mainly to the emerging infectious diseases caused by newly evolved pathogen strains such as multi-drug-resistant tuberculosis (MDR-TB) or chloroquine-resistant malaria (Figure 1) [2]. In Europe alone, the prevalence of MDR-TB cases increased from 12% in 2009 to 13.7% in 2010 [3]. In certain cases, increased prevalence of infections is also attributed to climate changes that have resulted in resurgence of disease vectors within animal reservoirs such as mosquito-borne West Nile virus (WNV) and Dengue virus (DENV) infections (Figure 1) [4,5]. The emergence of these pathogens and their subsequent spread causes a significant impact on global health economy and as such requires immediate attention.

Figure 1. Emergence and re-emergence of infectious diseases globally. The world representation of newly emerging or re-emerging diseases is shown by filled circles. Numbers indicate the sum of people living with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) in 2002 in specific regions. Figure adapted from Racaniello, V. R., *J Clin Invest.* 2004;113(6):796–798.

To prevent the spread of infectious diseases, it is critical that cost-effective preventative methods, such as vaccines, are developed. In order to develop successful and effective vaccines, it is important to understand the dynamic relationship between the immune system and the invading microorganisms, which continually evolve to overcome the immune response. Our current understanding of the immune system is growing quickly,

and we are continuously acquiring new information concerning what constitutes a protective immune response against a pathogen. This, in fact, will aid in the development of therapies that will enhance the function of the immune system to eliminate a specific microorganism.

Among the most susceptible populations to emerging and re-emerging infections are the elderly (>65 years of age) due to their waning immune system, a phenomenon referred to as immunosenescence [6]. It is thought that immunesencence leads to changes in the immune system that result in decreased responsiveness to infectious pathogens and, thus, increased morbidity and mortality to infections [7,8]. The aging population is growing globally and a lack of effective vaccines contributes to the problem of epidemics and has a great impact on the health economy. As such, it is critical to study and characterize the aging immune system and its response to emerging pathogens in order to develop new and improved vaccination platforms.

1.0 The immune system

In biological terms, immunity is a state enabled by the hosts' immune system that ensures resistance to invading microbes. The immune system serves to protect the human body from infectious agents and harmful substances through the complex interaction and function of various effector cells and molecules [9]. The immune system is composed of

various subsets of immune cells, which arise from pluripotent hematopoietic stem cells. The hematopoietic stem cell differentiates into common lymphoid progenitors or myeloid stem cells which undergo further differentiation into mature lymphocytes (B cells, T cells, natural killer (NK) cells and NK-T cells) and lineage specific precursor cells (neutrophils, monocytes, eosinophils, basophils, mast cells, megakaryocytes, and erythrocytes), respectively [10]. These cells can be discriminated morphologically and phenotypically by the presence of glycoprotein differentiation antigens, assigned specific cluster of differentiation (CD) numbers, which are displayed on their cell membranes [11].

Based on the specific features of the immune response such as the effector cell types involved, timing of the response and specificity of the effector cells the immune system can be separated into two arms: 1) Innate and, 2) Adaptive immunity. The innate immune response provides a first line of defense against invading organisms following detection of conserved molecular patterns shared by many pathogens [12]. Adaptive immunity is initiated after the engagement of innate immunity and it involves recognition of invading pathogen by highly specific antigen receptors on B and T lymphocytes [13]. Pattern recognition receptors (PRRs) responsible for the activation of innate immunity are germline-encoded in fully functional form, whereas the antigen-specific B and T cell receptors are encoded by gene elements which undergo somatic rearrangement and yield millions of different receptors from a few hundred genes [11]. Neutrophils, differentiated monocytes (macrophages), and dendritic cells (DCs) are some of the innate effector cells which express PRRs and, upon recognition of an invading pathogen, these cells rapidly

clear pathogen [14]. However, if the pathogen evades the innate immune responses and is not cleared within hours following infection, an adaptive immune response is initiated. DCs bridge the innate and the adaptive immune response by serving as antigen-presenting cells (APCs) to T helper lymphocytes in the context of major histocompatiblity complex (MHC) known as human leukocyte antigen (HLA); these are surface glycoproteins that "present" antigenic peptides [10,15]. This interaction, with the aid of co-stimulatory molecules and cytokines, leads to the activation of B and T cells which undergo proliferation and differentiation into an effector cell population capable of pathogen killing [15,16]. Following pathogen elimination, B and T cells undergo a contraction phase leaving a small subset of heterogeneous, long-lived memory lymphocytes [13]. The memory B and T cells rapidly engage their effector functions upon pathogen re-exposure and as such are an essential part of an efficient immune recall response.

Collectively, the immune system is comprised of two indispensible arms, innate and the adaptive, which work together to provide the most rapid, effective, and efficient protection from the invading microorganisms.

1.1 Adaptive immunity

The adaptive immune system is comprised of humoral immunity, mediated by serum antibodies produced by fully differentiated B cells (plasma cells), and cellular immunity,

mediated by T cells. Antibodies directly bind pathogenic microorganisms leading to their neutralization or killing by activation of the complement system and phagocytes [17,18]. T cells on the other hand, recognize pathogen-infected cells in the context of MHC molecules presenting pathogen derived peptides and initiate cytotoxic killing of the infected cell and/or cytokine production to inhibit pathogen replication [18,19]. The specificity of B cell receptors (BCRs) and T cell receptors (TCRs) is extremely diverse and results from random recombination of gene segments termed variable (V), diversity (D), and joining (J) segments, which become spliced together in various combinations to form the functional antigen-recognizing molecules [10,13]. Newly formed B and T cells undergo selection in the bone marrow and the thymus, respectively. Here, self-reactive cells expressing BCRs or TCRs that bind strongly to self-antigens are eliminated via apoptosis (central tolerance) or in the case of B cells can undergo receptor editing to alter their receptors such that they no longer exhibit specificity for self proteins [20,21]. Once in the periphery, BCR genes can undergo further somatic hypermutation to yield a receptor of better affinity and specificity.

Although B and T cells exhibit distinct effector functions, interactions between the two cell type results in their maturation and differentiation [18]. Specifically, T cells provide help for maturation of B cells into antibody-secreting plasma cells or memory B cells, whereas B cells can serve as antigen-presenting cells to the T cells [10,17,18,22]. The interaction of the two cell types ensures the development of long-lived memory cells which is a hallmark of the adaptive immune response [23]. Primary exposure to a

pathogen leads to the generation of cellular memory and persistence of lymphocyte clones, which, upon re-exposure to the same pathogen, rapidly engage effector functions [18,24,25]. Consequently, the adaptive immune system not only possesses a refined repertoire of recognition for non-self antigens but it ensures that the host is provided with life-long protection against the microbes that carry these antigens and thereby prolongs the life of the host by eliminating the possibility of recurrent infections. It is the goal of current vaccination platforms to elicit both B and T cellular responses that will lead to the development of life-long memory and as such provide prophylactic protection from invading pathogens. As such, protective immune responses entail both the humoral and the cellular arms of the immune system against invading pathogens; in the context of this thesis, I will focus on illuminating the immune response elicited by CD8+ T cells.

1.1.0 T cell development

The development of T cells occurs in the thymus following emigration of common lymphoid progenitors from the bone marrow and/or fetal liver. The recent emigrants undergo rapid proliferation driven by the cytokine interleukin (IL) -7, which is necessary for survival of developing T cells [26]. The specific pathway of IL-7-regulated T cell development is not completely understood; however, it is believed that IL-7 signaling enables rearrangement of specific TCR loci in T cells during development and as such is critically necessary [27]. The expansion of early thymocytes is accompanied by the

induction of several transcription factors which in turn induce the expression of proteins involved in genomic rearrangements to generate functional genes encoding the different polypeptide chains of the TCR, α and β, or γ and δ, resulting in αβTCRs or γδTCRs, respectively [13,28,29]. The β and δ gene loci are comprised of three gene segments, V (variable), D (diverse), and J (joining), whereas α and γ comprise only the V and the J gene segments. A single segment from each of the V, D and J loci are randomly spliced together by an enzyme termed V(D)J recombinase composed of two proteins encoded by the recombinase-activating genes 1 and 2 (*RAG1* and *RAG2*) [29,30]. The V(D)J recombinase along with other enzymes including DNA endonuclease, DNA repair enzymes, and ligase collaborate to cleave, repair and splice together the V, D, and J segments generating a large number of permutations of recombinantions that give rise to TCRs with various binding specificities [30,31]. This diversity in the TCRs is referred to as combinatorial diversity.

Rearrangements of two TCR genes followed by their surface expression of αβ or γδTCRs matures the pre-T cell into a "double positive" T cell, expressing surface molecules CD4 and CD8 [32]. These cells then undergo positive and negative selection based on the binding affinity of newly generated TCRs with the host specific MHC molecules and the peptides derived from self-proteins (self-peptides) presented on the MHC [32]. Double positive T cells bearing TCRs that recognize and bind host-specific MHCs incur positive selection while the rest are eliminated. Next, the double-positive T cells undergo negative selection of TCRs exhibiting high binding affinity for self-peptide/MHC and

apoptose to prevent autoreactivity and autoimmune disease, a process referred to as central tolerance [32]. In case self-reactive T cells escape negative selection and migrate into the periphery, peripheral tolerance ensures this cell is dominantly suppressed; self-reactive T cells are rendered anergic (inactive) by regulatory T cells [33]. The self-peptides presented by the MHC comprise those of proteins expressed by the thymus but also other proteins of differentiated peripheral organs whose expression becomes induced in the thymus for the purpose of central tolerance [34]. This mechanism substantiates that T cells of broad self-reactivity are eliminated. Once the cells undergo positive and negative selection, they mature into single-positive CD4+ or CD8+ T cells by additional interactions with either thymic epithelial MHC class II or MHC class I molecules, respectively [13,35]. The single-positive CD4 and CD8 T cells then enter the circulation from the thymus as fully differentiated, antigen-naïve cells ready for activation and further differentiation into antigen-specific T cells with effector functions. Consequently, T lymphocytes represent a very specific antigen-binding cell that can efficiently engage its effector functions upon recognition of a cognate antigen.

1.1.1 T cell antigen recognition

CD4+ and CD8+ T cells utilize the specificity of their TCRs to recognize antigenic peptide fragments of proteolytically processed ingested proteins that are presented on MCH class II molecules, or peptide fragments of proteins synthesized within the cell and

presented on MCH class I, respectively [36]. As such, the two T cell subtypes generate responses to differentially processed antigens.

All nucleated cells express MHC class I proteins. The class I MHC is a heterodimer comprised of a polymorphic transmembrane chain (heavy α chain) in association with a non-polymorphic β2-microglobulin (light chain) protein [37]. The heavy chain consists of three extracellular domains (α1, α2, α3), a transmembrane domain and a short cytoplasmic tail that anchors the protein to the cell membrane [38]. The extracellular domain, α3, folds into an immunolglobulin-like structure and interacts with the CD8 co-receptor on T cells. The α1 and α2 domains associate with each other to form a groove where the antigenic peptide binds. The MHC class I α chain encodes three distinct class I proteins, HLA-A, HLA-B, and HLA-C, which are highly polymorphic in the amino acids comprising the peptide-binding groove. These polymorphisms result in a variety of different alleles for each of the three HLA molecules and exhibit a great diversity in the peptide binding ability within each individual [39]. The individual set of expressed HLA alleles on a chromosome (one from the mother, one from the father) is referred to as the HLA haplotype.

MHC class I binding groove accommodates a peptide of 8-10 amino acids [40]. The complex between the class I MHC and the antigenic peptide is the target of recognition by CD8+ T cells which require contact with both moieties to become activated. The TCR has no measurable affinity for the antigenic peptide alone or for the MHC bound to other

peptides giving rise to a phenomenon of "MHC restriction" which describes the specificity of the TCR towards the complex of antigenic peptide and the MHC it binds [37].

The antigenic peptides bound by class I HLA molecules are derived from proteins synthesized within the cell and are referred to as "endogenous" antigens [36]. Proteolytic degradation of intracellular proteins within the proteosome generates fragmented peptides which enter the endoplasmic reticulum (ER) via TAP (transporter associated with antigen processing) and, here, are loaded onto class I HLA molecules (Figure 2) [41]. The peptide-loaded HLA molecules then associate with the β2-microglobulin and the assembled peptide/MHC complex gets transported by the Golgi apparatus to the plasma membrane [42]. HLA class I molecules present antigenic peptides derived from intracellular pathogens such as viruses, as well as tumor-specific proteins to CD8+ T cells, and result in their direct activation [43,44].

MHC class I molecules can present exogenous peptides (derived from extracellular antigens) by a process termed cross-presentation [45]. This process is speculated to occur when phagosome-internalized antigens are released into the cytosol and, subsequently, degraded by proteosomes and transported to the ER via TAP where they bind MHC class I molecules; the phagosome-to-cytosol pathway. Alternatively, MHC class I molecules can acquire the peptides generated within phagosomes or endosomes possibly via their transport into the phagosome or upon phagosome fusion with the ER which is the site of

class I molecule assembly [45]. Cross-presentation of antigens and subsequent activation of naïve CD8+ T cells in referred to as cross-priming [46].

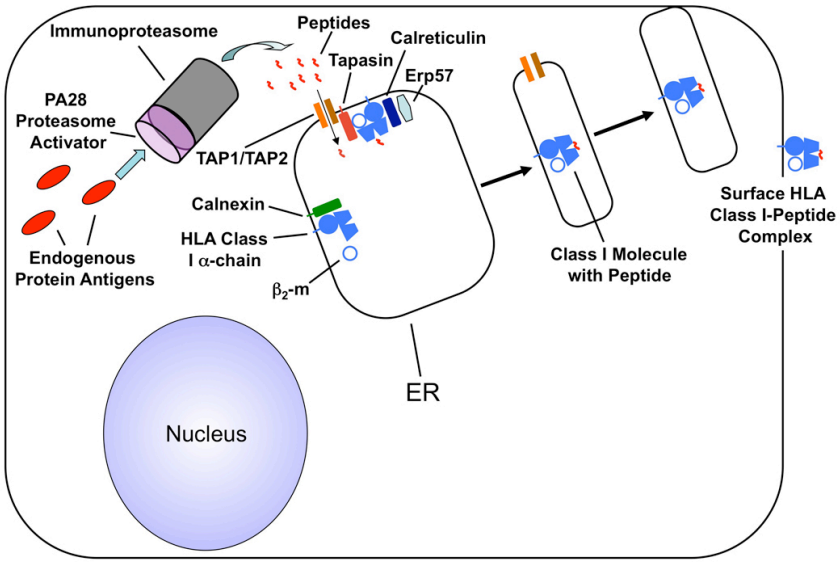

Figure 2. Exogenous antigen processing and presentation by MHC class I protein. TAP, transporter associated with antigen processing; HLA, human leukocyte antigen; ER, endoplasmic reticulum; β2-m, β2-microglobulin. Figure adapted from Chaplin, D.D., J Allergy Clin Immunol, 2010; 125.

MHC class II molecules are constitutively expressed by B cells, DCs, monocytes and macrophages among a few other cell types [36]. During inflammation and in presence of IFN-γ, additional cell types including epithelial and endothelial cells, can express MHC class II proteins on cell surfaces to present antigenic peptides to CD4+ T cells [36]. The MHC class II protein consists of one α and one β chain, each comprised of two domains that form a transmembrane portion, α2 and β2, and a peptide-binding groove, α1 and β1 [38]. The α2 transmembrane domain interacts with the CD4 co-receptor on T cells.

There are three major class II proteins in humans: HLA-DR, HLA-DQ, and HLA-DP. Similar to the class I proteins, these are highly polymorphic resulting in a diverse number of allelic variants for all three.

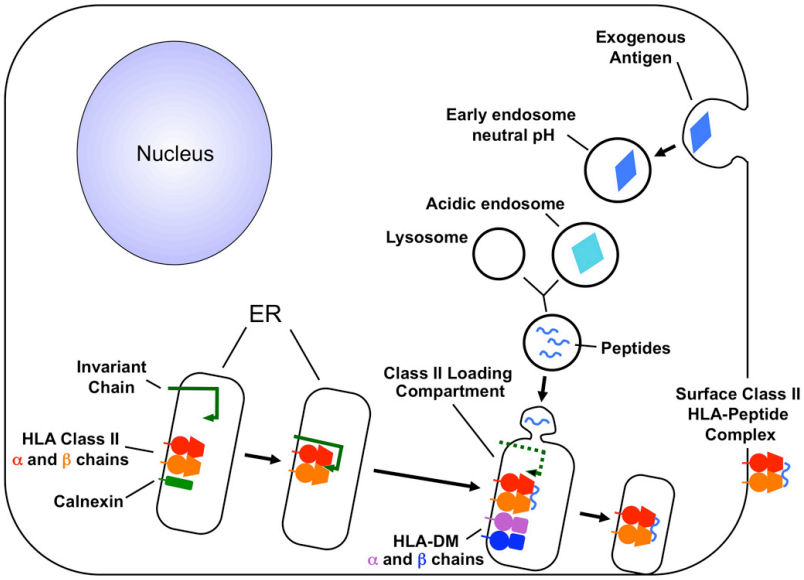

Figure 3. **Endogenous antigen processing and presentation by MHC class II protein**. HLA, human leukocyte antigen; ER, endoplasmic reticulum; β2-m, β2-microglobulin. Figure adapted from Chaplin, D.D., J Allergy Clin Immunol, 2010; 125.

MHC class II molecules present exogenous antigens to CD4+ T cells derived from the extracellular environment and can accommodate peptides of 13-18 amino acids within the peptide-binding groove [38,41]. Extracellular antigens enter the cell by phagocytosis (whole microorganisms, eg. bacteria) or receptor-mediated endocytosis (large polar molecules, eg. proteins) and form acidic vesicles of phagosomes and endosomes, respectively (Figure 3) [42]. Fusion of phagosomes with lysosomes results in the

degradation and processing of antigens into linear peptide fragments by means of proteolysis. These peptide fragments are loaded onto MHC class II molecules once the phagosome fuses with the MHC class II loading compartment vesicles. The MHC class II/peptide complexes are then transported to the cell surface. This pathway of antigen processing leads to the activation of CD4+ T cells and is directed against extracellular pathogens such as bacteria, parasites, and viral particles taken up by phagocytosis.

In summary, antigen processing and presentation to αβT cells occurs via two main pathways which deliver antigenic peptides to either MHC class I or MHC class II molecules and result in the activation of a specific TCR expressed on CD8+ or CD4+ T cells, respectively. The engagement of the TCR and the antigenic peptide/MHC complex enables the T cell to undergo proliferation and differentiation into an effector cell type.

1.1.2 T cell activation

In order to execute their effector functions, CD4+ and CD8+ T cells must first be primed. Naïve T cells undergo activation by "professional" APCs, primarily DCs [42]; however, B cells [47], and macrophages can also serve as APCs [48]. Professional APCs provide two critical signals to the naïve T cells: peptide/MHC complexes to engage the TCRs and co-stimulatory molecules that bind receptors on the T cell [49]. APCs that express peptide/MHC complexes without the costimulatory molecules are referred to as "non-professional" APCs and these can only trigger responses in already primed T cells. APCs

actively sample exogenous proteins by phagocytosis and endocytosis, and their activation is reliant upon recognition of pathogen-associated molecules (bacterial lipopolysaccharide (LPS), ds/ss RNA, DNA) via pattern recognition receptors (PRRs). Activated APCs upregulate MHC expression and emigrate from peripheral sites to the lymph nodes where they interact and activate naïve and memory T cells. T cell activation is initiated when a TCR recognizes a cognate peptide/MHC complex on an APC, leading to clustering of TCR-associated molecules at the T cell-APC junction, forming the so-called immunological synapse. The immunological synapse is comprised of three functional layers: the receptor interaction, signaling layer, and cytoskeletal transport [50].

The formation of a synapse between the TCR and the peptide/MHC complex is the first step required in the T cell activation process. The TCR activation process is reliant on adhesion, co-stimulatory and co-inhibitory, and co-receptor molecules. Adhesion molecules such as lymphocyte function-related antigen 1 (LFA-1) mediates the interaction of the T cell with the APC via ligation to the intracellular cell adhesion molecule 1 or 2 (ICAM-1 or ICAM-2) and bring the two cells in close proximity [51]. Co-stimulatory proteins (eg. CD28) and co-inhibitory proteins (eg. cytotoxic lymphocyte antigen 4, CTLA-4), regulate the TCR signaling positively and negatively via their interaction with CD80 and CD86, respectively [52]. These molecules can modulate T cell activation by increasing or decreasing the strength of TCR signaling and can initiate a specific T cell differentiation pathway [53,54]. Co-receptors directly assist the TCR in its recognition of the appropriate MHC class molecule; CD4 and CD8 co-receptors associate

with MHC class II and MHC class I molecules, respectively [55]. Co-receptors are directly involved in the intracellular signaling cascade of the TCR as they provide the cytoplasmic signaling domains that serve to transduce the signal once the TCR binds the peptide/MHC complex. CD3 co-receptor is a complex of protein chains that assemble to form a functional intracellular signaling domain of the TCR [56].

Collectively, activation of naïve T cells which requires recognition of an antigenic peptide/MHC complex, co-receptor ligation, and co-stimulatory molecules ultimately leads to cell proliferation, acquisition of effector functions and generation of long-lived memory T cells.

1.1.3 T cell differentiation

Following selection of CD4 and CD8 single positive T cells in the thymic medulla based on the interaction with MHC class II or MHC class I molecules, respectively, the mature, naïve T cells exit into the periphery to encounter cognate antigens presented by APCs and thereby differentiate into effector T cells [13]. The nature of the T cell response is highly dependent on the antigenic peptide, cytokine milieu, presence of co-stimulatory and adhesion molecules. CD4+ T cells differentiate into various subsets of T helper cells whose function is to enhance the responses of other effector cell types via secreted cytokines or direct cell-cell contact. CD4+ T cells provide help in the activation of cytotoxic CD8+ T cells or cytotoxic T lymphocyte (CTL) responses via the production of

IFN-γ, IL-12, and IL-2 [57,58]. In consideration of my thesis work which is solely focused on the CD8+ T cell immune response, the remainder of the introduction will be centered on CD8+ T cells.

CD8+ T cells are a major fraction of peripheral T cells whose function is to eliminate cells infected with intracellular pathogens, eg. viruses, and transformed cells. Naïve CD8+ T cells largely reside within the secondary lympoid organs and they are primed following encounter of antigenic peptides within the context of MHC class I molecules on APCs. The successful activation of CD8+ T cells is dependent on CD4+ T cell help. Besides providing the necessary cytokines, CD4+ T cells interact with the APCs via CD40-CD40L interaction and enable these cells to express the activation signals required by the CD8+ T cells [59]. Subsequently, activated CD8+ T cells undergo robust proliferation and then migrate to the periphery (the site of infection) and engage their effector functions to eliminate pathogen-infected cells [60]. Once pathogen is cleared, a select number of CD8+ T cell clones differentiate into memory cells that, upon re-exposure to the same pathogen, rapidly engage effector functions to prevent infection. In the absence of CD4+ T cell help, CD8+ T cells still undergo robust proliferation of antigen-specific population; however, the generation of long-lived memory is compromised since these "helpless" cells exhibit impaired functional responses upon re-infection [61,62].

CD8+ T cells play a very important role in control of viral infections and intracellular pathogen elimination. It was shown by various groups that effector CD8+ T cells provide optimal protection against *Mycobacterium tuberculosis* (Mtb) via the cytolytic pathways and production of cytokines [63,64,65]. Likewise, HIV-infected long-term non-progressors (LTNP) possess functional and highly proliferative HIV-specific CD8+ T cells that are believed to be responsible for efficient control of HIV replication and delay progression to acquired immunodeficiency syndrome (AIDS) [66,67]. Depletion of CD8+ T cells in animal models during infection with various viruses, such as hepatitis B virus (HBV), West Nile virus (WNV), cytomegalovirus (CMV), Epstein Barr-virus (EBV), has shown increased severity in the infection and robust dissemination of the infectious agent [68,69,70]. Consequently, CD8+ T cells play a very important role in the control of virus replication and progression of virus-related diseases such that current vaccination efforts are tailored at generating robust cellular immunity against pathogens.

1.2 West Nile virus infection

West Nile virus (WNV) is a neurotropic human pathogen that only recently became endemic in North America. Its first incidence was reported in the state of New York in 1999 and since then the virus has spread throughout the Western Hemisphere infecting more then 2.5 million people [71]. Humans are incidental hosts of WNV infection whose transmission occurs via mosquito vectors carrying the virus from virus-infected animals

[72]. Although the majority of WNV infections are asymptomatic, the severity of symptomatic diseases varies from West Nile fever to encephalitis and meningitis. The most severe cases of the infection result in acute flaccid paralysis and death. Aged and immunocompromised individuals are at a greater risk for the development of severe disease following WNV infection [73] presumably due to a defective immune system; however, no human studies have addressed this concept.

1.2.0 General overview of the virus

WNV belongs to the family *Flaviviridae* (genus *Flavivirus*) along with dengue virus (DENV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) [72]. Like most flaviviruses, WNV is maintained in an enzootic cycle between birds and mosquitoes with humans being the terminal hosts [74]. Flaviviruses are relatively small, consisting of 10.5 to 11 kb single-stranded RNA (ssRNA) genome of positive polarity [75]. The viral genome is translated into a single polyprotein that is cleaved by a viral serine protease (NS2b-NS3) and various host proteases into three structural (C-capsid, M-membrane, E-envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). Of the structural proteins, C binds viral RNA, premembrane (prM) protein blocks premature virus fusion and helps in E protein folding, while E mediates virus attachment, membrane fusion, and virus assembly [76,77]. The non-structural proteins function

together to regulate viral replication and transcription and evade host immune surveillance.

Flaviviruses enter the cell by receptor-mediated endocytosis, mediated by E protein following attachment to the surface of monocytes, macrophages and DCs [78]. Fusion of the viral and host cell membranes in the endosomal compartment leads to the release of nucleocapsid and viral RNA into the cytoplasm. The viral RNA is replicated in the rough endoplasmic reticulum (ER) and vesicle packets (VP) and is either packaged within progeny virions or used for viral protein translation [79]. Following assembly of immature flavivirus particles within the ER, infectious and mature virions are generated by cleavage of prM to M within the trans-Golgi network. The mature virions are released from the cell following vesicle fusion with the cell membrane where they infect the neighboring cells and propagate.

1.2.1 WNV Epidemiology

Clinical manifestations of WNV infection range in severity from influenza-like symptoms characterized by fever and general malaise to neuroinvasive disease such as encephalitis, meningitis and acute flaccid paralysis [77]. WNV was first identified in the West Nile province of Uganda in 1937 in an isolate of blood from a woman experiencing mild febrile illness [80]. However, WNV related encephalitis cases were reported much later, first in Algeria in 1994, and in Romania in 1996. Since then the virus has shown a wide

geographic range infecting the world's most populated regions with an increased incidence of severe disease. It is believed that the greatest contribution to WNV geographic spread occurs due to the migratory birds harboring the virus.

WNV emerged in North America in 1999 in New York and, over the next decade, it spread throughout the United States, and into Canada, Mexico, and the Caribbean [81]. To date, 37 088 cases of WNV infections were confirmed in the United States with 1549 associated deaths [82]. The virus transmission to humans occurs mostly via *Culex* mosquito vectors, although there have been reports of transmission through blood transfusions, organ transplantation, breast-feeding, or intrauterine exposure [83,84]. The majority of WNV infections are asymptomatic with an estimated 20% incidence of symptomatic disease, mainly the characteristic West Nile fever, and <1% of infections result in neuroinvasive diseases (encephalitis, meningitis, acute flaccid paralysis) [73,85]. Symptoms develop 2 to 14 days post-virus inoculation. Advanced age (>50 years of age) presents an increased susceptibility for the development of neuroinvasive disease and associated death, with an estimated 20-fold rise in occurrence [86,87]. Among these subjects, enhanced post-illness morbidity and mortality can ensue one to three years following acute illness [88,89]. Whereas the fatality rate for all hospitalized WNV-related encephalitis cases is approximately 10% [90], persons over the age of 70 present a case-fatality rate between 15% to 29% [91]. Among other risk factors for neurological complications following WNV infection are hypertension and an immunocompromised immune system [90,92,93]. As such, WNV represents a particular danger to the elderly

and the immunocompromised and, therefore, warrants a better understanding of the causes behind the increased prevalence of neuroinvasive disease to ensure a quality life for the globally growing aging population.

1.2.2 Immune response to WNV: the important role of CD8+ T cells

Human transmission of WNV occurs during a blood feeding by an infected mosquito vector. Following inoculation, WNV replicates in skin Langerhans DCs, which then traffic the virus to the draining lymph nodes [94]. Here, the virus undergoes further replication in cells of the monocytic lineage resulting in primary viremia which lasts up to a week [95]; however, the virus can spread to infect peripheral tissues such as the kidneys and the spleen. Detection of flaviviruses inside of a cell occurs via pathogen-recognition receptors (PRRs) which bind single-stranded or double-stranded viral RNA. These include Toll-like receptors 3 and 7 (TLR3 and TLR7), retinoic acid-inducible gene I (RIG-I), and melanoma differentiation associated gene 5 (MDA5) [96,97,98]. Virulent WNV strains have been shown to delay the activation of RIG-I early in the infection, giving the virus a replicative advantage by blocking the type I IFN response and thereby potentially promoting an overwhelming viral infection [99].

Viral clearance from the periphery is mediated by both the innate and the adaptive immune systems. IFN-α/β restricts viral translation and replication immediately after

infection, where as IgM antibodies modulate viral levels in serum and prevent early spread to the CNS (central nervous system) [79,100]. IFN-α/β also links the innate and the adaptive immune responses via stimulation of DC maturation [101], direct activation of B and T cells and their sustainment following activation [102,103]. Both, CD4+ and CD8+ T cells actively participate in viral clearance from the periphery, but if they fail to do so, the virus enters the circulation and migrates to the CNS via the blood-brain-barrier (BBB) resulting in severe neurological disease [104]. The mechanism by which WNV crosses the BBB is not fully understood; however, tumor necrosis factor alpha (TNF-α) has been implicated in mediating changes in the permeability of endothelial cells allowing the virus to cross into the CNS [105].

CD8+ T cells play a significant role in the control of WNV dissemination and clearance. CD8+ T cells produce IFN-γ which limits viral replication directly by inducing an antiviral state and indirectly by the activation of myeloid cells and CD4+ T cells and increasing the cell surface expression of MHC class I molecules [106,107]. IFN-γ limits early dissemination of WNV to the CNS as mice deficient in IFN-γ or its receptor endured an increased peripheral viral burden that resulted in a more rapid dissemination of the virus into the CNS and presented with increased lethality [108]. Upregulation of IFN-γ during the viremic phase in patients infected with WNV was correlated with lower viral loads, thus suggesting the important role of this cytokine in immediate anti-viral responses [109]. Murine experiments have directly shown the importance of CD8+ T cells in controlling WNV infection. Upon recognition of WNV-infected cells via MHC

class I molecule, CD8+ T cells release proinflammatory cytokines IFN-γ and TNF-α, and they engage cytotoxic responses which lead to the direct elimination of virus infected cells via the release of granzyme B (GrB) and perforin [110,111,112]. Mice lacking CD8+ T cells or MHC class I molecules showed sustained WNV loads in the CNS and exhibited greater mortality rates [70,113]. Moreover, mice with perforin-deficient CD8+ T cells also exhibited a more rapid CNS dissemination along with greater lethality of WNV in mice, thus signifying the important role of functional cytotoxic T cells in clearance of virus-infected neurons [114]. Adoptive transfer of wild-type CD8+ T cells in these mice was able to decrease viral burden and enhance survival. Consistent with the murine studies, humans with hematologic malignancies and impaired T cell function also show an increased risk towards the development of neuroinvasive WNV disease [90]. Conclusively, CD8+ T cells exhibit specific functions that control WNV dissemination to the CNS and elimination of virus-infected cells, and as such, their absence or impairment within the immune system has detrimental affects on the outcome of the infection. Thus, it is important to understand the molecular pathogenesis of WNV infection and how it evades the host immune surveillance for the development of preventative and therapeutic treatments for this infectious agent.

1.3 Herpesvirus infection

1.3.0 General overview of herpesvirus infection

Herpesviruses (family *Herpesviridae*) are very prevalent infectious agents that are endogenous to humans and a variety of eukaryotic animals. Herpesviruses establish a persistent infection that is either latent or recurring. As such, the immune system must provide life-long protection against these viruses and occurrences of severe disease in immunocompetent hosts are rare. Although the viruses differ in sequence and arrangements of their DNA they all comprise a relatively large double-stranded linear DNA genome contained within a capsid and an envelope expressing viral glycoprotein spikes [115]. Three subfamilies of herpesviruses exist based on their biologic properties including sequence similarity and collinear arrangement of their genes; alpha(α)-herpesviridae, beta(β)-herpesviridae, gamma(γ)-herpesviridae [116]. There are eight known human species of herpesviruses: herpes simplex virus 1 and 2 (HSV-1, HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (CMV), human herpersvirus 6 and 7 (HHV6, HHV7), and Kaposi sarcoma-associated herpesvirus (KSHV).

Infection with herpesviruses is characterized by three phases: acute infection, latency, and reactivation [116,117]. Acute infection is mostly asymptomatic or results in mild symptoms characterized by fever and rash. During this phase, the lytic virus replication at the epithelial surfaces is controlled by the adaptive immune response. Following acute

infection, a latency period without any over disease in immunocompetent persons occurs. Once latency is established, the viral genome is found inside the nucleus of infected cells but no detectable replication or gene expression is evident [118]. Reactivation of the virus seems to occur when immunity is suppressed. At this stage the viral gene expression is measurable and the virus elicits a robust inflammatory response leading to tissue damage and clinical disease.

The lifelong herpesvirus infection, specifically the viral reactivation, is kept under control by the adaptive immune system. This immune response must persist lifelong and robustly initiate the effector functions of CD8+ T cells to insure that viral replication is always kept in check.

1.3.1 Epstein-Barr virus (EBV)

Epstein-Barr virus (EBV) is a γ-herpesvirus that infects a very high percentage of humans by the time they reach adulthood and persist for their lifetime. Over 90% of adults are latently infected with EBV [119]. The virus is comprised of a 172 kb linear double-stranded (ds) DNA genome within an enveloped icosahedral capsid [120]. EBV has tropism for epithelial and B-cells, but it preferentially infects memory B-lymphocytes via binding of its envelope glycoprotein to surface B cell receptor, CD21, and HLA class II molecule as a co-receptor [121]. The infectious viral particles are transmitted by saliva and the initial site of viral replication occurs at the mucosal surfaces in nasopharyngeal

epithelial cells [122]. From there the virus enters the underlying tissues and latently infects resting memory B cells [123].

EBV infection can shift between an active lytic cycle and a latent state via the expression of specific viral genes involved in the two processes [124]. During primary EBV infection, the virus is in its lytic cycle where the viral DNA replicates and the majority of the viral genes become expressed. This process allows infectious virus to shed into saliva, infect B cells and epithelial cells, and transmit to new hosts. Following primary infection, EBV enters a latent state to prevent its detection by the host immune response. At this stage, the genomic DNA becomes circularized suppressing the expression of viral genes, and forms a closed circular plasmid that behaves like the host's chromosomal DNA [122]. As such, the virus is undetectable by the host immune system and is capable of persisting in infected B cells. Sporadic reactivation of EBV replication can occur in infected subjects, leading the virus to switch to the lytic cycle [124]. The triggers that enable this switch are unknown, however, differentiation of infected B cells into plasma cells might trigger the process [125]. The ability of EBV to reactivate poses a continuous challenge for the immune system by the invading pathogen.

EBV infection has been associated with a variety of human diseases including a febrile syndrome called infectious mononucleosis, which occurs if primary EBV infection is delayed until adolescence [126]. The immune system is able to control the acute viremia; however, it never completely eliminates latent EBV-infected B cells and as a result the

virus persists for life. The enduring EBV infection is asymptomatic in the majority of infected humans but malignant lymphomas often arise in immunodeficient or immunosuppressed hosts unable to control the recurring EBV replication [127]. EBV-associated epithelial and B cell malignancies can arise in immunocompetent hosts as in the case of nasopharyngeal carcinoma, gastric carcinoma, Burkitt Lymphoma, and Hodgkin Lymphoma [127,128]. It is possible that the EBV-infected cells present with an increased risk of genomic instability resulting in malignant transformation of virus-infected cells that escape immune monitoring by the host immune system and the aforementioned malignancies. As such, it is of great importance for the immune system to keep the persistent EBV infection in control by making the virus-specific memory cells available to eliminate any reactivating virus and thereby prevent virus-associated disease.

1.3.2 Cytomegalovirus (CMV)

Between 50 to 90% of healthy adults are chronically infected with cytomegalovirus (CMV); the largest ubiquitous herpesvirus belonging to the β subfamily [129]. CMV is composed of a dsDNA genome, 230 kb in size, enveloped by a protein rich matrix called the tegument, which is surrounded by a membrane containing viral glycoproteins [130]. The virus produces over two hundred proteins in three overlapping phases referred to as immediate-early, early, and late [131]. Synthesis of immediate-early and early genes is followed by viral replication and production of late genes, which mostly encode structural

proteins (matrix and tegument proteins and envelope glycoproteins). CMV infection has two modes; lytic and latent [132]. Primary CMV infection is lytic and it leads to virus replication and dissemination. It is always followed by latency where the virus remains dormant and resides in infected cells without causing any clinical disease in most healthy subjects. As such, an immunocompetent host can efficiently control lifelong CMV infection.

Transmission of CMV can occur via many different routes including saliva transmission, sexual contact, placental transfer, breastfeeding, blood transfusion, solid-organ transplantation or hematopoietic stem-cell transplantation. Primary CMV infection in immunocompetent adults is mainly asymptomatic or results in febrile disease that resolves following the activation of the adaptive immune response [133]. However, the development of humoral and the cellular immune responses against CMV do not eradicate the virus. Instead, CMV establishes a persistent infection in undifferentiated monocytes [134] and endothelial cells [135]. These cells then provide the reservoirs for viral shedding. In newborns, CMV is the leading cause of congenital infections ranging in clinical symptoms from vision loss, hearing loss, and neurological abnormalities to mortality [136].

The occurrence of CMV disease is almost entirely associated with immune suppression. Long-term immunosuppression induced by drugs in solid-organ or stem-cell transplantations, as well as obliteration of T cells during HIV infection, leads to

uncontrolled CMV replication and health complications that often result in life-threatening disease [137,138]. In the absence of a functional immune system, virus replication proceeds uncontrolled and leads to tissue pathology. The prevalence of CMV infection increases with age; in the United States 54% of adults aged 30-39 years showed CMV seropositivity whereas 91% of adults over the age of 80 were CMV seropositive [139,140]. Moreover, CMV infection has been correlated with an increased risk for mortality in the aging population. Specifically, in a cohort of community dwelling older women between the ages of 70-79, those having the highest CMV-specific antibody concentrations showed a higher risk towards frailty and mortality in comparison to CMV seronegative women [140]. In a population of Swedish octogenerians (80-89 years of age), CMV seropositivity was associated with a group of immune parameters that defined an "immune risk phenotype (IRP)" which predicted a two-year mortality in this population [141]. Consequently, the immune system appears to competently control CMV infection throughout most of an individual's life; however, the infection itself poses a higher incidence of mortality in old age for reasons that are not completely understood but are speculated to be related to the waning immune system.

1.3.3 CD8+ T cell responses to EBV and CMV

T cell mediated immune responses are critical in the control of herpesvirus replication. The initial immune response to CMV infection is dominated by functional CD8+ T cells

as was shown in CMV seronegative recipients who received CMV sereopositive renal allografts and developed primary viremia following the transplant [142]. The cytotoxic lysis of virus-infected cells by CD8+ T cells leads to their elimination and thereby prevents excessive and uncontrolled replication of the virus [143]. Moreover, CD8+ T cells produce the cytokines IFN-γ and TNF-α, each having direct anti-viral effects that interfere with viral replication [144]. During primary infections with both EBV and CMV, virus-specific CD8+ T cells undergo rapid expansion followed by a contraction and development of long-lasting memory T cells critical in controlling reactivating virus [145]. Following primary CMV infection, virus-specific memory CD8+ T cells accumulate in healthy individuals suggesting that CMV reactivation occurs frequently [146]. The oligoclonal expansion of CMV-specific CD8+ T cells ensures that the frequent virus reactivation is controlled and tissue pathology is limited. The epitope specificity of the CD8+ T cell response in CMV infected subjects is directed towards various proteins expressed by the virus [144], but often the highest magnitude of CD8+ T cell responses is elicited to epitopes within matrix protein pp65 [147]. Consequently, these epitope specific CD8+ T cell clones undergo expansions (oligoclonal) to mediate cellular immunity against CMV.

Loss of CD8+ T cell function in subjects infected with CMV and EBV commonly results in virus reactivation and uncontrolled replication that leads to various health complications. CMV disease in immune suppressed patients receiving bone marrow transplants was attributed to defective virus-specific CTLs and infusion of CMV-specific

CD8+ T cells post-transplant provided efficient virus control such that neither viremia nor disease was observed [148,149]. Likewise, an increased risk for posttransplant lymphoproliferative disease, an EBV-associated disease that occurs in transplant recipients, was correlated with low cellular immune responses [150]. Lack of functional virus-specific memory CD8+ T cells in patients with AIDS was correlated with increased risk for further health complications due to CMV or EBV reactivation, suggesting yet again the important contribution of CD8+ T cells in control of persistent virus infections [151,152].

1.4 The influence of virus infection on CD8+ T cell responses

1.4.0 CD8+ T cell effector functions

As stated previously, activated CD8+ T cells kill virus-infected cells indirectly via the production of specific cytokines or directly through cytotoxic mechanisms. The hallmark cytokines produced by CD8+ T cells are interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) whose function is to inactivate virus by interfering with the production of viral RNA [153,154]. Furthermore, IFN-γ has been shown to up-regulate the genes involved in the MHC class I and II antigen presentation pathways, as well as activate microbiocidal effector function of macrophages and attract immune cells to the site of inflammation via upregulation of chemokines and adhesion molecules [107]. TNF-α plays roles in all of these processes and is very important for macrophage

activation. Upon activation, CD8+ T cells are also know to express interleukin-2 (IL-2) and the high affinity IL-2 receptor [155]. IL-2 is necessary for CD8+ T cell proliferative responses during the expansion phase as well as development of memory [156]. At the peak of the anti-viral response, all antigen-specific CD8+ T cells express IFN-γ and at least half of these cells express TNF-α [157]. Alternatively, IL-2 expression is transient following TCR stimulation and undergoes rapid down-regulation such that only a few effector T cells, less then 10%, express IL-2 at the peak of the response [158].

A very important function of CD8+ T cells is their ability to directly kill virus-infected cells. This cytototoxicity occurs via granule exocytosis and/or Fas ligation pathways [112]. Effector T cells express cytotoxic mediators, granzymes and perforin, and upon recognition of a target cell they release the cytotoxins stored within the lytic granules [159]. These cells are commonly referred to as cytotoxic T lymphocytes (CTL). The CTLs form a point of contact that develops into an immunological synapse [160]. Degranulation of the lytic granules containing granzyme B (GrB, a serine protease which induces apoptosis of target cells via the caspase cascade) and perforin (forming pores in the target cell's plasma membrane, thereby enabling entry of GrB into target cells) leads to target cell killing [161]. Degranulation of the CTLs is marked by mobilization of CD107a molecule (also known as lysosomal-associated membrane protein 1 [LAMP1]) to the cell surface which become an established marker of degranulation of CD8+ T cells [162]. Upregulation of CD107a on the cell surface in combination with the detection of cytotoxins provides an indirect measure of CTL-mediated killing.

Alternatively, effector T cells express Fas ligand (FasL) and, upon ligation with a target cell expressing Fas on their surface, they induce cell killing by activation of caspases which cleave DNA and induce cell apoptosis [162]. Altogether, effector CD8+ T cells provide a protective immune response against various pathogens as well as malignant cells indirectly, via the secretion of cytokines or directly, by CTL-mediated killing.

1.4.1 Differentiation of CD8+ T cell memory subsets

An important characteristic of the adaptive immune response is the ability of T and B cells to generate immunological memory such that, upon reinfection with the same pathogen, a robust immune response is initiated leading to pathogen clearance. At the peak of the adaptive immune response antigen-specific CD8+ T cells undergo rapid expansion followed by a contraction phase, which leaves a selection of heterogeneous T cell clones to differentiate into a memory T cell pool [163]. Memory T cells have the ability to persist in an antigen-independent manner by slowly dividing under homeostatic signals of cytokines IL-7 and IL-15 [164]. This process is termed homeostatic turnover and it insures that the memory T cells are kept in circulation in case of secondary infections.

Several different memory T cell subsets exist within the memory T cell pool with distinct effector functions and homing potential [165,166]. Most broadly characterized of these subsets are the central memory (T_{CM}) and the effector memory (T_{EM}) T cell populations

[167]. Differentiation status of memory T cells can be defined by the expression of surface molecules such as CD45R0/CD45RA; isoforms of the transmembrane protein CD45 containing a large cytoplasmic domain with protein tyrosine phosphatase activity [168], CCR7; a chemokine receptor required for T cell migration to the secondary lymphoid tissues [169], and CD62L (L-selectin); a cell adhesion molecule involved in lymphocyte-high endothelial venule (HEV) extravasations [170]. Lymphocyte rolling along the HEV is initiated by the binding of CD62L to peripheral node addressins followed by CCR7 binding to chemokine ligand 21 (CCL21) found on HEV cells, and consequently, enabling extravasation of the cells through the vessel wall [171]. T_{CM} are characterized by CD45R0+ CCR7+ CD62L+ surface molecule expression where as T_{EM} lack the expression of CCR7 and are heterogeneous for CD62L (CD45R0+ CCR7- CD62L-/+). Naïve T cells express both CCR7 and CD62L but carry the CD45RA isoform.

Functionally distinct, T_{CM} express little effector functions but display robust proliferation, IL-2 production and differentiation into effector T cells in response to antigen [166,167]. Additionally, they posses the exclusive capability to home to the secondary lymphoid organs. T_{EM} on the other hand, are considered to provide protective memory by displaying immediate effector functions at the site of inflamed peripheral tissues characterized by the production of large IFN-γ amounts, perforin release and potent cytolytic activity [166]. CD8+ T_{EM} cells can further differentiate to become terminally differentiated memory T cells (T_{EMRA}) upon re-expression of CD45RA [172]. These cells

retain full effector functions and possess the greatest amount of perforin but they display impaired proliferative ability [173].

The relative distribution of the memory T cell populations varies within tissues; a greater proportion of T_{CM} can be found in the lymph nodes and tonsils whereas T_{EM} are excluded from the lymph nodes and, instead, populate the periphery (spleen, skin, lung, liver and the gut) [174,175]. The distinct functions and homing potential of the memory T cell subsets makes them preferentially suitable for different routes of viral infections. For example, since the T_{EM} mainly reside in the periphery, they present the first line of defense against systemic pathogens [176]. T_{CM} persist in the lymph nodes and provide protection against pathogens that infect via the lymphatics. Pulmonary infection with a common intestinal pathogen, rotavirus, generated memory T cells that were less efficient at clearing intestinal infection than memory T cells generated after oral rotavirus infection due to migratory and functional differences between the two memory T cell subsets [177]. Moreover, a mouse model infection with lymphocytic choriomeningitis virus (LCMV) showed that transfer of LCMV-specific T_{CM} into naïve mice provided better protection than T_{EM} upon LCMV challenge, as determined by a greater reduction in virus load [165]. Memory CD8+ T cells isolated from HIV LTNP patients exhibited virus inhibition *in vitro* and expressed a predominant T_{CM} phenotype, thus suggesting that T_{CM} might be better at mediating protection against HIV [67]. However, in persistent CMV infection the predominant cell type is that of T_{EMRA} (CR45RA+ CCR7-) and, although the virus is never eliminated by the immune system, it is kept under constantant surveillance by

T_{EMRA} cells which are robust effectors and cytotoxic killers [178,179]. Differentiation of memory T cells is a dynamic process where T_{CM} can differentiate into T_{EM} but the reverse may also be possible once antigen is cleared [165,180,181]. This plasticity enables the immune system to respond most efficiently to an invading pathogen and elicit specific T cells responses that are pathogen specific but also depended on the route and resolution of the antigen.

How memory T cells arise and persist in the tissues has been a topic of debate for some time. Whether these have inherent longevity or if they derive from a long-lived antigen-specific precursor population are questions under investigation. There are currently several models to propose the mechanisms for the differentiation of memory T cells [182]. The first model, termed *Uniform Potential (Linear Differentiation)*, suggests that memory T cells progress from naïve→T effectors (T_{EFF})→T_{EM}→T_{CM}, instead of being distinct subsets [183]. The second model, *Decreasing Potential*, suggests that T cells differentiate and acquire full effector functions and with each successive stimulation lose the ability to become memory cells [184]. The third model, *Fixed Lineage*, states that the commitment to the various memory cell subsets is defined very early after T cell activation and suggests that memory as well as effector T cells are present at the beginning of the response [185]. Lastly, the *Fate Commitment with Progressive Differentiation* is a model stating that the cell-fate decision of memory precursor effector cells (MPECs) to become long-lived T_{CM} is determined by the signal strength comprised of antigen effects, co-stimulation and inflammation [186]. Recent reports [187,188] from

human data and non-human primate animal models suggests that immunological memory arises from a stem cell-like memory population with self-renewing properties and the ability to differentiate into effector T cells upon antigen encounter. These memory cells, referred to as memory stem cells (T_{SCM}), resemble the phenotype of naïve T cells in their expression of CD45RA+, CD62L+, CCR7+, CD28+, CD27+, and IL-7Rα+ [187,188]. T_{SCM} were generated in the acute phase of viral infection and, upon antigenic stimulation, they exhibited self-renewal and differentiation into T_{CM} and T_{EM} having full effector functions (IFN-γ and TNF-α secretion). As such, T_{SCM} provided a long-lasting memory T cell pool that was capable of continuously replenishing the effector memory T cells such that they can eliminate invading pathogens while maintaining specific clones in the T cell repertoire.

1.4.2 Current understanding of protective CD8+ T cell responses

The specific correlates of protective immunity against a particular pathogen are not well understood. Current knowledge of what constitutes a protective human CD8+ T cell responses is derived from studies of vaccinees and humans infected with pathogens who do not succumb to clinical diseases. The most successful vaccine to date has been the smallpox vaccine, originally developed in 1796 by Edward Jenner, to prevent variola virus infection, the causative agent of devastating smallpox disease [189]. Recent studies showed that smallpox vaccine elicited functional memory CD4+ and CD8+ T cells that

persisted for decades suggesting that cellular responses are important correlates of protection [190,191]. Although the majority of currently available vaccines induce antibody production that is correlated with protection [192,193], it is known that cell-mediated immunity leads to viral clearance, termination of early viral replication and reduction in the severity of symptoms following infection [194,195].

It was previously thought that efficient control of viral replication and tumor growth by antigen-specific CD8+ T cells is solely correlated with the frequency of infiltrating effector cells; however, current understanding of cellular responses deems that rather than having a large quantity of infiltrating CD8+ T cells, better protection against various pathogens is correlated with the CD8+ T cells ability to simultaneously execute multiple effector functions [196,197]. Such CD8+ T cells are termed "polyfunctional", and the responses they elicit are considered qualitative. Flow cytometric analyses of T cell functional parameters has operationally characterized "polyfunctional" CD8+ T cells as capable of: i) release of cytokines, IFN-γ and TNF-α, and/or IL-2, ii) surface mobilization of CD107a molecule as an indirect measure of degranulation, and/or release of GrB and perforin, and iii) secretion of chemokines such as macrophage inflammatory protein 1 beta (MIP-1β) also known as CCL4 [198]. Chemokine MIP-1β is a potent proinflammatory protein that mediates recruitment of additional immune cells *i.e.* macrophages, to the site of infection and is also crucial in T lymphocyte chemotaxis from the circulation to inflamed tissue [199].

The correlates of protective CD8+ T cell responses following vaccination with the two most successful live vaccines, the smallpox vaccine (Dryvax comprised of vaccinia virus (VV)) and the yellow fever virus (YFV)-17D were characterized from human vaccinees [200]. Acute infection with both VV and YFV vaccines induced robust, antigen-specific CD8+ T cell expansions, production of IFN-γ, expression of GrB and perforin, followed by the generation of long-lived memory T cells. The comprehensive analyses of CD8+ T cell memory responses following vaccination, showed that the memory T cells were polyfunctional and, as such, capable of degranulation and production of cytokines IFN-γ, TNF-α, IL-2 and MIP-1β [201]. The polyfunctional long-lasting memory CD8+ T cells were correlated with vaccine efficacy and protection from VV and YFV.

Protective CD8+ T cell responses against *Mycobacterium tuberculosis*, the primary etiological agent of tuberculosis (TB), have also been illustrated in humans [202]. Mycobacteria antigen-specific CD8+ T cells secrete IFN-γ, TNF-α, and exhibit cytolytic killing of infected cells to prevent bacterial dissemination and, therefore, the onset of clinical disease [203,204]. In the quest to understand effective T cell responses, much attention has been given to a population of HIV-infected subjects, long-term non-progressors (LTNP) or "elite controllers (EC)", who, although infected, do not progress to develop AIDS [205]. Studies comparing immune responses between LTNP and disease progressors showed no difference in the antibody production but focused upon the qualitative difference of CD8+ T cells; LTNP patients exhibited higher cytotoxicity and proliferative potential of CD8+ T cells [206,207]. Furthermore, LTNP and EC patients

exhibited polyfunctional CD8+ T cells characterized by efficient degranulation of GrB and perforin [208,209], and production of multiple cytokines including IFN-γ, TNF-α, IL-2, and MIP-1β, a response that was lacking in HIV-progressors [210,211].

Understanding all aspects of what constitutes a protective antigen-specific CD8+ T cell response has proved to be remarkably difficult since all efforts towards the development of new vaccines for emerging and re-emerging pathogens, such as HIV, malaria, TB, and DENV have only yielded limited success [212,213,214,215]. As such, there is much more to be learned about CD8+ T cell immunity and the interaction of the invading pathogen with the immune system. Characterization of antigen-specific memory CD8+ T cells following efficiently cleared natural infections and/or effective vaccines revealed that polyfunctional CD8+ T cell responses could provide a new correlate of cellular immune protection.

1.4.3 Acute versus chronic viral infections

Viral infections can be classified into acute and chronic depending on whether the virus is eliminated or if it persists, respectively [216]. Acute viral infections elicit immense proliferation of CD8+ T cells that differentiate and acquire effector functions resulting in control of viral infection and elimination of antigen [217]. Once antigen is cleared, the effector T cell population diminishes in size (i.e. contracts) leaving a small number of

memory CD8+ T cells to persist independent of antigen and maintained by cytokine-driven homeostatic proliferation [164,218]. The memory T cell population produced by acute infection is typically heterogeneous and composed of a constellation of phenotypes but typically dominated by T_{CM}. Persistent viruses establish a lifelong infection and coexist with their host. Although the primary response to persistent viruses is similar to acute infections, the virus is never completely eradicated [219]. Persistent viruses have developed diverse strategies that enable them to hide from the immune system and enter latency, characterized by perseverance of functional genome without the production of infectious virions [220]. Several viruses are known to establish a persistent human infection that can be further divided into latent, "smoldering", and chronic, depending on whether the virus enlists a periodic reactivation, such as the herpesviruses, or continuous reactivation (e.g. HIV, hepatitis C virus [HCV]), respectively [216]. The term "smoldering" was used to describe infections such as CMV that persist at low levels but endure ongoing viral replication [221,222]. The ongoing antigenic presentation is believed to continuously recruit naïve T cells into the memory T cell pool such that life-long CMV infection has been correlated with a shrinking naïve T cells pool [223]. Chronic infections depict high-level viral replication with ongoing viremia resulting from inefficient primary containment of the virus [224]. This type of infection is characterized by inflammation, sustainment of prolonged antigenic presentation that leads to dysfunctional memory T cells and progression to disease [225,226]. The memory T cell population produced by persistent infections also display a constellation of phenotypes but are dominated by T_{EFF} and T_{EM}.

The repetitive antigenic stimulation associated with persistent infections has been shown to render T cells dysfunctional. Such a phenomenon was first shown in mice infected with chronic lymphocytic choriomeningitis virus (LCMV) clone 13, a rodent-borne virus that causes persistent infection [227]. In this study, virus-specific CD8+ T cells displayed impaired function in the production of effector cytokines, which was attributed to functional exhaustion due to over-stimulation by the viral antigens [227]. Since then, exhaustion of distinct CD8+ T cell functions was found to occur in a hierarchical manner where ability to produce IL-2, proliferate, and execute cytolysis wanes at an early stage, followed by loss of TNF-α production, and finally at the stage of extreme exhaustion IFN-γ production is lost [225,228,229]. In human studies, CD8+ T cells from patients infected with chronic HIV-1 revealed a progressive loss of T cell function marked by inability to execute cytotoxic killing of target cells, followed by loss of IFN-γ production [230,231]. The exhaustion of HIV-specific CD8+ T cells was correlated with disease progression to AIDS in several infected subjects. As such, chronic infections and persistent antigenic presentation may have deleterious effects on CD8+ T cells by causing terminal differentiation, exhaustion and cell dysfunction and these effects seem to be related to antigen load.

1.5 The aging immune system

1.5.0 Immunosenescence

The immune system undergoes age-associated changes that result in reduced ability to contain infections and reduced responsiveness to vaccination. Advanced age increases the susceptibility to a variety of infectious pathogens with consequences of higher morbidity and mortality in the elderly [232,233]. Specifically, novel infections represent a high disease burden in the elderly and this has been associated with waning of the immune system of the aged and associated dysfunction of the immune cells, a phenomenon referred to as "immunosenescence" [6,234]. Although the effects of immunosenescence are imposed on both the innate and the adaptive arms, the changes associated with aging T lymphocytes have been more extensively characterized and are the focus of my thesis.

The most prominent feature of immunosenescence is a substantial decline in the production of naïve T cells and the diversity of the circulating T cell pool [235]. Aging leads to involution of the thymic epithelium and subsequent decreased thymic output of naïve T cells [236]. As the thymus involutes, maintenance of naïve T cell numbers becomes dependent on post-thymic expansion of the existing cells resulting in reduced TCR diversity and restricted oligoclonal TCR repertoire in aged [237]. Furthermore, IL-7 production also declines with age. Given the importance of this cytokine necessary for naïve T cells survival, it is believed that loss of IL-7 further contributes to the decline of

naïve T cells in the aged [238]. The declining numbers of naïve T cells in the aging immune system are believed to increase the susceptibility of this human population to novel infections.

The naïve T cells that persist in old age are believed to sustain impaired function due to decreased exposure to survival signals (i.e. IL-7, IL-15) and exposure to oxidative stress in the periphery [239]. Mouse studies showed that naïve CD4+ T cells from old mice produced less IL-2, expanded less, and differentiated less into cytokine producing effector cells, than CD4+ T cells from young mice [240]. Induction of newly generated naïve CD4+ T cells in old mice, however, restored their function and their ability to provide help to activate other cell types [241]. Considering the necessity of functional CD4+ T helper cells in the development of effective CD8+ T cell responses it is conceivable that their impairment leads to the impairment of CD8+ T cell function.

The age-associated reduction of naïve T cells in the elderly is accompanied by an increased homeostatic proliferation of the memory T cell pool [242,243]. The consequence of such an event results in the terminal differentiation of memory T cells, characterized by the loss of co-stimulatory molecule CD28 [244,245]. CD28- CD8+ T cells preferentially accumulate and occupy the available immunological space due to their resistance to apoptosis [246]. Loss of CD28 on T cells has been associated with replicative senescence and reduced vaccine responsiveness (as measured by antibody production) in the elderly [223,247,248]. Often, the CD8+ CD28- T cell population in

the elderly comprises oligoclonal expansions of cells specific for chronic viruses such as CMV, suggesting that chronic infections can consume the attention of the CD8+ T cell pool and limit the diversity of the TCR repertoire in the elderly [223,249,250]. Moreover, it has been proposed that persistent infections such as CMV, result in the chronic elevation of inflammatory cytokines (IL-1, IL-6, and TNF-α) during aging, a process referred to as "inflammaging", that contribute to immunosenescence and have detrimental effects on longevity [140,251,252].

A major challenge for managing infectious diseases in the aging population is the evidence of reduced vaccine responsiveness in this group that has been associated with immunosenescence. Many commercially available vaccines that elicit protective immunity in young individuals are not able to induce protective immunity in a large number of elderly persons [253,254]. Whereas influenza vaccine efficacy in the young, healthy adults ranges from 47-86% in reducing virus confirmed illness [255], vaccine efficacy in older adults (>65 years of age) has been estimated between 17-53% at reducing pneumonia and influenza hospitalizations [254,256,257]. Similarly, administration of the live attenuated varicella zoster virus (VZV) vaccine in the elderly only partially decreased disease burden severity [258,259]. Collectively, these studies point to a critical need for new and effective vaccines for the globally expanding aging population.

1.5.1 CD8+ T cell responses in old age

The memory CD8+ T cell pool displays some evidence of a progressive loss of functionality with age. CD8+ T cells from older adults demonstrate decreased proliferative responses following antigenic stimulation of memory cells [260]. However, exogenous administration of IL-2 was able to restore T cell function following activation of the TCR in mice [240]. Other mouse models of viral infections have shown that the polyfunctionality of antigen-specific memory CD8+ T cell deteriorates with age leaving more monofunctional T cells to preside [261,262]. Polyfunctional CD8+ T cell responses in these models were correlated with better disease protection but the direct link has not been established. The functional defects of CD8+ T cells in the aged were extended to include impaired cytotolytic ability. Influenza vaccinees over the age of 65, expressed substantially lower GrB and perforin production by effector CD8+ T cells in comparison to the young, suggesting that these virus-specific cells were less adapt to eliminate virus-infected cells [263]. In summary, the variability of functional defects in CD8+ T cells associated with immunosenescence reveal suboptimal protective ability of these cells in the elderly.

1.5.2 The effects of persistent infections on the immune system

An ensuing body of literature suggests that persistent infections, such as the highly prevalent herpesviruses CMV and EBV, have deleterious effects on the aging immune

system. The term "smoldering" was used to describe infections such as CMV that persist at low levels [221,222]. The ongoing presentation of virus proteins is believed to continuously recruit naïve T cells into the memory T cell pool such that life-long CMV infection has been correlated with a shrinking naïve T cells pool [223]. Moreover, the size of the CMV-specific memory T cell pool is often very large and contributes to a substantial fraction of memory CD8+ T cells in healthy seropositive humans [264,265]. It has also been demonstrated that the frequency of CMV-specific T cells in circulation continuously increases throughout life, presumably as a result of persistent activation and proliferation of memory cells, such that the CMV-specific T cell population often dominates the T cell repertoire in the elderly [266,267]. Longitudinal analyses of age-related changes in the immune system of very old persons (>80 years of age) identified an immune risk phenotype (IRP) that was predictive of subsequent 2-4 year mortality and was correlated with persistent CMV infection and an increase in the number of fully differentiated CD8+ CD28- [141,250,268]. It is hypothesized that CMV-specific CD8+ T cells increase throughout life to contain the persistent infection but ultimately become functionally exhausted due to excessive antigenic stimulation and the functional impairment of the T cells subsequently results in onset of disease and mortality.

Similar to CMV infection, EBV infection is believed to drive repeated expansions of memory CD8+ T cells resulting in increased numbers of EBV-specific T cells as the infected host ages [269]. Interestingly, the EBV-specific CD8+ T cell population seems to remain stable in CMV-seropositive donors while the EBV-specific CD8+ T cell

population increases with age in CMV-seronegative donors, thus suggesting that the CD8+ T cell pool may be limited in its ability to deal with persistent infections [267]. Sandalova *et. al*. demonstrated that during acute infection with either influenza, Dengue virus (DENV), or adenovirus, the expansion of virus-specific CD8+ T cells to acute infecting viruses were accompanied by the expansions of CMV and/or EBV specific CD8+ T cells [270]. These results indicate that the persistent herpesvirus infections might indeed accelerate the loss of naïve T cells observed in the elderly population due to consistent recruitment of naïve T cells to control the persistently replicating viruses and, as such, contribute to immunosenescence.

1.5.3 Increased susceptibility of the aged to emerging infections

The most prominent feature of immunosenescence is a progressive loss of naïve T cells [253]. Naïve T cells are critical for the generation of adaptive immune responses to *de novo* pathogens and subsequently their loss is correlated with decreased functional immunity to new infections in the aging population [242,243,261]. For example, when a novel coronavirus, severe acute respiratory syndrome (SARS), appeared in 2002, it affected people of all ages but increased mortality was observed in persons over 50 years of age [271]. This increase in disease related morality was correlated with age-related defects in the immune system and inability to contain the replicating virus [272]. Similarly, the emergence of WNV in the Northern hemisphere presented a novel infection

with an increased prevalence and disease severity in the aging population. It was estimated that humans above the age of 50 had a twenty-fold increased risk for development of neuroinvasive disease [86]. Mouse studies of WNV infection, also demonstrated that aged mice succumbed to infection at a greater rate then the young animals [262,273]. Protective WNV immune response in mice was correlated with functional virus-specific CD8+ T cells capable of perforin release, cytokine production and migration into the brains of infected animals [70,108,114,274]. Currently no studies have been performed to address the question of immunosenescence and susceptibility to severe WNV infection in the elderly human population. However, considering the age-associated changes in the immune system it is conceivable that the immune response to this newly emergent virus is dysfunctional in the elderly and, as such, predisposes this population to infectious diseases and related disease severity.

Collectively, emerging and re-emerging infectious pathogens present a significant disease burden in the elderly, which are believed to result from inadequate immune protection. Considering the fact that the global population of the elderly is on a steady rise, it is critical that we develop a better understanding of immunesenescence to enable the development of improved vaccination strategies for this age group to prevent pathogen spread and reduce disease severity.

1.6 Scope of thesis research

CD8+ T cells provide critical immune control that limit viral infections and clinical disease to intracellular pathogens [163,198,220]. The elevated incidence of infections and related diseases in the elderly population implies that age-associated changes within the immune system underlie an impaired ability to control pathogens. West Nile virus was recognized in North America for the first time in 1999, and has since, spread across the Northern hemisphere infecting over 2.5 million people presenting the greatest disease burden in the elderly [71]. As such, WNV infection provides a great opportunity to study the development of immune responses to a novel virus in the elderly.

1.6.0 Research objectives

The goal of my PhD research was to provide a better understanding of the effects of aging on the development of memory CD8+ T cell responses to newly acquired and persistent viral infections in a cohort of naturally-infected humans. The work described in the results chapters of my thesis pertains to the following research objectives:

Objective 1 Identify WNV-specific CD8+ T cell epitopes using three different epitope mapping techniques **(Chapters 2, 3, 4)**. Investigate the distribution of WNV-specific CD8+ T cell epitopes across the viral polyprotein and their reactivities in the patient cohort **(Chapter 2)**. The results of the objective were published in:

> Parsons R, Lelic A, Hayes L, Carter A, Marshall L, Evelegh C, Drebot M, Andonova M, McMurtrey C, Hildebrand W, Loeb MB, Bramson JL. The memory T cell response to West Nile virus in symptomatic humans following natural infection is not influenced by age and is dominated by a restricted set of CD8+ T cell epitopes. J Immunol. 2008 Jul 15;181(2):1563-72.
>
> McMurtrey CP, Lelic A, Piazza P, Chakrabarti AK, Yablonsky EJ, Wahl A, Bardet W, Eckerd A, Cook RL, Hess R, Buchli R, Loeb M, Rinaldo CR, Bramson J, Hildebrand WH. Epitope discovery in West Nile virus infection: Identification and immune recognition of viral epitopes. Proc Natl Acad Sci U S A. 2008 Feb 26;105(8):2981-6.
>
> Larsen MV, Lelic A, Parsons R, Nielsen M, Hoof I, Lamberth K, Loeb MB, Buus S, Bramson J, Lund O. Identification of CD8+ T cell epitopes in the West Nile virus polyprotein by reverse-immunology using NetCTL. PLoS One. 2010 Sep 14;5(9):e12697.

Objective 2 Characterize the influence of age on the development of polyfunctional virus-specific memory CD8+ T cell responses to WNV, a model of a novel and acute viral infection, and CMV and EBV, common persistent viral infections, within a cohort of WNV-naturally infected subjects **(Chapter 5)**. These results were summarized in the following manuscript:

> Lelic A, Verschoor CP, Ventresca M, Parsons R, Evelegh C, Bowdish D, Betts MR, Loeb MB, Bramson JL. The polyfunctionality of human memory CD8+ T cells elicited by acute and chronic virus infections is not influenced by age. PLoS Pathog. 2012;8(12):e1003076.

— CHAPTER 2 —

THE MEMORY T CELL RESPONSE TO WEST NILE VIRUS IN SYMPTOMATIC HUMANS FOLLOWING NATURAL INFECTION IS NOT INFLUENCED BY AGE AND IS DOMINATED BY A RESTRICTED SET OF CD8+ T CELL EPITOPES

The memory T cell response to West Nile virus in symptomatic humans following natural infection is not influenced by age and is dominated by a restricted set of CD8+ T cell epitopes

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Preface: The data presented in this manuscript were generated by Robin Parsons, a laboratory technician in our lab, and myself. I processed many of the blood samples collected from WNV-infected subjects and isolated PBMCs that were cryopresereved and stored. I carried out the experiments to identify WNV reactive peptides (epitope mapping) using the overlapping peptide library and subsequently identified the corresponding CD8+ T cell minimal epitopes. Furthermore, the analysis of T cell reactivities following WNV peptide stimulations were performed by me, using the ELISPOT assays. Specifically, I contributed much of the data that were used to generate Figures 2, 3, 5, 7 and 8. Dr. Bramson prepared the manuscript, supervised my work and provided experimental guidance to the interpretation of the results.

The Memory T Cell Response to West Nile Virus in Symptomatic Humans following Natural Infection Is Not Influenced by Age and Is Dominated by a Restricted Set of CD8$^+$ T Cell Epitopes[1]

Robin Parsons,* Alina Lelic,* Lisa Hayes,* Alexandra Carter,* Laura Marshall,* Carole Evelegh,* Michael Drebot,† Maya Andonova,† Curtis McMurtrey,‡ William Hildebrand,‡ Mark B. Loeb,* and Jonathan L. Bramson[2]*

We examined the West Nile virus (WNV)-specific T cell response in a cohort of 52 patients with symptomatic WNV infections, including neuroinvasive and non-invasive disease. Although all virus proteins were shown to contain T cell epitopes, certain proteins, such as E, were more commonly targeted by the T cell response. Most patients exhibited reactivity toward 3–4 individual WNV peptides; however, several patients exhibited reactivity toward >10 individual peptides. The relative hierarchy of T cell reactivities in all patients showed a fixed pattern that was sustained throughout the 12-mo period of the current study. Surprisingly, we did not observe any relationship between age and either the breadth or magnitude of the T cell response following infection. We also did not observe a relationship between disease severity and either the breadth or magnitude of the T cell response. The T cell epitopes were distributed in a non-random fashion across the viral polyprotein and a limited number of epitopes appeared to dominate the CD8$^+$ T cell response within our cohort. These data provide important new insight into the T cell response against WNV in humans. *The Journal of Immunology,* **2008, 181: 1563–1572.**

West Nile virus (WNV)[3] emerged in North America as a significant human pathogen following an outbreak in New York in 1999. Since that time, annual outbreaks of WNV have occurred across the continent. In the U.S., there were 4,256 documented cases of WNV infection in 2006 and ~24,000 cases and 1,000 fatalities have been reported since 1999 (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm). Approximately 20% of those infected with WNV develop symptoms, which can range from "West Nile fever," characterized by symptoms including headaches, myalgias, rash, and fever, to neuroinvasive disease including meningitis, encephalitis, and acute flaccid paralysis. Since it has been estimated that <1% of all WNV infections produce neurological complications, many cases of WNV infection go unnoticed or are ascribed to other causes. As such, WNV likely infects a much broader population than can be appreciated by the clinical reports. As an example, although there were only 9,862 documented reports of clinical infection with WNV in the U.S. during 2003, surveillance data from blood donor screening programs estimated that there may have been as many as 735,000 infections in that same period, the majority of which went undiagnosed (1). Given the burden of WNV infection, greater understanding of the pathobiology of this infection is necessary to develop preventive and therapeutic strategies.

T cells play a major role in controlling virus infections and data from murine models support an important role for both CD8$^+$ and CD4$^+$ T cells in the resolution of WNV infection. CD8$^+$ T cells control viremia following infection with WNV and mediate clearance of the virus from the CNS (2–7). CD4$^+$ T cells are also necessary in these processes through their function as helpers for B cell and CD8$^+$ T cell development (8). Although most reports from murine models support a protective role for CD8$^+$ T cells during WNV infection, evidence from studies with the Lineage II Sarafend strain indicates that CD8$^+$ T cells may also contribute to immunopathology in the CNS (3). Characterization of T cell immunity in naturally infected patients who experienced mild and severe illness following WNV infection will provide new insight into the potential role of T cells in disease outcome and pathology. Such information may also be of value to other flavivirus infections, such as dengue, where T cells have similarly been associated with protection and immunopathology (9, 10).

Advanced age is a key risk factor for the development of severe pathology following WNV infection (11). Although the mechanisms that underlie this age-associated outcome are unknown, it is quite possible that immune deficits are the cause. Immune senescence is a well-described phenomenon where the innate and adaptive immune systems show progressive impairment with age (12). These immunological impairments manifest themselves as reduced

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[3] Abbreviations used in this paper: WNV, West Nile virus; cRPMI, complete RPMI 1640; SFC, spot forming cell.

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Table I. *Details of patient characteristics*[a]

ID	Sex	Age	Diagnosis	Date of Onset	Date of First Draw	WNV PRNT	Dengue PRNT	SFC/10^6 at 3–4 mo	No. Reactive Minipools	Dominant Pool
05001	M	82	neuro	8/28/2003	9/18/2004			870	5	E-22
05201	M	39	non-neuro	9/6/04	11/25/2004			260	3	E-22
07001	M	66	neuro	9/13/2003	9/22/2003			1180	2	M-6
08001	F	73	neuro	9/11/03	10/1/03				2	E-1
08201	M	54	neuro	9/24/2004	10/26/2004			1540	4	E-1
09001	M	82	neuro		10/10/03				7	E-1
10201	F	45	neuro		8/9/04				6	NS5–11
10202	M	32	neuro	8/6/04	9/2/04			3240	5	E-1
11201	M	64	neuro	9/7/04	10/20/2004			250	4	M-6
44301	M	69	neuro	8/9/05	9/13/2005	>1:160	neg		4	NS3–19, NS5–27
44302	M	42	neuro	8/7/05	9/27/05	1:160	neg	460	4	E-20, NS4B-11
44303	M	45	non-neuro	8/22/05	10/11/05			590	6	NS2A-2, NS4B-1
55301	F	42	non-neuro	6/18/2005	9/6/05	>1:160	neg	220	3	M-6
55302	M	65	neuro	8/3/05	8/25/2005	≥1:160	neg	7100	8	E-22, M-6
55303	F	53	non-neuro	8/7/05	9/2/05	>1:160	neg	350	2	E-1
55304	M	34	non-neuro	7/30/2005	9/2/05	>1:160	neg	270	4	NS3–19
55305	F	49	non-neuro	8/7/05	9/7/05	≥1:160	neg		3	E-22, NS5–17
55306	M	48	non-neuro	8/8/05	9/12/05	>1:160	neg		4	E-22, NS4B-1
55307	F	55	non-neuro	8/7/05	9/7/05	1:160/320	neg	320	2	M-6
55309	F	64	non-neuro	8/14/2005	9/14/2005	1:160	neg	660	12	E-22
55310	F	64	non-neuro	8/21/05	9/16/05	>1:160	neg		7	M-6
60001	M	71	neuro		10/22/2003	1:80/160	neg		7	E-22
66301	F	50	neuro	8/8/05	9/1/05	1:80	neg	330	4	NS2A-2
77302	M	29	neuro	8/1/05	8/25/05	1:160	neg		11	NS3–19
77303	F	57	neuro	8/13/2005	8/26/2005	1:160	neg			
77304	F	30	neuro	8/1/05	8/29/2005	1:80/160	1:160/320	1990	3	NS3–19, NS5–10
77305	M	43	non-neuro	8/22/2005	9/2/05	1:640	1:160/320			
77306	M	55	non-neuro	8/15/2005	9/6/05	1:160	1:640	120	4	NS2A-7
77307	F	55	neuro	8/15/2005	9/6/05					
77308	F	42	neuro	8/8/05	9/12/05	1:80	neg			
77309	F	40	non-neuro	8/9/05	9/5/05			450	5	NS2A-6
77310	M	40	non-neuro	8/8/05	9/13/2005	1:80	neg	510	6	M-6, NS3–19
77311	M	64	neuro	8/17/2005	9/9/05	≥1:160	neg	470	8	E-1
77312	F	45	neuro	9/1/05	9/26/05	1:80	neg			
77313	F	55	neuro	8/18/2005	9/15/2005	1:40/80	neg	2170	6	NS2A-7
77315	F	41	neuro	8/22/2005	9/15/2005	>1:160	neg	470	8	NS3–19, NS4B-13
77316	M	45	non-neuro	9/2/05	9/19/05	1:80	neg	3890	4	E-22
77317	M	55	non-neuro	9/1/05	9/26/05	>1:160	neg		10	M-6
77318	F	65	neuro	8/21/05	9/22/05	1:160	neg			
77319	F	41	non-neuro	7/31/05	9/26/05	>1:160	neg	980	4	E-22
77320	F	49	neuro	8/23/05	9/28/05	>1:160	neg			
77321	F	40	non-neuro	9/7/05	10/3/05	1:1280	1:160/320			
77322	F	55	non-neuro	9/19/05	10/5/05	1:160	neg	150	5	E-22, NS2A-6
77323	F	30	non-neuro	9/3/05	10/19/05	1:80/160	neg		2	E-1
77324	M	39	non-neuro	9/3/05	10/19/05	>1:160	neg		3	E-22, C-2
77325	F	46	neuro	8/18/05	10/19/05	≥1:160	1:40			
77326	F	59	non-neuro	9/7/05	11/3/05	1:80	1:40		7	NS5–17
77327	M	47	non-neuro	8/14/05	10/25/05	>1:160	1:40	230	2	NS4B-1
77328	F	50	non-neuro	9/15/05	11/15/05	≥1:160	neg		3	E-22, NS4B-1
77329	M	77	non-neuro	8/29/05	10/25/05	>1:160	neg		4	NS3–19, NS4A-3
77330	F	45	non-neuro	7/19/05	10/26/05	>1:160	1:40/80			
77331	M	57	non-neuro	9/30/05	10/31/05	>1:160	neg			
77332	F	60	neuro	6/?/05	11/10/05	1:80	neg			

[a] This table contains key gender and age characteristics for the patients in our study. The table also contains information regarding the day of disease onset and first sampling of PBMC following accrual to our study. PRNT results for WNV and dengue Abs are shown (neg = seronegative). The magnitude of the T cell response as reflected by the total number of SFC at 3–4 mo is shown. Similarly, we have included information regarding the number of minipools to which the patient exhibited positive reactivity and which minipool dominated the T cell response.

responsiveness to vaccination and increased susceptibility to infection. With regard to T cell-mediated immunity, it is believed that the ability to respond to new pathogens declines with age due to reduced frequencies of naive T cells that results from a number of age-related changes including decreased thymic output (13) and oligoclonal expansions of memory T cells (14, 15). In consideration of the important role of T cells in virus clearance from the CNS (2–8), it is possible that the increased incidence of neurological complications following WNV infection in older individuals may be related to lack of sufficient T cell immunity to control virus in the CNS.

To date, there have been no published reports of T cell immunity to WNV in humans. In the current manuscript, we describe changes in T cell immunity within the peripheral blood of a cohort of 52 symptomatic WNV-infected patients using a comprehensive approach that permits the examination of most, if not all, epitopes present within the viral polyprotein. Our patient population included individuals with both mild (no neurological complications) and severe disease (encephalitis, meningitis, meningoencephalitis, and acute flaccid paralysis) ranging in age from 29 to 82 years, allowing us to explore the relationship between age, disease pathology, and T cell immunity.

A Set-up of mini-pools (example)

Mini-Pool	C-1	C-2	C-3	C-4	C-5
Peptides	C#1, C#2, C#3, C#4, C#5, C#6	C#7, C#8, C#9, C#10, C#11, C#12	C#13, C#14, C#15, C#16, C#17, C#18	C#19, C#20, C#21, C#22, C#23, C#24	C#25, C#26, C#27, C#28, C#29, C#30, C#31, C#32

B Set-up of large pools (example)

Pool	A	B	C	D	E	F
1	C-1	C-2	C-3	C-4	C-5	E-1
2	E-2	E-3	E-4	E-5	E-6	E-7
3	E-8	E-9	E-10	E-11	E-12	E-13
4	E-14	E-15	E-16	E-17	E-18	E-19
5	E-20	E-21	E-22	E-23	E-24	M-1
6	M-2	M-3	M-4	M-5	M-6	M-7

FIGURE 1. The library was aliquoted into pools to simplify analysis and examples of the pooling procedure are given in this figure. *A*. Five minipools were prepared that contained all 32 overlapping peptides spanning C. The peptides contained in each minipool are listed. Comparable minipools were prepared for all the WNV protein. *B*. The minipools were arranged in a 2-dimensional matrix as shown. Larger pools were prepared that contained the minipools to permit screening of a large number of peptides. Stimulatory minipools were identified by coincident reactivity to two larger pools each carrying the specific minipool. As an example, Pool A contained minipools C-1, E-1, E-8, E-14, E-20, and M-2, and Pool 1 contained minipools C-1, C-2, C-3, C-4, C-5, and E-1. If a given patient sample showed reactivity to pools A and 1, then the stimulatory peptide was most likely contained with minipool C-1.

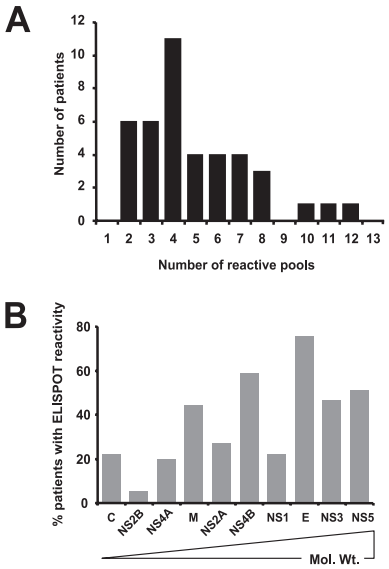

FIGURE 2. All the patients in our study displayed reactivity to at least two separate minipools and no patient exhibited reactivity to more than 12 minipools. *A.* This histogram represents the number of patients with reactivity to 2, 3, 4, etc. . . minipools. *B.* ELISPOT results were clustered based on individual proteins and the frequency of reactivity to specific proteins is shown. The proteins have been arranged on the *x*-axis in ascending order by molecular mass.

Materials and Methods

Cell culture materials

All plasticware used for cell culture was purchased from Falcon. RPMI 1640 powder was purchased from Invitrogen and prepared under sterile conditions at McMaster University. Cells were cultured in complete RPMI 1640 (cRPMI) consisting of RPMI 1640 with 10% FBS, 2 mM L-glutamine, 50 μM 2-ME, 10 μM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. *Staphylococcus* enterotoxin B was purchased from Toxin Technologies and used for T cell stimulation at a final concentration of 0.5 μg/ml.

Patients and PBMC preparation

Fifty-two patients were enrolled into the study following detection of serum WNV IgM by public health laboratories after presenting with symptoms of WNV infection. This trial was reviewed and approved by the Research Ethics Board at McMaster Univeristy. Patients were recruited into this study over three seasons (2003–2005). Serology for WNV and dengue virus was assessed by plague reduction neutralization test as described previously (16). In general, patients were entered into our study within 1 mo following the onset of symptoms (median = 28 days; ranging from 9 to 99 days post-onset of symptoms). In brief, the population consisted of 27 females and 26 males with an average age of 51.8 years (median = 49 years; ranging from 29 to 82 years). Details of the patient characteristics are available in Table I. HLA genotypes were determined using DNA sequence analysis at the Hamilton Health Sciences Histocompatibility Laboratory (Hamilton, ON) and Pure Transplant Solutions (Austin, TX). Blood samples were drawn into heparanized tubes once the patients were enrolled in our study and monthly thereafter for the 12 mo of the study. PBMC were isolated from the blood samples by centrifugation on Ficoll (Amersham Pharmacia) and cryopreserved in RPMI 1640 containing 12.5% human serum albumin (Sigma-Aldrich) and 10% DMSO according to the method described by Disis et al. (17).

Peptides

The sequences for a library of 847 15-mer overlapping peptides spanning the full WNV polyprotein were generated by the PeptGen application (www.hiv.lanl.gov/content/sequence/PEPTGEN/Peptgen.html). Separate peptide sets were produced for each individual protein (C, E, M, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The peptides were generated with 11 residues overlapping for all proteins except NS3, where the overlap was 10 residues, and NS5, where the overlap was 9 residues. We also generated a peptide set covering the C-terminal/N-terminal junctions between the individual viral proteins in the event that epitopes might be generated from the polyprotein before processing (termed the junction pool). A library comprising 91 MHC class I epitopes from EBV with broad

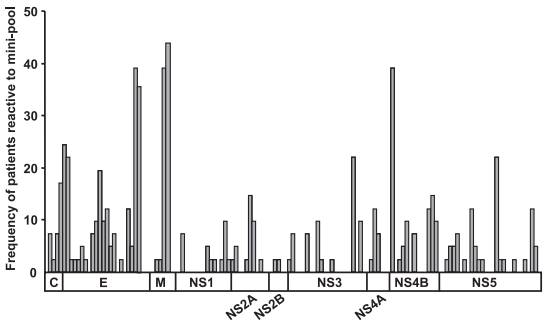

FIGURE 3. Reactivity was confirmed by stimulation of PBMC with individual minipools and the frequency of specific reactivities within our patient cohort ($n = 41$) is shown. Each bar represents a single minipool and the minipools have been arranged on the *x*-axis based on their relative position in the WNV polyprotein (shown as the *x*-axis).

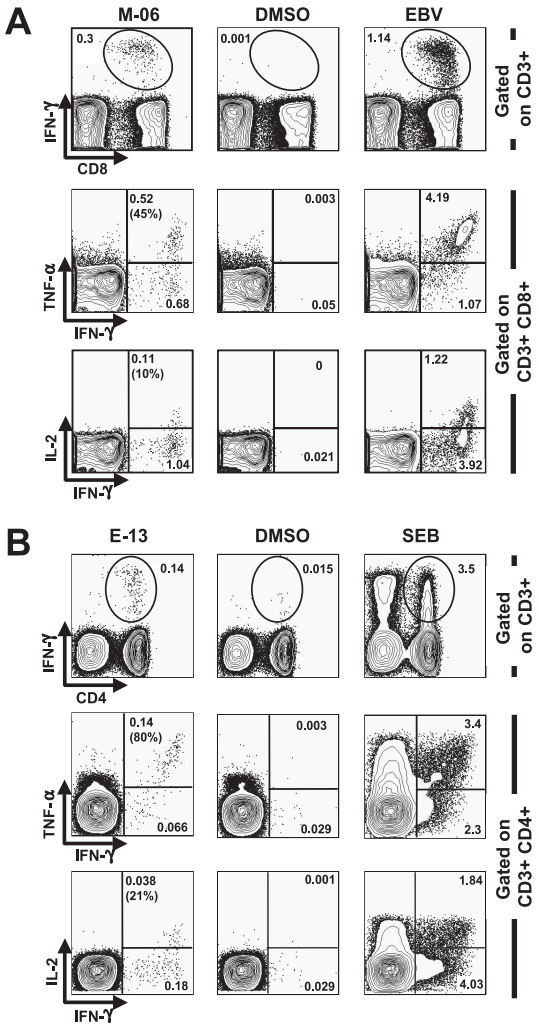

FIGURE 4. Both $CD8^+$ and $CD4^+$ T cells were identified in our screens. *A*, PBMC from patient 11201 were restimulated with minipool M-6, DMSO (negative control), or a pool of defined $CD8^+$ T cell epitopes from EBV (positive control). The samples were subsequently stained with Abs to CD3, CD8, CD4, IFN-γ, TNF-α, and IL-2 as described in *Materials and Methods*. *Top row*, The data was gated on live cells and CD3. The number in the left-hand corner reflects the % IFN-γ^+CD8^+ cells of total $CD3^+$ cells (shown by the elliptical gate). *Middle row*, The data was gated on live cells, CD3 and CD8. The number in the upper, left-hand quadrant is the % IFN-γ^+TNF-α^+ cells of total $CD8^+$ T cells; the bracketed number in the upper, left-hand quadrant is the % IFN-γ^+TNF-α^+ cells of total IFN-γ^+ cells; the number in the lower, left-hand quadrant is the % IFN-γ^+TNF-α^- cells of total $CD8^+$ T cells. *Bottom row*, The data was gated on live cells, CD3 and CD8. The number in the upper, left-hand quadrant is the % IFN-γ^+IL-2^+ cells of total $CD8^+$ T cells; the bracketed number in the upper, left-hand quadrant is the % IFN-γ^+IL-2^+ cells of total IFN-γ^+ cells; the number in the lower, left-hand quadrant is the % IFN-γ^+IL-2^- cells of total $CD8^+$ T cells. *B*, PBMC from patient 77313 were restimulated with minipool E-13, DMSO (negative control), or *Staphylococcus* enterotoxin B (positive control). The samples were subsequently stained with Abs to CD3, CD8, CD4, IFN-γ, TNF-α, and IL-2 as described in *Materials and Methods*. *Top row*, The data was gated on live cells and CD3. The number in the left-hand corner reflects the % IFN-γ^+CD4^+ cells of total $CD3^+$ cells (shown by the elliptical gate). *Middle row*, The data was gated on live cells, CD3 and CD4. The number in the upper, left-hand quadrant is the % IFN-γ^+TNF-α^+ cells of total $CD4^+$ T cells; the bracketed number in the upper, left-hand quadrant is the % IFN-γ^+TNF-α^+ cells of total IFN-γ^+ cells; the number in the lower, left-hand quadrant is the % IFN-γ^+TNF-α^- cells of total $CD4^+$ T cells. *Bottom row*, The data was gated on live cells, CD3 and CD4. The number in the upper, left-hand quadrant is the % IFN-γ^+IL-2^+ cells of total $CD4^+$ T cells; the bracketed number in the upper, left-hand quadrant is the % IFN-γ^+IL-2^+ cells of total IFN-γ^+ cells; the number in the lower, left-hand quadrant is the % IFN-γ^+IL-2^- cells of total $CD4^+$ T cells.

HLA coverage was prepared as a positive control for $CD8^+$ T cell reactivity (described by Bihl et al. (18)). All of the library peptides were synthesized by PepScan Systems (Lelystad) at 70% purity, resuspended in DMSO, and stored at −20°C.

We also produced a series of 10-mer and 9-mer peptides to define the minimal epitopes for library peptides E#4, M#32, NS3#113, and NS4B#5. The sequences of these peptides are listed in the text and the peptides were produced by Biomer Technologies.

ELISPOT

IFN-γ ELISPOTs were performed using kits purchased from BD Biosciences and conducted according to the manufacturer's instructions. PBMC were thawed and placed immediately into cRPMI prewarmed to 37°C. The cells were aliquoted into the ELISPOT plate at 1–2 × 10^5 cells/well and peptides were added at a final concentration of 2 μg/ml per peptide. The plates were incubated for 18–20 h at 37°C in a humidified incubator with 5% CO_2 and the assay was completed according to the manufacturer's directions. Spots were enumerated using an ImmunoSpot 3B analyzer (Cellular Technology). Positive reactivity was defined as responses that were at least 2-fold above background and a minimum of 50 spot forming cells (SFC)/10^6 cells.

The ELISPOT method was used as the preliminary screen to demonstrate T cell reactivity. To facilitate our analyses, the peptides were grouped into minipools of six consecutive peptides (see Fig. 1*A*) spanning a region of ~40 amino acids that we reasoned would most likely contain only a single epitope (although we cannot rule out the possibility of multiple epitopes within these minipools). We subsequently grouped the minipools into larger pools (six minipools per larger pool) using a 2-dimensional matrix as shown in Fig. 1*B*. This enabled us to screen the entire WNV polyprotein using 47 pools. Coincident reactivity between two large pools identified candidate minipools containing putative T cell epitopes. T cell reactivity was subsequently validated by restimulation of PBMC from the same patients with individual minipools. In some circumstances, we further deconvoluted the minipools by examining reactivity to individual peptides. To avoid confusion in nomenclature between the individual peptides and the minipools, we have used the number sign "#" between the protein name and the peptide number for individual peptides and we have used a hyphen "-" between the protein name and pool number for the minipools. Therefore, the first peptide in the set of overlapping peptides spanning C is named C#1, the second peptide is C#2, etc. . . The minipool containing C#1, C#2, C#3, C#4, C#5, and C#6 is named C-1 (see Fig. 1*A*).

Intracellular cytokine staining

PBMC were thawed and placed immediately into cRPMI prewarmed to 37°C and cultured overnight in cRPMI at 37°C. The cells were subsequently harvested, counted, and viability was assessed by trypan blue exclusion. Cells were aliquoted (1–2 × 10^6 cells/tube) into Falcon 2057 tubes, peptides were added to a final concentration of 2 μg/ml, anti-CD28 and anti-CD49d (BD Pharmingen) were added to a final concentration of 1 μg/ml, and the cells were incubated for 2 h. Brefeldin A was then added to a final concentration of 5 μM and the cells were incubated 4 h further. At the end of this period, cells were pelleted and washed in 10 μM EDTA. The cells were subsequently surface stained with either anti-CD8-PE-Cy7 or anti-CD4-PE-Cy7 and anti-CD3-PE-Cy5, permeabilized with Cytofix/Cytoperm, and intracellular cytokines were identified using anti-IL-2-FITC, anti-TNF-α-PE, and anti-IFN-γ-allophycocyanin [Note: all flow cytometry reagents were obtained from BD Pharmingen]. In some cases, samples were stained with both anti-CD8-PE-Cy7 and anti-CD4-allophycocyanin-Cy7. Fluorescence data were acquired using a FACSCanto and 200,000 events based on the live lymphocyte gate were collected per sample.

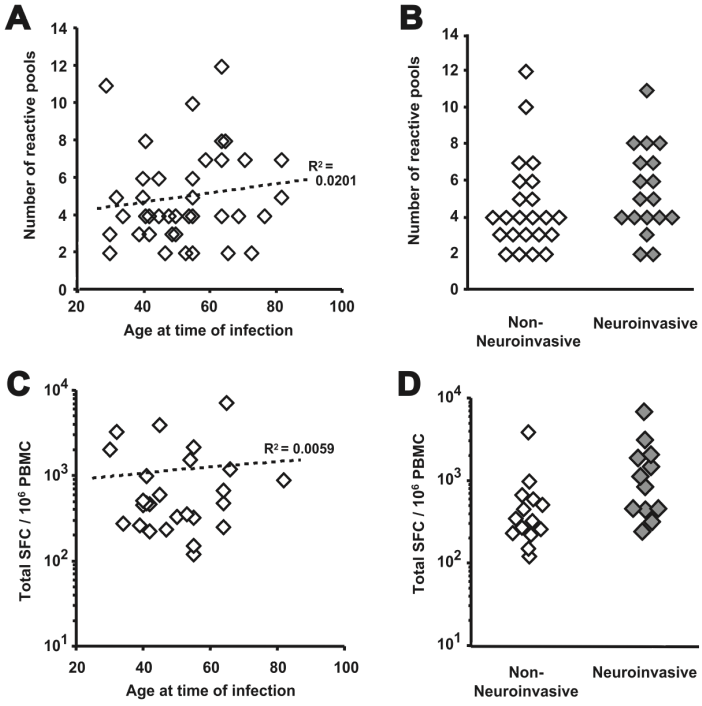

FIGURE 5. The breadth and magnitude of the T cell response does not correlate with age or disease pathology. *A*, The scattergram reflects the number of minipools recognized by individual patients as a function of their age. Each point represents a single patient. The dotted line represents a linear regression curve modeled on the data. *B*, The scattergram reflects the number of minipools recognized by individual patients separated into groups defined by disease severity. Each point represents a single patient. *C*, The scattergram reflects the magnitude of the T cell response at 3–4 mo post-onset of symptoms defined as the total number of SFC as a function of age. Each point represents a single patient. The dotted line represents a linear regression curve modeled on the data. *D*, The scattergram reflects the magnitude of the T cell response at 3–4 mo post-onset of symptoms defined as the total number of SFC separated into groups defined by disease severity. Each point represents a single patient.

Statistical analysis

The data are presented as mean ± SEM. All statistics (SEM, Students *t* test and regression analysis) were calculated using Microsoft Excel 2004 for Mac.

Results

Characterization of the T cell response to WNV

The design of the ELISPOT pools is shown in Fig. 1. Using this strategy, we were able to screen the entire WNV polyprotein on a single 96-well ELISPOT plate. We observed that the results of this screen were highly reproducible and stable over time. A total of 15 patients were screened with the full library using 2 different samples obtained at least 30 days apart (data not shown). For 12 patients, the pattern of reactivity was the same at both time points. For the remaining three patients, the reactivity measured with the later samples was too weak to compare with the data from the earlier time point. In no case did we observe a change in the pattern of reactivity. In general, we

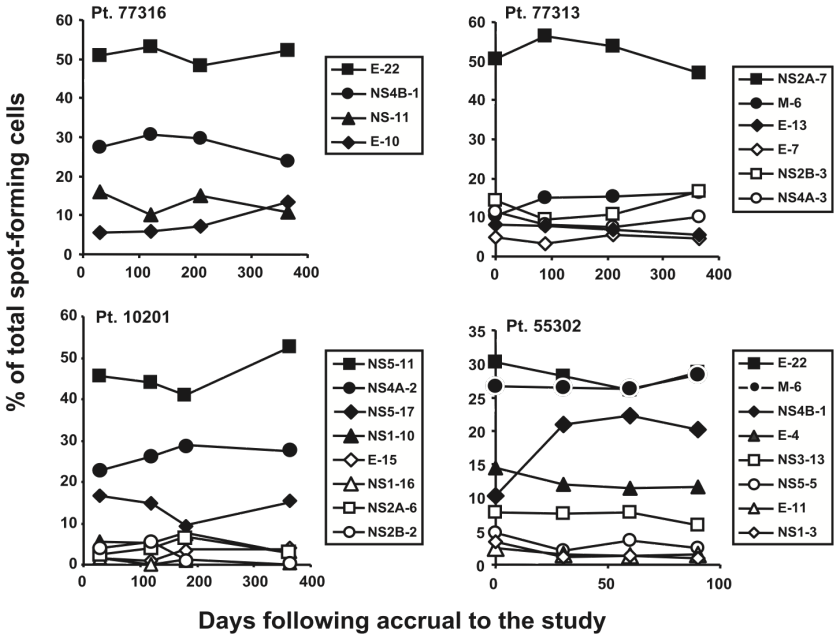

FIGURE 6. The hierarchy of dominant T cell responses remains stable for at least 1 year following WNV infection. PBMC from patients 77316, 77313, 10201, and 55302 were isolated at various times post-onset of symptoms and restimulated with individual minipools identified by screening with the entire polyprotein library. The data presented represent the proportion of SFC stimulated by each individual minipool relative to the total number of SFC produced by all the minipools combined. Each data point represents a specific time point, and the "% of total SFC" is calculated based on the total number of SFC at that specific time point. Each symbol reflects an individual minipool as defined by the legend associated with the panel.

observed the strongest ELISPOT results with the samples drawn closest to the onset of symptoms.

Forty-one patients were screened with the entire peptide library. The results were subsequently deconvoluted and individual minipools containing putative epitopes were selected for further screening. Each minipool comprised an average of six consecutive overlapping peptides (described in *Materials and Methods* and Fig. 1), which span a region of 40–50 residues of the polyprotein. All of the patients showed reactivity to at least two independent minipools. The median number of minipools for which a patient showed reactivity was 4 with a range of 2 to 12 (Fig. 2*A*). It should be noted that when a patient showed reactivity to neighboring pools, we counted this as only one reactive pool since, in all cases, we later discovered that the target epitope was shared by both pools (see below).

We found that not all proteins were equally targeted by the T cell response (Fig. 2*B*). Peptides from the E protein produced reactivity in the highest frequency of patients as almost 80 of the population exhibited some responsiveness against E. By contrast, NS2B was the protein least frequently targeted by the T cell response and only 2 of the 41 patients displayed some reactivity to NS2B. Although it appears that protein size may be related to immunogenicity, other factors must also be responsible (Fig. 2*B*). For example, NS4B, which is 255 residues long, elicited responses in a greater number of individuals than NS1 (355 aa), NS3 (619 aa), and NS5 (905 aa) (Fig. 2*B*). Likewise, the M protein, which is only 167 residues in length, elicited immunity in a comparable number of individuals as NS3 and NS5, which are 3.5 and 4.5 times larger (Fig. 2*B*).

When we examined reactivities to individual minipools, we observed two things: 1) there were many regions of the polyprotein that did not produce any reactivity in the ELISPOT assay and 2) several minipools were stimulatory in an unexpectedly high proportion of individuals (Fig. 3) [NOTE: throughout these studies, we observed that patients reactive to minipool E1 were also reactive to minipool E-2. Likewise patients reactive to E-21 were reactive to E-22 and patients responsive to M-5 were also responsive to M-6. Therefore, for the remainder of the text, we refer to these pools as E-1/-2, E-21/-22, and M-5/-6]. E was clearly the most immunogenic protein as three separate minipools (E-1/-2, E-11, E-21/-22) were stimulatory in >20% of the patients. In all other cases of common reactivity, only a single minipool from any of the other proteins was stimulatory in >20% of the individuals (C-5, M-5/-6, NS3–19, NS4B-1, and NS5-17). Strikingly, we identified minipools in E, M, and NS4B (E-21/-22, M-5/-6, and NS4B-1, respectively) that were stimulatory in ~40% of the individuals that we tested (Fig. 3).

The T cells that were responsive to E-21/-22, M-5/-6, and NS4B-1 were determined to be $CD8^+$ using flow cytometry (an example of the flow cytometry data is shown in Fig. 4*A*). In fact, most of the minipools identified by the ELISPOT assay contained $CD8^+$ T cell epitopes (data not shown), although we did identify minipools containing $CD4^+$ T cell epitopes (an example of the flow cytometry is shown in Fig. 4). The $CD4^+$ T cells differed from the $CD8^+$ T cells in terms of their cytokine production. At the time points shown (1–3 mo following onset of symptoms), only a fraction of the IFN-γ-secreting $CD8^+$ T cells also produced TNF-α (<50%) and fewer still produced IL-2 (<10%) (Fig. 4*A*, *middle* and *lower rows*). By contrast, most of the IFN-γ-secreting $CD4^+$ T cells produced TNF-α (>80%) and a larger fraction produced IL-2 (20%) (Fig. 4*B*, *middle* and *lower rows*). Thus, we can distinguish between $CD8^+$ and $CD4^+$ T cells based on both phenotype and cytokine production at early time points. At later time points (6 mo following infection and beyond), the West Nile-specific $CD8^+$ T cells secreted high levels of both IFN-γ and TNF-α, although they remained poor producers of IL-2 (data not shown).

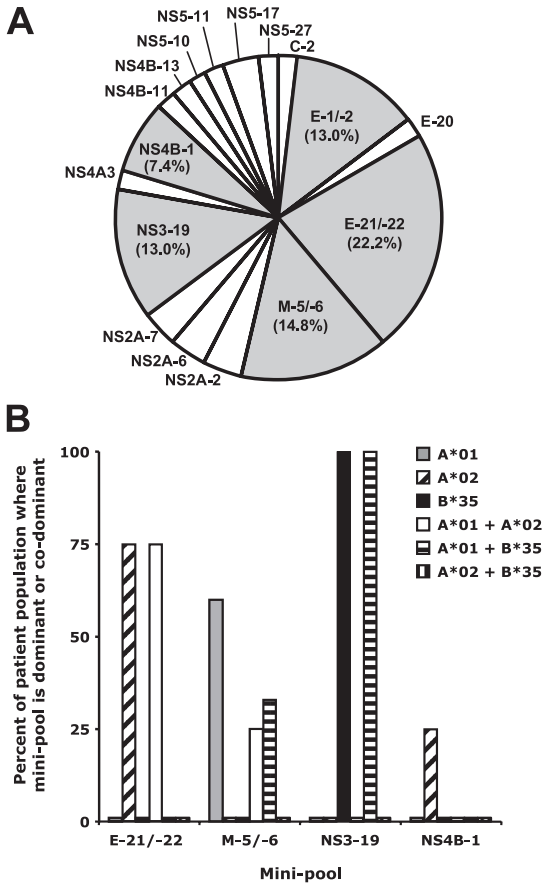

FIGURE 7. The pool of dominant $CD8^+$ T cell epitopes from WNV is composed of a restricted set of epitopes. *A*, The pie chart shows the relative frequency of a specific minipool being dominant within the cohort of 41 patients who were screened for reactivity to the entire polyprotein. Two minipools were considered codominant if the number of SFC produced by the two individual minipools were within 10% of each other. The gray shading reflects the five most commonly dominant minipools. *B*, The frequency of specific dominant minipools is presented as a function of the patient's HLA. The minipools are listed along the *x*-axis. Gray bars, Patients were HLA-A*01$^+$, HLA-A*02$^-$, HLA-B*35$^-$; diagonally hatched bars, patients were HLA-A*01$^-$, HLA-A*02$^+$, HLA-B*35$^-$; solid bars, patients were HLA-A*01$^-$, HLA-A*02$^-$, HLA-B*35$^+$; open bars, patients were HLA-A*01$^+$, HLA-A*02$^+$, HLA-B*35$^-$; horizontally hatched bars, patients were HLA-A*01$^+$, HLA-A*02$^-$, HLA-B*35$^+$; vertically hatched bars, patients were HLA-A*01$^-$, HLA-A*02$^+$, HLA-B*35$^+$.

Investigating the influence of age on T cell immunity to WNV

A key hypothesis that we sought to address is whether aging is associated with a decline in the T cell response to WNV infection, which, in turn, leads to increased severity of the illness. Within our cohort, the incidence of neuroinvasive disease was 30% among the patients aged <40 years old, 42% among the patients aged 41–50 years, 50% among the patients aged 51–60 years, and 85% among the patients >61 years old. Despite a clear relationship between age and disease severity, we did not observe any relationship between age and the diversity of T cell response (Fig. 5*A*). Even the

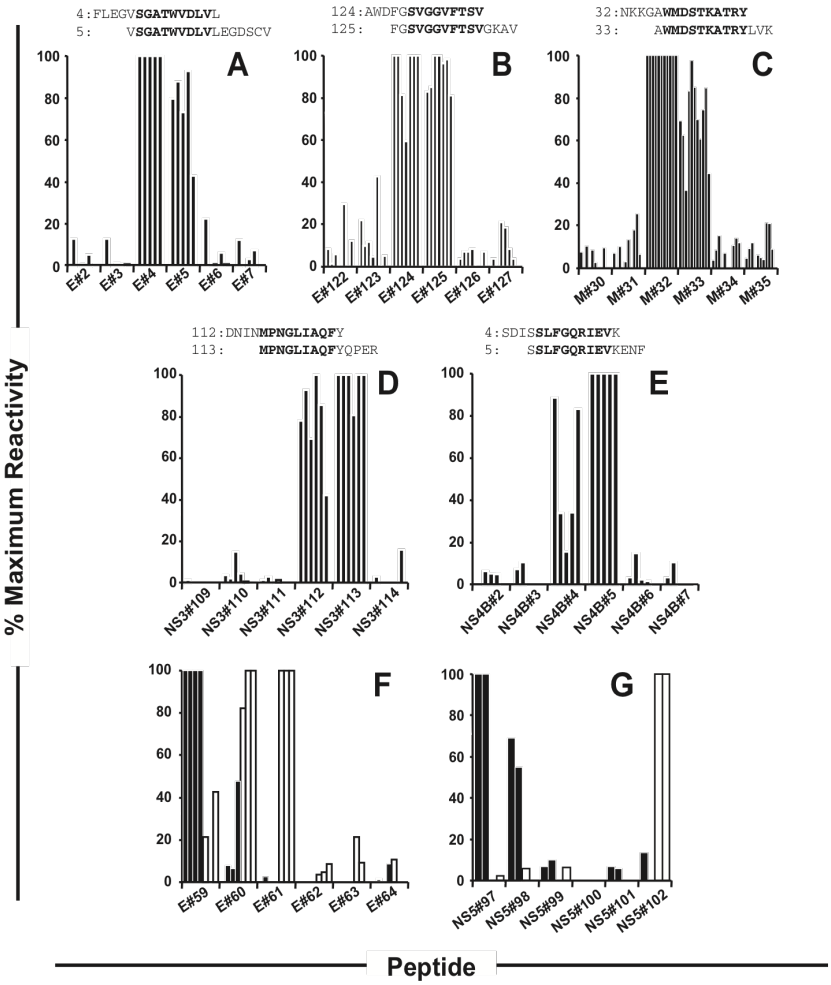

FIGURE 8. Patients with reactivities to dominant minipools were responding to the same peptides. PBMC from patients with reactivity to minipools E-1/-2 (*A*), E-21/-22 (*B*), M-5/-6 (*C*), NS3-19 (*D*), NS4B-1 (*E*), E-11 (*F*), and NS5-17 (*G*) were stimulated with individual peptides from those minipools. The data was normalized to the maximum signal produced by any peptide within the set for an individual PBMC sample. Each bar represents a single patient. Peptides are listed on the *x*-axis. *A–E*, the sequences listed correspond to the two peptides with the greatest stimulatory activity and the bolded letters reflect the minimal epitope as described in Table II. *F* and *G*, two discrete sets of peptides appeared to be stimulatory within each minipool. Open bars and solid bars discriminate between the distinct reactivities.

two most elderly individuals, aged 82 years, were reactive to five and seven distinct minipools (median for the population was four minipools; see Fig. 2). When we modeled these data, the point estimate of the linear regression coefficient was 0.018 with 95% confidence intervals ranging from −0.042 to 0.078. At the extremes of the confidence interval, a slope of −0.042 would represent a negligible reduction in reactive minipools with age, while at the other extreme 0.078 would be mild positive relationship. Likewise, we did not observe any relationship between disease severity and the diversity of the immune response (Fig. 5*B*). We also compared the magnitude of the T cell response to patient age and disease severity. However, this analysis was constrained by the fact that patients were accrued at different times following symptom onset. The earliest time point where we had obtained sufficient samples to carry out this analysis on the majority of our cohort was 3 mo following onset of symptoms. The total number of SFC for a given patient were tallied using samples obtained between 3 and 4 mo following onset of symptoms, and, again, we did not observe any relationship between age and the magnitude of the ELISPOT results (Fig. 5*C*). When we modeled these data, the point estimate of the regression coefficient for SFC was 0.009 and the 95% confidence intervals ranged from −0.037 to 0.056. Similarly, we did not observe a significant difference between patients with West Nile fever and neuroinvasive disease, although we did observe a trend toward greater total reactivity in the patients with neuroinvasive disease (Fig. 5*D*).

Characterization of epitope dominance and HLA-restriction

As expected, we observed a hierarchy of reactivity where a small number of epitope-specific T cell populations dominated the ELISPOT response in individual patients and represented a higher fraction of the total response. Fig. 6 shows an example of the dominance patterns in four individual patients over time. We found that the patterns were stable over the first year following infection for the dominant reactivities. In all cases, the predominant T cell populations that accounted for the highest fraction of the SFC were identified as $CD8^+$ by flow cytometry (data not shown). In some cases, we observed codominance where T cell populations with two distinct reactivities were present at similar frequencies (Fig. 6, *lower right panel*).

Surprisingly, immune dominance was restricted to a limited number of minipools (Fig. 7*A*). Only 18 minipools were dominant among the 41 patients we studied. Strikingly, dominant T cell responses to E peptides were observed in ~40% of the population (Fig. 7*A*) and T cell reactivity to an epitope contained within the E-21/-22 minipools was dominant in approximately one-quarter of

Table II. *Definition of minimal peptide epitopes for the most commonly recognized peptides*[a]

Stimulatory Peptides	Putative Epitope Peptides	Sequence	Reactivity	Restriction
E#4/E#5	E_{14-23}	EGVSGATW	−−	Undetermined
	E_{17-26}	SGATWVDLV	+++	
	E_{16-26}	VSGATWVDLV	++	
	E_{12-21}	FLEGVSGAT	−	
	E_{12-22}	FLEGVSGATW	−	
E#124/E#125	$E_{430-438}$	SVGGVFTSV	+++	HLA-A*02
M#32/M#33	$M_{111-120}$	WMDSTKATRY	+++	HLA-A*01
	$M_{112-120}$	MDSTKATRY	−	
	$M_{111-119}$	WMDSTKATR	−	
	$M_{110-118}$	AWMDSTKAT	−	
NS4B#4/NS4B#5	$NS4B_{17-26}$	FGQRIEVKEN	−	HLA-A*02
	$NS4B_{15-24}$	SLFGQRIEVK	++	
	$NS4B_{15-23}$	SLFGQRIEV	+++	
	$NS4B_{14-23}$	SSLFGQRIEV	+++	
NS3#112/NS3#113	$NS3_{501-509}$	MPNGLIAQF	+++	HLA-B*35
	$NS3_{501-510}$	MPNGLIAQFY	+++	
	$NS3_{500-508}$	NMPNGLIAQ	+	
	$NS3_{502-510}$	PNGLIAQFY	−	
	$NS3_{504-512}$	GLIAQFYQP	−	

[a] Stimulatory peptides were identified as described in Fig. 8. Putative epitopes were identified using predictive algorithms. The numbers in subscript represent the numerical position of amino acid residues within the protein sequence. Reactivity was defined as follows: +++, maximal reactivity; ++, 50–99% of maximal reactivity; +, <50% maximal reactivity; −, no reactivity. HLA restriction was defined based on the predicted binding of the epitope that produced the maximal response and the HLA genotype of the patients who were reactive to the defined epitope. This $E_{430-438}$ epitope was identified by mass spectrometry.

the population. Furthermore, five minipools were responsible for >70% of the dominant reactivities in this population (Fig. 7*A*, gray area). Cumulatively, these results demonstrate that the T cell response following WNV infection is somewhat constrained (Fig. 3) and dominated by a limited number of epitopes (Fig. 7*A*). Samples from an additional 11 patients (making a total of 52 patients) were evaluated for reactivity to the most common minipools (C-5, E-1/-2, E-11, E-21/-22, M5/-6, NS2A-6, NS3-19, NS4B-1, NS4B-11, NS5-10, and NS5-17). The incidence of minipool reactivity in this extended cohort was consistent with the data presented in Fig. 3 (data not shown).

In several cases, we found a clear association between HLA expression and responsiveness to the specific minipools, suggesting strongly that the patients were reactive to a common epitope within these pools. This association revealed an unexpected dominance of minipools E-21/-22 and NS3-19. Reactivity to the E-22 pool was the predominant response in 70% of the HLA-A*02-positive individuals that were tested with the entire library (n = 17; Fig. 7*B*). By contrast, responsiveness to the other pool containing a putative HLA-A*02-restricted epitope, NS4B-1, was found to be dominant in only a small fraction (3/17) of the HLA-A*02-positive individuals. Reactivity to NS3-19 was found to be either dominant or codominant in six of the seven HLA-B*35-positive patients that were tested with the full library (Fig. 7*B*). With respect to the M-5/-6 minipools, reactivity to this minipool dominated in 7 of 16 HLA-A*01-positive patients. Interestingly, reactivity the E-21/-22 and NS3-19 epitopes seemed to dominate over reactivity to M-5/-6 epitope in HLA-A*01-positive patients who also express HLA-A*02 or HLA-B*35 (Fig. 7*B*). Overall, these data demonstrate that the response to WNV appears to be strongly influenced by the HLA of the patient and is dominated by a limited number of $CD8^+$ T cell epitopes.

Identification of the minimal epitopes present in the most commonly reactive minipools

To confirm that a single peptide within the minipools was responsible for the observed T cell reactivity, we examined reactivity to single peptides within the most commonly reactive minipools. For these investigations, we limited our study to minipools that were found to be stimulatory in ≥20% of the study population (E-1/-2, E-11, E-21/-22, M-5/-6, NS3-19, NS4B-1, and NS5-17). Where samples were available, we restimulated PBMC with individual peptides from the respective minipools. For pools E-1/-2, E-21/-22, M-5/-6, NS4B-1, and NS3-19, we determined that each minipool contained a specific stimulatory peptide that was common to all responsive patients (Fig. 8, *A–E*). Interestingly, although minipools E-11 and NS5-17 were recognized by >20% of the total population, we did not find that a single peptide was responsible for this reactivity. Rather, it appeared that at least two epitopes on distinct peptides were present in each pool (Fig. 8, *F* and *G*). To facilitate the interpretation of Fig. 8, *F* and *G*, we separated the two patterns of reactivity by using closed bars to reflect the cluster of patients responsive to one of the two immunoreactive peptides within the minipool and open bars to reflect the patients responsive to the other peptide.

We subsequently used the computational algorithms available at the Immune Epitope Database (tools.immuneepitope.org/analyze/html/mhc_binding.html) to predict putative epitopes within the peptides that were identified in Fig. 8 and we successfully defined minimal epitopes for minipools E-1/-2 (residues 17–26 of E), M-5/-6 (residues 111–120 of M), NS3-19 (residues 501–509 of NS3-19), and NS4B-1 (residues 15–23 of NS4B-1) (Table II). The minimal epitope present in peptide E#124 (residues 430–438) was identified through a parallel study using mass spectrometry to sequence HLA-A*02-bound peptides on WNV-infected cells (C. McMurtrey, A. Lelic, P. Piazza, A. K. Chakrabarti, E. J. Yablonski, A. Wahl, W. Bardet, A. Eckerd, R. I. Cook, R. Buchli, M. Loeb, C. R. Rinaldo, J. Bramson, and W. H. Hiltebrand, submitted for publication). Using that approach, we also independently confirmed that the minimal epitope in NS4B ($NS4B_{15-23}$) was presented by HLA-A*02 on WNV-infected cells. Although we successfully mapped a minimal epitope contained in minipools E-1/-2 (Table II), we were unable to associate this peptide with an HLA restriction. These patients did not carry a common HLA-A or HLA-B allele, nor did they carry HLA-A or HLA-B alleles common to defined superfamilies. We do not have complete HLA-C

typing for all these individuals, so it is possible that this epitope is either carried by HLA-C or exhibits promiscuous binding activity. Based on a recent report demonstrating broad promiscuity among well-defined HIV and EBV epitopes (19), we suspect that E_{17-26} is likely a dominant epitope with promiscuous behavior.

Discussion

Most previous studies of $CD8^+$ T cell immunity following flavivirus infection in humans have focused on dengue virus infection, although there have also been some reports from yellow fever virus 17D vaccinees and children infected with Japanese encephalitis virus. From the dengue literature, it is clear that $CD8^+$ T cell responses develop against most viral proteins, and, indeed, $CD8^+$ T cell epitopes have been identified in E, NS1, NS2A, NS4A, NS4B, and NS5 (9, 10, 20–24). Likewise, a study of four volunteers immunized with the yellow fever virus vaccine demonstrated CTL reactivity against E, NS1, NS2A, NS2B, and NS3 (25). Thus, it is clear that the $CD8^+$ T cell response following flavivirus infection targets against both structural and non-structural proteins. Although our data are consistent with the previous reports, our study has also revealed an unexpected bias in the specificities of the dominant $CD8^+$ T cell responses. The results presented herein suggest that E is the most immunogenic WNV protein (Fig. 2) and reactivity to two peptides from E (E_{17-26} and $E_{430-438}$) dominated the $CD8^+$ T cell response in 40% of our cohort (Fig. 7). These data are in marked contrast to the results in dengue infection where T cell responses to NS3 are more common (9, 20, 21). This difference may be explained by either the nature of the T cell response in the infected cohorts (i.e., primary responses to WNV but secondary responses to dengue) or differences in the immunobiology of the viruses. Interestingly, reactivity to NS3 was also found to dominate the memory response following Japanese encephalitis virus infection (26, 27), suggesting that dominance of NS3 reactivity is not likely due to secondary responses. It is equally possible that ethno-geographic differences may influence the outcome, although a recent report in HIV-infected individuals found that neither geography nor ethnicity influenced the dominance of $CD8^+$ T cell responses against specific viral proteins (28).

The distribution of $CD8^+$ T cell epitopes across the polyprotein was relatively constrained and $CD8^+$ T cells specific for five epitopes dominated the response in 70% of our patients (Fig. 5*A*) was unexpected. Additionally, we found that reactivity to $E_{430-438}$, $M_{111-120}$, $NS3_{501-509}$, and $NS4B_{15-23}$ occurred in almost every patient expressing HLA-A*02, -A*01, -B*35, and -A*02, respectively. In an examination of vaccinia virus immunity, it was observed that a broad spectrum of epitopes was uncovered without any specific epitope showing dominance (29); a phenomenon described by Yewdell as "immunodemocratic" (30). Similarly, an immunodemocratic $CD8^+$ T cell response was observed among four HLA-identical siblings infected with EBV (31). A key difference between the previous reports and the current one is that the other studies involved large DNA viruses, whereas the current study was focused on a small RNA virus with only 3433 amino acids. As an example of common reactivities produced by less complex viruses, almost all HLA-A*02-positive individuals exhibit $CD8^+$ T cell reactivity to the $M1_{58-66}$ epitope from influenza A (32). Similarly, a comprehensive analysis of $CD8^+$ T cell determinants in HIV-infected subjects found three peptides that produced reactivity in >30% of the study population (57 patients) including a peptide in Nef that was recognized by 47% of the population, similar to the $M_{111-120}$ peptide described herein (33), although the relative dominance of $CD8^+$ T cells specific for the common HIV peptides was not assessed. Thus, the somewhat restricted distribution of T cell epitopes in the WNV polyprotein may reflect its small size.

As discussed in the introduction, it is generally believed that older individuals have a diminished capacity to mount $CD8^+$ T cell responses toward novel pathogens due to the diminished availability of naive T cells and the somewhat dysfunctional nature of the available $CD8^+$ T cells (12). Yet, in our study, age did not appear to influence any of the measured immunological parameters. It is notable that much of the existing literature that characterizes the influence of age on T cell immunity has focused on either chronic/latent herpesvirus infections (i.e., CMV, EBV, and Varicella zoster virus) or responses to recurrent infections, notably influenza. As such, the data may be skewed since they focused on memory responses that are likely influenced by multiple rounds of antigenic stimulation over the lifetime of the individual. By contrast, very few individuals in Canada, the source of our cohort, have been exposed to flaviviruses. Indeed, the majority of the patients in our cohort were found to be seronegative for dengue exposure, as defined by PRNT, and those who were found to have dengue-specific Abs actually had poorer responses to WNV than those who were seronegative. Therefore, the immune response produced by WNV appears to reflect a primary response to a novel agent thereby providing a unique opportunity to examine immune function in the elderly against novel agents. Our data demonstrates that it is possible to elicit robust $CD8^+$ T cell immunity against a novel agent in the elderly with a breadth and magnitude equivalent to younger individuals. It should be noted that the earliest samples we could analyze were obtained on average 30 days after the onset of symptoms and our comparisons of magnitude were based on samples obtained 3–4 mo after infection. We compared these parameters based on the assumption that the breadth and magnitude of the $CD8^+$ T cell population during the early memory phase is a direct reflection of the breadth and magnitude of the population at the peak of the acute response. Thus, it remains possible that age-related differences in the WNV-specific $CD8^+$ T cell population occurred at the peak of the response but escaped our analysis due to limitations in the availability of patient material. Additionally, since only 20–30% of WNV infections produce symptoms of illness, it is possible that the younger individuals within our cohort have an immunological defect akin to the defect in aged individuals. To address this possibility, we would need to examine age-matched asymptomatic individuals. Unfortunately, we have no method to identify asymptomatic individuals at early points following infection, so we cannot definitively address this issue. Additionally, we cannot comment on possible age-related defects in $CD4^+$ T cell immunity to WNV at this time since we only identified a few $CD4^+$ T cell epitopes. It has been suggested that failure to produce Abs in aged mice and humans may be a result of a defect in type 2 differentiation due to excessive production of type 1 cytokines (34, 35). Since type 1 polarization supports the development of antiviral $CD8^+$ T cell responses, it is possible that age is not a factor since the immune system in older individuals is driven toward type 1 already. Oligoclonal expansion of $CD8^+CD27^-$ cells has been associated with poor Ab responses to influenza vaccination (35, 36). Such $CD8^+$ T cell populations may be reactive to chronic infections (CMV or EBV) or represent an autoreactive population. Whether such expansions in humans impact upon the primary response to novel agents is not known. In a murine model of HSV-1 infection where the $CD8^+$ T cell response is dominated by TCRs bearing Vβ8 or Vβ10 rearrangements, diminished anti-HSV-1 immunity was observed when age-related clonal expansions occurred within T cell pools bearing Vβ8 or Vβ10 although clonal expansions of $CD8^+$ T cells bearing other Vβ segments did not impact the $CD8^+$ T cell response as severely

(37). In that regard, oligoclonal expansions may only affect T cell responses that depend upon $CD8^+$ T cells bearing specific TCR rearrangements. Since the T cell response to WNV involves an average of four distinct epitopes, it is quite possible that comparable expansions may have occurred within our cohort but the T cell populations maintain adequate diversity to respond to new Ags. As stated previously, whether WNV-specific $CD8^+$ T cell immunity reflects novel activation of naive precursors or reactivation of cross-reactive memory cells is unknown. Current investigations in our laboratory are addressing the relationship between general diversity of T cell clones within the elderly patients in our cohort compared with the younger patients. We are also examining the number of individual clonotypes within a given pool with a common reactivity as it is possible that the Ag-specific populations in the aged have expanded to a similar magnitude but represent a more clonally restricted population.

We have presented the first description of the T cell response to WNV in naturally infected humans. Although these investigations failed to demonstrate a relationship between the $CD8^+$ T cell response and disease pathology, we did observe a number of unexpected findings which have more general implications for antiviral $CD8^+$ T cell immunity.

Disclosures

The authors have no financial conflict of interest.

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— CHAPTER 3 —

EPITOPE DISCOVERY IN WEST NILE VIRUS INFECTION: IDENTIFICATION AND IMMUNE RECOGNITION OF VIRAL EPITOPES

Epitope discovery in West Nile virus infection: Identification and immune recognition of viral epitopes

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Preface: The research presented in this manuscript represents a collaboration between Curtis McMurtrey, a former PhD candidate with Dr. William Hildebrand (University of Oklahoma), and myself. Curtis utilized mass spectroscopy (MS) sequencing to identify WNV-specific peptides from soluble HLA molecules of WNV-infected cells. He was responsible for all the work that entailed the identification and subsequently the synthesis of WNV-specific peptides. I determined the immuno-reactivity of MS-identified peptides, sent to us by Dr. Hildebran's laboratory, in PBMCs of WNV-infected specimens by ELISPOT and ICS. I generated and analyzed the data for Figure 3, and I contributed data for Figure 4. Furthermore, I wrote a part of the manuscript. My work was supervised by Dr. Bramson, who provided experimental guidance and interpretation of the results.

Epitope discovery in West Nile virus infection: Identification and immune recognition of viral epitopes

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Cytotoxic T lymphocytes (CTL) play an important role in the control and elimination of infection by West Nile virus (WNV), yet the class I human leukocyte antigen (HLA)-presented peptide epitopes that enable CTL recognition of WNV-infected cells remain uncharacterized. The goals of this work were first to discover the peptide epitopes that distinguish the class I HLA of WNV-infected cells and then to test the T cell reactivity of newly discovered WNV epitopes. To discover WNV-immune epitopes, class I HLA was harvested from WNV (NY99 strain)-infected and uninfected HeLa cells. Then peptide epitopes were eluted from affinity-purified HLA, and peptide epitopes from infected and uninfected cells were comparatively mapped by mass spectroscopy. Six virus-derived peptides from five different viral proteins (E, NS2b, NS3, NS4b, and NS5) were discovered as unique to HLA-A*0201 of infected cells, demonstrating that the peptides sampled by class I HLA are distributed widely throughout the WNV proteome. When tested with CTL from infected individuals, one dominant WNV target was apparent, two epitopes were subdominant, and three demonstrated little CTL reactivity. Finally, a sequence comparison of these epitopes with the hundreds of viral isolates shows that HLA-A*0201 presents epitopes derived from conserved regions of the virus. Detection and recovery from WNV infection are therefore functions of the ability of class I HLA molecules to reveal conserved WNV epitopes to an intact cellular immune system that subsequently recognizes infected cells.

epitope hierarchy | human leukocyte antigen | immunodominance | major histocompatibility complex | mass spectrometry

West Nile virus (WNV) is a single-stranded positive sense RNA flavivirus that is related to other human pathogens such as yellow fever virus and dengue fever virus. In nature, WNV exists in an enzootic cycle between birds and mosquitoes, with other species like horses and humans acting as incidental terminal hosts (1, 2). In humans, WNV causes a nonspecific febrile illness with rare cases of fatal encephalitis (3). Like other flaviviruses, WNV translates its genome into a polyprotein of ≈3,400 aa that is proteolytically cleaved into three structural proteins and seven nonstructural proteins (4). In contrast to other RNA viruses, such as HIV and influenza, WNV exhibits a high degree of sequence conservation in its natural reservoir (5). Such sequence conservation makes WNV a promising target with regard to targeting humoral and cellular immune responses to conserved epitopes.

WNV elicits a strong immune response from innate and adaptive branches of the immune system (6). With regard to adaptive immunity, WNV infection results in the generation of neutralizing antibodies that can protect mice from lethal infection when given passively (7–9), whereas mice deficient for cytotoxic T lymphocytes (CTL) or class I major histocompatibility complex (MHC) exhibit increased viral burdens and increased mortality (10–13). A key component of the cellular antiviral immune response is the antigen-specific interaction of T cell receptors and the class I MHC–peptide complex. Human MHC class I, or the human leukocyte antigens (HLA), are a complex consisting of a polymorphic α chain, β_2-microglobulin, and a peptide ligand of 8–13 residues. Class I HLA molecules sample peptides that are representative of the intracellular proteome and present their peptide cargo on the cell surface. During viral infection, the proteome of the infected cell is altered as are the peptides presented to CTL by class I HLA. Although CTL recognize infected cells, the WNV epitopes presented by class I HLA have not been reported. The goals of this work were to identify peptide epitopes unique to infected cells and to assess T cell recognition of these epitopes.

To discover WNV immune epitopes, class I HLA molecules were gathered from infected and uninfected cells; then peptide epitopes from infected and uninfected cells were comparatively mapped, and peptides unique to infected cells were sequenced (14). Using this direct epitope discovery approach, we identified viral-encoded epitopes uniquely presented by HLA-A*0201 molecules of WNV-infected cells. We found A*0201 to be extremely effective in its interaction with WNV: highly conserved, high-affinity amino acid sequences dispersed throughout the viral proteome were sampled by class I HLA-A*0201. The discovered WNV epitopes were then tested for cellular reactivity in an IFN-γ ELISPOT assay in WNV-positive HLA-A*0201 patients. A hierarchy emerged whereby the conserved high-affinity epitopes were dominant cellular immune targets. These data represent a characterization of class I HLA-presented WNV epitopes and indicate that cellular immune responses focus on a conserved subset of the available WNV epitopes.

Results

Production and Purification of HLA-A*0201-Specific Peptide Pools from WNV-Infected Cells. The first objective of this work was to understand the number and nature of class I HLA-presented peptide epitopes unique to WNV-infected cells. Secreted class I HLA proteins (sHLA) were used to produce a sufficient quantity of peptides for comparative proteomics. To confirm that one set of peptides in the comparative analysis were derived from infected cells, we tested for viral RNA in the cells and in the sHLA-containing culture supernatant. Florescence *in situ* hybridization

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The authors declare no conflict of interest.

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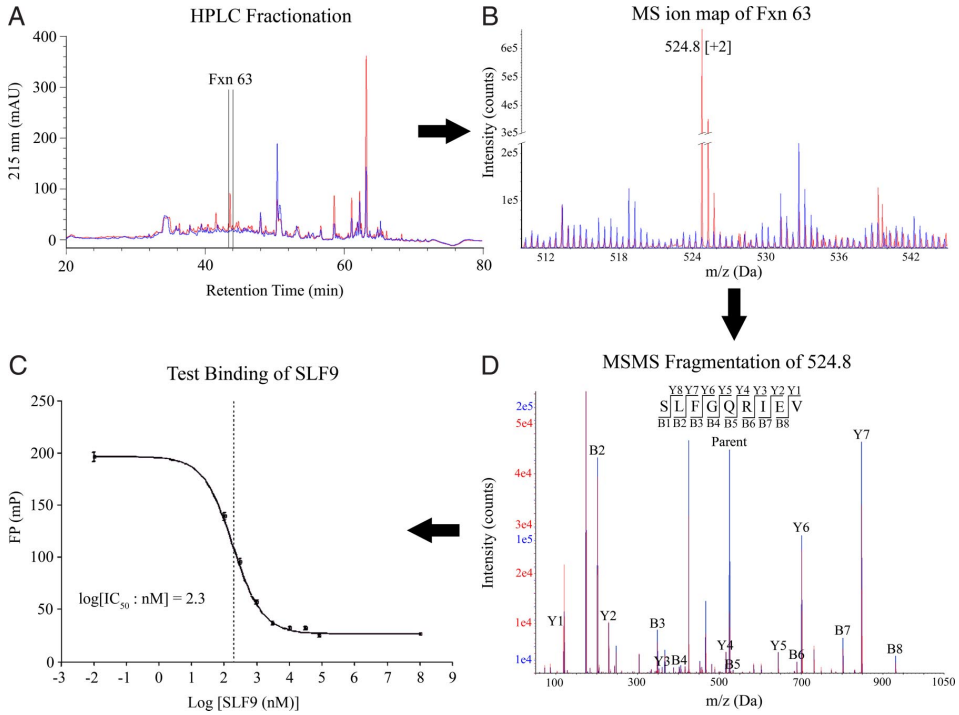

Fig. 1. Direct epitope discovery of WNV epitope SLF9. (*A*) RP-HPLC profile of HLA-A*0201 peptides from uninfected (blue) and WNV-infected (red) cells. (*B*) MS ion spectra of HPLC fraction 63 from uninfected (blue) and infected (red) cells. MS data were recorded for 300 scans. (*C*) Overlay of the MS/MS fragmentation pattern of ion 528.4 in the infected HPLC fraction 63 (identified as the SLF9 peptide; red) with the MS/MS fragmentation pattern for the SLF9 synthetic peptide (blue). (*D*) Florescence polarization competitive binding assay with the SLF9 synthetic peptide and HLA-A*0201.

(FISH) and real-time PCR (RT-PCR) confirmed the presence of viral RNA in the cells and supernatant, respectively. Viral RNA was detected 12 h after infection in the cells and in the supernatant (data not shown). Optimally infected cell supernatant from days 0.5 to 5.5 after infection were pooled for affinity chromatography purification. Infected and uninfected sHLA purified was 9.1 and 11 mg, respectively, as determined by ELISA.

Comparative Ion Mapping Identifies Six WNV-Derived Peptides. Pools of peptides eluted from class I HLA molecules contain thousands of different peptides. To reduce the complexity of the peptides before comparative analysis, peptides were separated by reverse-phase HPLC (RP-HPLC) into fractions containing ≈200–250 peptides each (Fig. 1*A*). Similar HPLC elution patterns for uninfected and infected peptide pools were obtained (Fig. 1*A*). MS ion maps were generated for corresponding infected and uninfected HPLC fractions. These ion maps were compared to identify ions unique to infected fractions. The vast majority of ions were shared in the infected and uninfected MS ion maps (Fig. 1*B*). Tandem mass spectrometry (MS/MS) sequence analysis of shared ions in the infected and uninfected ion maps produced like sequences (data not shown), confirming the alignment of HPLC fractions and overlap in the peptide preparations. Fig. 1*B* shows an ion map generated from HPLC fraction 63, where ion 524.8 was identified as unique to the WNV-infected peptide pools. MS/MS fragmentation was performed on ions unique to infected cells and individual peptide sequences were determined (Fig. 1*C*). MS/MS fragmentation at the same location in the corresponding uninfected fraction (and neighboring uninfected fractions) confirmed the absence of this peptide in uninfected cells. Fragmentation patterns between the newly discovered WNV-derived peptides were compared with synthetic peptide patterns to ensure sequence identity. Fig. 1*C* shows the MS/MS fragmentation pattern of ion 524.8 (identified as WNV-derived SLF9 peptide) overlaid with a synthetic peptide demonstrating a correct sequence assignment. Finally, WNV-derived peptides were assayed for their binding affinity to HLA-A*0201. Fig. 1*D* demonstrates that SLF9 is a high-affinity HLA-A*0201 ligand with an IC_{50} of 204 nM.

By using this direct comparative approach, six HLA-A*0201 WNV-derived peptides were identified from five different viral proteins (E, NS2b, NS3, NS4b, and NS5) (Fig. 2 and Table 1). Five peptides were nonamers and one a decamer. Three of the peptides (RLD10, SLF9, and SLT9) had a common A*0201 P2 leucine anchor residue, peptides YTM9 and ATW9 had a threonine P2 anchor, and SVG9 had a valine at P2. At their C terminus, A*0201 peptide ligands prefer a leucine or valine anchor, and only SLT9 (C-terminal alanine) did not have a common A*0201 C-terminal anchor. These putative WNV epitopes fit the reported A*0201-binding motif. When tested for binding to HLA-A*0201, these six WNV peptides were found to be high-affinity binders with SLF9, SVG9, and YTM9 being the strongest binders, having an IC_{50} of 204, 247, and 291 nM, respectively (Table 1). The other peptides, SLT9, ATW9, and RLD10, had comparatively lower affinities, with IC_{50} values of 503, 780, and 847 nM, respectively. The fluorescence polarization assay used to assess A*0201 binding categorizes peptides with IC_{50} values <5,000 nM as high affinity (15). In

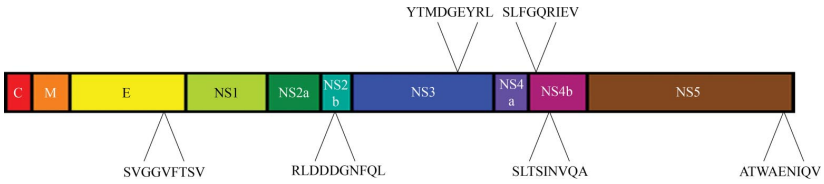

Fig. 2. Location of A*0201 WNV peptide epitopes on the WNV polyprotein. C, nucleocapsid; M, membrane; E, envelope glycoprotein; NS, nonstructural.

summary, we see that HLA-A*0201 presents six high-affinity peptides derived from five different proteins throughout the WNV proteome (Fig. 2).

CTL from Infected Individuals Recognize WNV Peptides. These WNV peptide epitopes were tested for recognition by CTL from HLA-A*0201 WNV-infected individuals. Peripheral blood mononuclear cells (PBMCs) from WNV-infected patients were tested in ELISPOT assays with the six identified WNV peptides. To assess CTL responsiveness over time, we tested three sets of CTL from HLA-A*0201 individuals: (*i*) the early set, PBMCs isolated from individuals who had recently recovered from infection (mean, 26.9 days after infection); (*ii*) the late set, PBMCs isolated from individuals ≈1 year after infection; and (*iii*) PBMCs from WNV-negative healthy controls. In both the WNV early and late infection sets, PBMCs responded strongly to SVG9 [mean = early, 125.3; late, 13.4 spot-forming units (SFU) per 10^5 PBMCs] and SLF9 (mean = early, 48.0; late, 9.8 SFU per 10^5 PBMCs) with a moderate but significant response in the early set to ATW9 (mean = 13.6 SFU per 10^5 PBMCs) compared with healthy controls. The WNV peptide epitopes RLD10, YTM9, and SLT9, as a group, demonstrated no significant activity above the healthy controls in the both early and late sets (Fig. 3). These data demonstrate an epitope hierarchy whereby SVG9>SLF9>ATW9>RLD10-SLT9-YTM9.

Although the early and late groups demonstrate a CTL response to WNV epitopes, a significant decrease in the magnitude of the CTL response is observed at the latter time point. For CTL that respond to the SVG9 peptide, there is a 9.3-fold decrease in average SFU from early to late, and for the SLF9 peptide there is a 4.9-fold decrease, suggesting a reduction in the number of CTL available to respond to specific WNV peptides over time (Fig. 3). The magnitude of CTL recall needed to confer immunity to subsequent infection is unknown. These data demonstrate a hierarchical CTL response to these newly discovered HLA-A*0201-restricted epitopes in both early and late populations, with a reduction in response at a later time after infection.

Heterogeneity Among Responses to HLA-A*0201-Restricted WNV Epitopes. Having identified common trends in epitope hierarchy and response strength (early and late; Fig. 3), we then analyzed CTL response heterogeneity in three ways: (*i*) the magnitude of each individual's response to each epitope; (*ii*) each individual's epitope hierarchy; and (*iii*) the total number of epitopes recognized by each individual's CTL (Fig. 4). Responders were categorized as high responders [>100 SFU per 10^5 PBMCs (Fig. 4*A*)], medium responders [10–100 SFU per 10^5 PBMCs (Fig. 4*B*)], and low responders [<10 SFU per 10^5 PBMCs (Fig. 4*C*)]. Eight individuals, six early and two late, were high responders (Fig. 4*A*). Fifteen medium responders were identified, seven of which followed the SVG9>SLF9>ATW9 hierarchy (Fig. 4*B*). Twelve low responders were identified, and although a hierarchy was not clear in many of these weak responders, responses to SVG9 and SLF9 predominated (Fig. 4*C*). Five infected individuals did not respond to WNV epitopes above background, although two of these nonresponders did not recognize the positive control peptides (data not shown); only 3 of 40 infected A*0201 individuals with measurable CTL responses failed to recognize the peptides discovered here.

Heterogeneity was observed in immune epitope hierarchies. (Fig. 4*D Left*). The largest grouping of donors (26 in total) responded to SVG9 (red) as the dominant epitope. Of the 26 donors that predominantly recognized SVG9, 19 recognized SLF9 (blue), and 5 recognized ATW9 (green) as a secondary epitope. Of the 19 donors that responded to SVG9 followed by SLF9, most (10 of 19) responded to ATW9 as their third epitope. Five of the donors that responded to SVG9>SLF9>ATW9 responded to RLD10 (yellow) as their fourth. For 7 donors, the dominant epitope was SLF9 of which 5 reacted to SVG9 as their second most dominant epitope. Finally, donors were grouped by the number of epitopes to which they responded (Fig. 4*D Right*). Few donors (2 of 40) responded to only one epitope with four epitopes most commonly recognized (11 of 40 donors).

Table 1. Identified WNV-derived peptides

Peptide	Sequence†	Protein	Location†	IC_{50}, nM
SVG9	SVGGVFTSV	Env	430–438	247
RLD10	RLDDDGNFQL	NS2b	78–87	847
YTM9	YTMDGEYRL	NS3	518–526	291
SLF9	SLFGQRIEV	NS4b	68–76	204
SLT9	SLTSINVQA	NS4b	15–23	503
ATW9	ATWAENIQV	NS5	862–870	780

†WNV NY99 polyprotein accession no. AAF20092.

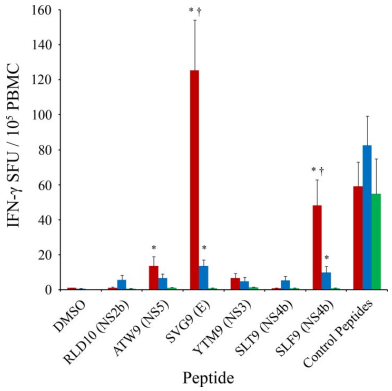

Fig. 3. CTL reactivity of identified WNV epitopes. Shown are IFN-γ ELISPOT assays with WNV peptides and PBMC isolated early (red) and late (blue) after infection from WNV-positive individuals and healthy controls (green). *, significant increase from healthy controls; †, significant increase from the late set (blue). Significance was determined by Kruskal–Wallis one-way ANOVA followed by Dunn's method; $P < 0.05$.

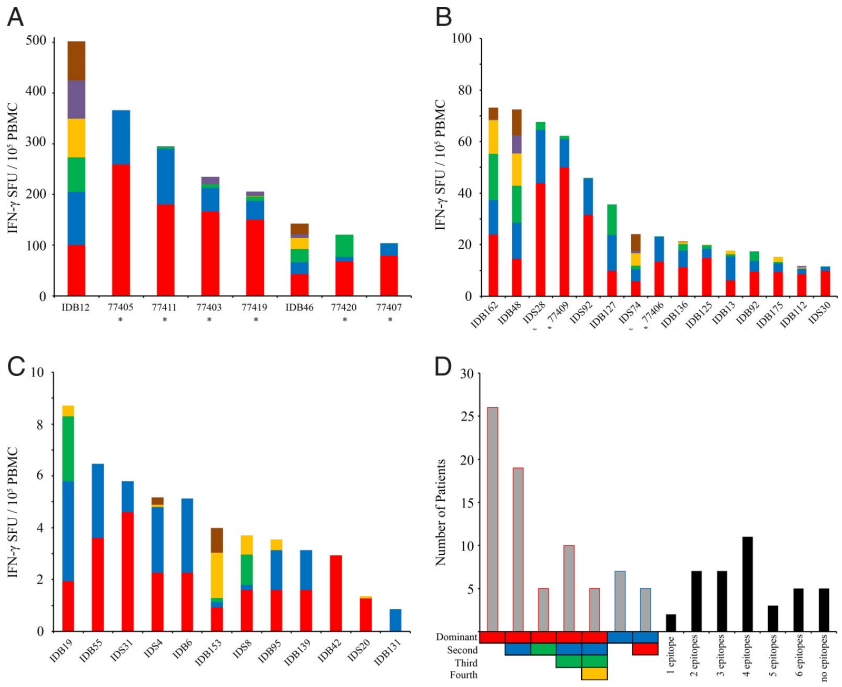

Fig. 4. Heterogeneity among CTL responses. Patient-specific responses to WNV epitopes SVG9 (red), SLF9 (blue), ATW9 (green), RLD10 (yellow), YTM9 (purple), and SLT9 (brown). (*A*) High responders, >100 SFU. (*B*) Moderate responders, 1–100 SFU. (*C*) Low responders, <10 SFU. Histograms represent the average number of SFU per patient minus the healthy control (plus 2× SEM) and DMSO control. *, patient is from the early group. (*D*) (*Right*) Number of patients with a specific epitope hierarchy. (*Left*) Number of epitopes to which each patient responded. Colored boxes, WNV epitopes as stated.

Viral Sequence Homology of the Epitopes. We next examined sequence variability among reported WNV isolates to assess conservation and divergence for these newly discovered A*0201 peptide epitopes. Of the dominant epitopes, SVG9 was the most conserved, with 97.2% (600 of 617 sequences) encoding the epitope sequence we identified in the NY99 strain. Within SVG9, 1.6% (10 sequences) displayed a conservative valine to isoleucine substitution at P2. The SLF9 and ATW9 epitopes demonstrated 86.1% (118 of 137) and 86.4% (171 of 198) homology among reported WNV sequences. Variants of the SLF9 epitope altered 3 or 4 of the 9 residues in the epitope, whereas the most common variation of the ATW9 epitope (11.1%) was a glutamate to histidine at P8. The YTM9, SLT9, and RLD10 epitopes displayed 97.8% (134 of 137), 100% (137 of 137), and 97.1% (135 of 139) sequence homology with reported WNV isolates, respectively. These data show that the WNV peptide epitopes sampled by A*0201 are highly conserved among hundreds of viral isolates.

Discussion

Several experimental observations can be gleaned from the endogenously processed WNV epitopes reported here. First is that the WNV-encoded peptides clearly distinguish themselves by MS intensity, as shown in Fig. 1*B*; the WNV peptides found here were high-abundance peptides compared with numerous unchanged host ligands. Another observation is that the six viral peptides were derived from multiple proteins in the WNV proteome. We observed that the class I molecule HLA-A*0201 was able to sample epitopes from half of the viral proteins, both structural and nonstructural, during infection. These peptides were of high affinity for A*0201 and were conserved among numerous WNV isolates.

An immune hierarchy emerged when these viral epitopes were assayed by IFN-γ ELISPOT. As a group, three WNV epitopes were recognized by CTL significantly above background: SVG9>SLF9>ATW9 (Fig. 3*A*). Epitopes RLD10 and YTM9 displayed moderate reactivity, and SLT9 was the least immunogenic. Furthermore, there was a significant decrease in CTL reactivity to WNV peptides in blood collected from infected individuals at a later time point, indicating a loss of CTL specific for WNV epitopes over time. Individual immune responses were heterogeneous in their level of response, epitope hierarchy, and number of total epitopes recognized. Moreover, there were no statistically significant associations of age, gender, race, duration of infection, and severity of disease with level of responses (data not shown). Cumulatively, these data show that six WNV epitopes were presented by A*0201, that most individuals recognized a dominant target and an auxiliary epitope, and that a small group of patients responded to five to six epitopes. Until now, this type of immune response hierarchy has been impossible to envision because a thorough survey of epitopes presented for recognition by the cellular immune system had not been completed. Here, the direct identification of WNV epitopes and subsequent immunogenicity testing provides metrics for how HLA molecules reduce viral proteins into a handful of optimal CTL targets.

The epitope hierarchy presented coincides with that seen in mice. Two dominant epitopes have recently been identified in mice during WNV infection. An E glycoprotein-derived epitope is presented by the mouse class I MHC molecule H-2K^b, and an NS4b epitope is restricted to H-2D^b (16, 17). In the Brien *et al.* work (16), the dominant H-2D^b peptide epitope accounted for 50–70% of the reactivity, and the remaining

CTL reactivity was dispersed among three other WNV peptide epitopes. These murine data fit the observations presented here in that we find a dominant SVG9 epitope along with subdominant SLF9 and ATW9 epitopes presented by A*0201. Moreover, these mouse studies reported a trend of dominant epitopes having the highest binding affinities, of having multiple viral proteins sampled, and for having epitope sequences conserved among numerous viral isolates (data not shown). The discovery of conserved/dominant WNV epitopes presented by human MHC molecules in this work is therefore consistent with observations in mice: dominant viral epitopes are presented by MHC class I and efficiently targeted by CTL.

One would be hard pressed to arrive at a more eloquent model of antigen presentation and cellular immune reactivity: multiple epitopes in half of the viral proteins are sampled by A*0201, a cellular immune response recognizes several of the available viral epitopes, and conserved epitopes of high affinity for A*0201 are preferentially targeted. These data are quite powerful for two reasons. First, understanding how the immune response successfully samples WNV epitopes provides a comparative model as we try to elucidate host–pathogen points of interaction with more elusive viruses such as HIV. Second, we have confirmed that conserved WNV epitopes are endogenously loaded and presented to CTL by the class I of infected cells. CTL-eliciting vaccines that use endogenous WNV epitopes can be tested, and the presentation of confirmed WNV epitopes by various HLA-A2 supertype members can be ascertained. In summary, the direct discovery and subsequent confirmation of viral CTL epitopes described here provide a solid foundation for viral antigen presentation studies and CTL vaccine design. These data provide an immune system "success story" whereby the nature of viral epitopes that are endogenously sampled has been determined and the immune response to these viral epitopes has been confirmed.

Materials and Methods

Virus and Cell Culture. WNV strain WNV NY99 was propagated on Vero E6 cell monolayers [American Type Culture Collection (ATCC) CRL-1586]. Infected cell supernatant was cleared of cell debris by centrifugation at $3,000 \times g$ for 15 min and stored at −80°C. HeLa (ATCC CCL-2) and Vero cells were subcultured according to ATCC instructions in DMEM (Caisson Laboratories, Inc.), 10% FBS (Serum Source International), and 1% penicillin/streptomycin (Invitrogen).

sHLA-Secreting Transfectant Cell Line. HLA-A*0201 cDNA was amplified by using a reverse oligonucleotide primer that truncated the 3′ end of exon 4, deleting the transmembrane and cytoplasmic domains. Furthermore, the 3′ reverse oligonucleotide primer included a VLDLr purification epitope tag (SVVSTDDDLA) that is recognized by the anti-VLDLr mAb (ATCC CRL-2197) (18). The resulting PCR product was cloned into pcDNA 3.1 (Invitrogen) and transfected by FuGENE 6 reagent (Roche) into HeLa cells. Quantification of sHLA in supernatant was performed by using a sandwich ELISA, where an antibody against the VLDLr epitope was the capture antibody, the primary detector antibody was directed against β_2-microglobulin (Dako Cytomation).

Viral Detection. WNV plaque assays were performed on both Vero and HeLa monolayers as described in ref. 19. Rapid detection of WNV RNA in supernatant was completed by using a TaqMan-based reverse-transcriptase RT- PCR. The primers and probes used have been reported to detect the E region of WNV genome (20). RT-PCR was performed by using the Applied Biosystems 7500 RT-PCR system under universal cycling conditions. Approximate plaque-forming units (pfu) were determined from the C_t value using a standard curve [$(y = -1.1145 \text{ Ln}(x) + 29.630)$; $R^2 = 0.9899$, x range $= 1.2 \times 10^1$ to 1.2×10^6 pfu/ml].

Class I HLA from WNV-infected cells was required to detect epitopes unique to infected cells. To confirm that HeLa cells were virus-infected, a FISH assay and flow cytometry were performed (21). The probes used in this work were 18S rRNA (5′-[Cy5]TCTAGCGGCGCAATACGAAT-3′) as a positive control, 18S rRNA reverse compliment (5′-[Cy5]ATTCGTATTGCGCCGCTA-3′) as a negative control, and WNVE (5′-[Cy5]GCCCGACCATGGGAGAAGCTC-3′). Hybridized cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Production and Isolation of HLA-A*0201 Peptides. sHLA-A*0201 peptide complexes were produced in a bioreactor format as described in refs. 22–24. To obtain sHLA from WNV-infected cells, bioreactors containing HeLa cells were infected with 1.5×10^8 pfu (as determined by HeLa plaque assay) in a total volume of 525 ml and recirculated in the bioreactor for 2 h before the harvest of sHLA-containing supernatant was initiated. Supernatant from days 0.5 to 5.5 after infection were pooled for purification because we needed 12 h to flush uninfected supernatant from the system. sHLA complexes were purified by affinity chromatography with the anti-VLDLr epitope antibody. Acid-eluted peptides were lyophilized and then fractionated by RP-HPLC with a Jupiter Proteo 90 Å, 4 μm, 150×2 column (Phenomenex) (14, 25).

MS Analysis and Peptide-Binding Assay. MS ion maps were generated for HPLC fractions containing peptides. Fractions were ionized by using nano-electrospray capillaries (Proxeon) into a QSTAR elite (Applied Biosystems) electrospray quadrupole time-of-flight mass spectrometer. Peptide sequences were determined manually by using the MASCOT software package (Matrix Science). Peptide binding to HLA-A*0201 was determined by using the florescence polarization method (Pure Protein L.L.C.) (16). WNV peptide epitope sequence alignments were made by using CLC Free Workbench 3 (CLC Bio).

WNV Patient Samples. For the early sample group, peripheral blood was collected from consenting donors (four men, four women) acutely infected with WNV in heparinized tubes in accordance with a protocol approved by Research Ethics Board at McMaster University. The median patient age was 62.5 years, with a range of 23–81 years (mean = 57.9 ± 7.1 years). The median time between onset of disease and the blood draw was 28.5 days, with a range of 12–46 days (mean = 26.9 ± 4.0 days). PBMCs were isolated and cryopreserved in RPMI containing 12.5% human serum albumin (Sigma) and 10% DMSO (Sigma) (26). HLA-A*02 expression was determined by flow cytometry by using phycoerythrin-conjugated HLA-A*02 antibody (clone BB7.2; Becton Dickinson).

For the late sample group, peripheral blood was collected from 32 WNV antibody-positive subjects, recruited from high-incidence areas in the central and southwest health districts of the State of Idaho. All persons had been diagnosed with WNV in the summer of 2006, with confirmatory testing conducted by the Idaho State Health Department. Subjects were recruited between March and June 2007, and all gave informed consent following approved Pittsburgh University IRB protocols. The median age of the Idaho cohort was 50 years, and the range was 31–88 years. After collection, the PBMC fraction was isolated on Ficoll–Hypaque density gradients and cryopreserved in 10% DMSO. Negative controls were anonymous donors obtained from Pittsburgh Central Blood Bank. All subjects were prescreened for HLA-A*02 by staining with antibody (clone BB7.2; Becton Dickinson) and confirmed by HLA DNA-based typing.

IFN-γ ELISPOT. For the early sample group, ELISPOT assays were carried out by using the human IFN-γ ELISPOT set (BD Biosciences) according to the manufacturer's instructions with 2 μg/ml peptide. As a positive control, PBMC samples were restimulated with peptide pools containing class I restricted viral peptides specific for Epstein–Bar virus (EBV; 91 peptides), cytomegalovirus CMV; 40 peptides), or influenza (47 peptides), respectively. The flu peptides were selected from the Immune Epitope database (27). The EBV and CMV peptides were described by Bihl *et al.* (28). For the late sample group, human IFN-γ ELISPOT assays were carried out as described in ref. 29. Briefly, PBMC were thawed, allowed to recover overnight at 37°C, and then added to nitrocellulose-coated filter plates (Millipore) at 1×10^5 cells per well (triplicate wells per treatment). After -overnight incubation with WNV peptides (10 μg/ml) or control pool of 4 CMV, 15 EBV, and 12 influenza virus (CEF) peptides (1 μg/ml) representing a cross-section of MHC class I dominant epitopes (30) or medium control, the plates were developed, and spots were counted with an automated ELISPOT reader (AID GmbH). All data are presented as SFUs per 10^5 PBMCs.

Statistical Analysis. For Fig. 3, significance was determined by Kruskal–Wallis one-way ANOVA followed by Dunn's method. A P value of <0.05 was considered significant. Statistical analysis was performed by using SigmaStat 3.0 software (SPSS, Inc.).

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— CHAPTER 4 —

IDENTIFICATION OF CD8$^+$ T CELL EPITOPES IN THE WEST NILE VIRUS POLYPROTEIN BY REVERSE-IMMUNOLOGY USING NETCTL

Identification of CD8$^+$ T Cell Epitopes in the West Nile Virus Polyprotein by Reverse-Immunology Using NetCTL

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Preface: The research presented in this manuscript represents a collaboration between the Bramson, Lund and Buss laboratories. Mette Voldby Larsen utilized a bioinformatics approach (NetCTL) previously designed by the Lund laboratory to identify cytotoxic T lymphocyte (CTL) epitopes. NetCTL predicted 112 WNV-specific peptides with broad WNV strain coverage and with various HLA-restriction predictions. The "*in-silico*" predicted peptides were synthesized by Kasper Lamberth in the Buss laboratory, tested for HLA affinity and all peptides with an affinity of less than 500nM were sent to us for T cell reactivity analysis. Consequently, I performed the experiments to determine the immunogenicity of the predicted peptides in PBMCs of WNV-infected subjects by ELISPOT and ICS. I generated and analyzed the data presented in Figure 1 and, together with Robin Parsons, I generated and analyzed the data presented in Figure 2. I also contributed to the writing of the manuscript. This work was supervised by Dr. Bramson, who also provided experimental guidance.

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Abstract

Background: West Nile virus (WNV) is a growing threat to public health and a greater understanding of the immune response raised against WNV is important for the development of prophylactic and therapeutic strategies.

Methodology/Principal Findings: In a reverse-immunology approach, we used bioinformatics methods to predict WNV-specific CD8$^+$ T cell epitopes and selected a set of peptides that constitutes maximum coverage of 20 fully-sequenced WNV strains. We then tested these putative epitopes for cellular reactivity in a cohort of WNV-infected patients. We identified 26 new CD8$^+$ T cell epitopes, which we propose are restricted by 11 different HLA class I alleles. Aiming for optimal coverage of human populations, we suggest that 11 of these new WNV epitopes would be sufficient to cover from 48% to 93% of ethnic populations in various areas of the World.

Conclusions/Significance: The 26 identified CD8$^+$ T cell epitopes contribute to our knowledge of the immune response against WNV infection and greatly extend the list of known WNV CD8$^+$ T cell epitopes. A polytope incorporating these and other epitopes could possibly serve as the basis for a WNV vaccine.

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Introduction

West Nile virus belongs to the family *Flaviviridae*, along with other human pathogens like *Yellow fever virus* and *Dengue fever virus*. It is an enveloped, spherical virus containing a single strand of RNA that is translated into a continuous polypeptide of approximately 3,400 amino acids. The polypeptide is post-translationally cleaved into ten distinct proteins including three structural proteins; capsid (C) protein, envelope (E) protein, and pre-membrane (prM) protein, and seven non-structural (NS) proteins; NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [1]. The virus is transmitted to humans by infected mosquitoes and causes West Nile fever in about 20% of infected people. The symptoms of West Nile fever are fever, headache, tiredness, and body aches that can last for a few days to several weeks. Less than one in 100 infected people will develop severe West Nile disease that may lead to fatal encephalitis [2]. The first incidents of WNV infection in the western hemisphere were detected in 1999 during an outbreak of encephalitis in New York City. Since then, the virus has spread across North America and is now a serious threat for public health in the United States, especially for immunocompromised recipients of transplanted organs [1]. Currently, no specific therapy is available for treatment and no vaccine has been approved for prevention of WNV infection in humans [3].

CD8$^+$ Cytotoxic T Lymphocytes (CTLs) of the immune system have the capacity to eradicate virus-infected host cells. CTL activation is achieved when peptides originating from virus proteins are presented at the surface of infected cells in complex with Human Leukocyte Antigen (HLA) class I molecules. Several studies have shown that CTLs indeed play a role in the cellular antiviral response against WNV infection in mice and humans [4–7].

Although the important role of CTLs in combating WNV is well-established, only a limited number of WNV CD8$^+$ T cell epitopes have so far been identified in humans. De Groot et al. applied a bioinformatics approach for predicting HLA-B*07 restricted WNV CD8$^+$ T cell epitopes [8]: Out of 16 predicted epitopes, 12 were confirmed to bind HLA-B*07 *in vitro*, but the peptides' ability to induce T-cell responses was not tested. Recent reports from our group and collaborators have described two different strategies for identifying CD8$^+$ T cell epitopes in WNV. In the first case, a mass spectroscopy method developed by the Hildebrand laboratory successfully identified four HLA-A*0201 restricted WNV CD8$^+$ T cell epitopes [9]. In a second study, we

used a shotgun approach, employing overlapping peptides spanning the entire WNV polyprotein and identified additional epitopes restricted by HLA-A*01 and HLA-B*35, as well as several epitopes for which the HLA restriction was not ascertained [10]. In a study by Lanteri et al., overlapping peptides spanning all WNV proteins were likewise tested for their ability to induce T cell responses and led to the discovery of eight frequently recognised WNV peptides [5]. Three of the responses were associated with particular HLA class I types (A*0101, A*0201, and Cw*0303/Cw*0304). In the current study, our objective is to extend the discovery of WNV $CD8^+$ T cell epitopes to additional HLA class I alleles, while also considering the sequence variability of WNV proteins. Koo et al. have recently identified regions of the WNV polyprotein that are fully conserved across all analysed WNV sequences and examined whether these regions contain experimentally confirmed or predicted $CD8^+$ T cell epitopes [11]. The variability of the WNV proteome is, however, unevenly distributed across the proteome with the structural proteins being most variably. At the amino acid level, the C protein has up to 23% differences across examined sequences, while the NS4b protein has the lowest diversity with at most 8% differences [11]. Accordingly, the majority of the conserved regions identified by Koo et al. were found in the non-structural proteins, while the C protein had none, and the two other structural proteins, prM and E, had the third and fourth least number of conserved regions [11]. It is likely that the structural proteins contain highly immunogenic epitopes that are missed when focusing solely on fully conserved regions. Previous studies have even suggested that the E protein is one of the most immunogenic proteins [5,10]. It is also possible that the structural proteins experience high variability precisely because it is a selective advantage for the virus to modify these proteins in response to the host immune system. The aim of the present study was therefore to discover novel WNV $CD8^+$ T cell epitopes that give a broad coverage of different WNV strains without necessarily being fully conserved across all strains. We employed a two-step bioinformatics reverse-immunology approach: First we used the *NetCTL* method [12,13] for predicting WNV $CD8^+$ T cell epitopes. The *NetCTL* method has previously proven successful in identification of $CD8^+$ T cell epitopes in Influenza [14,15], HIV [16], and Orthopoxvirus [17]. We then selected a subset of the predicted epitopes with a broad coverage of 20 fully-sequenced WNV strains. We were able to confirm that 26 of the predicted epitopes were indeed WNV $CD8^+$ T cell epitopes, when tested with a cohort of WNV-infected patients.

Materials and Methods

Bioinformatics search strategy for prediction and selection of HLA class I restricted WNV $CD8^+$ T cell epitopes

In 2006 when the study was initiated, only 20 WNV polyproteins were available in the GenBank [18] and RefSeq [19] databases (GenBank acc. no. AAM81752.1, AAM81753.1, AAP22088.1, AAP22089.1, AAP22086.1, AAP22087.1, AAQ55854.1, AAR-84614.1, AAT02759.1, AAU00153.1, AAV68177.1, AAT95390.1, AAV52687.1, AAV52688.1, AAV52689.1, AAV52690.1, AAW-81711.1, AAX09982.1, AAW28871.1, and RefSeq ID NC_001563). Each genome corresponds to a single long polyprotein of approximately 3,400 amino acids. The 20 polyproteins have an average %identity of 96.2% (range 87.0%–99.9%). Using the *NetCTL* method [12,13] (available at www.cbs.dtu.dk/services/NetCTL), $CD8^+$ T cell epitopes were predicted for each of the 12 HLA class I supertypes defined by Lund et al. in [20] (A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, B62). In practice, putative epitopes for a given HLA class I supertype were identified by predicting which peptides are presented by a specific HLA class I allele that represents the entire supertype (for example, HLA-A*0201 represents the A2 supertype, while HLA-A*0101 represents the A1 supertype). In the *NetCTL* method, each nonameric peptide in a protein is assigned a score based on a combination of predictions of proteasomal cleavage, Transporter Associated with antigen Processing (TAP) transport efficiency, and HLA class I affinity. The reliability of *NetCTL* has previously been shown to be as high as or higher than other publicly available methods for $CD8^+$ T cell epitope predictions [12,13]. For predictions of HLA class I affinity, *NetCTL* employs the *NetMHC* method [21,22], which has been judged to be one of the two best methods in a comparative study of the performance of 30 methods for HLA class I affinity prediction [23]. For each of the 12 HLA class I supertypes and each of the 20 WNV polyproteins, we selected the 17 nonameric peptides with the highest *NetCTL* score (the top 0.5%) as the predicted epitopes. This resulted in a total of 4,080 predicted epitopes of which 484 were unique. To reduce this set, we used the EpiSelect algorithm (previously described in [16]). In short, the EpiSelect algorithm aims, in an iterative procedure, at selecting a given number of predicted epitopes in a way that maximises the coverage of the viral strain with the smallest number of epitopes. Using this algorithm, 16 predicted epitopes were selected for each of the 12 HLA class I supertypes, resulting in a total of 192 peptides. The selected peptides are listed in Supplementary Figure S1 under the reference sequence with RefSeq ID: NC_001563. Note that 17 of the peptides are predicted to be restricted by more than one HLA class I allele, resulting in a total of 175 unique peptides. We are aware that when selecting only a relatively small fraction of the peptides with the highest *NetCTL* scores as the predicted epitopes, we will risk missing some important WNV epitopes. However, due to limited resources, we were not able to test all possible epitopes.

Bioinformatics methods for identifying possible HLA class I restriction

We investigated to what extent the recognised epitopes could be explained directly in terms of restriction by one of the patient's six HLA class I alleles. For this analysis, the pan-specific *NetMHCpan* prediction method [24,25] was used. Note that we here use *NetMHCpan*, and not the previously used *NetCTL* method, since the *NetCTL* version that was available when this analysis was performed, only allowed predictions for the 12 HLA class I alleles that represent the 12 HLA supertypes.

It has become apparent that HLA molecules do not present peptides at the same binding threshold [26,27]. Using a fixed binding affinity threshold would hence result in a bias in the predictions towards HLA molecules with low binding affinity thresholds. Instead, we use the *NetMHCpan* %rank score (previously described in Hoof et al. [28]). The *NetMHCpan* %rank score aims at removing the bias caused by the diverging binding affinity thresholds and placing binding scores for all HLA molecules on an equal scale. In practice, for a given HLA class I molecule, the predicted binding affinity of the identified epitope was ranked along with the predicted binding affinities of a common set of 1,000,000 random, natural, 9meric peptides for the same HLA molecule. A %rank score of, e.g., 5% thus means that only 5% of random peptides are predicted to bind the HLA molecule with an affinity stronger than the identified epitope. The %rank score is calculated for each of the six possible epitope:HLA class I pairs of a patient, and if the lowest %rank score was below 5%, we assigned this HLA class I allele as the restricting HLA, and say that we can successfully explain the epitope restriction. A study by Rao et al. [27] justifies the %rank score threshold of 5%: Rao et al. found that the binding fraction of 9mers among all possible 9mers in the human proteome is ~5% for HLA-A alleles and

~2% for HLA-B alleles. Among viral and bacterial proteoms, the binding fraction of peptides is even higher.

Calculating the epitope conservation

Since initiating the study, additional fully sequenced WNV genomes have become available. For calculating the epitope conservation of the identified epitopes, we examined their frequency in 140 fully sequenced WNV genomes from [11].

Calculating the epitope coverage

HLA population coverage data was obtained from dbMHC (http://www.ncbi.nlm.nih.gov/gv/mhc/). For each of the 11 epitope:HLA pairs, we first calculated their individual coverage based on the genotype frequency (also called the allele frequency) of the HLA allele and the conservation of the epitope in the 140 examined WNV strains:

$$f_i = g_i * c_i$$

where f_i is the coverage of epitope$_i$:HLA$_i$, g_i is the genotype frequency of HLA$_i$, and c_i is the conservation of epitope$_i$.

For each of the three HLA loci (A, B, and C), the coverage can be calculated separately as follows:

$$F_A = 1 - (1 - f_i - f_j - f_k)^2$$
$$F_B = 1 - (1 - f_l - f_m)^2$$
$$F_C = 1 - (1 - f_n - f_o)^2$$

where F_A, is the coverage of epitope:HLA-A pairs and f_i, f_j, and f_k are the coverage of the individual epitope:HLA-A pairs.

Where F_B, is the coverage of epitope:HLA-B pairs and f_l and f_m are the coverage of the individual epitope:HLA-B pairs.

Where F_C, is the coverage of epitope:HLA-C pairs and f_n and f_o are the coverage of the individual epitope:HLA-C pairs.

The total coverage, F, of all 11 epitope:HLA pairs can be calculated as:

$$F = 1 - (1 - f_i - f_j - f_k)^2 * (1 - f_l - f_m)^2 * (1 - f_n - f_o)^2$$

The total coverage was calculated separately for all populations in the following areas: Australia, Europe, North Africa, North America, North-East Asia, Oceania, South America, South-East Asia, South-West Asia, and Sub-Saharan Africa.

Biochemical peptide-HLA class I binding assay

The biochemical assay for peptide-HLA class I binding was performed as previously described [29,30]. Briefly, denatured and purified recombinant HLA heavy chains were diluted into a renaturation buffer containing HLA heavy chain, β_2-microglobulin and graded concentrations of the test peptide, and incubated at 18°C for 48 h allowing equilibrium to be reached. The concentration of generated peptide–HLA complexes was measured in a quantitative enzyme-linked immunosorbent assay and plotted against the concentration of peptide offered [29]. Because the effective concentration of HLA (3–5 nM) used in these assays is below the equilibrium dissociation constant (K_D) of most high-affinity peptide–HLA interactions, the peptide concentration leading to half-saturation of the HLA is a reasonable approximation of the affinity of the interaction. An initial screening procedure was employed whereby a single high concentration (20,000 nM) of peptide was tested. If no complex formation was found, the peptide was assigned as a non-binder to the HLA molecule in question; conversely, if complex formation was found in the initial screening, a full titration of the peptide was performed to determine the affinity of binding.

Peptides

Peptides were synthesised as previously described [15]. Briefly, the peptides were synthesised by standard 9-fluorenylmethyloxycarbonyl (FMOC) chemistry, purified by reversed-phase high-performance liquid chromatography (at least 80%, usually >95% purity) and validated by mass spectrometry (Shafer-N, Copenhagen, Denmark). Peptides were distributed at 20 μg/vial and stored lyophilised at −20°C until use. Peptides were dissolved just before use.

WNV patient study subjects

Thirteen patients infected with WNV were recruited into our study cohort over three seasons (2003–2005) (Table 1). We specifically selected patients who carried HLA-A*0101 or HLA-A*0201 to examine the immunogenicity of the peptides predicted to be restricted by these alleles, since we in our previous report have identified dominant HLA-A*0101 and HLA-A*0201 epitopes [10]. The patients were enrolled following detection of serum WNV IgM (IgM-MAC) by public health laboratories after presenting symptoms of WNV infection. This trial was reviewed and approved by the Research Ethics Board at McMaster University. Written informed consent was obtained from all participants. Serology for WNV and dengue virus was assessed by PRNT as described previously [31]. HLA genotypes were determined using DNA sequence analysis at the Hamilton Health Sciences Histocompatibility Laboratory (Hamilton, ON) and Pure Transplant Solutions (Austin, TX). Blood samples were drawn into heparanised tubes, Peripheral Blood Monocytes (PBMC) were isolated from the blood samples by centrifugation on Ficoll (Amersham Pharmacia) and cryopreserved in RPMI 1640 containing 12.5% human serum albumin (Sigma) and 10% DMSO.

IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay

PBMCs were screened in an initial IFN-γ ELISPOT assay to demonstrate peptide reactivity without *a priori* knowledge of patient HLA types. 112 putative epitopes with measured K_D<500 nM were assembled into 20 peptide pools according to a 2-D grid, where each peptide was present in only two pools. Coincident reactivity between two pools identified candidate peptides containing putative T cell epitopes. T cell reactivity was subsequently validated by restimulation of PBMCs from the same patient with individual peptides. IFN-γ ELISPOTs were performed using kits purchased from BD Biosciences and carried out according to the manufacturer's instructions. PBMCs were thawed and placed immediately into cRPMI pre-warmed to 37°C. The cells were aliquoted into the ELISPOT plate at $1–2\times10^5$ cells/well and peptides were added at a final concentration of 2 μg/ml per peptide. The plates were incubated for 18 to 20 hours at 37°C in a humidified incubator with 5% CO_2, and the assay was completed according to the manufacturer's directions. Spots were enumerated using an ImmunoSpot 3B analyser (Cellular Technology Ltd, Cleveland, OH). Positive reactivity was defined as responses that were at least two-fold above background and a minimum of 50 SFC/10^6 PBMCs. As a positive control for $CD8^+$ T cell reactivity, all samples were stimulated with a collection of WNV-specific $CD8^+$ T cell epitopes that was previously found to be frequently

Table 1. Characteristics of WNV-infected patients.

Patient ID	Age; sex	Time from onset of symptoms to PBMC collection (days)	HLA-A		HLA-B		HLA-Cw	
44401	54; F	29	0101	0201	0702	1517	0701	0702
44405	65; F	22	0101	0201	0702	1501	0303	0702
55302	65; M	32	01	02	57	40	ND	ND
55307	55; F	40	0101	0301	3701	4429	0501	0602
55308	33; F	55	0101	0301	0801	4701	0602	0701
55309	64; F	120	0201	0301	3503	4403	0401	0401
55310	64; F	31	0101	0101	0801	0801	0701	0701
55405	47; F	73	0101	0301	0702	0801	0701	0702
55407	63; M	66	0101	0301	1302	3503	0401	0602
55410	51; M	93	0201	0201	4001	4402	0304	0501
55413	51; M	120	0101	0201	0801	4402	0701	0501
55414	57; M	135	0201	3101	0702	5601	0102	0702
55415	43; F	136	0201	0201	2702	5601	0102	0202

Note that the HLA-A and -B alleles of patient #55302 were only determined by low-resolution serological typing. ND: Not Determined.
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recognised in any given patient [10]. This pool of peptides is termed "pool of dominant WNV epitopes" in the text and consists of the following sequences: SGATWVDLV, SVGGVFTSV, WMDSTKATRY, SLFGQRIEV, MPNGLIAQFY, GTKTFL-VHREWFMDL, FLVHREWFMDLNLPW, LGLQKLGYIL-REV, DTAGWDTRITRADL. Note that we here use the term *dominant* to describe epitopes that are frequently recognised in any given patient (as opposed to epitopes that elicit a strong immune response). None of the peptides in the WNV peptide pool were also in the set of predicted, selected epitopes described in the subsection *Bioinformatics search strategy for prediction and selection of HLA class I restricted WNV CD8$^+$ T cell epitopes*.

ICS validations

Intracellular cytokine staining (ICS) was employed to confirm that IFN-γ secreting cells identified by ELISPOT were actually CD8$^+$ T cells. Given the limiting amount of patient material available to our group, we chose to employ a recently described method for unbiased amplification of CD8$^+$ T cells to expand our frozen PBMCs prior to analysis [32]. Briefly, K64-4-1BBL cells were loaded with anti-CD3 and anti-CD28 and irradiated at 10,000 rads. Freshly thawed PBMCs were incubated with the loaded, irradiated K64-4-1BBL cells at a ratio of 2:1. We routinely observed 5 to 10 fold expansion in CD8$^+$ T cell numbers in the period of 10 to 12 days. The cultures were subsequently collected, washed, and used immediately for ICS. This initial, unbiased expansion step greatly increases the number of CD8$^+$ T cell effectors capable of recognising specific epitopes. Most importantly, this method does not alter the hierarchy of epitope reactivity (Supplementary Figure S2). Therefore, this method allowed us to both confirm the specificity of the epitope and define the reactivity as dominant or subdominant in terms of magnitude of response.

The ICS protocol was conducted as previously described [10] with some modifications. Briefly, cells were aliquoted ($1–2\times10^6$ cells/well) into 96-well U-bottomed plates. Peptides were added to a final concentration of 2 μg/ml and the cells were incubated for 2 hours. Brefeldin A was then added to a final concentration of 5 μM and the cells were incubated 4 hours further. At the end of this period, cells were pelleted and washed in 10 μM EDTA. The cells were subsequently surface stained with either anti-CD8-PE-Cy7 or anti-CD3-PE-Cy5, permeabilised with Cytofix/Cytoperm and intracellular cytokines were identified using anti-TNF-α-PE and anti-IFN-γ-APC (Note: All flow cytometry reagents were obtained from BD Pharmingen). Fluorescence data were acquired using a FACSCanto or an LSRII and 200,000 events based on the live lymphocyte gate were collected per sample.

Results

Prediction and selection of HLA class I restricted CD8$^+$ T cell epitopes

To identify WNV CD8$^+$ T cell epitopes with a broad coverage of WNV strains, we first used the *NetCTL* method [12,13] to predict HLA class I supertype restricted epitopes. We then selected a subset of 175 predicted epitopes that constitutes broad coverage of 20 WNV strains as described in *Materials and Methods*. Of the 175 predicted epitopes, 14 could not be synthesised. To determine whether the remaining 161 peptides were indeed binders to the relevant HLA class I molecules, they were tested in a biochemical *in vitro* binding assay. Overall, 112 peptides (70%) had a binding affinity (K_D) of 500 nM or less for the relevant HLA class I molecule (Supplementary Table S1). It has previously been shown that the vast majority of HLA class I restricted epitopes bind their relevant HLA molecule with a K_D of 500 nM or less [33].

Immunogenicity of the predicted epitopes

In the first round of analysis, the 112 peptides identified as binding with a K_D of 500 nM or less for the relevant HLA class I molecule, were tested for their ability to stimulate CD8$^+$ T cells from a study population of 11 WNV-infected patients. As shown in Figure 1, we were able to confirm that 18 predicted epitopes were recognised by CD8$^+$ T cells from these naturally-infected patients.

As a positive control, we used a pool of WNV epitopes that we have previously found to be recognised frequently in any given patient [10]. We denote this peptide collection the "pool of dominant WNV epitopes". Figure 1 shows that the positive control generally evoked a higher T cell response than the individual epitopes, which is not surprising, since the positive

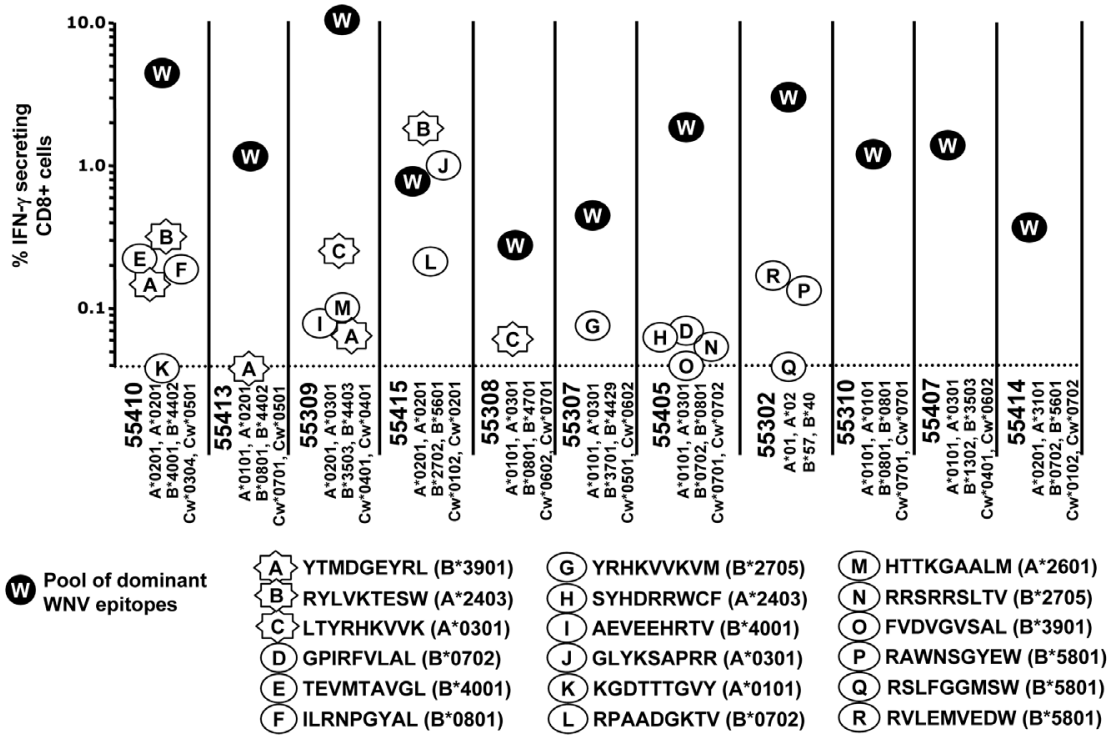

Figure 1. Immunogenicity of the predicted epitopes. One-hundred-and-twelve predicted epitopes were screened for reactivity by IFN-gamma ELISPOT using samples from 11 patients. Patient ID is listed on the X-axis along with the HLA alleles of each patient. Each circle/star represents an individual peptide. Circles correspond to peptides that displayed reactivity in only one patient. Stars correspond to peptides that displayed reactivity in more than one patient. As a positive control for these analyses, a pool of nine dominant WNV epitope peptides (labeled W) was included in each analysis.
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control contained *nine* previously identified WNV epitopes. Eight of the eleven patients exhibited reactivity to at least one of the predicted epitopes. These responses were typically subdominant in terms of magnitude of response, with the exception of RYLVKTESW and GLYKSAPRR in patient #55415. Three epitopes (Figure 1; star shaped) were recognised by more than one patient.

It is possible that technical complications resulting from the peptide pooling method may have obscured reactivity towards some putative epitopes. To address this possibility, we carried out a second round of analysis. This time we used only the peptides predicted to bind HLA-A*0101 or HLA-A*0201 because our previous study [10] had revealed that both of these alleles present epitopes (WMD10 = WMDSTKATRY restricted by HLA-A*0101 and SVG9 = SVGGVFTSV restricted by HLA-A*0201) that are both dominant with regards to frequency of recognition (they were recognised in all HLA-matched patients) and magnitude of response. Accordingly, the seven patients carrying either HLA*0101, HLA*0201, or both provided us with a robust method of characterising the predicted epitopes relative to previously defined epitopes. Two of the seven patients (patient #44401 and #44405) had not been tested in the first round of analysis. As seen in Figure 2, all patients possessed $CD8^+$ T cell reactivity to the pool of dominant WNV epitopes. Furthermore, all patients carrying the HLA-A*0101 allele exhibited robust reactivity to WMD10 (WMDSTKATRY) and all patients carrying HLA-A*0201 exhibited reactivity to SVG9 (SVGGVFTSV).

The analysis did unveil some reactivities that were not identified in the first round of analysis. Five of the nine HLA-A*0201 peptides and four of the seven HLA-A*0101 peptides evoked some degree of reactivity in the patient cohort. For the most part, responses to these peptides were subdominant both in terms of magnitude of response and in frequency of recognition (none of the peptides were recognised uniformly by all of the patients in our cohort). Two HLA-A*0101-binding peptides, MTKEEFTRY and KGDTTTGVY, were recognised at levels comparable to the known epitope WMD10 (WMDSTKATRY). Surprisingly though, peptide KGDTTTGVY only stimulated responses in patients who were HLA-A*0101-negative.

Compiling the results presented in Figure 1 and 2, three patients (#55310, #55407, and #55414) had no response to any of the tested peptides. Two patients (#55307 and #55308) each only responded against one of the tested peptides, while the highest number of responses was found using PBMC from patient #55410: Here, responses against seven different epitopes were detected. The average number of responses per patient was 3.6. For clarity, Supplementary Table S2 compiles the results from Figure 1 and 2 and lists them per identified epitope. In total, 26 epitopes were identified. They gave rise to 36 responses in ten WNV infected patients.

None of the identified epitopes induced a response in all patients expressing the predicted restricting HLA class I allele. At most, any individual epitope elicited a response in four different patients.

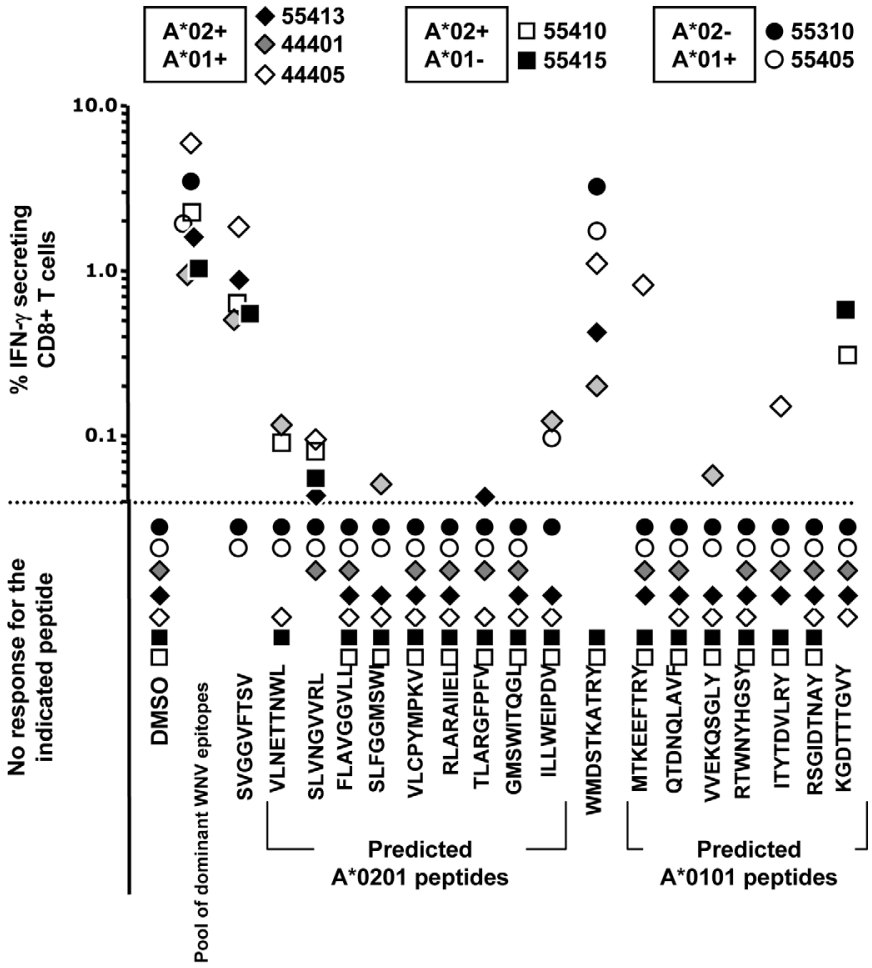

Figure 2. Immunogenicity of predicted HLA-A*0201 and HLA-A*0101 epitopes. Nine predicted epitopes confirmed to bind HLA-A*0201 and seven predicted epitopes confirmed to bind HLA-A*0101 were screened for reactivity by ICS assay using samples from seven patients that expressed either HLA-A*0201, HLA-A*0101, or both (listed on the X-axis). Each dot represents an individual patient. Patients expressing both HLA-A*0201 and HLA-A*0101 are represented by diamonds; patients expressing only HLA-A*0201 are represented by squares; patients expressing only HLA-A*0101 are represented by circles. Test peptides are listed on the X-axis. As a positive control for these analyses, a pool of nine dominant WNV epitopes (labeled W) was included in each analysis. Furthermore, SVGGFTSV is a dominant epitope restricted by HLA-A*0201, while WMDSTKATRY is a dominant epitope restricted to HLA-A*0101. Please note that the part of the graph below the dotted line consists of donors with no response to the indicated peptides.
doi:10.1371/journal.pone.0012697.g002

For instance, VLNETTNWL-A*0201 was stimulatory for $CD8^+$ T cells from patients #44401 and #55410, but not from #55413, #44405, or #55415, even though all five patients carry HLA-A*0201. The lack of conservation of epitopes in different WNV strains may explain some of these observations. For instance, the epitope AEVEEHRTV is only found in 8% of the 140 fully sequenced WNV strains, which might explain why patient #55410 did not exhibit a response to this peptide, although he carry the predicted restricting HLA class I allele, HLA-B*4001. However, this argument cannot explain all of our observations. As an example, the HLA-A*0301 restricted epitope LTYRHKVVK is found in all 140 examined WNV strains, but did not evoke a response in patient #55307, #55405, or #55407, although these patients all express HLA-A*0301.

Distribution of the epitopes

The identified epitopes and T cell responses are distributed across the WNV proteins as illustrated in Figure 3.

Most validated epitopes were found in the NS3 and NS5 proteins, which are indeed the two longest proteins containing the highest number of tested predicted epitopes (34 for NS3 and 50 for NS5). Epitopes in these proteins likewise gave rise to most T cell responses. No epitopes were found in NS4A, which is one of the shortest proteins with only five tested predicted epitopes. Six of the epitopes were found in structural proteins including one in the C protein, three in prM, and two in the E protein. Based on the present study, we were not able to observe a particular bias towards epitope location in certain WNV proteins, besides what can be explained by the mere size of the protein. We were thus not

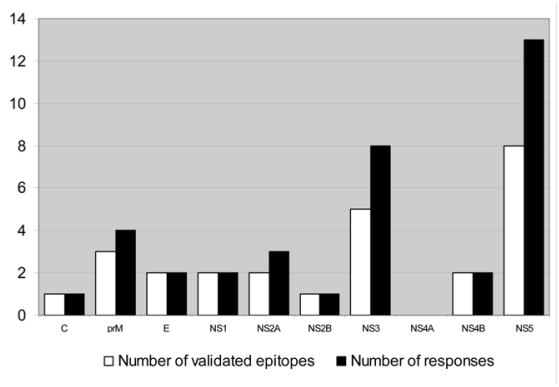

Figure 3. Location of the identified epitopes. The figure shows the number of validated epitopes in each of the ten WNV proteins and the number of responses that they evoked. C: Core protein, prM: Membrane protein, E: Envelope protein, NS1: Non-structural protein 1, NS2A: Non-structural protein 2A, NS2B: Non-structural protein 2B, NS3: Non-structural protein 3, NS4A: Non-structural protein 4A, NS4B: Non-structural protein 4B, NS5: Non-structural protein 5.
doi:10.1371/journal.pone.0012697.g003

able to confirm findings of other studies [5,10] that protein E is more commonly targeted by T cell responses.

Suggested HLA class I restriction

Table 2 lists the 36 observed epitope responses identified in Figure 1 and 2 and compares them with the HLA class I types of the patients as listed in Table 1.

Almost half (16 out of 36) of the observed responses can be explained by a direct match between the patient HLA class I type and the HLA class I allele used for selecting the given epitope (see Table 2). This result reflects that we started out by predicting epitopes restricted by the 12 HLA class I alleles that represent the major class I supertypes [20], but tested the predicted epitopes in all patients, regardless of whether the patients carried any of these specific 12 HLA class I supertype representative alleles or, for example, another HLA class I allele belonging to the same supertype. In accordance with this, the fraction of explainable epitope responses improved to 58% (21 out of 36), when also considering HLA class I supertype matches between HLA class I alleles expressed by the patients and the HLA class I alleles used for selecting the epitopes. For instance; AEVEEHRTV was selected for binding to HLA-B*4001, which represents the B44 supertype, but induced a response in patient #55309, who does not carry HLA-B*4001. However, patient #55309 carry HLA-B*4403, which is also a member of the B44 supertype. Likewise, RPAADGKTV was selected for binding to HLA-B*0702, but induced a response in patient #55415, who does not carry HLA-B*0702, but HLA-B*5601, an allele belonging to the B7 supertype [20].

For some of the identified epitopes there was more often a complete match between the HLA class I allele used for selecting the epitope and the HLA class I type of the patient displaying the response. For instance, nine of the epitopes that were predicted to be presented by the representative of the A2 supertype, HLA-A*0201, induced a response in HLA-A*0201-positive patients. In contrast, none of the epitopes that were predicted to be presented by the representative of the B39 supertype, HLA-B*3901, induced a response in B*3901-positive patients for the simple reason that none of the patients carry HLA-B*3901.

Since not all responses can be explained in terms of a direct match between the HLA class I allele used for selecting the epitope and the HLA class I alleles carried by the patient, nor by the supertype association of one of the HLA class I alleles carried by the patient, an alternative approach for identifying the most likely restricting allele in each responding patient was applied. We used a pan-specific peptide:HLA binding prediction algorithm called *NetMHCpan* [24,25] for investigating whether the recognised epitopes could be explained in terms of binding to one of the patient HLA class I alleles. Note that the *NetCTL* method [12,13], which was used for the initial epitope predictions, could not be used for this analysis, since *NetCTL* only allows predictions for the 12 HLA class I alleles that represent the 12 HLA class I supertypes. The summary of the analysis is shown in Table 2. The analysis did not include the three responses detected in patient #55302, since the HLA types of this patient were only determined by low-resolution serotyping. In short, the analysis was performed by calculating the *NetMHCpan* %rank score for each of the six possible epitope:HLA class I pairs as described in *Material and Methods*. If the lowest %rank score was below 5%, we assigned the HLA class I allele that resulted in this score as the restricting HLA and say that we can successfully explain the epitope restriction. Using this definition, we assigned 82% (27 out of 33, see Table 2) of the detected epitope specific T cell responses to a specific HLA class I allele.

As seen in Table 2, six responses remain unexplainable. For instance, ILLWEIPDV was selected for binding to HLA-A*0201, but induced a response in patient #55405, who does not express HLA-A*0201. Among the HLA class I alleles of #55405, HLA-A*0301 resulted in the lowest %rank score, 32, but this is well over the defined threshold of 5%. However, two of these six responses can be explained in terms of nested 8mer peptides. For instance, the 8mer peptide GDTTTGVY nested within KGDTTTGVY is predicted to bind within the 5% rank to the HLA-B*4402 allele.

Disregarding the six cryptic restrictions mentioned above including the nested peptide restrictions, we suggest that the 26 identified WNV CTL epitopes are restricted by 11 different HLA class I alleles (A*0101, A*0201, A*0301, B*0702, B*0801, B*2702, B*4001, B*4403, B*5601, Cw*0304, Cw*0602) covering 7 of the 12 major HLA-A and HLA-B supertypes. Table 3 lists the genotype frequency of these alleles in different areas of the world.

We are aware that the suggested restricting HLA class I alleles represent only the most likely restricting element, and that these assignment are merely based upon predictions.

Population coverage

Since a key objective of this study is to identify $CD8^+$ T cell epitopes that collectively have a broad coverage of WNV strains and thereby are of particular interest for vaccine development, we next examined the theoretical population coverage in different areas of the World with a minimal epitope set consisting of the 11 epitopes marked in bold in Table 2. These 11 epitopes were selected because they each are restricted by one or two of the 11 suggested restricting HLA class I alleles. If more than one epitope could be selected for the same HLA class I allele, we chose the more conserved epitope. Although additional WNV epitopes are known from previous studies [5,9,10] and others are likely still undiscovered, the analysis illustrates the coverage that could be obtained by a small set of epitopes.

We hypothesise that although we could not detect a response against all epitopes in all HLA class I matched patients even for 100% conserved epitopes, *immunising* with the epitopes will lead to $CD8^+$ T cell activation in all HLA class I matched individuals. This hypothesis is supported by a study by Assarsson et al., where

Table 2. Predicted HLA class I restriction of the 36 observed responses.

Patient	Sequence	Selecting HLA	Restricting HLA	%Rank	Direct HLA match	Super-type match	Explainable by %rank
44401	ILLWEIPDV	A*0201	A*0201	0.30	X	X	X
44401	VLNETTNWL	A*0201	A*0201	1.00	X	X	X
44401	SLFGGMSWI	A*0201	A*0201	0.80	X	X	X
44401	VVEKQSGLY	A*0101	A*0101	0.15	X	X	X
44405	**MTKEEFTRY**	A*0101	A*0101	0.40	X	X	X
44405	SLVNGVVRL	A*0201	A*0201	3.00	X	X	X
44405	ITYTDVLRY	A*0101	A*0101	0.10	X	X	X
55302	RAWNSGYEW	B*5801	B*57	NA		X	NA
55302	RSLFGGMSW	B*5801	B*57	NA		X	NA
55302	RVLEMVEDW	B*5801	B*57	NA		X	NA
55307	**YRHKVVKVM**	B*2705	Cw*0602	1.50			X
55308	**LTYRHKVVK**	A*0301	A*0301	0.30	X	X	X
55309	**AEVEEHRTV**	B*4001	B*4403	1.50		X	X
55309	HTTKGAALM	A*2601	NA	6.00			*
55309	LTYRHKVVK	A*0301	A*0301	0.30	X	X	X
55309	YTMDGEYRL	B*3901	A*0201	1,00			X
55405	FVDVGVSAL	B*3901	B*0702	3,00			X
55405	**GPIRFVLAL**	B*0702	B*0702	0.30	X	X	X
55405	ILLWEIPDV	A*0201	NA	32.00			
55405	**RRSRRSLTV**	B*2705	B*0801	4.00			X
55405	SYHDRRWCF	A*2403	B*0801	3.00			X
55410	SLVNGVVRL	A*0201	A*0201	3.00	X	X	X
55410	**ILRNPGYAL**	B*0801	Cw*0304	1.50			X
55410	KGDTTTGVY	A*0101	NA	32.00			**
55410	RYLVKTESW	A*2403	NA	6.00			
55410	**TEVMTAVGL**	B*4001	B*4001	0.15	X	X	X
55410	VLNETTNWL	A*0201	A*0201	1.00	X	X	X
55410	**YTMDGEYRL**	B*3901	A*0201	1.00			X
55413	YTMDGEYRL	B*3901	A*0201	1.00			X
55413	SLVNGVVRL	A*0201	A*0201	3.00	X	X	X
55413	TLARGFPFV	A*0201	A*0201	0.10	X	X	X
55415	KGDTTTGVY	A*0101	NA	32.00			
55415	SLVNGVVRL	A*0201	A*0201	3.00	X	X	X
55415	GLYKSAPRR	A*0301	NA	50.00			
55415	**RPAADGKTV**	B*0702	B*5601	0.80		X	X
55415	**RYLVKTESW**	A*2403	B*2702	0.80			X

The columns lists: **Patient:** Patient ID, **Sequence:** Epitope amino acid sequence, **Selecting HLA:** The HLA class I allele used for selecting the epitope, **Restricting HLA:** The HLA class I allele by which the epitope is predicted to be restricted in this patient using the *NetMHCpan* method. NA indicate that none of the patient's HLA molecules were predicted to present the peptide with a %rank score less than or equal to 5. **%rank:** The rank of the epitope among 1,000,000 random, natural, 9meric peptides based on the predicted binding affinities to the restricting HLA, **Direct match:** The patient carries the HLA class I allele for which the epitope is selected, i.e., the selecting and restricting HLA class I alleles are identical. **Supertype match:** The patient does not carry the HLA class I allele for which the epitope is selected, however, the selecting and restricting HLA class I allele belong to the same HLA class I supertype. **Explainable by %rank:** The patient does not carry the HLA class I allele for which the epitope is selected, but another HLA class I allele, which is also predicted to present the epitope (the %rank value for the restricting HLA is below 5.00).

Note that the rank-analysis was not performed for patient #55302, since the HLA-A and -B alleles of this patient was only determined by low-resolution serological typing and the HLA-C alleles are undetermined. The three epitopes that are recognised in this patient are, however, all well presented by B*5701 (by serological typing it is known that patient #55302 carries B57) with %rank scores between 0.1% and 0.2%.

*This peptide can be presented as an 8mer, HTTKGALL, to B*3503 with a %rank score of 5%.

**This peptide can be presented as an 8mer, GDTTTGVY, by B*4402 with a %rank score of 5%.

Epitopes marked in bold are used for the population coverage calculations.

doi:10.1371/journal.pone.0012697.t002

Table 3. Allele frequencies of 11 HLA class I alleles in different areas of the world.

HLA	Australia	Europe	North-East Asia	North America	Oceania	South-East Asia	South-West Asia	South America	Sub-Saharan Africa	North Africa
A*0101	0,022	0,164	0,059	0,042	0,003	0,007	0,094	0,002	0,056	0,137
A*0201	0,127	0,272	0,153	0,145	0,144	0,069	0,158	0,221	0,103	0,176
A*0301	0,014	0,141	0,037	0,037	0,005	0,006	0,048	NA	0,051	0,040
B*0702	0,011	0,139	0,054	0,038	0,003	0,007	0,026	0,006	0,044	0,029
B*0801	0,012	0,118	0,004	0,022	NA	0,003	0,043	NA	0,042	0,077
B*2702	NA	0,005	0,001	0,002	NA	NA	0,001	NA	NA	0,007
B*4001	0,092	0,049	0,045	0,022	0,149	0,165	0,007	0,002	0,004	0,007
B*4403	0,001	0,049	0,047	0,018	0,005	0,015	0,029	NA	0,035	0,099
B*5601	0,161	0,004	0,005	0,004	0,005	0,016	0,001	NA	0,003	NA
Cw*0304	0,009	0,065	0,098	0,218	0,108	0,173	0,009	0,238	0,048	NA
Cw*0602	0,009	0,091	0,086	0,042	0,005	0,014	0,117	0,002	0,145	NA

HLA population coverage data was obtained from dbMHC (http://www.ncbi.nlm.nih.gov/gv/mhc/).
NA: Not available.
doi:10.1371/journal.pone.0012697.t003

$CD8^+$ T cell responses were detected against all previously identified epitopes after immunising transgenic mice with the epitopes [34]. Whether or not the memory $CD8^+$ T cells will later recognise cells infected with WNV depends on which epitopes the infecting WNV strain contain. The population coverage of the 11 epitope:HLA class I pairs is accordingly calculated by considering both the HLA class I allele frequencies and the epitope conservation as described in *Material and Methods*. Table 4 summarises the coverage of the 11 epitope:HLA class I allele pairs in ten areas of the World. Considering only the 11 restricting HLA class I alleles identified in this study, more than half of the population is covered in nine out of the ten areas of the World. For the North American population the coverage is 72%, while the coverage is 93% for the European population. The smallest coverage is found in Australia, where 48% of the population is covered.

Table 4. Epitope coverage in ten areas of the World.

Area	Coverage
Australia	0.48
Europe	0.93
North Africa	0.67
North America	0.72
North-East Asia	0.69
Oceania	0.59
South America	0.65
South-East Asia	0.62
South-West Asia	0.67
Sub-Saharan Africa	0.66

HLA class I allele frequencies were obtained from dbMHC (http://www.ncbi.nlm.nih.gov/gv/mhc/). Coverage is calculated as described in the subsection *Calculating the epitope coverage* in *Materials and Methods*. A Coverage of 1 corresponds to maximum (full) coverage.
doi:10.1371/journal.pone.0012697.t004

Discussion

Using reverse immunology and employing bioinformatics methods, we have discovered 26 new WNV specific $CD8^+$ T cell epitopes, which significantly extends the repertoire of known WNV $CD8^+$ T cell epitopes. We suggest that the newly discovered epitopes are restricted by 11 different HLA class I alleles.

When we initiated our study, only 20 fully-sequenced genomes from WNV strains were publically available, and they form the basis of our predictions. Since then, additional WNV strains have been sequenced and the WNV variability has been analysed at a larger scale [11]. Our approach included selecting predicted WNV epitopes that experience broad coverage of the 20 originally sequenced WNV strains. It is likely that we would select a different set of broadly covering predicted epitopes, if we were to repeat the study using data from all presently available WNV strains. Nevertheless, our results indicate that selecting predicted epitopes with a broad coverage of WNV strains - in contrast to 100% conserved epitopes - enables identification of more epitopes in the structural WNV proteins. These proteins vary the most and hence contain the fewest fully conserved regions [11].

In the present study, we observed that the number of predicted epitopes is a direct function of protein size. However, in our recent study, we observed that the interindividual patterns of $CD8^+$ T cell dominance (the frequency of recognition) do not correlate with protein size but rather with the individual's HLA. As an example, individuals expressing HLA-A*0201 were primarily reactive to an epitope in E and an epitope in NS4b [9,10], while individuals expressing HLA-A*0101 displayed a $CD8^+$ T cell response directed against prM. Thus, protein size alone is not sufficient to explain dominance within individuals or between individuals. Furthermore, we and our collaborators have recently reported a direct survey of WNV peptides bound by HLA-A*0201 in infected cells [9] and did not observe a correlation between protein size and natural loading of HLA class I. It should be noted that we identified a number of epitopes in the present study that evoked more robust responses in some of the patients than were observed with our previously identified collection of "dominant" epitopes. These observations highlight the complexities of antigen processing and stress the importance of using combined methodologies (*in silico*, *in vitro*, and *in vivo*) for epitope discovery.

The complex epitope recognition pattern in the WNV infected patients showed that not all peptides that induce a CTL response in one patient do so in all patients expressing the restricting HLA class I allele. This is true even for epitopes that are fully conserved across all analysed WNV strains. It is, however, inevitable that not all patients expressing the appropriate HLA allele will respond to a given epitope restricted by this allele due to factors like dominance, competition, "holes" in the T cell repertoire etc. In fact, in a recent work we show that only 34–50% of patients expressing an appropriate HLA allele will respond to an epitope restricted by this allele [35]. The fact that not all patients expressing a given allele respond to all epitopes restricted by this allele is thus not an indication of a faulty prediction method, but rather a result of factors we cannot control.

Unlike the results of our recent study identifying WNV $CD8^+$ T cell epitopes, where reactivity to four dominating epitopes were found in almost all patients expressing the restricting HLA class I allele [10], the $CD8^+$ T cell epitopes identified in the present study maximally induced response in about 25% of patients bearing the appropriate HLA. It seems that the $CD8^+$ T cell response against WNV includes both a few epitopes recognised in the majority of infected individuals - interindividually dominant epitopes - as well as a broad response against interindividually subdominate epitopes that each are recognised in some infected individuals, but not in others. Similar observations are apparent for other small RNA viruses, e.g., Influenza A virus: Almost all HLA-A*0201 positive individuals were found to respond against the epitope $M1_{58–66}$ in a study from 1995 [36], while $CD8^+$ T cell epitopes identified in a later study were responsive in only some patients carrying the restricting HLA class I alleles [15,37]. $CD8^+$ T cell responses against HIV have also been found to contain both interindividually dominant and subdominant epitopes [16,38,39].

We tested all peptides with an *in vitro* determined HLA class I binding affinity below (i.e. better than) 500 nM in all the WNV infected patients. Half of the responses were found in patients not expressing the predicted restricting HLA class I allele. The concordance between predictive and actual HLA class I restriction could be slightly improved by taking into account the supertype association of the patient HLA class I alleles. In contrast, only 18% (six responses) remained unexplainable when applying a pan-specific HLA peptide binding prediction method for calculating the %rank score of the epitope to each of the responding patient's six HLA class I alleles and considering the allele with a %rank score below 5 as the restricting allele. These results confirm recent findings that HLA class I supertypes often provide an oversimplification of the HLA class I specificity space [24,40,41]. Moreover, and maybe more importantly, this analyses shows that the majority of these immune responses are indeed predictable using advanced bioinformatics methods for pan-specific HLA-peptide binding and that cellular responses are hence directly explained in terms of peptide binding to one of the patients HLA molecules in accordance with earlier work by for instance Hoof et al. [28].

Despite the complex epitope recognition pattern observed, we hypothesised that all of the newly identified WNV epitopes will induce a CTL response in all individuals carrying the restricting HLA class I allele, if the individuals were to be *immunised* with the epitopes. This hypothesis is supported by a study concerning the repertoire of $CD8^+$ T cell epitopes recognised after Vaccinia Virus infection [34]. Here it is shown that all Vaccinia Virus $CD8^+$ T cell epitopes identified in a previous study in the context of natural infection [42] were able to elicit CTL responses in mice immunised with the epitopes. Similar immunological analysis is required to verify that the WNV epitopes identified in the present study are able to induce a successful antiviral response in a host. Nevertheless, we performed a theoretical analysis, in which we assembled a minimal set of 11 epitopes suggested restricted by 11 different HLA class I alleles. We then calculated the population coverage, if one was to use this set of epitopes for immunising populations in different areas of the World. We found very high population coverage. The population coverage would be even higher, if we had also considered HLA class I alleles that bind the epitopes as strong as or stronger than the restricting HLA class I allele identified in the present study. Although our discovery of WNV epitopes is based on relatively few patients and could be strengthened by further immunological follow-up experiments, the results indicate that very few epitopes are sufficient for covering the majority of the human population. In the context of an epitope based vaccine against WNV, a larger set of epitopes is, however, preferable to prevent the virus from producing escape variants not containing any of the epitopes. The final composition of an epitope based WNV vaccine in terms of, e.g., subdominant contra dominant epitopes, adjuvant and $CD4^+$ T cell epitopes is not dealt with in this study, but clearly these issues also need to be resolved before a vaccine can become a reality.

In conclusion, using advanced bioinformatics methods for $CD8^+$ T cell epitope prediction, we have discovered 26 new WNV epitopes that we suggest are restricted by 11 different HLA class I alleles. These epitopes contribute to our knowledge of the immune response against WNV infection and extend the list of known WNV $CD8^+$ T cell epitopes.

Supporting Information

Figure S1 Location of the selected, predicted $CD8^+$ T cell epitopes. The 192 selected, predicted epitopes are listed under the reference sequence with RefSeq ID: NC_001563. The HLA class I supertype restriction is listed in parenthesis after the sequence of the epitope. Please note that 17 of the epitopes are predicted to be restricted by more than one HLA class I allele, resulting in a total of 175 unique peptides.
Found at: doi:10.1371/journal.pone.0012697.s001 (0.03 MB PDF)

Figure S2 *In vitro* expansion prior to analysis increases sensitivity and does not impact epitope hierarchy. Cryopreserved PBMC from patient #55302 were thawed and rested overnight prior to stimulation for ICS assay (upper panels). A portion of the thawed cells were also subjected to a round of *in vitro* expansion using K64-4-1BBL cells as described the subsection ICS validations of Materials and Methods prior to analysis by ICS assay (lower panels). The numbers reflect the percentage of IFN-γ-positive cells of total live lymphocytes.
Found at: doi:10.1371/journal.pone.0012697.s002 (1.28 MB TIF)

Table S1 Measured binding affinity. Of the 175 predicted $CD8^+$ T cell epitopes, 161 were synthesised and their in vitro binding affinity to the predicted restricting HLA class I allele was measured. The table lists the 112 peptides that experience a KD below 500 nM.
Found at: doi:10.1371/journal.pone.0012697.s003 (0.01 MB PDF)

Table S2 The 26 identified WNV CD8+ T cell epitopes. The columns lists: Sequence: Amino acid sequence of the epitope, Selecting HLA: The HLA class I allele used for selecting the epitope, Protein: Source protein of the epitope, Position: Starting position of the epitope in the source protein, Conservation: Conservation of the epitope in 140 fully sequenced WNV strains obtained from (Koo et al., 2009), Number of responses: The number of responses that were observed against this epitope in this

study, Responders: The patients that responded against this epitope. The HLA alleles of each patient are written in subscript after patient ID number. HLA alleles marked in bold are alleles by which the epitope is predicted to be restricted in this patient (see the paragraph "Suggested HLA class I restriction and Table 3 for details), Figure: The figure that illustrates the response.
Found at: doi:10.1371/journal.pone.0012697.s004 (0.01 MB PDF)

Author Contributions

Conceived and designed the experiments: MVL MN MBL SB JB OL. Performed the experiments: MVL AL RP MN IH KL JB. Analyzed the data: MVL AL RP MN IH KL MBL SB JB OL. Contributed reagents/materials/analysis tools: MVL RP MN MBL JB OL. Wrote the paper: MVL MN JB.

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Figure S1


10 60
NC_001563: MSKKPGGPGKNRAVNMLKRGMPRGLSLIGLKRAMLSLIDGKGPIRFVLALLAFFRFTAIA
GRGPIRFVL(B39)
VLALLAFFR(A3)
AIAPTRAVL(B7)
RFVLALLAF(A24)
FVLALLAFF(A26)
VLSLIGLKR(A3)
GPIRFVLAL(B7)
70 120
NC_001563: PTRAVLDRWRGVNKQTAMKHLLSFKKELGTLTSAINRRSTKQKKRGGTAGFTILLGLIAC
VMIGLIASV(A2)
GVNKQTAMK(A3)
130 180
NC_001563: AGAVTLSNFQGKVMMTVNATDVTDVITIPTAAGKNLCIVRAMDVGYLCEDTITYECPVLA
FLCDDTITY(B62)
YLCEDTITY(B62)
IPTAAGKNL(B7)
190 240
NC_001563: AGNDPEDIDCWCTKSSVYVRYGRCTKTRHSRRSRRSLTVQTHGESTLANKKGAWLDSTKA
HSRRSRRSL(B7)
RRSRRSLTV(B27)
250 300
NC_001563: TRYLVKTESWILRNPGYALVAAVIGWMLGSNTMQRVVFAILLLLVAPAYSFNCLGMSNRD
ILRNPGYAL(B8)
LVKTESWIL(B8)
RYLVKTESW(A24)
LLVAPAYSF(B62)
LLLLVAPAY(B62)
310 360
NC_001563: FLEGVSGATWVDLVLEGDSCVTIMSKDKPTIDVKMMNMEAANLADVRSYCYLASVSDLST
STKAACPTM(A26)
370 420
NC_001563: RAACPTMGEAHNEKRADPAFVCKQGVVDRGWGNGCGLFGKGSIDTCAKFACTTKATGWII
GWGNGCGLF(A24)
430 480
NC_001563: QKENIKYEVAIFVHGPTTVESHGKIGATQAGRFSITPSAPSYTLKLGEYGEVTVDCEPRS
SYTLKLGEY(A24)
TVSPSAPTY(B62)
SGIDTNAYY(A26)
RSGIDTNAY(A1)
490 540
NC_001563: GIDTSAYYVMSVGEKSFLVHREWFMDLNLPWSSAGSTTWRNRETLMEFEEPHATKQSVVA
REWFMDLNL(B44)
KSFLVHREW(B58)
VHREWFMDL(B39)
550 600
NC_001563: LGSQEGALHQALAGAIPVEFSSNTVKLTSGHLKCRVKMEKLQLKGTTYGVCSKAFKFART
KLQLKGTTY(A3)
KLKLKGTTY(A3)
TYGVCSKAF(A24)
TYGVCAKAF(A24)
QEGALHQAL(B44)
KCRVKMEKL(B8)
610 660
NC_001563: PADTGHGTVVLELQYTGTDGPCKVPISSVASLNDLTPVGRLVTVNPFVSVATANSKVLIE
GRLVTVNPF(B27)
VPISSVASL(B7)
GTVVLELQY(A1)
GHGTVVLEL(B39)
670 720
NC_001563: LEPPFGDSYIVVGRGEQQINHHWHKSGSSIGKAFTTTLRGAQRLAALGDTAWDFGSVGGV
LAALGDTAW(B58)
730 780
NC_001563: FTSVGKAIHQVFGGAFRSLFGGMSWITQGLLGALLLWMGINARDRSIAMTFLAVGGVLLF
RSLFGGMSW(B58)
GMSWITQGL(A2)
SLFGGMSWI(A2)
FLAVGGVLL(A2)
790 840
NC_001563: LSVNVHADTGCAIDIGRQELRCGSGVFIHNDVEAWMDRYKFYPETPQGLAKIIQKAHAEG
YHPETPQGL(B39)
ETPQGLAKI(A26)
850 900
NC_001563: VCGLRSVSRLEHQMWEAIKDELNTLLKENGVDLSVVVEKQNGMYKAAPKRLAATTEKLEM
GLYKSAPRR(A3)
RRLAATTEK(B27)
VVEKQSGLY(A1)
KENGVDLSV(B44)
VSRLEHQMW(B58)


910 960
NC_001563: GWKAWGKSIIFAPELANNTFVIDGPETEECPTANRAWNSMEVEDFGFGLTSTRMFLRIRE
KAWGKSIIF(B58)
970 1020
NC_001563: TNTTECDSKIIGTAVKNNMAVHSDLSYWIESGLNDTWKLERAVLGEVKSCTWPETHTLWG
1030 1080
NC_001563: DGVLESDLIIPITLAGPRSNHNRRPGYKTQNQGPWDEGRVEIDFDYCPGTTVTISDSCEH
YCPGTTVTL(B39)
1090 1140
NC_001563: RGPAARTTTESGKLITDWCCRSCTLPPLRFQTENGCWYGMEIRPTRHDEKTLVQSRVNAY
YQTDSGCWY(A1)
WYGMEIRPL(A24)
1150 1200
NC_001563: NADMIDPFQLGLMVVFLATQEVLRKRWTAKISIPAIMLALLVLVFGGITYTDVLRYVILV
ITYTDVLRY(A1)
VLRKRWTAK(A3)
FQLGLLVVF(B27)
YVILVGAAF(A26)
1210 1260
NC_001563: GAAFAEANSGGDVVHLALMATFKIQPVFLVASFLKARWTNQESILLMLAAAFFQMAYYDA
QPAFMVASF(B7)
ILLMLAAAF(B62)
MLAAAFFQM(A2)
QPVFMVASF(B7)
1270 1320
NC_001563: KNVLSWEVPDVLNSLSVAWMILRAISFTNTSNVVVPLLALLTPGLKCLNLDVYRILLLMV
ILLWEIPDV(A2)
MVGVGSLVK(A3)
WMILRAISF(A24)
EIPDVLNSL(A26)
1330 1380
NC_001563: GVGSLIKEKRSSAAKKKGACLICLALASTGVFNPMILAAGLMACDPNRKRGWPATEVMTA
TEVMTAVGL(B44)
EVMTAVGLM(A26)
AAKKKGASL(B7)
1390 1440
NC_001563: VGLMFAIVGGLAELDIDSMAIPMTIAGLMFAAFVISGKSTDMWIERTADITWESDAEITG
AIVGGLAEL(A2)
ISGKSTDMW(B58)
1450 1500
NC_001563: SSERVDVRLDDDGNFQLMNDPGAPWKIWMLRMACLAISAYTPWAILPSVIGFWITLQYTK
LAVSAYTPW(B58)
GAPWKIWML(B8)
LAISAYTPW(B58)
LMNDPGAPW(B62)
1510 1560
NC_001563: RGGVLWDTPSPKEYKKGDTTTGVYRIMTRGLLGSYQAGAGVMVEGVFHTLWHTTKGAALM
VLWDTPSPK(A3)
YRIMTRGLL(B27)
MTRGLLGSY(A1)
WHTTKGAAL(B39)
HTTKGAALM(A26)
MTRGILGSY(A1)
KGDTTTGVY(A1)
VYRIMTRGL(A24)
1570 1620
NC_001563: SGEGRLDPYWGSVKEDRLCYGGPWKLQHKWNGHDEVQMIVVEPGKNVKNVQTKPGVFKTP
RLCYGGPWK(A3)
1630 1680
NC_001563: EGEIGAVTLDYPTGTSGSPIVDKNGDVIGLYGNGVIMPNGSYISAIVQGERMEEPAPAGF
MEEPAPAGF(B44)
GVIMPNGSY(B62)
1690 1740
NC_001563: EPEMLRKKQITVLDLHPGAGKTRKILPQIIKEAINKRLRTAVLAPTRVVAAEMSEALRGL
MLRKKQITV(B8)
1750 1800
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GYISTRVEL(A24)
REHSGNEIV(B44)
MSPHRVPNY(A1)
CHATLTHRL(B39)
1810 1860
NC_001563: YIATKVELGEAAAIFMTATPPGTSDPFPESNAPISDMQTEIPDRAWNTGYEWITEYVGKT
EYVGKTVWF(A24)
RAWNSGYEW(B58)
1870 1920
NC_001563: VWFVPSVKMGNEIALCLQRAGKKVIQLNRKSYETEYPKCKNDDWDFVITTDISEMGANFK
KSYETEYPK(A3)
VIQLNRKSY(B62)
1930 1980


NC_001563: ASRVIDSRKSVKPTIIEEGDGRVILGEPSAITAASAAQRRGRIGRNPSQVGDEYCYGGHT
TEGEGRVIL(B39)
HTNEDDSNF(A26)
RVIDSRKSV(B7)
1990
2040
NC_001563: NEDDSNFAHWTEARIMLDNINMPNGLVAQLYQPEREKVYTMDGEYRLRGEERKNFLEFLR
EERKNFLEL(B44)
KVYTMDGEY(A3)
YTMDGEYRL(A2)
YRLRGEERK(B27)
YQPEREKVY(B62)
2050
2100
NC_001563: TADLPVWLAYKVAAAGISYHDRKWCFDGPRTNTILEDNNEVEVITKLGERKILRPRWADA
RPRWADARV(B7)
KVAAAGVSY(B62)
NEVEVITKL(B44)
SYHDRRWCF(A24)
RRWCFDGPR(B27)
2110
2160
NC_001563: RVYSDHQALKSFKDFASGKRSQIGLVEVLGRMPEHFMVKTWEALDTMYVVATAEKGGRAH
WEALDTMYV(B44)
GGRAHRMAL(B7)
RVYSDHQAL(B7)
2170
2220
NC_001563: RMALEELPDALQTIVLIALLSVMSLGVFFLLMQRKGIGKIGLGGVILGAATFFCWMAEVP
VMTMGVFFL(A2)
LGAATFFCW(B58)
GVFFLLMQR(A3)
2230
2280
NC_001563: GTKIAGMLLLSLLLMIVLIPEPEKQRSQTDNQLAVFLICVLTLVGAVAANEMGWLDKTKN
QTDNQLAVF(A1)
2290
2340
NC_001563: DIGSLLGHRPEARETTLGVESFLLDLRPATAWSLYAVTTAVLTPLLKHLITSDYINTSLT
SLYAVTTAV(A2)
LYAVTTAVL(A24)
RESFGVESF(B44)
TSDYINTSL(B39)
2350
2400
NC_001563: SINVQASALFTLARGFPFVDVGVSALLLAVGCWGQVTLTVTVTAAALLFCHYAYMVPGWQ
SLARGFPFV(A2)
TLARGFPFV(A2)
FVDVGVSAL(B39)
2410
2460
NC_001563: AEAMRSAQRRTAAGIMKNVVVDGIVATDVPELERTTPVMQKKVGQIILILVSMAAVVVNP
RRTAAGIMK(B27)
RAAQRRTAA(B7)
2470
2520
NC_001563: SVRTVREAGILTTAAAVTLWENGASSVWNATTAIGLCHIMRGGWLSCLSIMWTLIKNMEK
ITAAAVTLW(B58)
2530
2580
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LTKEEFTRY(A1)
FTRYRKEAI(B8)
MTKEEFTRY(A1)
2590
2640
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LRWLVERRF(B27)
RRFLEPVGK(B27)
KRVQEVRGY(B27)
KRVQEVKGY(B27)
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WLHRGPKEF(B8)
AEVEEHRTV(B44)
RVLEMVEDW(B58)
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2760
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RRYGGGLVR(B27)
KVLCPYMPK(A3)
KVIEKMEVL(B8)
VLCPYMPKV(A2)
FCIKVLCPY(A26)
2770
2820
NC_001563: TSQVLLGRMEKKTWKGPQFEEDVNLGSGTRAVGKPLLNSDTSKIKNRIERLKKEYSSTWH
TRAVGKPLL(B39)
KIRNRIERL(B8)
AHYEEDVNL(B39)
GRMEKKTWK(B27)
KIKNRIERL(B8)
2830
2880


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RTWNYHGSY(A1)
SLVNGVVRL(A2)
KPTGSASSL(B7)
2890
2940
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AMFEEQNQW(B62)
VLNETTNWL(A2)
ETTNWLWAF(A26)
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3000
NC_001563: EEQNQWKNAREAVEDPKFWEMVDEEREAHLRGECNTCIYNMMGKREKKPGEFGKAKGSRA
SRAIWFMWL(B27)
GSRAIWFMW(B58)
3010
3060
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WFMWLGARF(A24)
LEFEALGFL(B44)
3070
3120
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RPAVGGKTV(B7)
YRHKVVKVM(B27)
RPAADGKTV(B7)
RLARAIIEL(A2)
NEAKVLELL(B44)
LTYRHKVVK(A3)
3130
3180
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GPKVRTWLF(B8)
GVKVRVWLF(B8)
3190
3240
NC_001563: GEERLSRMAVSGDDCVVKPLDDRFATSLHFLNAMSKVRKDIQEWKPSTGWYDWQQVPFCS
WYDWQQVPF(A24)
3250
3300
NC_001563: NHFTELIMKDGRTLVVPCRGQDELIGRARISPGAGWNVRDTACLAKSYAQMWLLLYFHRR
YFHRRDLRL(B8)
DTACLAKSY(A1)
YAQMWQLLY(A1)
KSYAQMWLL(B58)
YAQMWLLLY(A1)
3310
3360
NC_001563: DLRLMANAICSAVPANWVPTGRTTWSIHAKGEWMTTEDMLAVWNRVWIEENEWMEDKTPV
RVWIEENEW(B58)
3370
3420
NC_001563: ERWSDVPYSGKREDIWCGSLIGTRTRATWAENIHVAINQVRSVIGEEKYVDYMSSLRRYE
REDIWCGSL(B44)
3430
NC_001563: DTIVVEDTVL

Figure S2

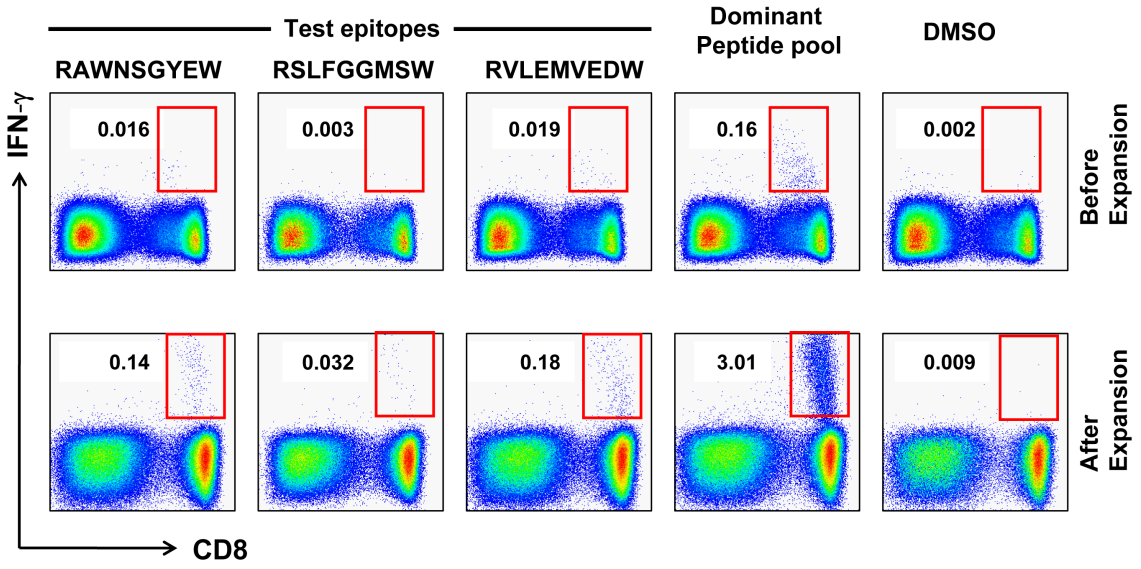
Test epitopes
RAWNSGYEW
RSLFGGMSW
RVLEMVEDW
Dominant Peptide pool
DMSO
IFN-γ
CD8
0.016
0.003
0.019
0.16
0.002
0.14
0.032
0.18
3.01
0.009
Before Expansion
After Expansion

Table S1

Sequence	HLA	Measured K_D (nM)
ITYTDVLRY	A*0101	3
VVEKQSGLY	A*0101	6
RSGIDTNAY	A*0101	66
QTDNQLAVF	A*0101	168
RTWNYHGSY	A*0101	237
MTKEEFTRY	A*0101	246
KGDTTTGVY	A*0101	472
SLFGGMSWI	A*0201	1
GMSWITQGL	A*0201	1
ILLWEIPDV	A*0201	1
VLNETTNWL	A*0201	2
FLAVGGVLL	A*0201	3
RLARAIIEL	A*0201	3
TLARGFPFV	A*0201	3
SLVNGVVRL	A*0201	5
VLCPYMPKV	A*0201	21
GLYKSAPRR	A*0301	37
VLRKRWTAK	A*0301	43
LTYRHKVVK	A*0301	94
VLWDTPSPK	A*0301	117
MVGVGSLVK	A*0301	380
VLSLIGLKR	A*0301	481
RYLVKTESW	A*2403	4
WYDWQQVPF	A*2403	7
LYAVTTAVL	A*2403	8
SYHDRRWCF	A*2403	9
WFMWLGARF	A*2403	10
TYGVCAKAF	A*2403	13
TYGVCSKAF	A*2403	25
EYVGKTVWF	A*2403	26
GYISTRVEL	A*2403	45
WMILRAISF	A*2403	120
SYTLKLGEY	A*2403	143
RFVLALLAF	A*2403	176
ETTNWLWAF	A*2601	2
DTITNVTTM	A*2601	4
EVMTAVGLM	A*2601	5
ETTNWLWTF	A*2601	5
MTRGLLGSY	A*2601	8
DTACLAKSY	A*2601	8

HTTKGAALM	A*2601	23
FCIKVLCPY	A*2601	314
AIAPTRAVL	B*0702	2
RPRWADARV	B*0702	9
RAAQRRTAA	B*0702	11
GPIRFVLAL	B*0702	13
AAKKKGASL	B*0702	15
VPISSVASL	B*0702	25
KPTGSASSL	B*0702	27
RPAVGGKTV	B*0702	36
HSRRSRRSL	B*0702	47
GGRAHRMAL	B*0702	63
QPAFMVASF	B*0702	71
RVIDSRKSV	B*0702	97
RPAADGKTV	B*0702	97
IPTAAGKNL	B*0702	150
RVYSDHQAL	B*0702	205
MLRKKQITV	B*0801	35
FTRYRKEAI	B*0801	39
GPKVRTWLF	B*0801	126
KIRNRIERL	B*0801	254
ILRNPGYAL	B*0801	483
AMFEEQNQW	B*1501	17
GVIMPNGSY	B*1501	29
LMNDPGAPW	B*1501	40
TVSPSAPTY	B*1501	68
YQPEREKVY	B*1501	80
LLLLVAPAY	B*1501	87
VIQLNRKSY	B*1501	105
LLVAPAYSF	B*1501	137
KVAAAGVSY	B*1501	165
YLCEDTITY	B*1501	197
RRWCFDGPR	B*2705	11
RRTAAGIMK	B*2705	24
YRHKVVKVM	B*2705	33
YRIMTRGLL	B*2705	35
RRLAATTEK	B*2705	37
RRFLEPVGK	B*2705	50
RRYGGGLVR	B*2705	62
GRLVTVNPF	B*2705	75
LRWLVERRF	B*2705	98
GRMEKKTWK	B*2705	103

RRSRRSLTV	B*2705	385
WHTTKGAAL	B*3901	17
FVDVGVSAL	B*3901	52
CHATLTHRL	B*3901	64
TRAVGKPLL	B*3901	141
YTMDGEYRL	B*3901	154
GRGPIRFVL	B*3901	162
RESFGVESF	B*4001	1
REHSGNEIV	B*4001	1
KENGVDLSV	B*4001	3
REDIWCGSL	B*4001	7
REWFMDLNL	B*4001	9
TEVMTAVGL	B*4001	10
QEGALHQAL	B*4001	14
WEALDTMYV	B*4001	27
NEAKVLELL	B*4001	28
NEVEVITKL	B*4001	80
AEVEEHRTV	B*4001	130
VEGLGLQKL	B*4001	230
RVLEMVEDW	B*5801	4
RSLFGGMSW	B*5801	5
KSYAQMWLL	B*5801	8
LAVSAYTPW	B*5801	11
KSFLVHREW	B*5801	11
RAWNSGYEW	B*5801	16
KAWGKSIIF	B*5801	42
VSRLEHQMW	B*5801	56
LAALGDTAW	B*5801	66
GSRAIWFMW	B*5801	87
ISGKSTDMW	B*5801	91

Table S2

Sequence	Selecting HLA	Protein	Pos.	Cons. (%)	No. of responses	Responders	Figure
YTMDGEYRL	B*3901	NS3	518	97	3	55410**A*0201-A*0201**-B*4001-B*4402-Cw*0304-Cw*0501 55413A*0101-**A*0201**-B*0801-B*4402-Cw*0701-Cw*0501 55309**A*0201**-A*0301-B*3503-B*4403-Cw*0401-Cw*0401	1
RYLVKTESW	A*2403	prM	119	99	2	55410A*0201-A*0201-B*4001-B*4402-Cw*0304-Cw*0501 55415A*0201-A*0201-**B*2702-**B*5601-Cw*0102-Cw*0202	1
LTYRHKVVK	A*0301	NS5	573	100	2	55309A*0201-**A*0301**-B*3503-B*4403-Cw*0401-Cw*0401 55308A*0101-**A*0301**-B*0801-B*4701-Cw*0602-Cw*0701	1
GPIRFVLAL	B*0702	C	42	96	1	55405A*0101-A*0301-**B*0702**-B*0801-Cw*0701-Cw*0702	1
TEVMTAVGL	B*4001	NS2B	5	99	1	55410A*0201-A*0201-**B*4001**-B*4402-Cw*0304-Cw*0501	1
ILRNPGYAL	B*0801	prM	128	100	1	55410A*0201-A*0201-B*4001-B*4402-**Cw*0304**-Cw*0501	1
YRHKVVKVM	B*2705	NS5	575	100	1	55307A*0101-A*0301-B*3701-B*4429-Cw*0501-**Cw*0602**	1
SYHDRRWCF	A*2403	NS3	557	89	1	55405A*0101-A*0301-B*0702-**B*0801**-Cw*0701-Cw*0702	1
AEVEEHRTV	B*4001	NS5	154	8	1	55309A*0201-A*0301-B*3503-**B*4403**-Cw*0401-Cw*0401	1
GLYKSAPRR	A*0301	NS1	95	1	1	55415A*0201-A*0201-B*2702-B*5601-Cw*0102-Cw*0202	1
KGDTTTGVY	A*0101	NS3	15	96	2	55410A*0201-A*0201-B*4001-B*4402-Cw*0304-Cw*0501 55415A*0201-A*0201-B*2702-B*5601-Cw*0102-Cw*0202	1/2
RPAADGKTV	B*0702	NS5	584	8	1	55415A*0201-A*0201-B*2702-**B*5601**-Cw*0102-Cw*0202	1
HTTKGAALM	A*2601	NS3	51	100	1	55309A*0201-A*0301-B*3503-B*4403-Cw*0401-Cw*0401	1
RRSRRSLTV	B*2705	prM	88	99	1	55405A*0101-A*0301-B*0702-**B*0801**-Cw*0701-Cw*0702	1
FVDVGVSAL	B*3901	NS4B	112	99	1	55405A*0101-A*0301-**B*0702**-B*0801-Cw*0701-Cw*0702	1
RAWNSGYEW	B*5801	NS3	343	94	1	55302A*01-A*01-**B*57**-B*40	1
RSLFGGMSW	B*5801	E	447	99	1	55302A*01-A*01-**B*57**-B*40	1
RVLEMVEDW	B*5801	NS5	163	99	1	55302A*01-A*01-**B*57**-B*40	1

VLNETTNWL	A*0201	NS5	375	100	2	44401$_{A*0101\text{-}\mathbf{A*0201}\text{-}B*0702\text{-}B*1517\text{-}Cw*0701\text{-}Cw*0702}$ 55410$_{\mathbf{A*0201\text{-}A*0201}\text{-}B*4001\text{-}B*4402\text{-}Cw*0304\text{-}Cw*0501}$	2
SLVNGVVRL	A*0201	NS5	321	100	4	44405$_{A*0101\text{-}\mathbf{A*0201}\text{-}B*0702\text{-}B*1501\text{-}Cw*0303\text{-}Cw*0702}$ 55410$_{\mathbf{A*0201\text{-}A*0201}\text{-}B*4001\text{-}B*4402\text{-}Cw*0304\text{-}Cw*0501}$ 55415$_{\mathbf{A*0201\text{-}A*0201}\text{-}B*2702\text{-}B*5601\text{-}Cw*0102\text{-}Cw*0202}$ 55413$_{A*0101\text{-}\mathbf{A*0201}\text{-}B*0801\text{-}B*4402\text{-}Cw*0701\text{-}Cw*0501}$	2
SLFGGMSWI	A*0201	E	448	99	1	44401$_{A*0101\text{-}\mathbf{A*0201}\text{-}B*0702\text{-}B*1517\text{-}Cw*0701\text{-}Cw*0702}$	2
TLARGFPFV	A*0201	NS4B	105	96	1	55413$_{A*0101\text{-}\mathbf{A*0201}\text{-}B*0801\text{-}B*4402\text{-}Cw*0701\text{-}Cw*0501}$	2
ILLWEIPDV	A*0201	NS2A	124	87	2	44401$_{A*0101\text{-}\mathbf{A*0201}\text{-}B*0702\text{-}B*1517\text{-}Cw*0701\text{-}Cw*0702}$ 55405$_{A*0101\text{-}A*0301\text{-}B*0702\text{-}B*0801\text{-}Cw*0701\text{-}Cw*0702}$	1/2
MTKEEFTRY	A*0101	NS5	19	94	1	44405$_{\mathbf{A*0101}\text{-}A*0201\text{-}B*0702\text{-}B*1501\text{-}Cw*0303\text{-}Cw*0702}$	2
VVEKQSGLY	A*0101	NS1	89	1	1	44401$_{\mathbf{A*0101}\text{-}A*0201\text{-}B*0702\text{-}B*1517\text{-}Cw*0701\text{-}Cw*0702}$	2
ITYTDVLRY	A*0101	NS2A	49	98	1	44405$_{\mathbf{A*0101}\text{-}A*0201\text{-}B*0702\text{-}B*1501\text{-}Cw*0303\text{-}Cw*0702}$	2

— CHAPTER 5 —

THE POLYFUNCTIONALITY OF HUMAN MEMORY CD8+ T CELLS ELICITED BY ACUTE AND CHRONIC VIRUS INFECTIONS IS NOT INFLUENCED BY AGE

The polyfunctionality of human memory CD8+ T cells elicited by acute and chronic virus infections is not influenced by age

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Preface: All of the data described in this manuscript were generated and analyzed by me, with the exception of the phenotyping displayed in Figure 1, which was carried out by Robin Parsons. With the help of my supervisor Dr. Bramson, I designed all of the experiments and was responsible for optimizing the flow cytometic assays. Dr. Chris Vershoor and Dr. Mario Ventresca contributed their expertise in the analysis of high dimensional data sets using specific statistical and mathematical models shown in Figures 4 and 5B. I wrote the manuscript and addressed the reviewer's comments with the assistance of Dr. Bramson. This work was supervised by Dr. Bramson, who provided experimental guidance and expertise in the analyses and interpretation of the results.

The Polyfunctionality of Human Memory CD8+ T Cells Elicited by Acute and Chronic Virus Infections Is Not Influenced by Age

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Abstract

As humans age, they experience a progressive loss of thymic function and a corresponding shift in the makeup of the circulating CD8+ T cell population from naïve to memory phenotype. These alterations are believed to result in impaired CD8+ T cell responses in older individuals; however, evidence that these global changes impact virus-specific CD8+ T cell immunity in the elderly is lacking. To gain further insight into the functionality of virus-specific CD8+ T cells in older individuals, we interrogated a cohort of individuals who were acutely infected with West Nile virus (WNV) and chronically infected with Epstein Barr virus (EBV) and Cytomegalovirus (CMV). The cohort was stratified into young (<40 yrs), middle-aged (41–59 yrs) and aged (>60 yrs) groups. In the aged cohort, the CD8+ T cell compartment displayed a marked reduction in the frequency of naïve CD8+ T cells and increased frequencies of CD8+ T cells that expressed CD57 and lacked CD28, as previously described. However, we did not observe an influence of age on either the frequency of virus-specific CD8+ T cells within the circulating pool nor their functionality (based on the production of IFNγ, TNFα, IL2, Granzyme B, Perforin and mobilization of CD107a). We did note that CD8+ T cells specific for WNV, CMV or EBV displayed distinct functional profiles, but these differences were unrelated to age. Collectively, these data fail to support the hypothesis that immunosenescence leads to defective CD8+ T cell immunity and suggest that it should be possible to develop CD8+ T cell vaccines to protect aged individuals from infections with novel emerging viruses.

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Introduction

CD8+ T cells can provide robust protection against pathogens and tumors. As a result, significant effort has been invested into developing vaccines that elicit protective CD8+ T cell memory responses. It is generally believed that immunological function decreases with advanced age, a phenomenon known as *immunosenescence*, rendering older individuals at higher risk of infection. While vaccination would seem to be an appropriate intervention to improve protective immunity, several reports have demonstrated that older individuals mount impaired responses to conventional vaccines, suggesting that alternate platforms or strategies may be required. Notably, antibody responses to influenza and tick-borne encephalitis vaccines were impaired in the elderly [1,2,3]. With regard to T cell immunity, recent reports from a large-scale immunization study with a live vaccine against varicella zoster demonstrated that while it is possible to boost zoster-specific CD4+ T cells to a protective level in individuals >60 years of age, vaccine responsiveness did appear to wane in individuals >75 years of age [4,5]. These data support the concept that immunosenescence may be an issue to overcome in the development of effective vaccines for elderly individuals; however, further research is required to truly understand the extent of immune dysfunction in older humans.

Alterations in the CD8+ T cell compartment are among the most common characteristics in the elderly T cell repertoire and are thought to reflect an impaired ability to control infection [6,7]. The aged CD8+ T cell population is characterized by a high proportion of CD28− cells (often co-expressing NK markers, such as CD57), which are believed to reflect highly differentiated T cells that lack the capacity to proliferate [1,8]. In some cases, the CD8+ CD28− T cell population comprises an oligoclonal expansion of CMV-reactive cells, suggesting that chronic infections may preoccupy the immune response in the elderly, leading to a CD8+ T cell repertoire with limited diversity [9,10,11,12]. The sum of these observations suggests that the CD8+ T cell population in the elderly is compromised in its capacity to respond to novel infections. However, the exact relationship between the global phenotypic changes in the CD8+ T cell compartment that appear with age and the functionality of antigen-specific CD8+ T cells is poorly defined. Further, there is a paucity of data regarding the ability of the elderly to mount CD8+ T cell responses to novel infections. Although it is generally assumed that age-associated

Author Summary

The prevalence and severity of viral infections increases with advanced age, a phenomenon associated with a defective immune system. The thymic output of naïve T cells declines as we age and it is this lack of naïve T cells that is believed to contribute to the inability of the aged to respond to novel infections and develop subsequent memory T cell responses. Here we show that individuals aged 60+ are capable of developing memory CD8+ T cells to West Nile virus, novel pathogen, indistinguishable in terms of polyfunctionality to those of subjects <60 years of age. Furthermore, we show that chronic and life-long infections with CMV and EBV result in similar polyfunctional virus-specific memory CD8+ T cell responses in subjects of all age groups. Our work demonstrates that aged individuals can elicit functional memory CD8+ T cell responses to a new pathogen while maintaining polyfunctional CD8+ T cells against recurrent chronic virus infections. Current vaccine platforms, which rely upon inactivated pathogens or recombinant subunits, are poorly effective in the aged. Our data suggest that live viruses may be more effective vaccine platforms in older humans.

changes in the CD8+ T cell compartment may explain the heightened risk of elderly individuals to infection, experimental data are sparse. Herein we provide one of the few studies in humans that demonstrate the impact of age on CD8+ T cell immunity to pre-existing and novel viral infections.

West Nile virus (WNV) emerged as a novel human pathogen in the Northern hemisphere in 1999, and since then has caused numerous viral outbreaks across North America [13,14,15]. From 2003–2008, we collected sequential blood specimens from >100 people acutely infected with WNV with an age distribution ranging from 19–85 years. Given this age range, we reasoned that our cohort would be suitable to study the relationship between age and the development of virus-specific CD8+ T cells following a novel acute infection. In our original report of this cohort, we observed that age did not influence the magnitude or breadth of the memory T cell response to WNV [16], suggesting that age may not impair the development of CD8+ T cell immunity against acute infections.

Our previous work did not address the longevity or functionality of CD8+ T cell memory that develops following WNV infection. Thus, it remained possible that the older members of our cohort failed to develop a CD8+ T cell memory pool that was functionally equivalent to the younger members. In this current report, we have examined the polyfunctionality of the WNV-reactive CD8+ T cell population at later time points post-infection. We have also examined memory responses to EBV and CMV within this cohort, as these lifelong infections may differentially impact the functionality of memory CD8+ T cells. Our results reveal that although the memory CD8+ T cells display distinct polyfunctional states that are virus-specific, we observed no impact of ageing on polyfunctionality. These studies have revealed that memory CD8+ T cell immunity in older individuals is intact and suggest that vaccine development should focus on other parameters that may be defective in the elderly.

Results

Ageing results in increased frequencies of highly-differentiated CD8+ T cells and decreased frequencies of naïve CD8+ T cells

For these studies, we have examined the CD8+ T cell memory responses from a cohort of 72 patients who were naturally infected with West Nile virus (WNV). We stratified our cohort into 3 groups: *young* (<40 years of age; n = 21), *middle-aged* (41–59 years of age; n = 25) and *aged* (>60 years of age; n = 26). To confirm that these cohorts displayed the expected age-associated changes in the CD8+ T cell compartment, we compared the phenotype of CD8+ T cells among the three different age groups. Significantly higher frequencies of CD8+ CD28− and CD8+ CD28− CD57+ cells were observed within the aged cohort (Figure 1A and 1B). Likewise, we noted that the CD45RA+ CD28+ CD8+ T cell population was significantly decreased in the naïve T cell pool in middle-aged and aged populations compared to the young population (Figure 1C). We also observed a significant reduction in the presence of naïve (CD45RA+ CCR7+) T cells in the aged subjects (Figure 1D). These observations confirm that our aged cohort displayed the expected immunosenescent phenotype within the CD8+ T cell compartment.

Age does not impact the frequency of functional virus-specific CD8+ T cells

We first sought to confirm our previous results showing that age did not impact the magnitude of the WNV-specific CD8+ T cell response. In our original study, we employed ELISPOT to monitor WNV-specific CD8+ T cells. However, cytokine production by ELISPOT cannot be attributed solely to CD8+ T cells. Therefore, in the current study, we employed flow cytometry to specifically identify cytokine-producing CD8+ T cells and provide a more accurate assessment of the functionality of the virus-specific CD8+ T cells. For these experiments, we used specimens obtained 6–7 months following WNV infection. Since our study population consisted of individuals with diverse HLAs, virus-specific CD8+ T cells were identified based on cytokine production following stimulation with a broad collection of immunodominant peptides that span the breadth of HLAs expressed by our cohort. Briefly, for these experiments, freshly thawed PBMCs were stimulated with pools of dominant epitope peptides derived from WNV, CMV or EBV and cytokine production (IFN-γ, TNF-α and IL-2) was measured on a per-cell basis using flow cytometry. While the CD8+ T cells produced varying amounts of cytokine following peptide stimulation, we did not observe any peptide-specific CD8+ T cells that could produce IL-2 or TNF-α in the absence of IFN-γ. Since all of our peptide-stimulated CD8+ T cells expressed IFN-γ, which is considered to be the cytokine that mediates the primary anti-viral response by the adaptive immune system [17], we defined "virus-specific" CD8+ T cells as those which produced IFN-γ following stimulation with specific peptide epitopes (see Table S1 for a complete list of measured IFN-γ frequencies). The frequencies of CD8+ T cells specific for either WNV, CMV or EBV was similar among all age groups (Figure 2). We did note a trend towards elevated frequencies of CMV- and EBV-specific CD8+ IFN-γ+ T cells in the middle-aged and aged cohorts relative to the young cohort, but this trend did not reach statistical significance (Figures 2B–D).

Stability of CD8+ T cell memory following acute infection is not influenced by age

While our data indicate that older individuals mount CD8+ T cell responses to acute infection (i.e. WNV) that are equivalent in magnitude to younger individuals, it is possible that the responses display different stability. To address this question, we measured the frequencies of WNV-specific CD8+ T cells in our cohort at 2 additional time points: baseline (early memory; average of 35 days after symptom onset) and 2–4 years post symptom onset (late memory). The magnitude of the WNV-specific CD8+ T cell

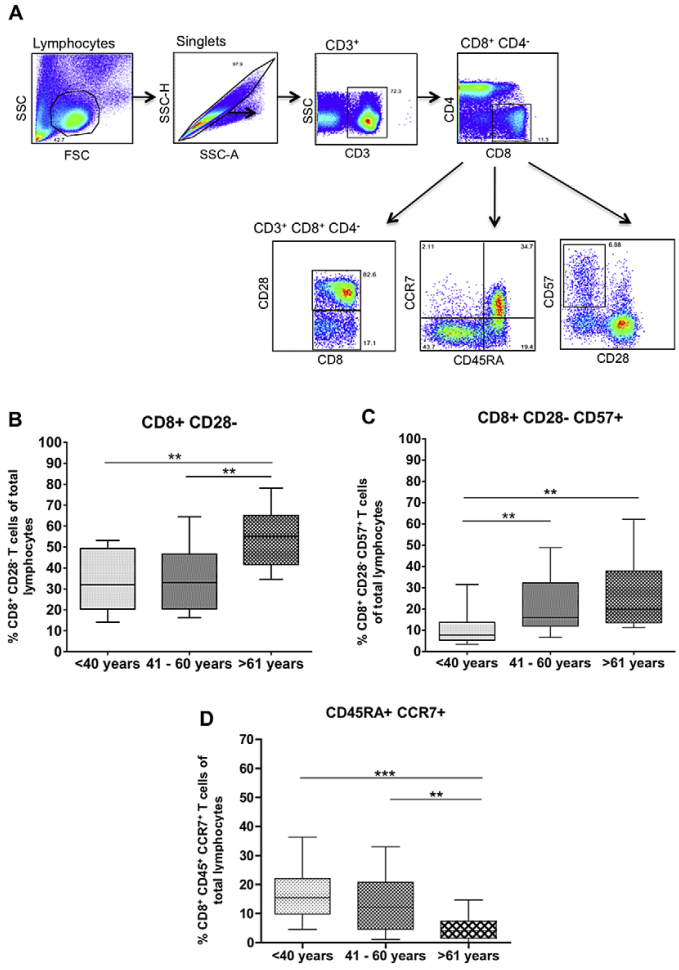

Figure 1. Ageing results in increased frequencies of highly-differentiated CD8+ T cells and decreased frequencies of naïve CD8+ T cells. A) Flow plots depicting gating strategy for phenotypic analysis. B) Percentage of CD8+ CD28− T cells within the peripheral blood lymphocyte pool; C) Percentage of CD8+ CD28− CD57+ T cells (terminally differentiated cells) within the peripheral blood lymphocyte pool; D) Percentage of CD45RA+ CCR7+ (naïve) T cells within the peripheral blood lymphocyte pool. Data are group according to stratification described in the Materials and Methods. Statistical analysis perfomed by one-way ANOVA with Tukey's mulitple comparison post-test. Box and whiskers plots are calculated at 95% confidenced interval.
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response was highest at 1 month and declined thereafter (Table 1). Importantly, the magnitude of the WNV-specific CD8+ T cells was equivalent among the various age cohorts at all 3 time points, suggesting that the longevity of the memory CD8+ T cell response is not age-dependent.

For these experiments, we also examined the production of TNF-α and IL-2 following peptide stimulation. As stated above, we did not observe any CD8+ T cells that produced TNF-α or IL-2 in the absence of IFN-γ following WNV peptide stimulation. We observed that 30%–50% of the WNV-specific CD8+ T cells were IFN-γ+ TNF-α+ double positive (Table 1). We also noted that only a fraction of WNV-specific CD8+ T cells could produce IL-2 and this did not increase with time. No difference was observed in the frequencies of TNF-α- or IL-2-producing WNV-specific CD8+ T cells among the 3 age groups at any time point (Table 1).

Defining polyfunctional T cell populations using FLOCK

In the previous paragraph, WNV-specific CD8+ T cells were crudely separated into 3 populations based on the expression of either IFN-γ, TNF-α, or IL-2. To gain further insight into the polyfunctional nature of the virus-specific CD8+ T cells, we also measured the cytotoxic capacity of the CD8+ T cells by granzyme B expression, upregulation of perforin and mobilization of CD107a (a measure of degranulation) following peptide stimulation of WNV samples obtained 6–7 months post symptom onset. Similar to our observations with cytokine production, we did not observe any peptide-specific CD8+ T cells that could upregulate perforin or mobilize CD107a in the absence of IFN-γ. Therefore, all functional parameters have been defined relative to the expression of IFN-γ. Polyfunctionality of antigen-specific CD8+ T cells was defined using a newly developed computational

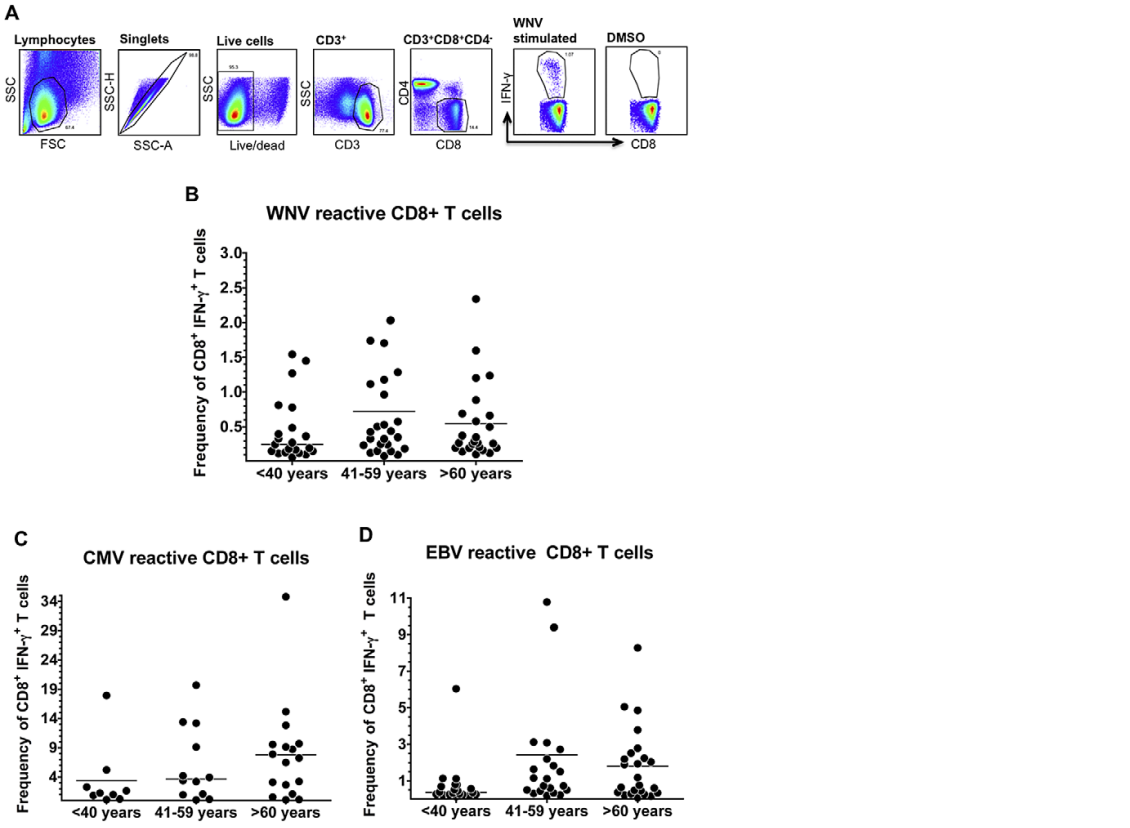

Figure 2. Age does not impact the frequency of functional virus-specific CD8+ T cells. A) Flow plots depict gating strategy for IFNγ+ CD8+ T cells. (B–D) Pooled WNV, CMV or EBV peptides were used to stimulated freshly thawed PBMCs isolated from WNV-naturally infected subjects 6–7 months post symptom onset. Scatter plots depict IFNγ+ CD8+ T cell responses only from reactive subjects (>0.05% and 3 fold above DMSO background); (B) WNV reactive subjects: 20/21 Young, 24/25 Mid-aged, 24/26 Aged; (C) CMV reactive subjects: 7/21 Young, 12/25 Mid-aged, 17/26 Aged; (D) EBV reactive subjects: 17/21 Young, 21/25 Mid-aged, 24/26 Aged. Means are displayed as horizontal lines. Statistical analysis performed by one-way ANOVA followed by Tukey's multiple comparison post-test.
doi:10.1371/journal.ppat.1003076.g002

analysis of flow cytometry data: FLOCK (FLOw Cytometry without K), publicly available in the Immunology Database and Analysis Portal – ImmPort (www.immport.org). FLOCK utilizes a density-based clustering approach and algorithms to define biologically relevant populations from multiparametric data sets without the bias of manual gating [18]. Using FLOCK, we identified 16 distinct functional populations from IFN-γ+ CD8+ T cells for all three antigens (WNV, CMV and EBV), that were defined as negative (neg), low (lo), intermediate (int), and high (hi), based on the signal intensity of each marker (Figure 3). All populations were derived from IFN-γ+ events, thus there were no IFN-γ negative events.

To address the question of whether advanced age impacted the development of polyfunctional memory CD8+ T cell responses, we analyzed the large data set comprising functional population frequencies (FLOCK identified) by Principal Component Analysis (PCA). PCA is a linear technique that transforms data of interrelated variables into a set of uncorrelated principal components (PCs) while maintaining the original variation of the data set in reduced dimensionality [19]. The polyfunctional analysis of antigen-specific CD8+ T cells was separated into two parameters: cytokine functional populations (C2–C7; Figure 4) and cytotoxic functional populations (C8–C17; Figure 4). Consequently, two PCA analyses were generated per antigen. PCA plots comprising cytokine functional populations for WNV, CMV and EBV were generated using the top two PCs that accounted for 78%, 86% and 78% of the overall variance, respectively. Functional populations C2 ($IFN\gamma^{lo}$ $TNF\alpha^{lo}$ $IL2^{lo}$) and C3 ($IFN\gamma^{lo}$ $TNF\alpha^{neg}$ $IL2^{neg}$); C4 ($IFN\gamma^{int}$ $TNF\alpha^{hi}$ $IL2^{neg}$) and C5 ($IFN\gamma^{int}$ $TNF\alpha^{hi}$ $IL2^{lo}$); C6 ($IFN\gamma^{hi}$ $TNF\alpha^{hi}$ $IL2^{hi}$) and C7 ($IFN\gamma^{int}$ $TNF\alpha^{int}$ $IL2^{int}$) tended to cluster, indicating a strong positive correlation (Figure 4). The vector clustering would suggest that these functional populations are the same or very similar. We observed no specific age clustering, suggesting no relationship between age and CD8+ T cell function based on cytokine production.

PCA plots depicting the cytotoxic functional phenotypes for WNV, CMV and EBV- specific CD8+ T cells were generated using the top two PCs and account for roughly 65% of the overall variance (Figure 4). It is important to note that the interpretation of the data did not change when we examined 3-D plots of the first 3 PCs (approximately 80% of the total variance) and for simplicity of interpretation we used biplots to explain these data. We

Table 1. Stability of WNV-specific CD8+ T cell memory response is not influenced by age[1].

	Young (<40 years of age)	Middle-aged (41–59 years)	Aged (>60 years of age)
BASELINE			
% WNV-specific CD8$^+$ T cells[2]	0.4102±0.3057	0.5553±0.6462	0.6118±0.7584
% TNFα$^+$ of total CD8$^+$ IFNγ$^+$ T cells[3]	35.69±11.87	42.11±17.82	39.22±16.85
% IL2$^+$ of total CD8$^+$ IFNγ$^+$ T cells[4]	3.363±3.564	1.565±1.416	2.635±2.213
6–7 MONTHS			
% WNV-specific CD8$^+$ T cells[2]	0.3577±0.3743	0.4158±0.5169	0.3260±0.3082
% TNFα$^+$ of total CD8$^+$ IFNγ$^+$ T cells[3]	47.51±17.17	56.59±19.85	42.95±15.61
% IL2$^+$ of total CD8$^+$ IFNγ$^+$ T cells[4]	1.595±1.589	2.713±1.613	1.977±1.872
>2 YEARS			
% WNV-specific CD8$^+$ T cells[2]	0.157±0.21	0.094±0.06	0.119±0.09
% TNFα$^+$ of total CD8$^+$ IFNγ$^+$ T cells[3]	34.94±14.50	41.22±19.39	29.90±15.38
% IL2$^+$ of total CD8$^+$ IFNγ$^+$ T cells[4]	3.698±2.125	2.639±3.274	2.822±2.307

[1]All data represent the mean +/− standard deviation.
[2]Percentage of CD3+CD8+ cells that produce cytokine in response to WNV peptides.
[3]Percentage of CD3+CD8+ IFNγ+ cells that also produce TNFα.
[4]Percentage of CD3+CD8+ IFNγ+ cells that also produce IL2.
doi:10.1371/journal.ppat.1003076.t001

observed high positive correlations between populations C9 (IFNγ^{int} GrB^{lo} Prf^{neg} CD107a^{hi}) and C11 (IFNγ^{hi} GrB^{lo} Prf^{lo} CD107a^{int}); C13 (IFNγ^{hi} GrB^{lo} Prf^{lo} CD107a^{hi}) and C16 (IFNγ^{hi} GrB^{lo} Prf^{neg} CD107a^{hi}), which suggests that they might belong to the same functional population but were segregated in into two based on automated binning by FLOCK analysis. We also find that population C10 (IFNγ^{hi} GrB^{int} Prf^{lo} CD107a^{int}) contributes very little to the overall variance of the system for WNV and CMV since its vector length is small relative to the other defined cytotoxic phenotypes. Furthermore, as observed for antigen-specific CD8+ T cell cytokine function, we found no evidence that CD8+ T cell cytotoxicity was affected by age. These results suggest that while different combinations of cytotoxic markers define virus-specific CD8+ T cell responses, they show no linear relationship with age.

CD8+ T cell polyfunctionality is virus-specific

Preliminary analysis of polyfunctional WNV, CMV, and EBV-specific IFN-γ+ CD8+ T cells (producing cytokines; IL2 and TNF-α, and mobilizing cytotoxic mediators; GrB, perforin and CD107a) revealed that WNV and CMV polyfunctional responses were more similar than EBV-specific polyfunctional CD8+ T cells (Figure 5A). On average, EBV-specific CD8+ T cells were better producers of IL2 but failed to upregulate Granzyme B or perforin in comparison to WNV and CMV-specific CD8+ T cells (Figure 5A). We next performed the Kolmogorov-Smirnov (KS) test to determine whether WNV, CMV or EBV-specific CD8+ T cell functional phenotypes defined by FLOCk were drawn from the same distributions. The KS test is based on the null hypothesis that the samples are drawn from the same distribution, thus larger p-values suggest that the two sets are similar. A comparison of the evaluated KS statistics between the different viral antigens for all functional phenotypes showed that WNV and CMV were more functionally similar than EBV (Figure 5B). For example, T cell phenotype identified in population C4 (IFNγ^{int} TNFα^{hi} IL2^{neg}) for WNV and CMV had a calculated p value of 0.889 suggesting a very similar distribution of this functional population, which was not observed for EBV. We further noted that more than 40% of the functional cells (IFNγ+) simultaneously produced TNFα+ for all three antigens, but there was a difference in the ability to produce IL-2. WNV-specific CD8+ T cells produced the least IL-2, EBV-specific CD8+ T cells produced the highest amounts of IL-2 and CMV-specific CD8+ T cells displayed an intermediate phenotype (Figure 5A).

The KS analysis of the cytotoxic functional populations revealed a striking similarity between CMV- and WNV-specific T cells, where populations C8 (IFNγ^{lo}GrB^{lo}Prf^{lo}CD107a^{hi}) and C16 (IFNγ^{hi}GrB^{lo}Prf^{neg}CD107a^{hi}) were distributed similarly ($p = 0.909$ and $p = 0.882$, respectively). Overall, a higher frequency of CMV- and WNV-specific memory T cells were cytotoxic and polyfunctional (GrB+ Prf+ CD107a+) in comparison to the EBV-specific T cells, which became CD107a^{hi} following peptide stimulation but remained low in terms of GrB and Perforin expression (Figure 5A).

Using PCA biplots, we were able to discriminate antigenic stimulation (WNV, CMV or EBV) based on the resultant functional phenotypes (Figure 5B). Corroborating the KS distribution analysis, the PCA showed that CMV and WNV are indiscriminant based on the above-mentioned functional populations, whereas EBV-specific functional phenotypes cluster separately. This effect of EBV segregating away from WNV and CMV was especially evident when cytotoxic populations were analyzed by PCA (Figure 5B). Altogether, it does not appear that age has an impact on the development of memory CD8+ T cells with the capacity to elaborate multiple functions. Rather, it appears that the polyfunctional profile of virus-specific CD8+ T cells appears to be a function of the pathogen.

Discussion

Contrary to the suggestion that susceptibility to new infections in the aged occurs due to insufficient CD8+ T cell immunity as a result of diminished frequencies of naïve CD8+ T cells and/or dysfunctional CD8+ T cell memory [20,21], we have shown that aged individuals mount CD8+ T cell memory responses to a novel viral agent that are equivalent to young individuals. In fact, extensive analysis of CD8+ T cell functional parameters revealed no relationship between age and the capacity to produce cytokines

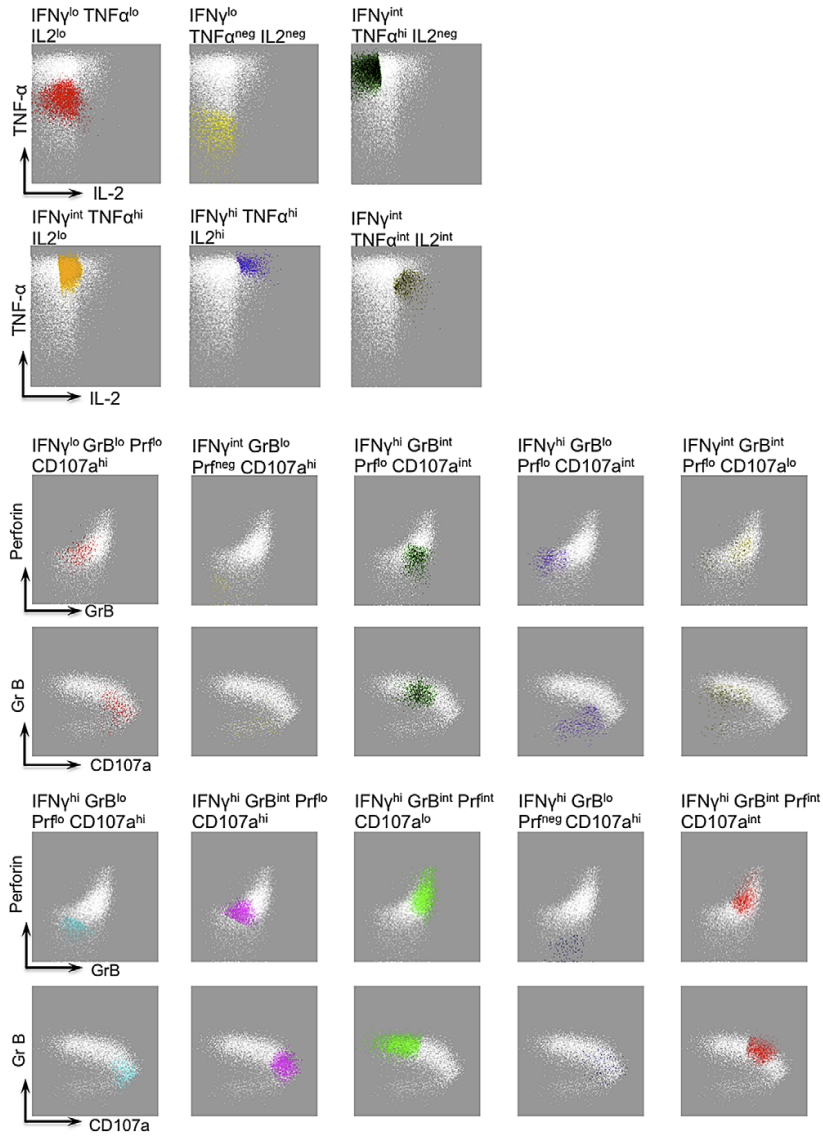

Figure 3. FLOCK gating strategy and description of IFN-g functional populations. A single representative sample, stimulated with CMV peptide pool, depicts the gating strategy by FLOCK, which identified the 16 functional populations (shown in various different colors and specified by title above the plot). WHITE dots in each panel depict the IFNγ+ CD8+ T cells and the functional populations (depicted by colours) are defined based on the level of expression of IFNγ and other cytokines (TNFα and IL2) or cytotoxic molecules (CD107a, GrB, and Perforin) as shown by the arrows. The same functional populations were applied in cross-sample comparison of WNV, CMV and EBV-specific CD8+ T cells.
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or mobilize cytotoxic mediators in response to stimulation by peptides derived from viruses responsible for both acute (WNV) and chronic (CMV, EBV) infections despite clear evidence of an immunosenescent phenotype in the bulk CD8+ T cell pool (elevated frequencies of CD28− CD57+ cells and decreased frequencies of CD45RA+ CCR7+ cells relative to the younger members of the cohort). Thus, although the members of our aged cohort displayed expected age-related changes in the composition of the CD8+ T cell compartment, these alterations did not manifest as a defect in functional virus-specific immunity, even when the primary virus infection occurred in old age, as in the case of WNV.

A recent study revealed that infection of middle-aged and old macaques with Rhesus CMV (RhCMV) produced RhCMV-specific CD8+ T cells with comparable functionality in both age groups [22], supporting the concept that anti-viral CD8+ T cell responses may not be dysfunctional in aged individuals. In contrast, immunization with modified vaccinia Ankara (MVA) elicited weaker CD8+ T cell responses in old macaques compared

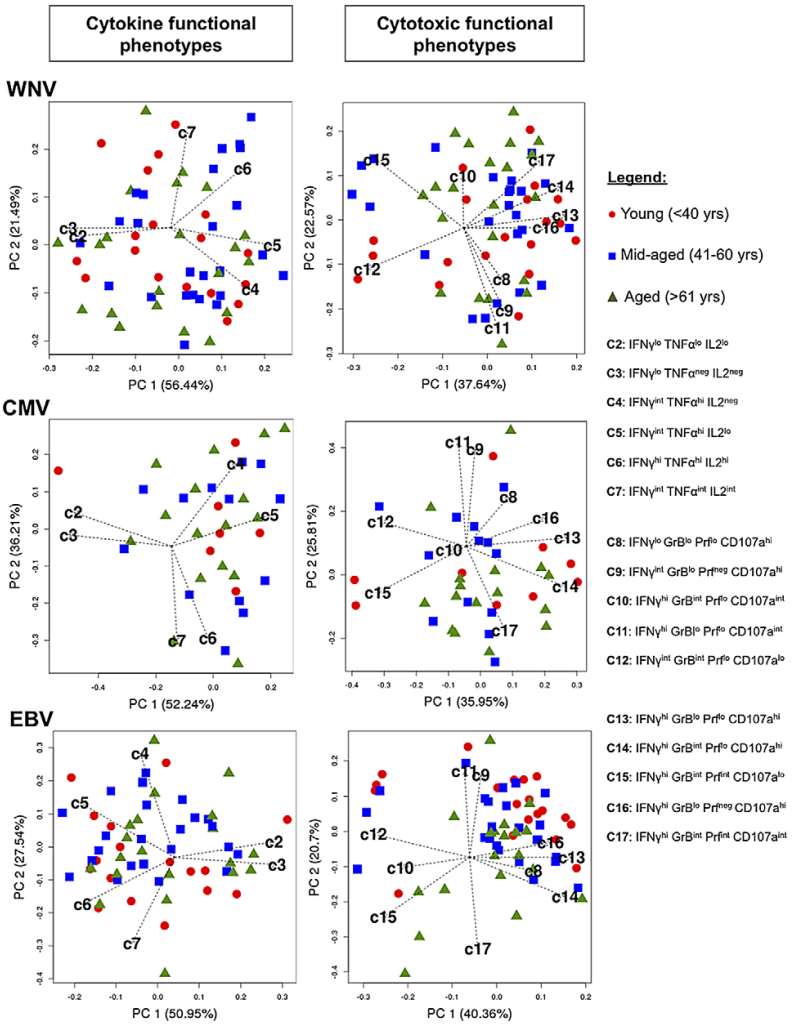

Figure 4. Principal Component Analysis of antigen-specific CD8+ T cell polyfunctionality shows no correlation with age. The Principal Component Analysis plots display biplots of top −2 principal components. The biplots show the samples (red circles = Young <40 years, blue squares = Mid-aged 41–59 years, green triangles = Aged >60 years) and the FLOCK defined functional CD8+ T cell phenotypes as a vector in a two-dimensional plane. The length of each vector indicates the approximate variance of the specific functional population. Lack of clustering of data points around specific vectors signifies lack of correlation between age and functionality of CD8+ T cells. Principal Component Analysis done by R version 2.14. ***PC1***, principal component 1; ***PC2***, principal component 2.
doi:10.1371/journal.ppat.1003076.g004

to young macaques [7]. While the results of the MVA experiments may seem at odds with our observations, the authors of this latter report employed live vaccinia virus to stimulate MVA-specific CD8+ T cells *in vitro* for their functional assays. In contrast, our current report and the report on RhCMV employed synthetic peptides that do not require additional processing for presentation to CD8+ T cells. Since stimulation of CD8+ T cells with live vaccinia virus relies upon the infection, expression and processing of antigen by the cells in the test sample, it is possible that the CD8+ T cell response was intact but antigen presentation by the cells used to present vaccinia antigens in the *in vitro* assay were defective in the aged monkeys. The authors argued that DCs were not affected by the age of the monkeys; however, they only investigated a limited number of parameters and they did not examine antigen processing through the classical MHC class I pathway. Therefore, we cannot discount a possible role for defective antigen presentation in their *in vitro* stimulation. Another possible explanation for the differences may stem from the nature of the immunogens. MVA is a variant of vaccinia virus that replicates poorly in primate cells, whereas RhCMV and WNV replicate effectively in primate cells. Therefore, effective stimulation of CD8+ T cells responses in the elderly may rely upon the nature of the infectious agent. This will be an important point to consider with regard to vaccine design.

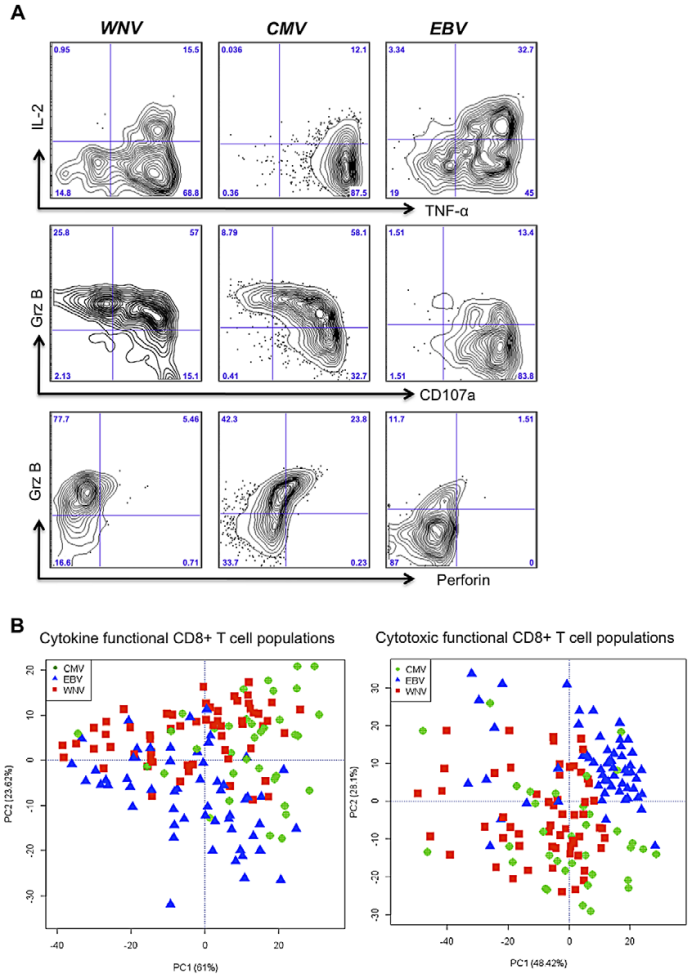

Figure 5. CD8+ T cell polyfunctionality is virus-specific. A) Flow plots depict representative samples following WNV, CMV, and EBV peptide stimulations and the cytokine and cytotoxic marker staining on IFNγ+ CD8+ T cells. B) Principal component analysis of FLOCK identified cytokine and cytotoxic functional phenotypes shows clustering of virus-specific functional T cells that are similar between WNV and CMV but different for EBV. Principal Component Analysis done by R version 2.14. ***PC1***, principal component 1; ***PC2***, principal component 2.
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It has been proposed that chronic CMV infection may drive immune senescence due to repeated oligoclonal expansions of CMV-specific CD8+ T cells leading to overpopulation of the memory T cell pool [7,11,23,24,25] and ultimately limiting the ability of the aging individual to combat previously encountered or novel viral infections [25]. However, a recent report has suggested that the size of the CD8+ T cell compartment may increase with age to accommodate expanding memory T cell populations without depleting CD8+ T cells with other specificities [26]. Interestingly, this phenomenon was not reflected within the peripheral blood where the expanding memory populations increased in frequency at the expense of T cells with other specificities. Rather, the expansion of antigen-specific memory CD8+ T cells was accommodated by increased numbers of CD8+ T cells present within the tissues, suggesting that measures of CD8+ T cell frequencies within the peripheral blood may not accurately reflect the true composition of the CD8+ T cell pool. Although it is relatively easy to measure CD8+ T cells present in the tissues in murine studies, addressing this concept in humans is not trivial. Nevertheless, in light of this recent report, the apparent decline in available naïve CD8+ T cells in the peripheral blood of individuals with evident expansion of CMV-specific CD8+ T cells may not truly reflect a corresponding decrease in the availability of naïve T cells in the lymphoid tissues, where primary responses to viruses are initiated.

Similar to previous reports, we have observed a trend towards higher frequencies of CMV- and EBV-reactive CD8+ T cells in the aged cohort. However, this trend did not achieve statistical significance and not all aged individuals displayed an expanded CMV- or EBV-specific CD8+ T cell pool. Similar results have been reported by others [27,28]. Importantly, in all of these reports, the functionality of the CMV-specific CD8+ T cells did

not change with age (the other reports did not investigate EBV-specific CD8+ T cells). It is notable that all of these reports employed functional analyses to define the CMV-specific CD8+ T cells. In contrast, when CMV- and EBV-specific CD8+ T cells were quantified using MHC multimers, it was noted that dysfunctional populations of CMV- and EBV-specific CD8+ T cells accumulate with age based on tetramer staining and IFN-γ production [29,30]. The implications of these dysfunctional cells are unclear as these aged individuals successfully control both CMV and EBV infections and, based on our results, are able to mount effective CD8+ T cell responses to novel infections. We noted a number of mid-aged and aged individuals with frequencies of CMV-reactive CD8+ T cells that represented more than 9% (9 subjects) of the circulating CD8+ T cell pool, but we did not observe any relationship between expanded CMV-specific CD8+ T cells and impaired generation of WNV-specific CD8+ T cells, indicating that CMV expansions do not limit the ability of the host to respond to a novel infection, consistent with the report of Vezys et al. [26].

Detailed comparison of the functional CD8+ T cell response between the different viruses (WNV, CMV and EBV) revealed interesting differences in functional profiles, corroborating previous reports examining virus-specific CD8+ T cell immunity in humans [31,32,33]. Striking similarities in both phenotype and cytotoxic profile were observed between memory WNV- and CMV-specific CD8+ T cells, despite the fact that the former is an acute infection and the latter is a chronic infection. The majority of WNV- and CMV-specific CD8+ T cells displayed a phenotype consistent with terminally-differentiated effectors (CD45RA+ CD28−) whereas EBV-specific CD8+ T cells were mostly less differentiated (CD45RA− CD28+) (data not shown). Consistent with the phenotype and the differentiation status, CMV-specific CD8+ T cells produced high levels of GrB and perforin but failed to produce IL-2, whereas EBV-specific CD8+ T cells failed to produce perforin and had less GrB but significantly more IL-2 (Figure 5A); a similar dichotomy in the production of perforin and IL-2 was described in our previous work with a smaller cohort of patients [33]. This is consistent with previous reports that show the expression of cytotoxic enzymes is related to cellular maturity, such that CD45RA+/− CD28− cells express high levels of cytotoxicity due to highly differentiated phenotype and CD45RA+/− CD28+ T cells express little cytotoxic attributes [33,34,35,36].

Collectively, we demonstrate here that aging individuals are capable of mounting polyfunctional memory CD8+ T cell responses to a novel pathogen, which has significant implications for vaccine development for the elderly. Most of our current understanding on the relationship of aging to vaccination has relied upon measurements of antibodies following vaccination and it is clear that the serological response in the elderly is attenuated [1,2,3]. In striking contrast, our results described herein and in our previous report [16] reveal that the aged can mount a robust, polyfunctional CD8+ T cell response to novel pathogens while sustaining a robust polyfunctional responses to chronic infections. Collectively, our data suggest that vaccination in older humans should focus on CD8+ T cell immunity and that live vaccines should be considered as the platform of choice. The results presented here entice our curiosity and desire to better understand the aging immune system for the purpose of developing much needed vaccines for our greatly expanding aging population.

Materials and Methods

Ethics statement

This research was approved by the Hamilton Health Sciences/ McMaster Health Sciences Research Ethics Board that operates in compliance with the ICH Good Clinical Practice Guidelines and the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans and Division 5 Health Canada Food and Drug Regulations. All patients in this study provided informed written consent.

WNV patient cohort and PBMC preparation

Seventy-two patients were enrolled into the study following detection of serum WNV IgM by public health laboratories after presentation of WNV-related symptoms. Serology for WNV was assessed by plaque reduction neutralization assay as described previously [37]. Recruitment of patients occurred over a period of 5 years (2003–2007). This trial was reviewed and approved by the Research Ethics Board at McMaster University.

Patients were entered into our study within 1 month following symptom onset (median = 30 days, ranging from 4–100 post symptom onset) and blood was collected on the first visit (baseline sample) and once every month thereafter for a period of one year. Twenty-five patients were contacted 2–4 years post symptom onset and their blood was collected at convalescence of disease. The population consisted of 37 men and 35 women ranging in age from 19 to 85 years. Patients were subdivided into three cohorts for these experiments based on age; young <40 years of age, mid-aged 41–59 years, and aged >60 years of age (Table S1).

Blood samples were drawn into heparanized tubes and PBMC were isolated from the blood by centrifugation on Ficoll (Amersham Pharmacia). PBMC were cryopresrved in RPMI 1640 containing 12.5% human serum albumin (Sigma-Aldrich) and 10% DMSO according to the method described by Disis et al. [38].

Peptide stimulations

WNV peptides used for the stimulation of PBMCs were identified previously [16] and 13 of commonly immunogenic peptides were pooled together for the purpose of having a single WNV stimulation that would encompass the vast majority of reactivities within the cohort. Peptides were either deconvoluted to a minimal epitope of 8–9 amino acids or were uses as a 15-mer. CMV stimulation consisted of 168 identified CMV-specific CD8+ T cells epitopes pooled into a single pool. Likewise, EBV stimulation consisted of 91 identified EBV-specific CD8+ T cell epitopes pooled together into a single pool.

Intracellular cytokine staining

PBMC were thawed and placed immediately into 37°C pre-warmed complete RPMI (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μM 2-ME, 10 μM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. Thawed PBMC were cultured overnight at 37°C incubator. The cells were subsequently harvested, counted, and viability was assessed by trypan blue exclusion. Cells were aliquoted ($2–2.5\times10^6$ cell/well) into round-bottom, 96-deep-well plate (Costar); peptides were added to a final concentration of 2 μg/ml and were incubated for 1 hr at 37°C. DMSO diluted in cRPMI was used as a peptide-non-specific negative control. Brefeldin A and Monensin A (BD Biosciences) were added to the cell/peptide mixture as per manufacturer's instructions and were incubated for an additional 4 hrs. At this point, cells were pelleted and washed in 10 μM EDTA. The cells were first stained with Near IR viability stain (Invitrogen, Molecular Probes) and subsequently with different antibody cocktails depending on the analysis. Cytokine analysis cocktail comprised of anti-human CD3-Qdot 605 (Molecular Probes), CD8-Alexa flour 700, CD45RA-PE Texas red (Beckman Coulter), CD28-PE Cy5, CD4-Pacific Blue, CD14-Pacific Blue, CD19-Pacific Blue surface antibodies. Cells were then permeabilized with Cytofix/Cytoperm

(BD Biosciences), and intracellular cytokines were identified using anti-human IFNγ-APC, TNFα-FITC, and IL-2-PE [Note: all flow cytometry reagents were obtained from BD Biosciences unless otherwise specified]. Fluorescence data were acquired using LSRII flow cytometer (BD Biosciences) and 500,000–1 000,000 events based on the live lymphocyte gate were collected per sample. Data were analyzed using FlowJo. A positive response was measured as the IFN-gamma frequency greater than 0.05 and three fold above DMSO background.

Cytotoxic analysis

PBMCs were cultured and stimulated as described above however in addition to the stimulatory peptides, anti-human CD107a-PE conjugated antibody (BD Biosciences) was added to the cells at the beginning of the stimulation for 1 hr. Brefeldin A and Monensin A (BD Biosciences) were added to the cell/peptide/CD107a mixture as per manufacturer's instructions and were incubated for an additional 4 hrs. The cells were subsequently stained with a Near IR viability stain (Invitrogen, Molecular Probes) as per manufactures instructions followed by the cytotoxic antibody cocktail: anti-human surface antibodies [CD8-PerCP Cy5.5 (eBiosciences), CD4-Alexa Flour700 (BD Biosciences), CD19 and CD14-Alexa Flour 700 (eBiosciences)] and intracellular anti-human antibodies [IFNγ-APC, Granzyme B-FITC and Perforin-Pacific Blue (conjugated to Pacific Blue in house using standard conjugation protocols)]. The perforin antibody detects *de novo* as well as preformed perforin and when used in conjuction with IFNγ following in-vitro peptide simulation we are able to determine the frequency of *de novo* formed perforin only. Fluorescent data was acquired using the LSR II as described above.

Phenotyping of PBMCs

An aliquot of thawed patient PBMCs ($0.5–1 \times 10^6$ cell/stain) was used for the purpose of phenotyping the cells. Cells were stained in round-bottom 96-well plates with anti-CD3-APC-H7, CD8-Alexa Flour 700, CD4-Pacific Blue, CD45RA-PE Texas Red (Beckman Coulter), CD28-PE, CD57-FITC, and anti-CCR7-PE Cy7 [Unless otherwise stated all antibodies were purchased from BD Biosciences]. Analysis of surface marker staining was done by LSR II flow cytometer and data was analyzed using FlowJo software.

FLOCK analysis

FLOCK is an automated computational approach publically available at the Immunology Database and Analysis Portal – ImmPort (www.immport.org), which utilizes algorithms and density-based clustering to identify cell subsets. FLOCK analysis is comprised of five components: data preprocessing, grid-based density clustering, cross-samples comparison, result visualization, and population statistics calculations. Detailed methodology for FLOCK analysis can be found in [18]. In summary, binary .fcs files specifically gated on live/singlet/CD3+/CD8+ CD4−/IFNγ+ events were converted to tab-delimited ACSII text format and exported from FlowJo (Tree Star) in a data matrix file. Samples (6–7 months post WNV symptom onset) were considered positive if following peptide stimulation they expressed IFNγ frequency above 0.05 and 3 fold above DMSO background and consequently were included in the FLOCK analysis. This was the means by which our data was normalized. The exported CD8+ IFNγ+ events were than subjected to density-based grouping based on expression of IFNγ, TNFα and IL2 for determination of cytokine populations; and IFN-γ, CD107a, GrB and Perforin for determination of cytotoxic populations depending on the distances between each point and where its coordinates lie in the defined grid. FLOCK identified 16 cell populations: 6 defining cytokine populations and 10 defining cytotoxic populations (Figure 3). Population centroids (the average of coordinates of a given set of points) were applied to multiple samples in a cross-sample analysis to enable population comparisons between WNV, CMV, and EBV-specific CD8+ memory T cell populations.

Statistical analysis

The data are presented as mean values. Simple descriptive statistics (means, standard deviations, Students t test and regression analysis) were calculated using GraphPad Prism version 1.0. Box and whiskers plots are calculated at 95% confidenced interval and generated using GraphPad Prism version 1.0.

The large data set comprising functional population frequencies was analyzed by Principal Component Analysis (PCA), which is a linear technique that transforms data of interrelated variation of the data set in reduced dimensionality [19]. To graphically reveal clustering, multi-collinearity and outliers of our data set following PCA we used a biplot consisting of top −2 PCs. The biplots show both the samples and features of the data set, where each sample is displayed as a point in a two-dimensional plane, and each functional population (defined by FLOCk clustering) is presented as a vector. The length of each vector indicates the approximate variance of the specific functional population. The distance between two points is an approximate of the Euclidean distance between their associated functional phenotype. Thus, samples that cluster together are interpreted as similar. Conversely, observing no clustering of points implies very little similarity among the data points. The correlation between any two functional phenotypes can be approximated by the angles between them, where angles of 90 or 270 degrees apart show correlations approaching zero, and angles of 0 or 180 degrees show a correlation of 1 or −1, respectively.

The Kolmogorov-Smirnov (KS) test was used to determine whether WNV, CMV or EBV-specific CD8+ T cell functional phenotypes were drawn from the same or different distributions. The KS statistic quantifies a distance between the empirical distribution functions of two samples and is based on the null hypothesis that the samples are drawn from the same distribution if the p value approaches 1.

Supporting Information

Table S1 Patient characteristics and reactivities. All patients associated with the studies in this manuscript are listed in this table using their anonymous identifiers. Age and sex (M = male, F = female) are listed. The percentage of CD3+CD8+ T cells that produced IFN-γ in response to the WNV, EBV, and CMV peptide pools is shown. Specimens used for measuring the virus-specific responses listed in this table were obtained 6–7 months following the onset of WNV infection. A positive response was defined as greater than 0.05% of CD3+CD8+ T cells and 3 fold above DMSO background. All the data shown in this table have been corrected for the DMSO background. Responses that were defined as negative are indicated by an asterisk (*).
(DOC)

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Author Contributions

Conceived and designed the experiments: AL JLB. Performed the experiments: AL RP CE. Analyzed the data: AL CPV MV. Contributed reagents/materials/analysis tools: MRB MBL. Wrote the paper: AL CPV MV DB JLB MRB.

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Table S1. Patient Characteristics and Reactivities. All patients associated with the studies in this manuscript are listed in this table using their anonymous identifiers. Age and sex (M = male, F = female) are listed. The percentage of CD3+CD8+ T cells that produced IFN-γ in response to the WNV, EBV, and CMV peptide pools is shown. Specimens used for measuring the virus-specific responses listed in this table were obtained 6-7 months following the onset of WNV infection. A positive response was defined as greater than 0.05% of CD3+CD8+ T cells and 3 fold above DMSO background. All the data shown in this table have been corrected for the DMSO background. Responses that were defined as negative are indicated by an asterisk (*).

			% IFN-γ+ of total CD3+ CD8+ cells		
Patient I.D.	**Age**	**Sex**	**WNV**	**CMV**	**EBV**
77408	19	F	0.811	5.220	1.121
77407	23	F	0.154	0.007*	0.254
44402	24	M	0.365	0.006*	0.009*
77302	29	M	0.779	0.001*	0*
77304	30	F	0.486	0.004*	0.456
77323	30	F	0.105	0.021*	0.335
77421	30	F	0.124	0.244	0.041*
10202	32	M	1.270	0.011*	0.130
55308	33	F	0.066	0.012*	0.804
77423	35	M	0.395	0.135	0.245
55313	36	F	0.135	0.017*	0.250
77404	36	F	0.333	0.046*	0.353
77413	37	M	1.542	0.07*	0.382
60002	38	M	0.250	1.650	0.520
5201	39	M	0.273	0.007*	0.03*
77324	39	M	0.174	0.008*	6.040
77419	39	M	1.449	0.015*	0.289
55420	40	F	0.120	1.260	0.270

77309	40	F	0.153	1.000	0.783
77310	40	M	0.185	2.285	0.565
77321	40	F	0.042*	0.053*	0.691
77315	41	F	0.238	0*	0.308
77319	41	F	0.150	0.014*	1.120
10201	45	F	0.250	4.201	3.120
77312	45	F	0.188	0.018*	2.198
77316	45	M	1.178	9.118	3.088
77401	45	F	0.129	3.939	0.499
77505	46	M	0.255	0.014*	0.605
77301	47	M	0.350	0*	0.06*
77327	47	M	0.100	13.190	0.720
77411	47	F	0.327	0*	0.004*
55405	47	F	1.115	0.005*	0.042*
77320	49	F	0.083	0.004*	0.502
55410	51	M	4.254	3.404	10.784
55413	51	M	0.504	0.009*	17.184
44410	52	M	0.964	0.027*	0.724
77422	52	M	1.285	0.04*	1.155
77307	55	F	1.704	19.674	0.022*
77313	55	F	0.530	0.02*	2.720
77317	55	M	0.438	13.398	1.638
77322	55	F	0.046*	0.088	0.228
55414	57	M	0.154	0.194	0.444
77303	57	F	1.740	1.087	1.507
77326	57	F	0.575	0*	0.335
77331	57	M	0.329	0.959	1.829
77424	58	F	0.426	3.226	0.196

55408	60	F	0.211	0.008*	0.481
77332	60	F	0.026*	1.093	0.643
77502	60	F	0.263	0.004*	0.753
77403	61	F	0.155	9.136	0.027*
77412	62	F	0.00*	9.560	4.860
55407	63	M	0.197	0.03*	1.897
77402	63	F	0.579	0.549	0.229
11201	64	M	0.690	6.490	8.280
55310	64	F	0.257	0.015*	5.057
77311	64	M	0.350	0.031*	0.290
77405	64	M	2.339	0*	2.189
44405	65	F	0.108	9.708	0.158
60003	65	M	0.200	0.100	0.053
7001	66	M	0.886	0.015*	2.516
77425	66	M	0.663	0.001*	0.007*
51002	67	M	0.374	0.154	0.079
77420	67	M	1.238	8.738	0.258
55311	69	M	0.003*	2.660	0.340
60001	71	M	0.271	7.241	0.461
51001	73	M	0.199	3.249	2.039
55401	75	M	0.147	15.157	3.787
77329	77	M	0.291	3.101	0.371
55316	78	F	0.166	34.786	2.786
77406	80	M	1.203	0.032*	2.243
77409	81	F	0.274	12.824	1.934
9001	82	M	0.050	7.887	0.607

— CHAPTER 6 —

GENERAL DISCUSSION

The overall goal of my thesis research was to utilize West Nile virus (WNV) infection in humans as a tool to investigate the effects of aging on the development of polyfunctional memory CD8+ T cell responses. The results presented herein were among the first studies to characterize the human immune response to WNV. The novel findings presented in my thesis demonstrate that, in contrast to popular belief, aged individuals are capable of eliciting robust, polyfunctional CD8+ T cells to a novel virus as well as maintaining functional CD8+ T cell immunity to life-long herpersvirus infections. I believe that these observations have important implications for vaccine development and suggest that efforts to develop novel vaccines for the elderly should focus on platforms that aim to evoke CD8+ T cell immunity.

In the following Discussion, I will address key elements of my research that have not been fully articulated in the Discussions associated with each chapter.

6.0 A new perspective on CD8+ T cell immunity in the aged

It is commonly believed that the aging immune system undergoes immunosenescence resulting in increased susceptibility of the aged individuals to infectious diseases [234,260]. Aging humans (60+ years of age) experience severe complications that are uncommon in young people following viral infections such as influenza, respiratory syncytial virus (RSV), and reactivated varicella zoster virus (VZV) [6,275,276]. The

currently available vaccines for the aforementioned viruses have shown great effectiveness in preventing childhood disease, yet vaccination of the elderly consistently yields only limited protection [277,278]. This reduced vaccine efficacy in the elderly is ascribed to a lack of neutralizing antibodies and dysfunctional T cell responses [253]. Age-associated changes in the immune system have been shown to result in defective B cell responses and failure to form new protective antibodies [279]. Central to humoral immunity, the naïve CD4+ T cells that provide help in the activation and differentiation of antibody secreting B cells become impaired with age [280]. Examination of CD4+ T cells from old mice exhibited reduced responsiveness to TCR stimulation and proliferation, aberrant cytokine production, and failure to provide proper cognate help to antigen-specific B cells thus resulting in reduced antibody production and germinal centre formation [281]. These observations were corroborated in elderly people immunized with the currently available vaccines against influenza, tetanus, and *Streptococcus pneumoniae*; effective prophylactic vaccines via humoral immune protection, which exhibited significantly lower neutralizing antibodies in the elderly when compared to young vaccinees [282,283,284]. The current vaccines to prevent VZV reactivation in the elderly induce a pronounced increase in the number of effector CD4+ cells that are only partially protective since the associated disease still ensues in many elderly vaccinees [285]. It is important to note that the vaccination platforms for both young adults and aging individuals consist of the same vaccine formulations, and thus have not been tailored to stimulate immune responses in a significantly age-altered immune environment [286]. The aging immune system displays a systemic increase in

inflammatory cytokines (referred to as inflammaging) [287]. As such, the threshold for immune activation may be heightened in the elderly due to the elevated basal level of inflammatory cytokines, and the adjuvants contained in the vaccines may not produce sufficient stimulation to overcome this threshold [288]. Consequently, current vaccines fail to protect the elderly due to their limited ability to induce prophylactic humoral and cellular immunity and because they have not been tailored to induce immune responses in the age-altered immune system. It is interesting to note that we also completed a pilot analysis of humoral responses in our WNV cohort through a collaboration with Mike Diamond (University of Washington) and observed no difference in antibody responses to key neutralizing epitopes in the E protein (data published in [289]). Collectively, our data suggest that both cellular and humoral responses to WNV are unaffected by age in humans. A major difference between our WNV studies and previous vaccine studies in humans is the nature of the immunogen. While vaccine studies rely upon a non-replicating immunogen, WNV is a fully active pathogen. Thus, perhaps live agents are required to overcome the threshold to achieve immunization in the elderly?

Animal studies have elucidated that old mice infected with WNV succumb more rapidly to lethal neuroinvasive disease due to a significant decrease in the frequency of naïve and functionally competent CD8+ T cells in comparison to young mice [262,273]. However, the results presented in my thesis contradict the hypothesis that WNV disease severity in the aged is attributed to dysfunctional CD8+ T cells. Although the aged cohort displayed characteristic markers of immunosenescence, the age-associated changes did not affect

the development of new and polyfunctional CD8+ T cell responses. These findings further imply that prophylactic immunizations against acute viruses should focus on developing vaccines specifically aimed at inducing CD8+ T cell responses. Functional CD8+ T cells have been correlated with protection against influenza [290] and VZV reactivation [277] in old people, thus suggesting that antigen-specific CD8+ T cells elicited by vaccines could provide prophylactic immunity against some pathogens.

Based on a number of aging human cohort studies, persistent CMV infection is believed to accelerate the effects of immunosenescence. Increased frailty and mortality have been observed in CMV-seropositive aged humans when compared to CMV-seronegative age-matched individuals [140,291,292]. It has been proposed that the overwhelming lifelong persistence of CMV in the elderly drives the virus-specific CD8+ T cells to exhaustion and results in an increased number of dysfunctional CD8+ T cells unable to control recurrent viral replication [268]. However, most of these studies focused on a limited number of CMV-specific CD8+ T cells and were restricted to specific HLAs. Conversely, I have shown that both young and aged naturally infected individuals express functionally consistent CMV-specific CD8+ T cell responses, as measured by cytokine production and mobilization of cytotoxic molecules. For my studies, I used a peptide pool of all known CMV epitope peptides that span multiple HLAs. Thus, the findings presented here, which take a more global view of the CMV-specific CD8+ T cell response, argue that CMV-specific immunity is maintained in old age and does not contribute to the frailty of the aging population. Similar observations were confirmed

using a rhesus macaque model of CMV infection in old animals, which displayed maintenance of CMV-specific T cell immunity, thus supporting the idea that CMV infection does not drive dysfunctional CD8+ T cells in old age [293].

Additionally, it is believed that persistent CMV infection further restricts the limited naïve T cell pool in the elderly by continuously recruiting naïve T cells into the expanding CMV memory pool [294]. As such, CMV infection in the elderly is believed to limit the host's ability to form new memory T cell responses to heterologous viral infections. This effect was shown in mice persistently infected with murine CMV who exhibited significantly weaker CD8+ T cell responses to superinfections with influenza and WNV [295]. However, I did not observe this effect in aged humans persistently infected with CMV, who upon infection with WNV generated functional memory CD8+ T cell responses that were equal in magnitude and breadth (as measured by the diversity of epitope reactivities) to the responses seen in young individuals. Consequently, persistent infections of both young and aged human subjects with either CMV or EBV did not limit the development of cellular immunity upon WNV co-infection.

Our understanding of the aging immune system is still very limited. Although aged people who live/lived a long life, infection-free have been studied, no specific correlates of healthy immune aging have been defined. The conclusions derived from studies of aging in human cohorts only provide correlates and may be pathogen specific. Humans present a great diversity in genetics, environment, and lifestyle, all which impact the

aging immune system. As such, it is important to note that the results presented here were derived from a genetically diverse human population of WNV-infected subjects with otherwise unknown health status. Despite this noted diversity in our cohort, all of the naturally infected persons over the age of 60 generated polyfunctional antigen-specific CD8+ T cell response to a novel virus. Considering the importance of CD8+ T cells in clearance of virus-infected cells, the implications of these findings suggest that vaccines aimed at inducing antigen-specific CD8+ T cells could impart protective immunity in the elderly and concomitantly reduce the incidence of morbidity and mortality associated with infectious diseases.

6.1 West Nile virus infection as a tool to study immunity in the aged

My research has demonstrated that WNV infection provides a useful tool to study primary immune responses to a novel pathogen in humans of all ages. As described above, the outcomes of my research have provided novel insight into the development of CD8+ T cell memory in the aged. The information garnered from the studies of WNV in the elderly may also translate to other flavivirus infections such as Yellow Fever virus (YFV). Vaccination of the elderly with the licensed YFV vaccine, YF-17D, is associated with adverse affects that were attributed to a delayed humoral response and viremia [296]. Consequently, characterization of the immune response to a related flavivirus could assist

in the development of better protective vaccines against YFV for the elderly who travel to endemic areas and experience primary exposure to this very pathogenic virus.

Although WNV infection provided a useful tool to characterize the peripheral CD8+ T cell immune responses to a novel virus, the question of whether these CD8+ T cell responses would be protective, still remains. Due to the nature of human immunology, I cannot ascertain whether these CD8+ T cell responses contributed to virus control during primary infection or whether they would protect the elderly individuals in a secondary virus exposure. For practical reasons, my studies were limited to PBMCs isolated from blood of WNV-naturally infected individuals. These samples represent the peripheral T cell response and do not allow a functional assessment of T cells at the site of ongoing WNV infection i.e. within the brain of patients presenting with neuroinvasive disease. Thus, it is conceivable that the peripheral circulating CD8+ T cells do not reflect the immune response in the brains of infected persons. In addition, all patient samples used in my research were collected following the onset of WNV symptoms and do not reflect the T cell response during the acute phase of viral infection. The WNV incubation period ranges from 2-14 days, and the collection of the first available blood sample occurred within 30 days of symptom onset [297]. Consequently, I studied memory CD8+ T cell responses, which I believe are a reflection of the primary CD8+ T cell responses. Indeed, this assumption is supported by the notion that the development of memory T cells bearing full functional potential is characteristic of an effective primary immune responses to pathogens [298]. However, the existence of WNV-specific CD8+ T cell

responses in WNV-immune patients does not guarantee protective immunity. Based on their polyfunctional properties and the current belief that qualitative (ie. polyfunctional) memory T cells correlate with protective anti-viral responses [201,299], I infer that the WNV-specific CD8+ T cells would also be protective. However, the immune response against WNV is multi-factorial, comprised of both humoral and cellular responses, thus making it impossible to ascertain if either one of the components alone, or if both together provide virus protection. Consequently, while the WNV infected patient cohort offered the ability to characterize CD8+ T cell responses in the elderly, it did not provide any further knowledge of correlates of immune protection in the elderly.

6.2 Methods for epitope discovery

Discovery of WNV-specific CD8+ T cell epitopes was performed using three different epitope mapping techniques including the application of overlapping peptide library, mass spectroscopy (MS) sequencing of peptides isolated from secreted HLA molecules, and the use of NetCTL peptide prediction algorithm. All techniques identified virus-specific epitopes but epitope mapping using overlapping peptide library was the most effective at identifying the greatest number of highly immunogenic peptides from PBMCs of naturally infected WNV patients.

WNV has a genome consisting of ~3400 amino acids and due to its smaller genome size in comparison to other very large viruses (eg. vaccinia virus, ~60 000 amino acids) it was feasible to generate a library of overlapping peptides that spanned the entire polyprotein. Using this method, thirteen WNV-specific CD8+ T cell peptides were uncovered from six of the ten viral proteins. The identified peptides were restricted by several different HLA alleles, which facilitated the analysis of T cell reactivities in a diverse population of WNV-infected patients. As such, this method did not focus on a specific HLA-restricting molecule as in the case of MS sequencing but it covered HLA-specificities of the entire patient cohort. Furthermore, identification of epitopes using the entire viral polyprotein displayed a hierarchy of reactivities and epitope dominance within the virus-infected PBMC specimens that were not revealed by the other methods. The MS-identified epitopes did show a hierarchy of HLA-A*0201-restricted peptide reactivities; however, the overlapping peptides presented a hierarchy of WNV-immunoreactive peptides across the entire viral polyprotein and of different HLA-specificities. NetCTL epitope prediction method was the least successful at identifying WNV immunoreactive peptides and furthermore it did not identify any of the peptides discovered by the other two methods.

The immunoreactive epitopes of which a small number dominated the CD8+ T cell response were strongly influenced by the HLA haplotype of the patient. The HLA-B*35-restricted $NS3_{501-510}$ epitope and the HLA-A*02-restricted $Env_{430-438}$ epitope exhibited the highest T cell reactivity, thus suggesting that these two epitopes alone can induce robust

CD8+ T cell responses in subjects carrying the restricting HLA molecules. This is very beneficial considering the high prevalence of HLA-A*0201 allele within most ethnic groups in the United States [300].

Both the overlapping peptide library and the MS sequencing epitope discovery methods independently identified the immunodominant $Env_{430-438}$ [SVG9] peptide as well as the $NS4B_{68-76}$ [SLF9] peptide. These findings propose that these two epitopes represent frequently occurring WNV-specific CD8+ T cells within the patient cohort that could be utilized for the generation of vaccines against WNV.

Of the one-hundred and twelve NetCTL-predicted WNV-specific CTL epitopes, only twenty-six were found to be weakly immunoreactive. The clear limitation of the NetCTL prediction method was its inability to identify highly immunogenic epitopes. Indeed, only four epitopes identified by NetCTL elicited T cell responses of a similar magnitude to the WNV epitopes discovered by the overlapping peptide library method. Disappointingly, NetCTL predicted none of the highly immunogenic peptides discovered by either MS sequencing or overlapping peptide libraries. Many of the NetCTL predicted HLA-A*0201-resctircted peptides were not found to be reactive in HLA-A*0201 expressing patients and highly immunoreactive HLA-A*0201-restricted $Env_{430-438}$ [SVG9] and $NS4B_{68-76}$ [SLF9] epitopes were not predicted by the software either. Thus, although this complex computer algorithm is very exciting and did yield some relevant WNV peptides, further refinements are clearly required as NetCTL failed to identify highly

immunoreactive CD8+ T cell epitopes and was unable to accurately select the restricting HLA of the predicted peptides.

In summary, epitope mapping using the overlapping peptide library method and direct stimulation of PBMC samples from WNV-immune subject identified highly reactive and immunodominant CD8+ T cell epitopes with a broad HLA specificity. These immunoreactive epitopes differentiate immunogenic regions of the virus that could be used in vaccines formulation to elicit robust WNV-specific T cell responses. In comparison to the other methods of epitope discovery, overlapping peptide library most successfully identified the greatest number of immunoreactive CD8+ T cell epitopes that were capable of eliciting polyfunctional CD8+ T cell responses.

6.3 Concluding remarks

Prevention of infectious disease and the associated increase in morbidity, mortality, hospitalizations, and overall cost of healthcare in the elderly should not be regarded as necessary but rather mandatory considering the global increase in the ageing population. Consequently, it is critical that we further our understanding of the aging immune system and the attributes of protective immunity for the elderly in order to develop prophylactic and age-specific vaccines. The findings of my research suggest that immunizing the elderly with vaccines tailored to elicit antigen-specific CD8+ T cell responses is possible,

and could mediate protective immunity against the overwhelming infectious diseases in this population. This will require the development of novel vaccination platforms, since the current effective vaccines for the young offer limited efficacy for the elderly.

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— APPENDIX I —

WNV-SPECIFIC CD8+ T CELL RESPONSES IN RELATION TO DISEASE SEVERITY OUTCOMES

Preface

The results presented herein are an extension of Chapter 5. I had hypothesized that disease severity following WNV infection is related to the magnitude of the CD8+ T cell

response in infected patients. As stated previously, we could not sample the patients at early time points following infection, thus, all of our data are derived from the early memory responses. Since the magnitude and functionality of the memory T cell should be a direct reflection of the magnitude and functionality of the acute T cell response, we believe it is fair to use these data to make assertions regarding the acute response to WNV. All of the specimens and assays (with the exception of the CFSE proliferation assay) have been described in Chapter 5.

Results

Quality of the polyfunctional WNV-specific memory CD8+ T cell response is not associated with disease severity

The initial assessment of cellular function from PBMCs of WNV-infected subjects was performed by IFN-γ ELISPOT analyses as described in Chapter 2. In summary, fifty-two PBMC specimens were segregated into two groups based on symptom severity, non-neuroinvasive and neuroinvasive disease, and the immune response following WNV peptide pool stimulation was analyzed *in vitro* as IFN-γ SFU (spot forming units)/10^6 PBMCs (Chapter 2, Figure 5). Comparison of the number of reactive minipools as well the magnitude of the IFN-γ response between the neuroinvasive and non-neuroinvasive PBMC specimens did not reveal differences in the cellular response.

To verify that the CD8+ T cells elicited the immune response following WNV peptide stimulation and to characterize this functional response in greater detail, ICS followed by flow cytometry studies were performed on PBMC specimens from non-neuroinvasive and neuroinvasive, symptomatic WNV-infected subjects. Although no difference in the production of IFN-γ by patient PBMCs was noted between the two cohorts, it remained possible that other functional parameters were disrupted in the CD8+ T cells of patients with neuroinvasive disease. It was my hypothesis that the ability of WNV-specific memory CD8+ T cells to generate multiple cytokines and execute cytotoxic function in response to viral infection would define the difference between non-neuroinvasive and neuroinvasive subjects.

PBMC specimens isolated 6-7 months post symptom onset were stimulated with a pool of 13 immunogenic WNV peptides indentified in Chapter 2, and the production of cytokines IFN-γ, TNF-α, and IL-2 by CD8+ T cells was quantified using ICS and flow cytometry. A total of fifty-seven WNV-infected PMBC specimens varying in disease severity were assessed within two groups: 1) non-neuroinvasive (twenty-eight specimens), and 2) neuroinvasive (twenty-nine specimens). Both groups had similar numbers of male and female subjects. Twenty-four of twenty-eight non-neuroinvasive specimens displayed CD8+ T cell IFN-γ+ responses (median value=0.146%) that were at least three-fold above the negative control measurement, whereas twenty-five of twenty-nine neuroinvasive specimens displayed IFN-γ+ CD8+ T cell responses (median value = 0.260%) above the negative control values (Figure 11A). Comparison of the median frequency of IFN-γ+

CD8+ T cells between the neuroinvasive and non-neuroinvasive WNV-infected specimens showed no statistically significant difference (p=0.218; Figure 1A). These data corroborated the initial observations from Chapter 2 showing that the magnitude of T cell response, as measured by IFN-γ ELISPOT analyses, was not correlated with disease severity.

The production of TNF-α from IFN-γ+ CD8+ WNV-specific T cells was compared between the two groups and it revealed no statistical difference (p=0.711) in the frequency of IFN-γ/TNF-α double positive CD8+ T cells (non-neuroinvasive = 45.5% median; neuroinvasive = 51.8% median) (Figure 1B). Although the production of IL-2 by virus-specific CD8+ T cells was minimal, the frequency of IFN-γ/IL-2 producing WNV-specific CD8+ T cells did not differ (p=0.857) between the non-neuroinvasive (median = 2.05%) and neuroinvasive (median = 1.53%) groups (Figure 1C). Collectively, the polyfunctional WNV-specific CD8+ T cells, capable of IFN-γ, TNF-α and IL-2 production, did not differentiate the PBMC responses from virus-infected individuals based on neurological symptoms.

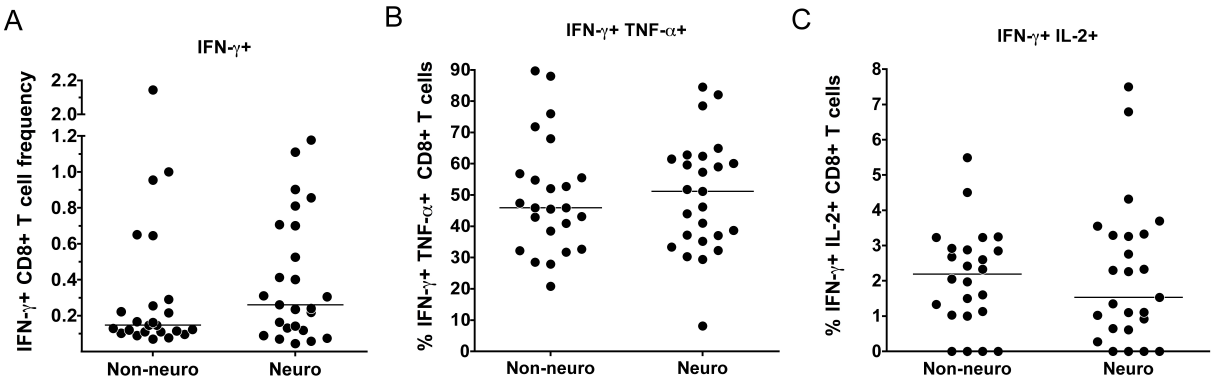

Figure 1. The polyfunctional cytokine response by WNV-specific memory CD8+ T cells does not correlate with disease severity. Peripheral blood mononuclear cell (PBMC) specimens collected 6-7 months post symptom onset were stimulated with a pool of WNV immunogenic peptides (Chapter 2, Table 1) and intracellular cytokine staining and flow cytometry analyses were performed following 5 hr of stimulation. The frequencies of A) IFN-γ+, B) IFN-γ+ TNF-α+, and C) IFN-γ+ IL2+, CD8+ T cells were compared between non-neuroinvasive (n=28) and neuroinvasive (n=29) WNV-infected subjects. Dimethyl sulfoxide (DMSO) stimulated PBMC specimens were used as negative controls. A significant IFN-γ response was operationally defined as three-fold above the background and a frequency of IFN-γ+ CD8+ T cells greater than 0.05%. Non-neuroinvasive group= twenty-four positive responses; neuroinvasive group= twenty-five positive responses. Each datum represents a single patient and the bars indicate the median values of the measured responses. Student's *t*-test was performed using GraphPad Prism 4.0.

Functional analyses addressed the cytotoxic ability of memory CD8+ T cells via mobilization of CD107a, and degranulation of granzyme B (GrB) and perforin following peptide stimulation. Sixty-four WNV-infected PBMC specimens obtained 4-6 months post symptom onset were analyzed, with thirty-two of these comprising each of the non-neuroinvasive and the neuroinvasive groups. The cytotoxic analyses examined IFN-γ producing CD8+ T cells since all WNV-reactive cells secreted IFN-γ upon peptide pool stimulation and, hence, were at least monofunctional. Comparative analyses of the ability

of WNV-specific IFN-γ+ CD8+ T cells to mobilize CD107a, and to degranulate GrB and perforin showed no statistically significant difference (p=0.538, 0.248) between non-neuroinvasive and neuroinvasive WNV-infected specimens (Figure 2). An increased frequency of GrB+ IFN-γ+ and GrB+ CD107a+ IFN-γ+ CD8+ T cells was noted in neuroinvasive infected patients (median values of 77% and 46%, respectively), but this increase did not reach significance (p=0.052, 0.080) compared to the non-neuroinvasive subjects (median values of 61% and 37%, respectively) (Figure 2 A,E). The frequency of IFN-γ+ GrB+ Perforin+ CD8+ T cells was similar (p=0.248) between non-neuroinvasive and neuroinvasive groups with median values of 4.3 and 5.2%, respectively (Figure 2D). Although perforin production by WNV-specific CD8+ T cells was low (below 5%), it was not significantly different between the two cohorts (p=0.268). Collectively, the cytotoxic WNV-specific CD8+ T cell response, as measured by mobilization of CD107a, and degranulation of GrB and perforin following antigenic peptide stimulation of PBMC specimens, was not significantly different between non-neuroinvasive and neuroinvasive WNV-infected subjects, thus suggesting that lack of cytotoxic CD8+ T cells was not correlated with diseases severity.

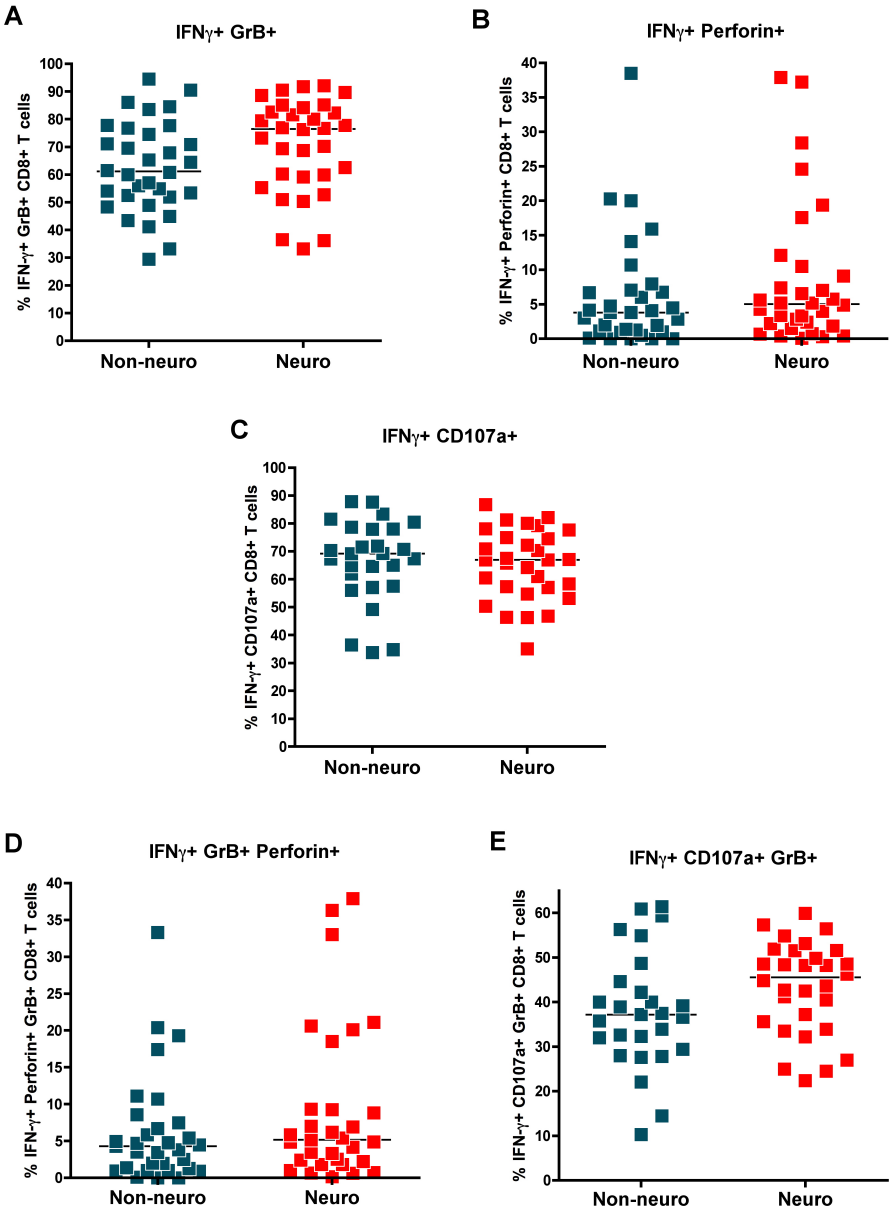

Figure 2. Cytotoxic polyfunctional WNV-specific CD8+ T cell response does not correlate with disease severity. Comparative analyses of A) IFN-γ+ Granzyme B+ (GrB+), B) IFN-γ+ Perforin+, C) IFN-γ+ CD107a+, D) IFN-γ+ GrB+ Perforin+, and E) IFN-γ+ CD107a+ GrB+ production by WNV-infected PBMC specimens isolated 4-6 months post symptom onset between non-neuroinvasive (n=32) and neuroinvasive (n=32) groups. Intracellular cytokine staining (ICS) and flow cytometry analyses were performed following WNV peptide pool stimulation. Each datum symbolizes an individual patient specimen and the horizontal bars represent median values. Statistical analyses were done using a student's *t*-test in GraphPad Prism 4.0.

Proliferative responses of virus-specific CD8+ T cells are not correlated with WNV disease severity

To assess the proliferative capacity of WNV-specific memory CD8+ T cells *in vitro*, carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMCs were incubated with WNV peptides and allowed to proliferate for 8 days. CFSE is an intracellular fluorescent dye that stably binds to proteins, thereby accumulating within lymphocytes. Following each cell division, the CFSE-fluorescence halves within each daughter cell, thus enabling an assessment of cell division. The CFSE dilution assay allows detection of up to 8 cell divisions before the fluorescence becomes too low to detect [301]. The fluorescence of CFSE dye is operationally defined as high or low; CFSE high (hi) events correspond to no proliferation whereas CFSE lo events correspond to T cells which have underwent division. Expanded cells were collected and surface stained for the presence of cell surface CD3 and CD8 molecules, and flow cytometry was used to determine the frequency of the expanded virus-specific CD8+ T cell population. Using this method, the proliferative abilities of PBMCs of WNV-infected non-neuroinvasive and neuroinvsive subjects were compared. Ten of twenty-four non-neuroinvasive specimens showed a CD8+ T cell specific proliferation in response to WNV peptides whereas sixteen of twenty-two neuroinvasive specimens exhibited CD8+ T cell proliferation as measured by the frequency of CFSE low (lo) events (Figure 3). Although it appeared that neuroinvasive PBMC specimens displayed better proliferative potential (since 73% of the analyzed samples proliferated in response to WNV peptides, compared with only 42% of the analyzed non-neuroinvasive specimens), the direct comparison of the frequency of

CFSE lo CD8+ T cells showed no statistically significant difference (p=0.385) between the two groups (Figure 3B). These findings indicated that the proliferative function of memory CD8+ T cells was not correlated with disease severity.

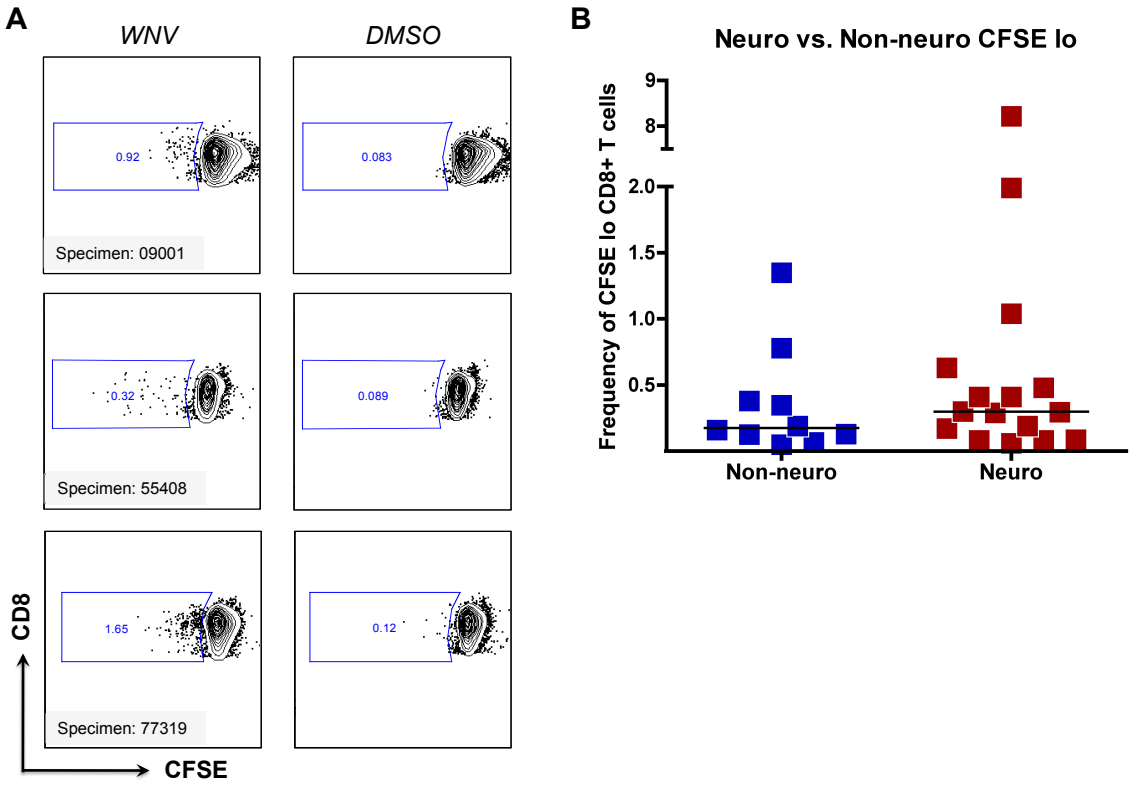

Figure 3. Proliferative ability of WNV-specific CD8+ T cells does not correlate with disease severity. A carboxyfluorescein succinimidyl ester (CFSE) dilution assay was performed on peripheral blood monocular cells (PBMCs) of WNV-infected non-neuroinvasive and neuroinvasive subjects. PBMCs were incubated with a pool of WNV identified peptides (Chapter 2, Table 1) or with dimethyl sulfoxide (DMSO; negative control) and were allowed to proliferate for 8 days. A) Flow cytometry plots depicting CD8+ T cell proliferation in response to WNV peptides and DMSO, as measured by CFSE dilution in three representative specimens (09001, 55408, and 77319). Numbers within plots represent frequencies of CFSE low (lo) events within each gate. CFSE lo cells correspond to proliferated cells. B) Comparisons between CFSE lo CD8+ T cells in non-neuroinvasive (n=24) and neuroinvasive (n=22) WNV-infected specimens. Each datum symbolizes a single patient. The horizontal bars represent median values. Statistical analyses were performed by student's *t*-test using GraphPad Prism 4.0.

Conclusions

I hypothesized that the severity of the disease following WNV infection is attributable to the impairment of CD8+ T cell functionality. To test this hypothesis, I compared the functional prolife of CD8+ T cells between non-neuroinvasive and neuroinvasive, symptomatic WNV-infected subjects. It was my goal to determine whether significant differences exist in the ability of WNV-specific CD8+ T cells to produce cytokines IFN-γ, TNF-α, IL-2, to mobilize CD107a, and degranulate GrB and perforin. Furthermore, the proliferative capacity of CD8+ T cells using CFSE dilution assay in the presence of WNV peptides was assessed between the two patient groups. Collectively, there were no statistically significant differences in the functionality of CD8+ T cells between the non-neuroinvasive and neuroinvasive symptomatic WNV subjects, thus suggesting that the function of virus-specific CD8+ T cells alone does not correlate with the severity of WNV infection; CD8+ T cell responses were qualitatively and quantitatively similar following peptide stimulation.

Contrary to my hypothesis, the frequency of IFN-γ producing CD8+ T cells also capable of mobilizing CD107a and degranulating GrB and perforin was not decreased in the neuroinvasive WNV-infected specimens when compared to the non-neurionvasive subjects. These data led to the interpretation that neuroinvasive cases of WNV infection could, in fact, be associated with increased cytotoxic CD8+ T cell function. This enhanced function could in turn mediate immune-related pathology where the immune system responds to the over-production of neurotropic virus by increasing the numbers of

CTLs to eliminate virus-infected cells, thus leading to over-engagement of the immune response and subsequent immunopathology. Clinical data have demonstrated that immunocompromised, WNV-infected subjects are more susceptible to neuroinvasive complications [86,302], thus suggesting that an intact immune system is necessary for the control and elimination of WNV infection. I showed here that the functionality of CD8+ T cells between non-neuroinvasive and neuroinvasive WNV-infected subjects was similar in cytokine production with slightly increased cytotoxicity, thus suggesting that a lack of functional CD8+ T cell responses does not contribute to the development of neuroinvasive disease.

Materials and Methods

All of the details for the methods and patients described in this Appendix can be found in Chapter 5 with the exception of the CFSE dilution analysis, which is described below.

CFSE dilution analysis: To address the proliferative ability of antigen-specific CD8+ T cells we employed the CFSE dilution assay. Following thawing of PBMC samples, cells were cultured in cRPMI as previously described, however, supplemented with heat-inactivated 10% human AB serum (Sigma) instead of FBS. Cells were stained with 5 uM Carboxy Fluroscein Succinimidyl Ester (CFSE) dye (eBiosciences) diluted in phosphate buffer solution (PBS) with 5% FBS for 10 minutes at room temperature. Following

staining, $2x10^5$ cells were aliquoted per well of a round bottom 96-well plate (BD Falcon, Franklin Lakes, NJ) and were stimulated with WNV peptide pool at a final concentration of 2 uM for 8 days in a 37 C humidified incubator. DMSO stimulated cells were used as a negative control for all patient samples while Staphylococcal enterotoxin B (SEB) stimulated cells were used as a positive control. After an 8-day cell culture in the presence of peptide ligands, we analyzed the proliferative potential of CD8+ T cells via CFSE dye dilution by flow cytometry and FlowJo software. Cells were additionally stained with anti-human CD3, CD4, and CD8 fluorescently conjugated antibodies.

— APPENDIX II —

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