Spring 5-2017

Elucidating the Role of Interleukin-17A in West Nile Virus Infection

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ELUCIDATING THE ROLE OF INTERLEUKIN-17A IN WEST NILE VIRUS INFECTION

by

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A Dissertation
Submitted to the Graduate School
and the Department of Biological Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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May 2017

Published by the Graduate School

ABSTRACT

ELUCIDATING THE ROLE OF INTERLEUKIN-17A IN

WEST NILE VIRUS INFECTION

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West Nile virus (WNV) is a neurotropic flavivirus of significant public health importance for which no therapeutics and vaccine are currently available. Interleukin-17A (IL-17A) is an inflammatory cytokine that regulates diverse immune functions, while its role is unclear in host's immune response to WNV. Furthermore, $CD8^+$ T cells are crucial components of immunity and play a vital role in recovery from WNV infection. Here, we report a previously unrecognized function of IL-17A in regulating $CD8^+$ T cell cytotoxicity. We show that WNV induces the expression of IL-17A in both mouse splenocytes and human peripheral blood mononuclear cells cultured *in vitro*, and in plasma of WNV-infected mice and humans. In a mouse model of WNV infection, we demonstrate that IL-17A deficient mice (*Il17a*$^{-/-}$) are more susceptible to WNV and develop a higher viral burden compared to wild-type (WT) mice. Interestingly, the $CD8^+$ T cells isolated from WNV-infected *Il17a*$^{-/-}$ mice are less cytotoxic and express lower levels of cytotoxic mediator genes, which can be restored by supplying recombinant IL-17A *in vitro* and *in vivo*. Moreover, treatment of WNV-infected mice with recombinant IL-17A, as late as day 6 post-infection, significantly reduces viral burden and increases survival, suggesting a therapeutic potential of IL-17A. In conclusion, we demonstrate a novel function of IL-17A in promoting

$CD8^+$ T cell cytotoxicity against WNV infection, which may have broad implications in other microbial infections and cancers.

ACKNOWLEDGMENTS

I am grateful to my Ph.D. advisor Dr. Fengwei Bai for his excellent supervision and providing a wonderful opportunity to accomplish my research in his lab. I appreciate his tremendous help, scientific expertise and valuable time. His encouragement and support considerably helped me to understand science, learn scientific techniques, and conduct research. I am also grateful to my research committee members Dr. Faqing Huang, Dr. Mohamed Elasri, Dr. Yanlin Guo, and Dr. Shahid Karim and like to greatly appreciate their scientific insight, expertise, and valuable time. I like to thank Dr. Penghua Wang (Yale University) for his assistance in preliminary studies.

I like to thank my lab mate Amber M. Paul for her assistance in performing experiments. I am also thankful to Biswas Neupane and all other lab members for their help and creating an excellent working environment. I like to thank Nick and Emily for their help in animal care.

I like to thank Dr. Dobrivoje S. Stokic and Dr. Arturo Leis from University of Mississippi Medical Center for their encouragement in conducting WNV research. I like to extend my sincere thank to Dr. Richard A. Flavell (Yale University) for providing IL-17A knockout mice, Dr. John F. Anderson (Connecticut Agricultural Experiment Station) for providing WNV (CT2471), and Dr. Michael S. Diamond (Washington University in St. Louis) for providing $MC57GL_{WNV\text{-}E}$ and $MC57GL_{vector}$ cells. Finally, I like to thank Mississippi IDeA Network of Biomedical Research Excellence (MS-INBRE) for providing excellent research facility.

DEDICATION

I dedicate this dissertation to my father Durga Datta Sharma Acharya and my mother Sharada Sharma Acharya, who are endless source of inspiration and encouragement throughout my life. I like to extend my gratitude to my beloved wife Ranjana Baral, and my sisters Rasmi and Roshani for their love and care. Finally, I like to thank all my friends for their motivation and encouragement.

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LIST OF ABBREVIATIONS

ACT-1	NF-κB activator 1
ANOVA	Analysis of variance
ATCC	American type culture collection
BBB	Blood brain barrier
BSL3	Biosafety level 3
cDNA	Complementary DNA
CHIKV	Chikungnya virus
CNS	Central nervous system
d.p.i.	Days post infection
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
Fig	Figure
FCB	Flow cytometry buffer
hPBMC	Human peripheral blood mononuclear cells
i.p.	Intraperitoneal
IFN	Interferon
IL	Interleukin
IL-17A	Interleukin-17A
IL-17R	Interleukin-17 receptor

IRB	Institutional review board
IACUC	Institutional animal care and use committee
LCMV	Lymphocytic choriomeningitis virus
MOI	Multiplicity of infection
mRNA	Messenger RNA
NK	Natural killer
NKT	Natural killer T
NS	Nonstructural
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PFU	Plaque-forming unit
qPCR	Quantitative polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RFC	Relative fold change
rIL-17A	Recombinant IL-17A
RNA	Ribonucleic acid
SEFIR	Similar expression to fibroblast growth factor and IL-17R
SEM	Standard error of mean
TLR	Toll-like receptor
TNF	Tumor necrosis factor
USM	The University of Southern Mississippi
WNV	West Nile virus

WNV-E	WNV envelop
WT	Wild-type

CHAPTER I - INTRODUCTION

1.1 West Nile Virus

West Nile virus (WNV), a neurotropic single-stranded RNA virus belonging to *flaviviridae* family, is generally transmitted to human by an infected mosquito bite, primarily of *Culex* species (Colpitts et al., 2012; Kuno and Chang, 2005). However, WNV can also be transmitted through other less frequent transmission routes, including blood transfusion (Pealer et al., 2003; Stramer et al., 2005), organ transplantation (Centers for Disease and Prevention, 2009), breastfeeding (Blazquez and Saiz, 2010), and congenital infections (Alpert et al., 2003). WNV infection in human can cause fever and may result in injury and death of neurons, the latter can lead to various neurological manifestations, such as encephalitis, flaccid paralysis, meningitis, chronic neurologic sequelae and possibly death, particularly in the elderly and immunocompromised individuals (Colpitts et al., 2012; Hayes et al., 2005; Samuel and Diamond, 2006).

Structurally, WNV is an enveloped virus with 50 nm diameter, which comprises an icosahedral nucleocapsid surrounded by a host-derived lipid envelope (Mukhopadhyay et al., 2003). The WNV genome contains a capped, single-stranded, and plus-sensed RNA genome of approximately 11 Kb in size. The WNV genome translates to a polyprotein precursor, which undergoes posttranslational processing by viral and cellular proteases to generate three structural (capsid [C], premembrane [PrM], and envelope [E]), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Among these proteins, the structural proteins form the structure of virion, whereas the

non-structural proteins are essential in genome replication, virion assembly, and viral pathogenesis (Chung et al., 2006; Liu et al., 2006; Mukherjee et al., 2011a).

1.1.1 WNV Is a Major Public Health Problem

WNV was first discovered in Uganda about 80 year ago and primarily maintained in a mosquito-bird-mosquito transmission cycle (Colpitts et al., 2012). Until 1990's it caused several sporadic outbreaks of minor public health importance in Africa, Asia, Europe and the Middle East with a limited capacity to cause neuroinvasive diseases (Draganescu, 1979; Kramer et al., 2008). The first report of neuroinvasive cases of WNV in Algeria in 1994 (Le Guenno et al., 1996), and a huge report of neurological impairments during subsequent WNV outbreaks in South-East of Romania (Cernescu et al., 1997), and other parts of southern Europe (Hubalek and Halouzka, 1999) brought this virus into a real public health concern. In northern hemisphere, WNV was first identified in New York in 1999 (Hayes, 2001; Mostashari et al., 2001), which subsequently spread throughout the USA within a few years (Benjelloun et al., 2015).

Currently, WNV is causing endemic diseases throughout the North America and has a wide geographical distribution in all continents of globe except Antarctica (Benjelloun et al., 2015). In the US alone, there have been over 40,000 cases of WNV reported between 1999 and 2014, out of which about 45% were classified as neuroinvasive cases (CDC, 2015). However, the actual burden of WNV is likely much higher than previously thought because only a proportion (about 20%) of infected individual develop a clinical disease and a majority of infected individuals remain asymptomatic (Mostashari et al., 2001). It has been

recently estimated that over 3 million individuals have been infected with WNV in the USA alone, out of which about 780,000 had a symptomatic illness (Petersen et al., 2013). Considering the worldwide circulation of WNV and supportive evidence of its capacity to change pathogenicity and transmission potential (Davis et al., 2005; Ebel et al., 2004; Moudy et al., 2007; Prow et al., 2014; van den Hurk et al., 2014), there is an urgent need to develop a safe and effective antiviral drug or vaccine against WNV infection (Martina et al., 2010). However, no licensed antiviral drug or vaccine is currently available for WNV infection in humans.

1.1.2 Pathogenesis of WNV Infection Is Not Well Understood

After a bite of WNV-infected mosquito inoculates infectious viruses into human skin, WNV first infects skin-resident cells such as dendritic cells (Langerhans cells) and keratinocytes. Infected dendritic cells can carry the viruses to draining lymph nodes, after which WNV circulates through lymphatic systems and enters the blood stream (Lim et al., 2011; Ye et al., 2011). WNV can infect various cells including macrophages, neutrophils, and other leukocytes leading to development of a transient viremia. Subsequently, WNV can disseminate to peripheral organs, such as liver and spleen, and then to the brain and spinal cord. In most of the immune-competent individuals, WNV symptoms range from unapparent infection to mild febrile illness (Colpitts et al., 2012). In a few symptomatic cases, particularly in the elderly and immunocompromised individuals, WNV can cause injury and apoptosis of neuron, which can potentially cause various neurological impairments and death (Colpitts et al., 2012). Viral

entry into the brain is considered a hallmark of WNV pathogenesis and disease severity. Although the mechanism of neurotropism and how WNV enters the central nervous system (CNS) is not clearly understood, hematogenous entry via disrupted blood-brain barrier (BBB) and transneural entry via retrograde axonal transport have been suggested to play a role (Suen et al., 2014; Suthar et al., 2013).

1.1.3 Host's Immunity Is Crucial in WNV Recovery

WNV can infects various type of immune cells and strongly activates host immune responses, which play important roles in controlling viremia, reducing viral dissemination to the CNS, and recovery from the disease (Samuel and Diamond, 2006). Clinical reports of WNV-infected human cases along with research using mouse models or *in vitro* approaches have identified critical roles of several components of immune system in WNV infections (Arjona et al., 2011; Suthar et al., 2013). In brief, type-I interferon (IFNs) (Lazear et al., 2011; Samuel and Diamond, 2005), the complement system (Mehlhop and Diamond, 2006), and humoral immunity (Diamond et al., 2003a; Diamond et al., 2003b) limit viremia and control WNV dissemination to CNS. Similarly, components of cell-mediated immunity, including $CD4^+$ (Sitati and Diamond, 2006) and $CD8^+$ (Shrestha and Diamond, 2004) T cells, have been shown to clear WNV from the CNS, limit viral persistence, and facilitate recovery. In contrast, the roles of neutrophils, NK cells, and γδ-T cells are still unclear (Bai et al., 2010; Shrestha et al., 2006a; Welte et al., 2008). Cytokine signaling of interleukin (IL)-23 (Town et al., 2009), interferon-γ (IFN-γ)(Shrestha et al., 2006b), and IL-1β (Ramos et al.,

2012) have been shown to protect against WNV infection, while cytokine such as IL-10 (Bai et al., 2009) and IL-22 (Wang et al., 2012) have been shown to favor WNV pathogenicity. The role of tumor necrosis factor-α (TNF-α) still remains elusive (Shrestha et al., 2008b; Wang et al., 2004a). However, the role of several other components of immune system yet remains unknown and the mechanism of WNV pathogenesis, including its tropism to neurons and CNS invasion, remains unclear. In addition, we have limited knowledge on viral or host factors that contribute to imbalance between viral pathology and host to WNV infection.

1.2 Interleukin-17A and Its Signaling

IL-17A, a major cytokine of IL-17 family (IL-17A, B, C, D, E, and F), was discovered in 1993 as the cytotoxic T-lymphocyte antigen-8 (Rouvier et al., 1993). $CD4^+$ Th17 (Th-17) cells are one of the most common sources of IL-17A. Besides Th-17 cells, a wide variety of immune cells, including $CD8^+$ T cells, γδ-T cells, and natural killer T (NKT) cells can also produce IL-17A under various pathological conditions (Gu et al., 2013). IL-17A signals through IL-17 receptor (IL-17R) complex composed of IL-17RA and IL-17RC subunits (Ho and Gaffen, 2010). Both subunit of IL-17R complex encode a SEFIR (Similar Expression to Fibroblast growth factor genes and IL-17 Receptor) domain that mediates various downstream signaling events through the adaptor protein NF-κB activator-1 (ACT1) (Gaffen, 2009; Ho and Gaffen, 2010). Specifically, the ACT1 recruits to IL-17R through interaction with SEFIR domain and activates TNF receptor-associated factor-6 (TRAF6) and TRAF3, which are essential upstream activators of the nuclear factor-κB (NF-κB) signaling pathway. In addition, ACT1

is also required for IL-17A mediated stabilization of mRNAs that encode several chemokines and cytokines. Moreover, IL-17A also signals through ACT1 independent pathway by activation of extracellular signal-regulated kinase (ERK) (Ho and Gaffen, 2010).

1.2.1 IL-17A Regulates Diverse Immune Functions

The finding that IL-23 drives expression of IL-17 in $CD4^+$ T cells led to the realization of a new paradigm that many functions formerly attributed to $CD4^+$ Th-1 cells were in fact mediated by a distinct, IL-17-producing T cell subset known as Th-17 cells (Aggarwal et al., 2003; Langrish et al., 2005). It is now well established that IL-1β, IL-6, TGF-β, and IL-21 drive development, while IL-23 stabilizes Th-17 cells (Korn et al., 2007; Nurieva et al., 2007; Sutton et al., 2006; Zhou et al., 2007).

IL-17A signaling regulates diverse immune functions including the expression of various inflammatory cytokines, chemokines and antimicrobial peptide, activation and recruitment of leukocytes, and production of antibodies (Gu et al., 2013; Song and Qian, 2013) during infection and immunity. For example, IL-17A has often been described as a mediator of inflammation (Witowski et al., 2004) with prominent roles in allergic and autoimmune diseases including multiple sclerosis (McFarland and Martin, 2007; Zepp et al., 2011), rheumatoid arthritis (van den Berg and Miossec, 2009), psoriasis (Raychaudhuri, 2013), asthma (Newcomb and Peebles, 2013), and Crohn's disease (Siakavellas and Bamias, 2012). Similarly, IL-17A also plays protective roles against some bacterial and fungal infections. For instance, IL-17A may enhance neutrophil

recruitment and protect against bacterial and fungal pathogens such as *Klebsiella pneumonia* and *Escherichia coli* (Happel et al., 2005; Shibata et al., 2007; Ye et al., 2001), *Listeria monocytogenes* (Hamada et al., 2008), *Mycobacterium tuberculosis* (Witowski et al., 2004), *Francisella tularensis* (Lin et al., 2009), *Chlamydia muridarum* (Zhang et al., 2009), *Candida albicans* (Conti et al., 2009; Huang et al., 2004) and *Pneumocystis carinii* (Rudner et al., 2007). Conversely, IL-17A signaling has been suggested to facilitate toxoplasmosis (Guiton et al., 2010) and certain fungal infections (Zelante et al., 2007).

1.2.2 Role of IL-17A in Viral Infections Is Unclear

The IL-17A has been originally identified as a homolog of virally encoded ORF13 gene of *herpesvirus saimiri* (Yao et al., 1995). This discovery generated interest to study the role of this cytokine in viral infection. It has been demonstrated that genetically constructed vaccinia virus (VV) expressing IL-17A ($VV^{IL\text{-}17A}$) caused more severe disease in mice (Patera et al., 2002), but $VV^{IL\text{-}17A}$ was also reported less virulent and IL-17 deficient ($Il17a^{-/-}$) mice were more susceptible to VV infection (Kohyama et al., 2007). Thus, the role of IL-17A during VV infection remains inconclusive.

Interestingly, it has been reported that type I IFNs potently suppress IL-17A expression (Curtis et al., 2009; Tilg et al., 2009), which further makes the functional role of IL-17A during viral infections more complex to understand. In most of the viral infections, type I IFN-mediated antiviral response primarily occurs during early phase of infection (Lazear et al., 2011; Pinto et al., 2011). It can be speculated that the IL-17A functions may be more dominant in the later

phase of infection when type I IFN expression levels become low. It has been shown that viral infections can induce expression of IL-17A (Mukherjee et al., 2011b; Town et al., 2009; Wang et al., 2013), which has been implicated in priming T cell responses during lymphocytic choriomeningitis virus (LCMV) hepatitis (Jie et al., 2014) and mediating immunopathogenicity of viral infections such as influenza virus (Crowe et al., 2009), respiratory syncytial virus (de Almeida Nagata et al., 2014; Mukherjee et al., 2011b), murine encephalomyelitis virus (Hou et al., 2009) and hepatitis B virus (Wang et al., 2013). However, the role of IL-17A in WNV infection in not clearly understood.

1.3 Possible Implication of IL-17A in WNV Infection

It has been previously reported that WNV infection induces Toll-like receptor-7 (TLR7) dependent production of IL-23 in mice (Town et al., 2009). IL-23 is known as a prime regulator for stabilization and maintenance of $CD4^+$ Th-17 cells, which are the major cell type secreting IL-17A (Aggarwal et al., 2003; McGeachy et al., 2009; Stritesky et al., 2008). Thus, it is likely that WNV infection induces IL-17A expression, which may play a role in WNV immunity. The following literature provides strong evidence for possible involvement of IL-17A signaling during WNV infection.

1.3.1 IL-17A Is Crucial in the CNS Inflammation

WNV primarily targets neurons and causes inflammation in the CNS, which play important role in its pathogenesis. Astrocytes and microglia up-regulate the expression of functional IL-17A receptor and respond to this cytokine during brain inflammatory conditions (Das Sarma et al., 2009). In addition, IL-17A

is also described as a mediator of CNS inflammation (McFarland and Martin, 2007; Zepp et al., 2011) and suggested as a blood-brain-barrier permeability factor (Kebir et al., 2007). Therefore, the WNV pathogenesis and host's immune response in the CNS are likely impacted by IL-17A.

1.3.2 IL-17A May Control Host's Immunity to WNV Infection

Considering the role of IL-17A in regulating diverse immune function, it can be speculated that this cytokine may play an important role in WNV immunity. For instance, IL-17A has been shown to regulate the expression of other cytokines including IL-1β, IFN-γ and TNF-α (Maione et al., 2009; Song and Qian, 2013), infiltration of leukocytes, and production of antibodies (Yao et al., 1995; Yuan et al., 2010). As these cytokines are previously described to modulate immunity to WNV (Ramos et al., 2012; Shrestha et al., 2006b; Shrestha et al., 2008b; Wang et al., 2004a), one can expect possible involvement of IL-17A in WNV immunity. Moreover, brain-infiltrating leukocytes are crucial in clearance of WNV (Bai et al., 2010; Shrestha and Diamond, 2004), which can be potentially controlled by IL-17A signaling. Furthermore, the humoral immune response, which plays an important role in controlling viremia and WNV dissemination to CNS (Diamond et al., 2003a), can be potentially regulated by IL-17A (Yuan et al., 2010).

Among the CNS-infiltrating leukocytes, $CD8^+$ T cells play crucial role in WNV immunity (Shrestha and Diamond, 2004, 2007; Shrestha et al., 2012; Shrestha et al., 2006a). Although the relation between IL-17A signaling and $CD8^+$ T cell cytotoxicity is not yet studied, several literatures support this novel axis

may play a role in WNV and other viral infections. First, both Th-17 cells and γδ-T cells, the major cells that produce IL-17A, have been previously shown to promote $CD8^+$ T cell cytotoxicity during intracellular infections (Hamada et al., 2009; Xu et al., 2010). Second, the Th-17 cells and IL-17 have also been shown to control $CD8^+$ T cell function in autoimmune diseases (Ankathatti Munegowda et al., 2011a). Third, the $CD8^+$ T cells have a crucial role in clearance of tumor cells (Benchetrit et al., 2002; Martin-Orozco and Dong, 2009). Several studies also demonstrated the expression of IL-17A in tumor cells (Benchetrit et al., 2002; Martin-Orozco and Dong, 2009). Interestingly, it has been shown that Th-17 cell and IL-17A induce cytotoxic function of $CD8^+$ T cells and control tumor progression (Benchetrit et al., 2002; Martin-Orozco and Dong, 2009), however, the mechanism for this IL-17A-$CD8^+$ T cell axis remains yet unknown. Therefore, considering the crucial role of $CD8^+$ T cells in WNV immunity, WNV is a suitable model to study IL-17A-$CD8^+$ T cell axis.

CHAPTER II – SIGNIFICANCE, HYPOTHESIS, AND INNOVATION

2.1 Significance

WNV infection is a significant public health problem, for which no therapeutics or vaccine are currently available. In addition, IL-17A and $CD8^+$ T cells regulate diverse immune functions during microbial infections, malignancies, and autoimmune diseases. In this study, we studied the function of IL-17A in WNV immunity and identified a potential therapeutic role of IL-17A in facilitating WNV clearance by promoting $CD8^+$ T cells cytotoxicity. Further understanding of the interplay between $CD8^+$ T cells and IL-17A axis may lead to identification of novel therapeutic strategies. Moreover, this novel function of IL-17A may have broad implications in deciphering the immunopathology of other viral infections and malignancies, where $CD8^+$ T cells functions are crucial.

2.2 Hypothesis

Despite intensive research over the past 15 years, WNV pathogenesis and host's immune response still remains unclear. IL-17A, a major cytokine in the IL-17 family regulates diverse immune functions including the expression of various inflammatory cytokines and chemokines, activation and recruitment of leukocytes, and production of antibodies (Gu et al., 2013; Song and Qian, 2013). Moreover, IL-17A has often been described as a mediator of inflammation (Witowski et al., 2004) with an important role in allergic and autoimmune diseases (McFarland and Martin, 2007; Newcomb and Peebles, 2013; Raychaudhuri, 2013; van den Berg and Miossec, 2009; Zepp et al., 2011), malignancies (Martin-Orozco and Dong, 2009; Murugaiyan and Saha, 2009), and

infections (Conti et al., 2009; Hamada et al., 2008; Happel et al., 2005; Huang et al., 2004; Lin et al., 2009; Rudner et al., 2007; Shibata et al., 2007; Ye et al., 2001; Zhang et al., 2009). However, the role of IL-17A signaling is not clear during viral infections and has been previously studied in WNV infection. As we described in the introduction (section 1.7), there are several evidences suggesting a possible role of IL-17A in controlling host's immune response to WNV. Therefore, *we hypothesized that IL-17A may play a crucial role in WNV immunity*. In this study, we tested this hypothesis under following four specific aims.

Specific Aim 1: Study the expression of IL-17A during WNV infection in vitro and in vivo. It has been previously reported that TLR7 mediates IL-23-dependent protective immune responses against WNV infection in mice (Town et al., 2009). IL-23 is known as a prime regulator for stabilization and maintenance of $CD4^+$ Th-17 cells, which are the major cell type secreting IL-17A (Aggarwal et al., 2003; McGeachy et al., 2009; Stritesky et al., 2008). Since IL-17A is also described as a mediator of CNS inflammation (McFarland and Martin, 2007; Zepp et al., 2011), and a factor contributing to blood-brain-barrier permeability (Kebir et al., 2007), we asked if WNV induces IL-17A production and whether it involves IL-23 signaling.

Specific Aim 2: Study the role IL-17A in WNV immunity in a mouse model. Based on the literature, it is likely that IL-17A may play either protective or pathogenic role during WNV infection. To dissect this question, we used a mouse

model of WNV infection and compared survival and viral burden of WNV infected IL-17A deficient (*Il17a*$^{-/-}$) and WT control mice.

Specific Aim 3: Study the immune functions of IL-17A during WNV infection. IL-17A has been shown to recruit leukocytes (Happel et al., 2005; Ye et al., 2001), control cytokine and chemokine expression (Maione et al., 2009; Song and Qian, 2013), and regulate humoral immune response (Grund et al., 2012; Tarlinton, 2008; Yuan et al., 2010). Under this specific aim, we asked how various immune functions were controlled by IL-17A during WNV infection.

Specific Aim 4: Study the mechanism of IL-17A mediated regulation of WNV immunity. From Aim 3, we identified a novel function of IL-17A in regulation of $CD8^+$ T cell cytotoxicity. Under Aim 4, we studied the mechanism of IL-17A mediated control of $CD8^+$ T cells cytotoxicity and its potential therapeutic role against WNV infection.

2.3 Innovation

Cytotoxic $CD8^+$ T cells are important component of immunity, particularly in clearance of intracellular infections and tumors. One of the effector mechanisms used by these cells is cytotoxic killing of target cells. However, regulators of $CD8^+$ T cell cytotoxicity, in particular, the expression of cytotoxicity mediators in these cells is poorly understood. In addition, functions of several molecules expressed in $CD8^+$ T cells remain uncharacterized. One such molecule is the IL-17A receptor (IL-17R), which is expressed on the surface of $CD8^+$ cells (Lindemann et al., 2008; Yao et al., 1995). In additions, subsets of $CD8^+$ cells can produce IL-17A (Hamada et al., 2009). Considering the role of IL-

17A in regulation of diverse immune functions during microbial infections, malignancies, and autoimmune diseases, it is likely that IL-17A signaling may play a role in biology of $CD8^+$ T cells. $CD8^+$ T cells are particularly important in controlling viral infections and have been shown to play a crucial role in clearance of WNV infection (Shrestha and Diamond, 2004). In this study, we studied the role of IL-17A in a mouse model of WNV infections and discovered a novel, previously unrecognized, function of IL-17A in promoting $CD8^+$ T cells cytotoxicity.

CHAPTER III – EXPERIMENTAL APPROACHES

3.1 Ethics Statement and Biosafety

Written informed consent was obtained from all human volunteers and human WNV cases prior to their enrollment in this study. The protocol for human subject has been reviewed and approved by the University of Southern Mississippi (USM) Institutional Review Board (IRB, protocol # CH-R11120601, Appendix A). All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at USM (protocol #12041201, Appendix A). All the *in vitro* experiments and animal studies involving infectious WNV were performed by the certified personnel in biosafety level 3 (BSL3) or animal biosafety level 3 (ABSL3) laboratories following standard biosafety protocols approved by USM Institutional Biosafety Committee.

3.2 Virus Stock and Animal Studies

Low-passaged WNV isolate CT2741 (Anderson et al., 1999) was provided by Dr. John F. Anderson at the Connecticut Agricultural Experiment Station. Low-passaged CHIKV LR OPY1 2006 strain was provided by Dr. Robert B. Tesh at the University of Texas Medical Branch. WNV and CHIKV stocks used in this study were prepared by propagating the viruses in Vero cells through a single passage and titrated in Vero cells by a plaque-forming assay as previously described (Bai et al., 2005). Vero cells (ATCC CCL-81) were cultured in a 37°C incubator with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS). Interleukin-17A deficient (*Il17a*$^{-/-}$) mouse breeding pairs (C57BL/6J background) were provided

by Dr. Richard A. Flavell at the Yale University School of Medicine and wild-type (WT) control mice (C57BL/6J) were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-23 deficient (*Il23p19*$^{-/-}$) breeding pairs on a mixed C57BL/6 × 129 background were obtained from the Mutant Mouse Regional Resource Center (MMRRC). Mice were housed under standard conditions in the animal facility at USM. Gender-matched and 7 to 9 weeks old *Il17a*$^{-/-}$ and WT control mice were infected with 1,000 plaque forming units (PFUs) of WNV by i.p. injection in 100 µl of phosphate buffer saline (PBS) containing 5% gelatin (Town et al., 2009). For footpad inoculation, 100 PFU of WNV in 50 µl PBS containing 1% FBS was injected into the mouse footpad after isofluorane anesthesia (Bai et al., 2009). Infected animals were observed twice daily for up to 21 days for morbidity and mortality.

3.3 Cell Culture and *In Vitro* Infection

Human peripheral blood mononuclear cells (hPBMCs) were isolated from blood of healthy human volunteers using Ficoll-paque™ PLUS (GE healthcare). To isolate murine splenocytes, healthy C57BL/6J mice (7-week old) were euthanized and spleens were collected to make single cell suspension. After red blood cell lysis, both mouse splenocytes and hPBMCs were purified and cultured in the Dulbecco's modified Eagle medium (DMEM, Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, and 1% non-essential amino acids. Cells were infected with WNV (MOI = 0.1, 1 or 5) and collected in TRIreagent (Molecular Research Center) at 24 h and 48 h for total RNA extraction.

3.4 Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from cells or animal tissues (i.e. blood, spleen, and brain) using the TRIreagent or RNeasy kit with on-column DNA digestion (Qiagen). First-stranded complementary DNA (cDNA) was synthesized using the iSCRIPT^{TM} cDNA synthesis kit (Bio-Rad). WNV-envelope (*WNVE*) RNA copy numbers were quantified using probe-based qPCR and normalized to cellular *β-actin* gene, as previously described (Bai et al., 2005). qPCR assays for cytokine, chemokine, and other immunological marker genes were performed using SYBR Green supermix (Bio-Rad) and data are presented either as the relative fold change (RFC) by ΔΔCT method using *β-actin* as a housekeeping gene or as a copy number ratio of target gene to cellular *β-actin*. Primer sequences for mice (Bai et al., 2005) and human (Kozaci et al., 2007) *β-actin* are previously described. Primers sequences for human *Il17a* (F, 5’-TGTGATCTGGGAGGCAAAGT-3’; R, 5’-GATCTCTTGCTGGATGGGGA-3’), and mouse *Il17a* (F, 5’-TCTCCACCGCAATGAAGACC-3’, R, 5’-TTTCCCTCCGCATTGACACA-3’); *perforin*-1 (F, 5'-TGTTCCTCCTGGGCCTTTTC-3'; R, 5'-CCATACACCTGGCACGAACT-3'), *granzyme-A* (F, 5'-CACGTGAGGGGGATCTACAAC-3'; R, 5'-TCTCCCCCATCCTGCTACTC-3'), *granzyme-B* (F, 5'-TGCTACTGCTGACCTTGTCTC-3'; R, 5'-CCATGTAGGGTCGAGAGTGG-3'), *fasL* (F, 5'-GAACTGGCAGAACTCCGTGA-3'; R, 5'-TGAGTGGGGGTTCCCTGTTA-3'), *Ifn*-α (F, 5'-TTCCCCTGACCCAGGAAGAT-3'; R, 5'-CTTCTGCTCTGACCACCTCC-3'), *Ifn*-β (F, 5'-

TGTCCTCAACTGCTCTCCAC-3'; R, 5'-ATCTCTGCTCGGACCACCAT-3'), *Act-1* (F, 5'-GAGGACGAGCATGGCTTACA-3'; R, 5'-TGGCATTTGGGAAGAGCACA -3') were designed using NCBI's primer designing tool and synthesized by Integrated DNA Technologies.

3.5 Enzyme Linked Immunosorbent Assay (ELISA)

IL-17A, IFN-β, IL-1β, IFNγ, TNFα, IL-6, IL-10, IL-12p40, and anti-WNV-E IgM antibody in plasma of WNV-infected mice (1,000 PFU, i.p.) were measured using ELISA kit (R&D Systems) following manufacturer's instructions. Level of IL-17A in culture media, and sera of human WNV cases and healthy controls was measured by ELISA kit from Enzo Life Sciences.

3.6 Confocal Microscopy

Brains were collected from WNV-infected mice (1,000 PFU, i.p.) after intracardial PBS perfusion, fixed overnight in 4% PFA at 4°C, and cryoprotected in sucrose. Para-median sagittal sections (25 μm) of brain were pre-blocked for 30 min at room temperature and then probed overnight at 4°C with a combination of primary antibodies against CD11b, CD45 and WNV antigen (anti-WNV antibody provided by John F. Anderson, other antibodies purchased from BD Biosciences). After a PBS wash, sections were probed with appropriate fluorescent labeled secondary antibodies for 1 h at room temperature, counterstained with DAPI (Invitrogen), and mounted in fluorescence mounting medium (ProLong Gold). Images were acquired in independent channels using a Nikon A1R confocal microscope.

3.7 Flow Cytometry

Brains were collected from WNV-infected (1,000 PFU, i.p.) mice after intracardial PBS perfusion and processed into a single cell suspension. Brain leukocytes were isolated using a discontinuous Percoll gradient (GE Healthcare) and probed with CD45, CD4, CD8, and CD11b (BD Biosciences or eBioscience). After staining, cells were washed two times in flow cytometry buffer (FCB, PBS with 2% FBS) and fixed in 4% paraformaldehyde (PFA) for 15 minutes. For intracellular staining, cells were permeabilized and probed with antibodies against perforin, and granzyme A (eBioscience). Cells were then washed and re-suspended in FCB. Data were acquired on a flow cytometer (BD LSRFortessa) and analyzed with FlowJo or FACSDiva^{TM} software (BD Biosciences).

3.8 CD8$^+$ T cell Isolation and Cytotoxicity Assay

Spleens were collected from WNV-infected (100 PFU, i.p.) mice at 10 d.p.i.. Splenic CD8$^+$ T cells were isolated by negative antibody selection with magnetic beads using the mouse CD8$^+$ T Lymphocyte Enrichment Set-DM (BD Biosciences). Purity of CD8$^+$ T cells was examined by flow cytometry after staining with fluorescent labeled anti-CD3 and anti-CD8 antibodies (eBioscience). Cytotoxicity of CD8$^+$ T cells was measured as described previously (Shrestha and Diamond, 2004), with some modifications. Briefly, purified CD8$^+$ T cells (~ 80-90% purity) were co-cultured for 4 h in 96-well plates with target cells expressing the ectodomain of *WNV-E* (MC57GL$_{WNV-E}$) or control cells containing the expression vector (MC57GL$_{vector}$) (gifted by Dr. Michael S. Diamond) with effector to target cell ratios of 50:1. CD8$^+$ T cell cytotoxicity was

measured using a LDH Cytotoxicity Detection Kit (Thermo Scientific). Transcripts of *perforin-1*, *granzyme A*, *granzyme B* and *Fas-ligand* (*FasL*) genes in purified $CD8^+$ T and $CD8^-$ cells were measured by qPCR, as described above.

3.9 *Ex Vivo* and *In Vivo* IL-17A Treatment Assays

For *ex vivo* studies, splenocytes were isolated from WNV-infected (100 PFU, i.p.) mice at 8 d.p.i. and splenic $CD8^+$ T cells were purified as described above. In some experiments, $CD4^+$ T cells were depleted from splenocytes using CD4 Magnetic Particles - DM (BD Biosciences). Splenocytes, purified $CD8^+$ T cells, or $CD4^-$ splenocytes were cultured for 24 h in the presence of mouse recombinant IL-17A (50 ng/ml, eBioscience). In some experiments, splenocytes were cultured for 24 h in the presence of mouse recombinant IL-17A (50 ng/ml) and subjected to $CD8^+$ T cell purification. Expression of *perforin-1*, *granzyme A*, *granzyme B* and *FasL* in splenocytes, $CD8^+$ T cells, and splenic $CD8^-$ cells were measured by qPCR, as described above.

For *in vivo* IL-17A treatment and survival studies, WT female mice (8-week old) were inoculated with WNV (100 PFU) via i.p. route. At day 6 p.i, mice were treated (2.5 μg/mouse) with i.p. administration of carrier-free mouse recombinant IL-17A (eBioscience) or PBS, and monitored daily for mortality and morbidity for up to 21 days. Randomly selected mice were euthanized at day 8 p.i. and WNV burden in brain and the expression of the cytotoxic mediators in splenic $CD8^+$ and $CD8^-$ T cells were measured by qPCR as described above.

3.10 Statistical Analyses

Data were analyzed using two-tailed student's *t*-test or analysis of variance (ANOVA) in GraphPad Prism (GraphPad Software, version 6), with $p < 0.05$ considered statistically significant.

CHAPTER IV – RESULTS

4.1 WNV Induces Expression of *Il17a* and *Il17ra* in Humans

We previously reported that WNV induces IL-23 production in mice in a TLR7-dependent manner (Town et al., 2009). Considering the role of IL-23 in Th17 cell stabilization and IL-17A production (Aggarwal et al., 2003), we hypothesized that IL-17A may play a role in WNV infection. To test this, we measured the expression of *Il17a* in human cells infected with WNV *in vitro*. Human peripheral blood mononuclear cells (hPBMCs) isolated from healthy human volunteers without a history of WNV infection were infected with WNV (MOI = 0.1 and 5) for 24 h and 48 h *in vitro*. WNV-infected hPBMCs were collected, total RNA isolated, and cDNA synthesis and quantitative real-time PCR (qPCR) was performed to measure transcripts of *Il17a* and cellular *β-actin* as a housekeeping gene. The qPCR results showed that *Il17a* gene expression was up-regulated in WNV-infected hPBMCs (Fig. 4.1A), which was further confirmed by measuring IL-17A production in hPBMC culture supernatants (Fig. 4.1B) by an enzyme-linked immunosorbent assay (ELISA).

To relate these *in vitro* results to WNV infection in humans, we used ELISA to measure the production of IL-17A in the sera of human cases with active WNV infection (fever or neuroinvasive disease), with a history of recovered neuroinvasive WNV diseases, and healthy controls who have no history of WNV infection. The cases with active and long-standing history of neuroinvasive WNV diseases showed a trend of higher levels of IL-17A in sera compared to WNV fever cases and healthy controls (Fig. 4.1C), with no difference between the latter

two. These results demonstrate that WNV infection induces a production of IL-17A in humans and suggest that this cytokine may play a role in WNV infection.

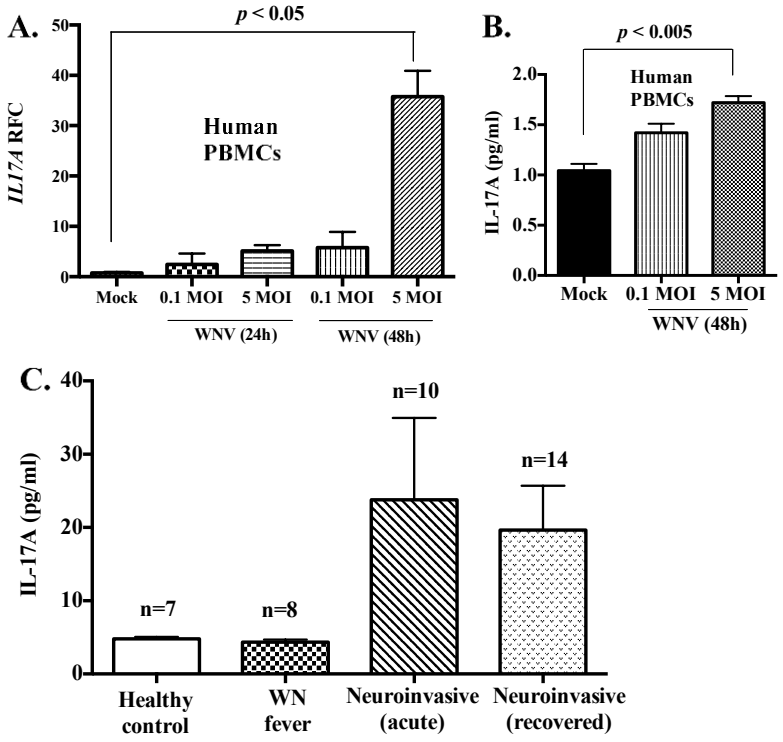

Figure 4.1 WNV induces expression of *Il17a* and *Il17ra* in humans

(A) *Il17a* transcripts were measured by qPCR and expressed as relative fold change (RFC) after normalization to cellular *β -actin* in human PBMCs infected with WNV for 24 h or 48 h. (B) IL-17A production in culture supernatant of WNV-infected hPBMC was measured by ELISA. (C) Levels of IL-17A in sera of human WNV patients and healthy controls were measured by ELISA. Error bars represent standard error of mean (mean ± SEM). Data in A and B represent two independent experiments performed in triplicates and analyzed by a one-way ANOVA. "*ns*" denotes no significant difference ($p > 0.05$).

4.2 WNV Induces Expression of *Il17a* and *Il17ra* in Mice

To expand upon the findings from human samples, we used a mouse model of WNV infection because it reflects various aspects of human WNV disease (Bai et al., 2009; Bai et al., 2005; Town et al., 2009). Splenocytes isolated from C57BL/6J mice were infected with WNV (MOI = 0.1) *in vitro* for 24 h

and 48 h, and the expression of *Il17a* gene was measured by qPCR. Similar to hPBMC, *Il17a* transcript levels were up-regulated at both 24 and 48 h.p.i. in mouse splenocytes infected with WNV *in vitro* (Fig. 4.2A).

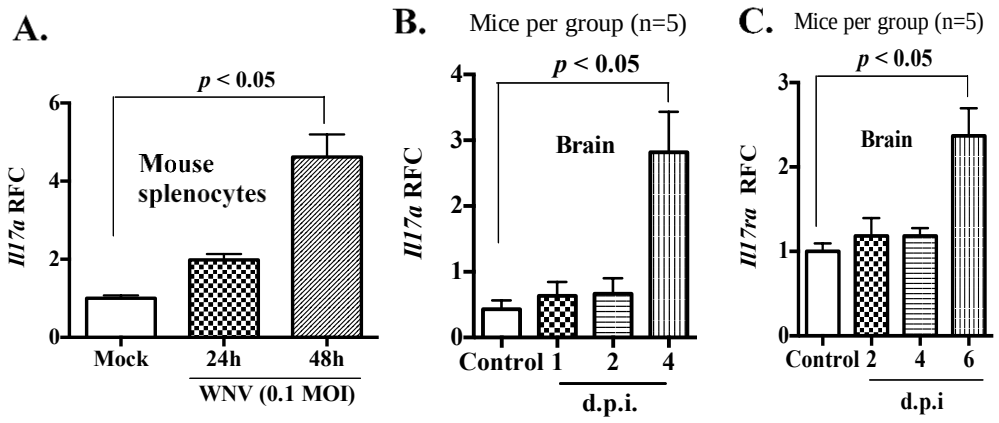

Figure 4.2 WNV induces expression of *Il17a* and *Il17ra* in mice

(A) RFC of *Il17a* transcripts after normalization to cellular *β -actin* in mouse splenocytes infected with WNV (MOI = 0.1) *in vitro*. (B to C) WT (C57BL/6) mice were infected with WNV (1,000 PFU, i.p.) and expression of *Il17a* (B) and *Il17ra* (C) transcripts were measured in brain tissue by qPCR. Error bars represent standard error of mean (mean ± SEM). Data in A represent two independent experiments performed in triplicates and analyzed by a one-way ANOVA. Data in B and C represent two independent experiments (n = 5 mice/group) and analyzed by two-tailed student's t-test; "*ns*" denotes no significant difference ($p > 0.05$).

Since astrocytes, microglia, and brain infiltrating immune cells express functional interleukin-17 receptor A (IL-17RA) in brain inflammatory conditions (Das Sarma et al., 2009), we measured the expression of *Il17a* and *Il17ra* genes in brain of WNV-infected mice. For this, we infected a group of WT mice with WNV (1,000 PFU, i.p.), sacrificed them at various time points to collect brains, and measured levels of *Il17a* and *Il17ra* transcripts by qPCR. Indeed, there was a significantly up-regulated expression of both *Il17a* (Fig. 4.2B) and *Il17ra* (Fig. 4.2C) genes in brain of WNV-infected mice compared to uninfected controls.

Collectively, these results indicate that WNV infection elevates the expression of both *Il17a* and *Il17ra* in mice, suggesting a possible role of IL-17A in WNV infection.

4.3 WNV-mediated IL-17A Production Depends on IL-23 Signaling

To further measure *Il17a* expression in mice and to test whether its production was IL-23 dependent, we intraperitoneally (i.p.) infected a group of wild-type (WT) littermates and IL-23 deficient (*Il23p19*$^{-/-}$) mice (both are in mixed C57BL/6×129 background) with 1,000 plaque-forming units (PFUs) of WNV and measured IL-17A protein in plasma by ELISA. The results showed that WNV infection in mice induced IL-17A production (Fig. 4.3), but the level of this cytokine was undetectable in serum samples from mock-infected control mice (data not shown).

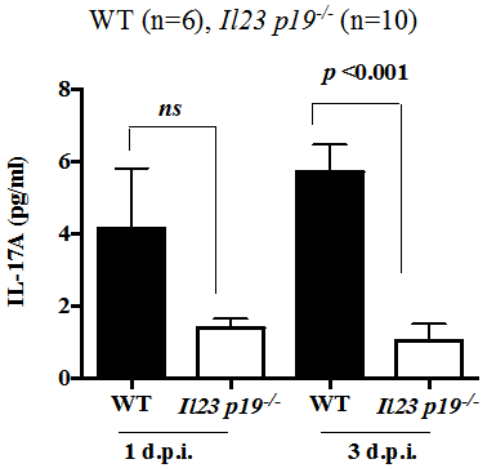

Figure 4.3 WNV-mediate IL-17A response in a IL-23 dependent manner

IL-17A production was measured in plasma of *Il23p19*$^{-/-}$ and their littermate WT control mice (7-9 week old) infected with WNV (1,000 PFU, i.p.) by ELISA. Error bars represent standard error of mean (mean ± SEM). Data represent two independent experiments (n = 3 to 5 mice/group) and analyzed by two-tailed student's t-test; "*ns*" denotes no significant difference ($p > 0.05$).

Moreover, there was approximately 80% reduction in *Il17a* expression in *Il23p19*$^{-/-}$ mice at 3 days post infection (d.p.i), suggesting that IL-17A production during WNV infection in mice largely depends on IL-23 signaling (Fig. 4.3).

4.4 IL-17A Protects Mice from Lethal WNV Infection

To further investigate the role of IL-17A in WNV immunity, we used a mouse model of WNV encephalitis (Town et al., 2009). IL-17A deficient (*Il17a*$^{-/-}$) and WT control mice (7-8 weeks old, sex-matched, strain C57BL/6J) were challenged *via* i.p. injection with 1,000 PFUs of WNV (Town et al., 2009), a dose that kills approximately 40 ~ 50% of WT animals. Morbidity and mortality was monitored twice daily for 21 days. We found strikingly greater susceptibility of *Il17a*$^{-/-}$ mice (20% survival) vs. WT control mice (60% survival) to lethal WNV infection (Fig. 4.4A).

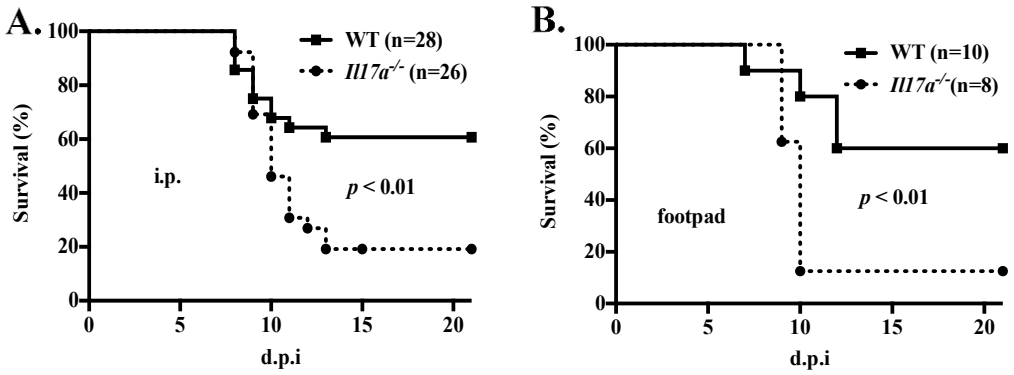

Figure 4.4 *Il17a*$^{-/-}$ mice are more susceptible to WNV infection

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ mice were infected with WNV via i.p. (1,000 PFU) or footpad (100 PFU) routes and monitored for mortality twice daily for 21 days; percentage (%) survival was compared using the Kaplan-Meier survival and log-rank test. (A) Survival curves after i.p inoculation, or (B) footpad inoculation.

To exclude the possibility of an inoculation route specific response, we also challenged *Il17a*$^{-/-}$ and WT mice with 100 PFUs of WNV *via* footpad (Bai et al., 2009), and performed survival analysis. Similar to the i.p. route, footpad inoculation showed that *Il17a*$^{-/-}$ mice were more susceptible to WNV infection (Fig. 4.4B). Together, these data suggest that IL-17A protects mice from lethal WNV infection.

4.5 Mice Deficient in IL-17A Develop Higher Viral Burden

To further expand the protective function of IL-17A in controlling WNV infection, we compared the virological profiles of WNV-infected *Il17a*$^{-/-}$ vs. WT mice in various tissues such as blood, spleen, liver and brain. Measurement of WNV viremia by qPCR revealed no difference at 2 d.p.i, however, a 3-fold increase in the transcript level of WNV envelope gene (*WNVE*) was observed at 4 d.p.i. in WNV-infected *Il17a*$^{-/-}$ mice compared to WT controls (Fig. 4.5A).

To assess the viral burden in peripheral organs, we sacrificed WNV-infected mice, collected liver, spleen and brain samples at the selected time points, and performed a qPCR analysis. When compared to WT controls, we found approximately 2-fold increase in *WNV-E* transcripts in the liver of *Il17a*$^{-/-}$ mice (Fig. 4.5B). Consistent with the survival results, there were about 6-fold (at 7 d.p.i.) and 30-fold (at 8 d.p.i.) increases in *WNVE* transcripts in the brain of WNV-infected *Il17a*$^{-/-}$ mice compared to WT controls (Fig. 4.5C). Although there was no difference in viral burden in spleens of WT vs. *Il17a*$^{-/-}$ mice at 4 d.p.i, *Il17a*$^{-/-}$ mice had significantly higher level (about 3-fold) of *WNVE* transcripts on 8 d.p.i (Fig. 4.5D).

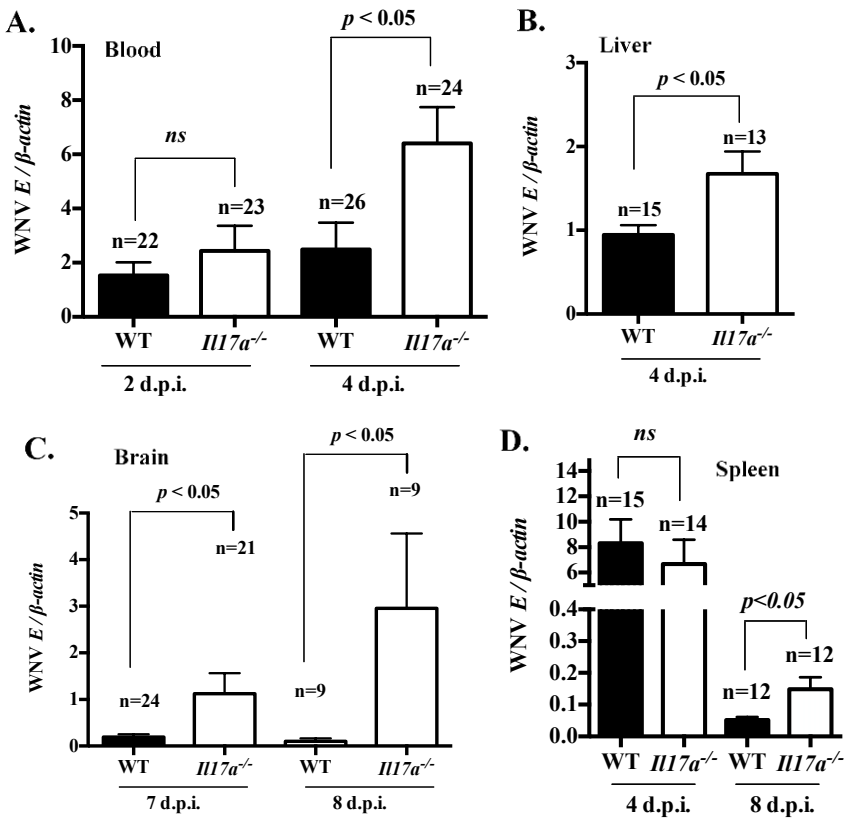

Figure 4.5 IL-17A deficiency results in deficient WNV clearance in mice

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ mice were infected with WNV via i.p. (1,000 PFU). qPCR was performed to measure *WNV-E* RNA in blood (A), liver (B), brain (C), and spleen (D) with viral burden expressed as the ratio of *WNV-E* RNA copies to cellular *β-actin* transcripts. The ratios of viral load between WT and *Il17a*$^{-/-}$ mice (mean ± SEM) were compared by two-tailed student's t-test; "*ns*" denotes no significant difference ($p > 0.05$).

These data demonstrate that mice deficient in IL-17A develop higher viral burden in blood and liver at day 4, and have deficient clearance of WNV from brain and spleen at day 8 p.i., leading to greater WNV susceptibility. Collectively, these results indicate that IL-17A plays a protective role during WNV infection.

4.6 CNS Leukocyte Slightly Elevates in WNV-infected *Il17a*$^{-/-}$ mice

IL-17A has been shown to recruit leukocytes including neutrophils during microbial infections (Happel et al., 2005; Ye et al., 2001), and inflammatory conditions (Griffin et al., 2012). Since we observed a higher WNV burden in brain

of *Il17a*$^{-/-}$ mice, we asked if this was related to IL-17A-mediated control of leukocyte infiltration into brain during WNV infection. For this, we performed confocal microscopy to detect WNV-E antigen, CD45 (pan-leukocyte marker), and CD11b (microglial and macrophage marker) in brain sections of WNV-infected *Il17a*$^{-/-}$ and WT mice sacrificed at 6 d.p.i. We focused on the olfactory bulb because we have shown that this brain region is most sensitive to WNV infection (Bai et al., 2009; Town et al., 2009; Wang et al., 2004a). Consistent with the qPCR measurement of *WNVE* RNA in brain tissue (Fig. 4.5C), the confocal imaging revealed more WNVE antigens in the brains of WNV-infected *Il17a*$^{-/-}$ mice compared to WT controls (Fig. 4.6A and B). Similar results were also obtained in other brain regions, including the cerebral cortex, brainstem, cerebellum, and striatum (data not shown). Unexpectedly, confocal imaging results showed more CD45^{+} (Fig. 4.6A) and CD11b^{+} leukocytes (Fig. 4.6B) in the brain of WNV-infected *Il17a*$^{-/-}$ mice than WT controls.

To confirm these data and further quantify brain infiltrating immune cells, we performed flow cytometric analysis of brain leukocytes isolated from WT and *Il17a*$^{-/-}$ mice infected with 1,000 PFU (i.p.) of WNV for 6 days. We characterized CD45^{+}, CD11b^{+}, CD3^{+}CD4^{+} and CD3^{+}CD8^{+} cell populations, as previously described (Town et al., 2009). Consistent with the confocal imaging results, we observed a trend towards elevation of all leukocyte population in the brain of WNV-infected *Il17a*$^{-/-}$ mice compared to WT controls (Fig. 4.6C).

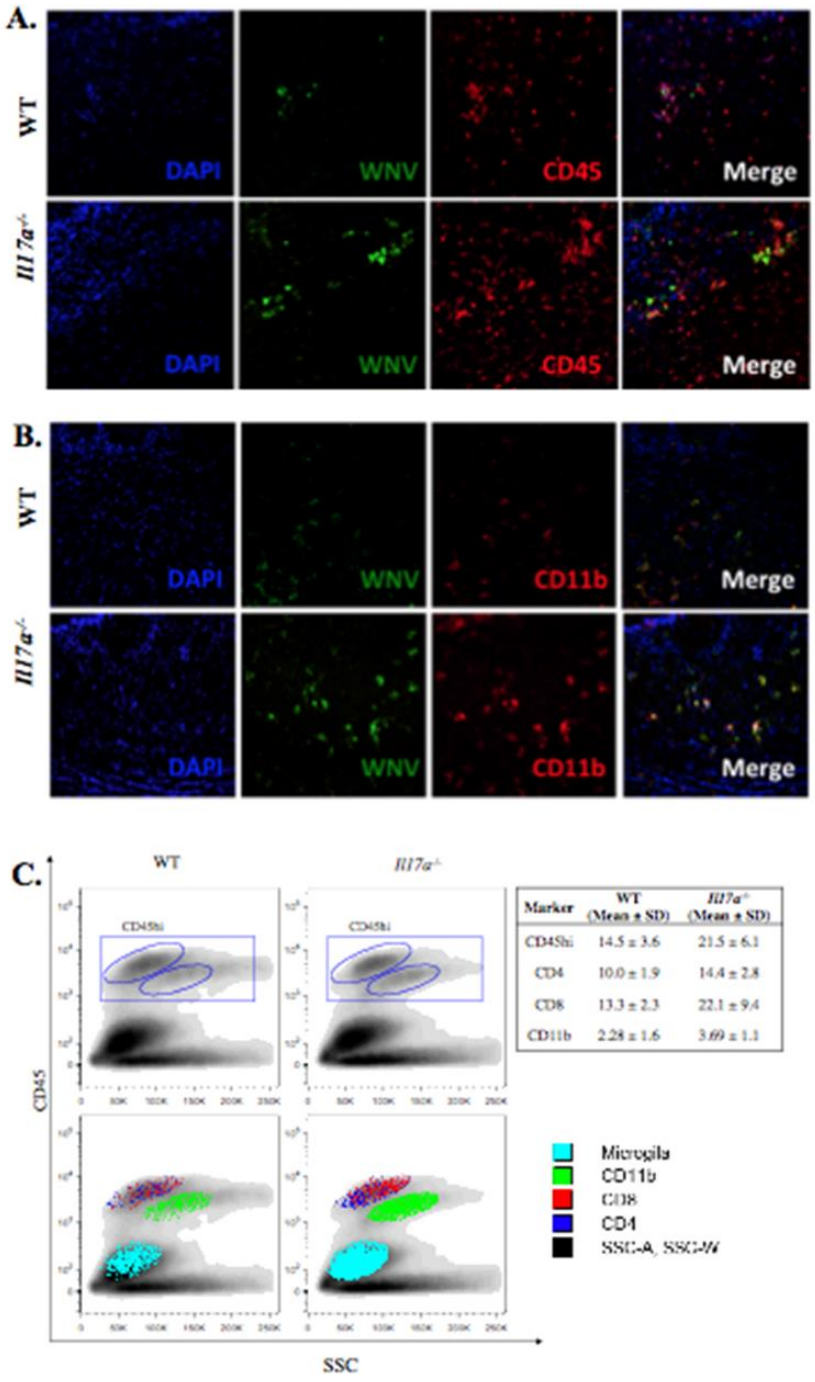

Figure 4.6 CNS leukocyte profile in WNV-infected *Il17a*$^{-/-}$ mice

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ mice were challenged with WNV (1,000 PFU) through i.p.. (A to B) PBS perfused brains were isolated on 6 d.p.i, and WNV antigen (green signal) and CD45 (leukocyte common antigen, red, panel A) or CD11b (macrophage and microglia marker, red, panel B) were

detected by Nikon A1R confocal microscope (original magnification = 20X). DAPI (blue signal) was used as a nuclear counterstain; representative images are shown. (C) Brain leukocytes isolated on day 6 d.p.i. were characterized by flow cytometry after probing with antibodies against WNVE, CD45, CD4, CD8, and CD11b. Signal color used in dot plots and numbers representing the percentages of positive cells within gated populations are shown (right). Data represent two independent experiments (n = 5 mice per group for each experiment).

To test whether the higher leukocyte infiltration into WNV-infected *Il17a*$^{-/-}$ mouse brains was affected by leukocyte expansion or differentiation in the periphery, we compared leukocyte populations in spleen of *Il17a*$^{-/-}$ and WT mice infected with WNV. There was no difference in leukocyte populations in spleens of WNV-infected *Il17a*$^{-/-}$ vs. WT mice (data not shown), suggesting that more leukocytes in brain may not be due to the possible effects of IL-17A on leukocyte expansion in the periphery.

4.7 WNV-infected *Il17a*$^{-/-}$ Mice Upregulate CCL5

To further dissect the mechanism by which more leukocytes migrate into the brains of WNV infected *Il17a*$^{-/-}$ mice, we performed qPCR to measure the expression of selected chemokines genes (*Cxcl1*, *Cxcl10*, *Ccl5*) known to mediate recruitment of leukocytes. There was significantly elevated expression of *Ccl5* (also known as RANTES) (Fig. 4.7A) and its receptor *Ccr5* (Fig. 4.7B) in blood of WNV infected *Il17a*$^{-/-}$ mice at 4 d.p.i., but no difference in expression of other chemokines, such as *Cxcl1* and *Cxcl10* (data not shown). In addition, there was a significant increase of *Ccl5* expression (Fig. 4.7C) in the brains of WNV-infected *Il17a*$^{-/-}$ mice at 8 d.p.i., but no difference in expression of other leukocyte recruiting chemokines or chemokine receptors, such as *Cxcl10* (Fig. 4.7D) and

Cxcr3 (Fig. 4.7E). These results may imply a link between deficient IL-17A and higher *Ccl5* expression that may contribute to more leukocyte homing to the brain of *Il17a*$^{-/-}$ mice during the course of WNV infection.

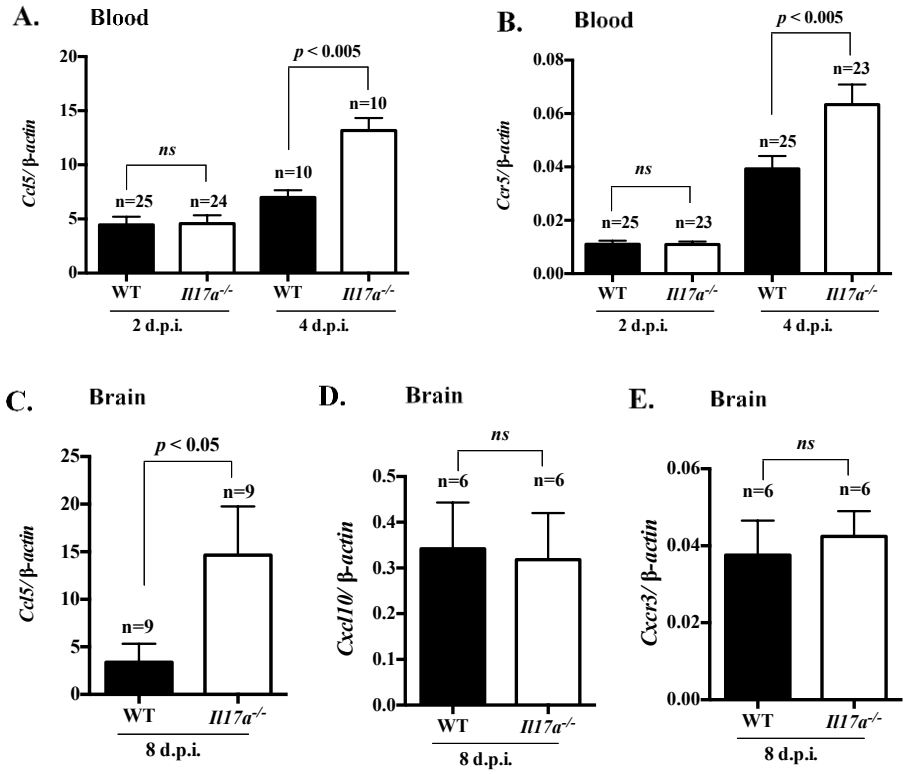

Figure 4.7 WNV infected Il17a$^{-/-}$ mice upregulate CCL5

Expressions of *Ccl5* (A) and *Ccr5* (B) gene at the mRNA levels were measured by qPCR in the blood of WNV-infected mice at 2 and 4 d.p.i.. mRNA levels of *Ccl5* (C), *Cxcl10* (D) and *Cxcr3* (E) genes were measured by qPCR in brain of WNV-infected mice at 8 d.p.i. Gene expression data were normalized to cellular *β-actin* mRNA and compared by two-tailed student's t-test; "*ns*" denotes no significant difference ($p > 0.05$).

4.8 IL-17A Does Not Affect Interferon Response and Inflammation

WNV infection induces potent type I IFN responses in mice, which plays a critical role in controlling both viremia and encephalitis (Samuel and Diamond, 2005). We tested whether deficient IL-17A alters type I IFN expression during

WNV infection by qPCR and ELISA. The qPCR results showed no difference in *Ifn-*α expression in blood of *Il17a*$^{-/-}$ *vs.* WT control mice at 4 d.p.i. (Fig. 4.8A). Similarly, no difference in expression of *Ifn-*β gene was observed in blood (Fig. 4.8B), spleen (Fig. 4.8C), liver (Fig. 4.8D), and brain (Fig. 4.8E) samples from WNV-infected *Il17a*$^{-/-}$ *vs*. WT control mice measured at various time points. To further confirm these results, we also measured IFN-β protein in plasma of WNV-infected WT and *Il17a*$^{-/-}$ mice at 3 d.p.i. by ELISA and found no difference in IFN-β expression (Fig. 4.8F). These results suggest that the type I IFNs response remains unaltered in *Il17a*$^{-/-}$ mice during WNV infection.

IL-17A cause potent inflammation and regulate expression of several cytokines including IL-1β, IFNγ and TNFα (Maione et al., 2009; Song and Qian, 2013). We assessed the possible role of IL-17A in inflammatory responses during WNV infection by measuring inflammatory cytokine expression in plasma by ELISA. Again, there was no difference in levels of IL-1β, IL-6, IL-10, IFN-γ, IL-12 p40, and TNF-α in plasma from WNV-infected *Il17a*$^{-/-}$ vs. WT control mice at both 1 and 3 d.p.i. (Fig. 4.8G-L). In addition, no significant difference in expression of these cytokines was detected by qPCR in brain of WNV-infected *Il17a*$^{-/-}$ vs. WT control mice at 8 d.p.i. (data not shown). Collectively, these results demonstrate that higher viral load in *Il17a*$^{-/-}$ mice is likely not due to altered production of interferon and other inflammatory cytokines.

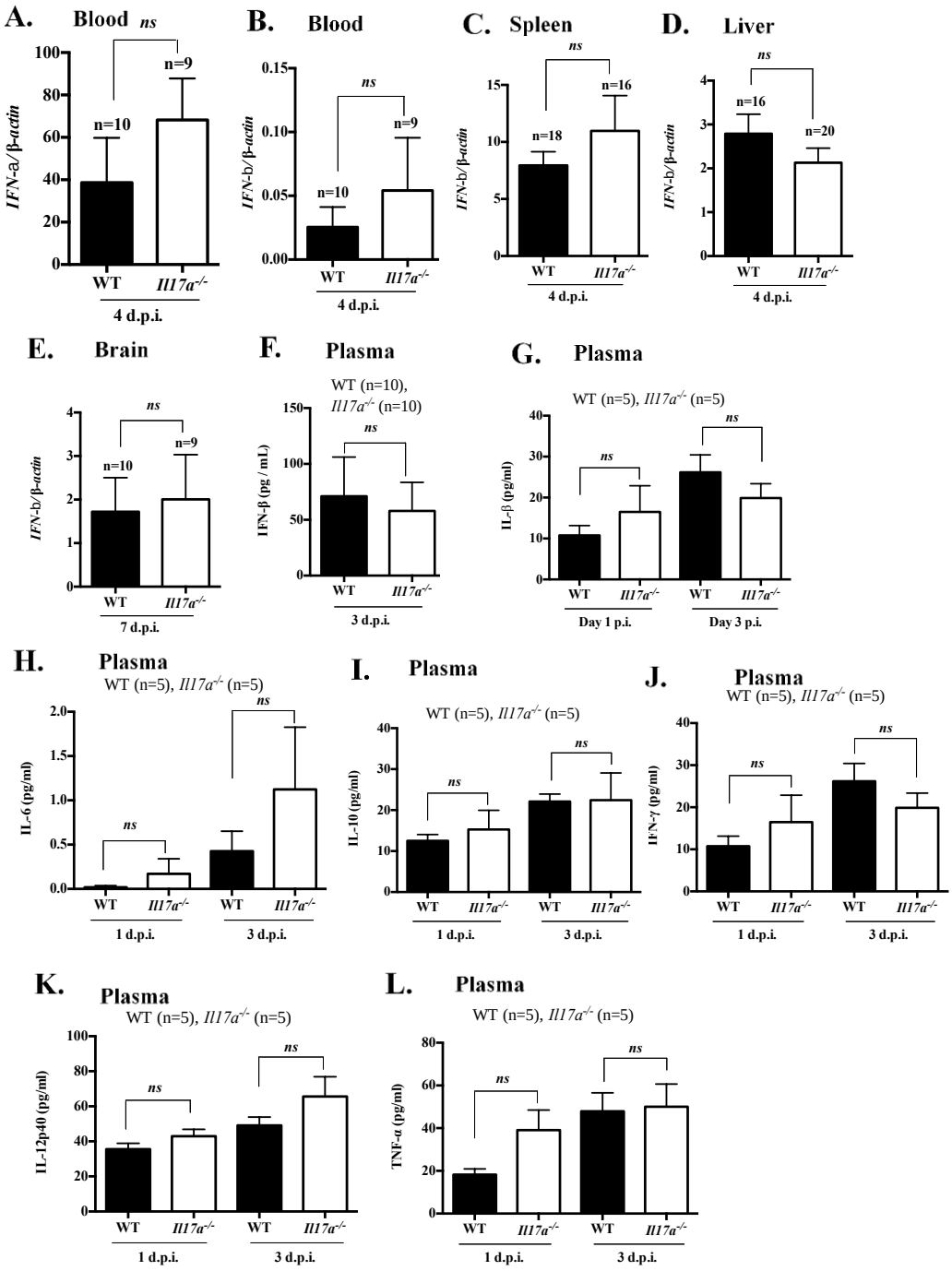

Figure 4.8 Antiviral and inflammatory responses of WNV-infected *Il17a*$^{-/-}$ mice

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ were infected with 1,000 PFU (i.p.) of WNV. Blood, plasma, and tissue samples were collected at the selected time points (d.p.i.) for cytokine and anti-WNV-E IgM measurement. Expression of interferon-□ (*Ifn-□)* gene transcripts in blood (A), and expression of interferon- *β* (*Ifn- β)* gene transcripts in blood (B), spleen (C), liver (D), and brain (E) were measured by qPCR (normalized to cellular *β -actin* mRNA). (F to L)

Protein level of IFN-β IL-1β IL-6, IL-10, IFN- γ, IL-12p40, and TNF-α in plasma were measured by ELISA. Data (mean ± SEM) represent at least two independent experiments performed in triplicates and analyzed by two-tailed student's t-test; "*ns*" denotes no significant difference ($p > 0.05$).

4.9 IL-17A Does Not Have a Direct Anti-WNV Effect

We next asked if IL-17A has a direct antiviral activity against WNV infection, which has been shown for some other cytokines, such as TNF-α (Ruggiero et al., 1989; Shrestha et al., 2008b) and IL-6 (Moore et al., 2012). However, no effect in replication of WNV was observed in Raw 264.7 (mouse macrophage, Fig. 4.9A) and Neuro 2a (mouse neuroblast, Fig. 4.9B) cells that were pretreated with mouse recombinant IL-17A (1 to 100 ng/ml), suggesting that IL-17A may not have a direct antiviral effect against WNV replication.

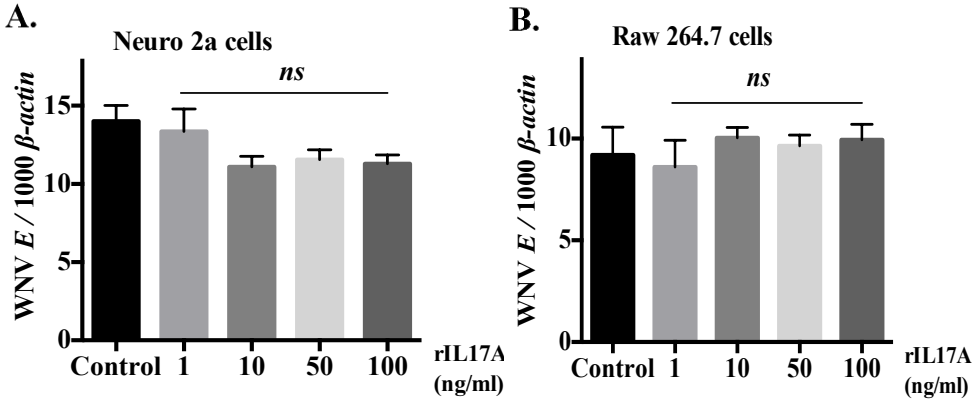

Figure 4.9 IL-17A does not directly control WNV replication

Replication of WNV was analyzed by qPCR in Raw 264.7 (A) and Neuro 2a (B) cells that were pretreated with mouse recombinant IL-17A (1 to 100 ng/ml) for 6 h followed by infection with WNV (1 MOI) for 24 h. Data (mean ± SEM) represent at least two independent experiments performed in triplicates and analyzed by one-way ANOVA; "*ns*" denotes no significant difference ($p > 0.05$).

4.10 IL-17A Does Not Control Humoral Immune Response

Besides type I IFN, humoral immune response also plays an important role in clearance of WNV from the blood and peripheral organs and limits viral

dissemination to the CNS (Diamond et al., 2003a). Although the role of IL-17A in humoral immunity is not well understood, it has been shown that B cells express IL-17RA (Yao et al., 1995), whereas Th17 cells (major IL-17A producers) promote B cells to produce antibodies (Yuan et al., 2010). To test the possible effect of IL-17A in humoral immune responses during WNV infection, we compared WNV-E specific IgM antibody production in WNV-infected *Il17a*$^{-/-}$ and WT mice by ELISA. Both *Il17a*$^{-/-}$ and WT mice produced similar levels of anti-WNV-E IgM when measured at 2, 3, and 5 d.p.i. (Fig. 4.10). These results demonstrate that higher viral load in *Il17a*$^{-/-}$ mice is likely, not due to altered antibody responses.

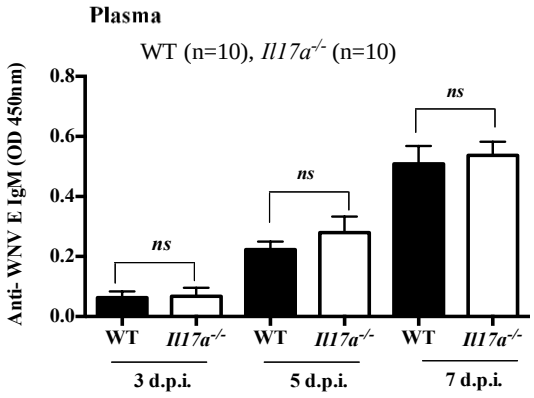

Figure 4.10 WNV-mediated IL-17A does not affect antibody production

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ were infected with 1,000 PFU (i.p.) of WNV. Anti-WNV-E IgM antibody levels in plasma were measured by ELISA. Data (mean ± SEM) represent at least two independent experiments performed in triplicates and analyzed by two-tailed student's t-test; "*ns*" denotes no significant difference ($p > 0.05$).

4.11 IL-17A Deficiency Causes the Reduced CD8$^+$ T Cell Cytotoxicity

Brain-infiltrating leukocytes play a vital role in clearing WNV from the CNS during WNV infection (Shrestha and Diamond, 2004; Sitati and Diamond, 2006; Town et al., 2009). In particular, CD8$^+$ T cells are crucial for clearance of WNV

from the CNS and spleens (Shrestha and Diamond, 2004, 2007; Shrestha et al., 2006a; Wang et al., 2004b). Despite a modest elevation trend of brain infiltrating $CD8^+$ T cells in *Il17a*$^{-/-}$ mice, viral burden in the brains of these mice was higher than in WT controls (Fig. 4.5C). In addition, *Il17a*$^{-/-}$ mice were also deficient in clearing WNV from spleen (Fig. 4.5D). Therefore, we hypothesized that $CD8^+$ T cells in *Il17a*$^{-/-}$ mice may be functionally defective in their ability to clear WNV-infected target cells. To test this, we infected WT and *Il17a*$^{-/-}$ mice through i.p. with a sub-lethal dose of WNV (100 PFU) to prolong the course of WNV infection. This is important because $CD8^+$ T cells play a major role in clearing WNV-infected cells during the later phase (day 8 to 12) of infection (Shrestha and Diamond, 2004; Wang et al., 2004b), and most of *Il17a*$^{-/-}$ mice infected with a higher dose (e.g. 1,000 PFU or more) develop severe diseases and die during this time period. At 10 d.p.i., mice were sacrificed and splenic $CD8^+$ T cells were purified using a negative antibody selection method. The purified effector $CD8^+$ T cells were then co-cultured with the target cells ($MC57GL_{WNV\text{-}E}$) or control cells ($MC57GL_{vector}$) for 4 h. The target cells express the ectodomain of the *WNVE* in a pcDNA3.1 vector, while the control cells only express the parent vector (Shrestha and Diamond, 2004). The cytotoxicity of effector $CD8^+$ T cells to WNV specific target cells was assessed by measuring the quantity of intracellular lactate dehydrogenase released into culture supernatants from the lysed target cells. Strikingly, the cytotoxicity assay showed about 2-fold reduction in cytotoxicity of $CD8^+$ T cells isolated from WNV-infected *Il17a*$^{-/-}$ mice in comparison to WNV-infected WT mice (Fig. 4.11A). These results demonstrate that $CD8^+$ T cells from

Il17a$^{-/-}$ mice failed to mount an effective target cell-specific cytotoxic response during WNV infection.

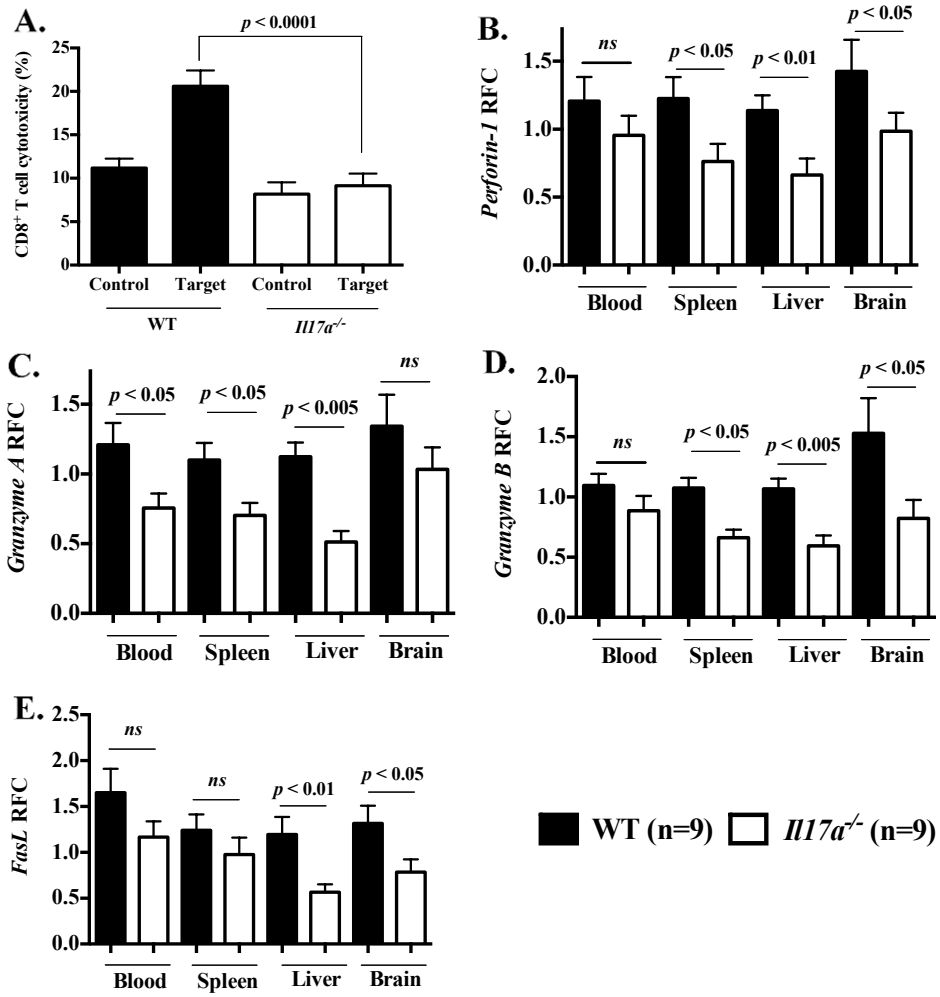

Figure 4.11 CD8$^+$ T cells from *Il17a*$^{-/-}$ mice have reduced cytotoxicity

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ mice were infected with WNV at 100 PFU (i.p.) for 10 days. (A) Purified splenic CD8$^+$ T cells were co-cultured with the target (MC57GL$_{WNV-E}$) or control (MC57GL$_{vector}$) cells at a 50:1 effector/target ratio for 4 h and cytotoxicity was assayed by measuring the release of intracellular lactate dehydrogenase in culture supernatants. (B to E) RFC in transcripts of *perforin-1* (B), *granzyme A* (C), *granzyme B* (D), and *FasL* (E) in indicated tissues of WT or *Il17a*$^{-/-}$ mice were measured by qPCR (normalized to cellular *β -actin* mRNA). Data (mean ± SEM) represent three independent experiments (n = 3 mice/group). "*ns*" denotes no significant difference ($p > 0.05$).

Cytotoxicity of CD8$^+$ T cells employ granule (e.g., perforin and granzyme) exocytosis and Fas-Fas ligand (FasL) dependent mechanisms to kill target cells

(Shresta et al., 1998). To test whether IL-17A regulates the expression of cytotoxicity mediator genes, we performed qPCR assay in blood, spleen, liver, and brain of WT and *Il17a*$^{-/-}$ mice that were infected with WNV (100 PFU) for 10 days. Interestingly, the expression levels of *perforin-1* (Fig. 4.11B), *granzyme A* (Fig. 4.11C), *granzyme B* (Fig. 4.11D) and *FasL* (Fig. 4.11E) were significantly lower in most of these tissues collected from *Il17a*$^{-/-}$ mice compared to WT controls.

4.12 *Il17a*$^{-/-}$ CD8^{+} T Cells Express Less Cytotoxicity Mediators

Although CD8^{+} cells are the major cells that express these cytotoxic mediators, other cells, such as NK cells, may also express these genes and contribute to cytotoxic effector function by the mechanism similar to CD8^{+} T cells. To specifically test if the attenuated cytotoxicity of CD8^{+} T cells in *Il17a*$^{-/-}$ mice was due to the lower expression of these cytotoxic mediator genes, we performed qPCR to measure the expression of *perforin-1*, *granzyme A*, *granzyme B* and *FasL* in CD8^{+} T cells purified from spleens of WNV-infected WT and *Il17a*$^{-/-}$ mice at 10 d.p.i.. Consistent with the cytotoxicity assay results, the qPCR showed that CD8^{+} T cells isolated from the *Il17a*$^{-/-}$ mice have significantly reduced expression of *perforin-1* (Fig. 4.12A), *granzyme A* (Fig. 4.12B), *granzyme B* (Fig. 4.12C) and *FasL* (Fig. 4.12D) compared to WT controls.

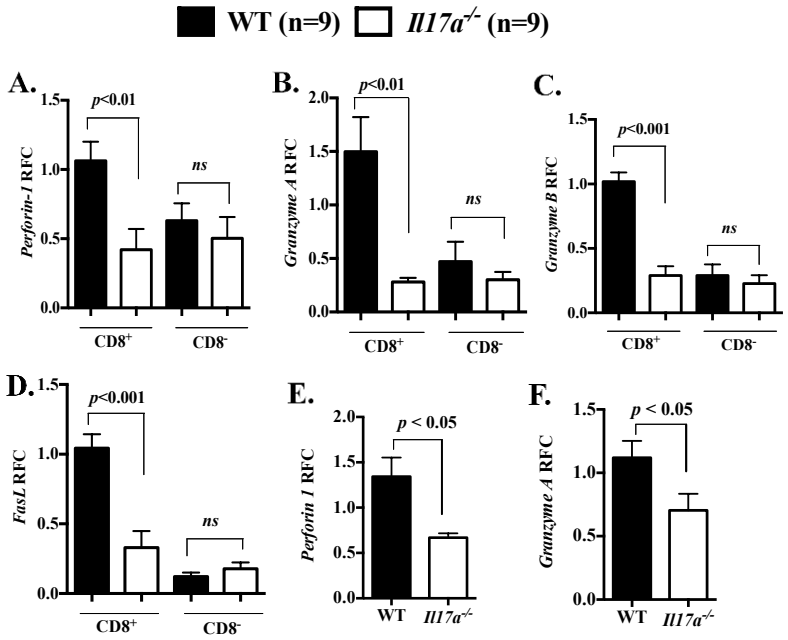

Figure 4.12 *Il17a*$^{-/-}$ CD8^{+} T cells express reduced cytotoxicity mediators

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ mice were infected with WNV at 100 PFU (i.p.) for 10 days and euthanized to obtain splenic CD8^{+} T cells. (A to D) RFC in expression of transcripts of *perforin-1* (A), *granzyme A* (B), *granzyme B* (C), and *FasL* (D) in splenic CD8^{+} T cells or CD8^{-} cells from WT or *Il17a*$^{-/-}$ mice were measured by qPCR (normalized to cellular *β -actin* mRNA). (E to F) RFC in the expressions of *perforin-1* (E), and *granzyme A* (F) in the spleens of CHIKV-infected (10^{5} PFU, i.p.) WT and *Il17a*$^{-/-}$ mice were measured on 12 d.p.i. by qPCR. Data (mean ± SEM) shown in A to D represent three independent experiments (n = 3 mice/group); and data in E to F represent two independent experiments (n = 3 mice/group). "*ns*" denotes no significant difference ($p > 0.05$).

To test if these cytotoxicity mediators were also less expressed in other immune cells in *Il17a*$^{-/-}$ mice, we performed qPCR assay on CD8^{-} splenocytes (mixed immune cells including NK cells) isolated from WNV-infected WT and *Il17a*$^{-/-}$ mice at 10 d.p.i. In contrast to the CD8^{+} cells, we found no difference in the expression of *perforin-1*, *granzyme A*, *granzyme B* and *FasL* genes in CD8^{-} splenocytes from *Il17a*$^{-/-}$ mice (Fig. 4.12A-D). Whereas in WNV-infected WT mice, the expression of these cytotoxicity marker genes was significantly higher in CD8^{+} than CD8^{-} cells, no such differences were detected between CD8^{+} and

$CD8^-$ cells isolated from *Il17a*$^{-/-}$ mice (Fig. 4.12A-D). In addition, there was no significant difference in the expression of these cytotoxicity marker genes between uninfected *Il17a*$^{-/-}$ and WT mice (data not shown).

To further test whether the IL-17A-mediated cytotoxicity mediator expression is specific to WNV, or common across viral infections, we measured the expression of cytotoxicity mediator genes in WT and *Il17a*$^{-/-}$ mice infected with chikungunya virus (CHIKV), a single-stranded RNA virus from genus *Alphavirus* of the family *Togaviridae*. Similar to WNV, we found significantly reduced expressions of *perforin*-1 (Fig. 4.12E) and *granzyme A* (Fig. 4.12F) in the splenocytes isolated from CHIKV-infected *Il17a*$^{-/-}$ mice compared to WT controls. Collectively, these results suggest that IL-17A promotes the expression of the cytotoxicity mediators and facilitate $CD8^+$ T cells cytotoxicity during WNV and other viral infections, such as CHIKV.

4.13 IL-17A Induces Cytotoxic Mediators Independent of $CD4^+$ T Cells

The role of IL-17A in regulating the expression of cytotoxic mediators in $CD8^+$ T cells has not been previously reported. To further investigate and confirm this, we isolated splenocytes from WNV-infected WT mice at 8 d.p.i., cultured them with recombinant IL-17A (50 ng/ml) *ex vivo* for 24 h, and measured the expression of *perforin-1*, *granzyme A*, *granzyme B* and *FasL* genes by qPCR assay. The treatment with recombinant IL-17A significantly up-regulated the expression of *perforin-1*, *granzyme A*, and *granzyme B*, but not *FasL* (Fig. 4.13A-D), in the splenocytes isolated from WNV-infected WT mice. Similar results were

obtained when splenocytes were isolated from WNV-infected *Il17a*$^{-/-}$ mice and treated with recombinant IL-17A *ex vivo* (Fig. 4.13A-D).

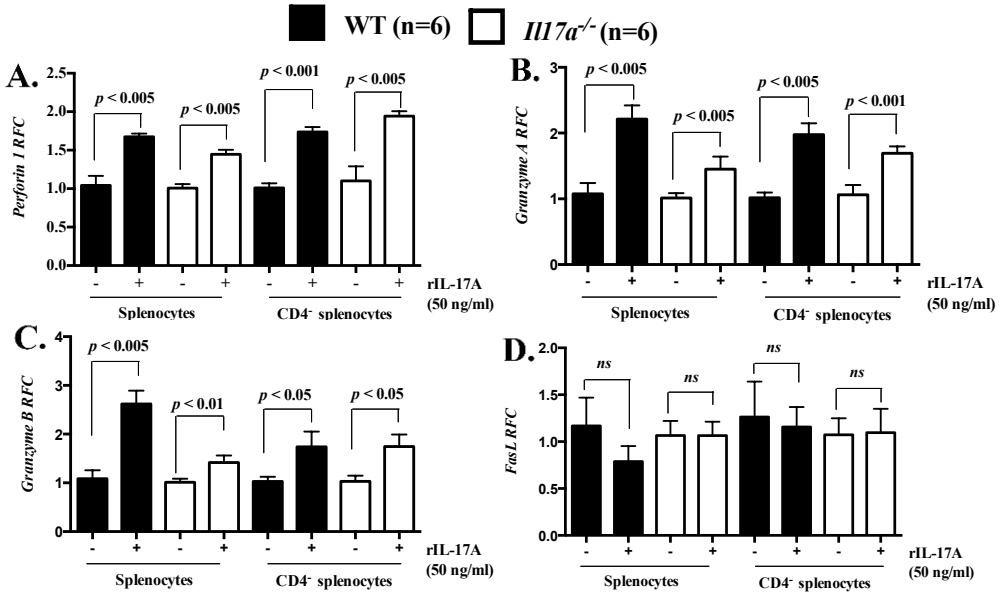

Figure 4.13 IL-17A promotes cytotoxic mediators independently of CD4$^+$ cells

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ mice (n = 6 mice/group) were infected with 100 PFU (i.p.) of WNV and sacrificed on 8 d.p.i. to isolate splenocytes and CD8$^+$ T cells. Splenocytes or CD4$^+$ T cells depleted splenocytes (CD4$^-$ splenocytes) were cultured with or without recombinant mouse IL-17A (50 ng/ml) for 24 h *ex vivo* and RFC of transcripts of *perforin-1* (A), *granzyme A* (B), *granzyme B* (C), and *FasL* (D) were measured by qPCR (normalized to cellular *β -actin* mRNA). All data (mean ± SEM) represent two independent experiments (n = 3 mice/group) and compared by two-tailed student's t-test. All data from WT and *Il17a*$^{-/-}$ mice were normalized to respective mock treated controls.

To assess the possible role of CD4$^+$ T cells in IL-17A-mediated expression of cytotoxic mediators, we depleted CD4$^+$ T cells from the splenoocytes isolated from WNV-infected *Il17a*$^{-/-}$ and WT mice on 8 d.p.i., and cultured CD4$^-$ splenocytes with recombinant IL-17A (50 ng/ml) *ex vivo* for 24 h. The supply of recombinant IL-17A induced expression of the cytotoxic mediators even in the absence of CD4$^+$ T cells (Fig. 4.13A-D), suggesting that IL-17A-mediated

induction of cytotoxic mediators expression in $CD8^+$ T cell is independent of $CD4^+$ T cells.

4.14 IL-17A-induced Cytotoxicity Mediators May Involve ACT-1 Signaling

IL-17A exerts its function through IL-17A receptor (IL-17R), which is a heterodimeric receptor complex of IL-17RA and IL-17RC. Although the signaling pathway downstream of IL-17R has not been completely understood, one common pathway that has been characterized is the activation of the classical NF-κB pathway (Gaffen, 2009; Gu et al., 2013). Upon binding to IL-17A, SEFIR domain of IL-17R recruits the signaling adaptor ACT-1, which further recruits an essential upstream activator of the classical NF-κB pathway called TRAF6 (Gaffen, 2009). To test if IL-17A signaling in WNV-infected cells occurs *via* ACT-1, we measured the expression of *Act-1* in splenocytes and $CD8^+$ T cells isolated from WNV-infected mice and treated *ex vivo* with recombinant IL-17A, as described above. The qPCR results showed that IL-17A treatment up-regulated the expression of *Act-1* gene in splenocytes (Fig. 4.14A) and $CD8^+$ T cells (Fig. 4.14B) isolated from WNV-infected mice.

To further test if IL-17A-mediated expression of cytotoxicity mediators involves NF-κB activation, we treated splenocytes isolated from WNV-infected WT mice with recombinant IL-17A in the presence of Bay-11-7082 (an inhibitor of NF-κB) and measured the expression of the cytotoxicity mediators, as described above. Consistent with previous reports (Huang et al., 2007; Ribaux et al., 2007), Bay-11-7082 inhibited the IL-17A mediated *Cxcl1* expression that involves NF-κB pathway. However, inhibition of NF-κB did not inhibit, but interestingly further up-

regulated the IL-17A-mediated expression of *perforin-1*, *granzyme A*, and *granzyme B* (Fig. 4.14C). These data indicate that IL-17A-mediated expression of cytotoxicity mediators may involve ACT-1, but may occur independently of NF-κB pathway, which requires further investigation.

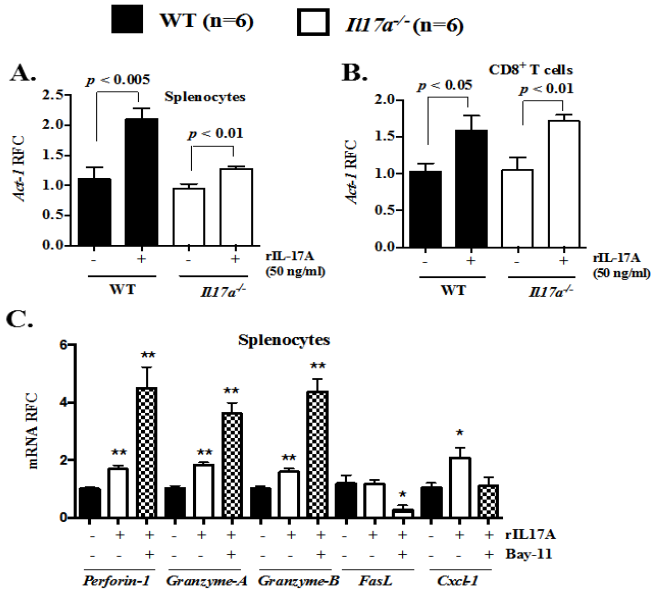

Figure 4.14 IL-17A induces the expression of ACT-1

Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ mice (n = 6 mice/group) were infected with 100 PFU (i.p.) of WNV and sacrificed on 8 d.p.i. to isolate splenocytes and CD8^{+} T cells. Splenocytes (A) or purified CD8^{+} T cells (B) were cultured *ex vivo* with or without recombinant IL-17A (rIL17A, 50 ng/ml) for 24 h, and expression (RFC) of *Act*-1 was by measured qPCR. (C) RFC of *Act*-1 in splenocytes cultured *ex vivo* for 24 h with or without rIL17A (50 ng/ml), in the presence of Bay-11-7082 (4 μM) or DMSO as vehicle control (< 0.05%). All data (mean ± SEM) represent two independent experiments (n = 3 mice/group) and compared by two-tailed student's t-test. All data from WT and *Il17a*$^{-/-}$ mice were normalized to respective mock treated controls. * denotes $p < 0.05$; ** denotes $p < 0.005$; and "*ns*" denotes not significant ($p > 0.05$).

4.15 IL-17A Directly Induces Cytotoxic mediators in CD8^{+} T Cells

IL-17R is expressed by virtually all cell types and tissues examined (Moseley et al., 2003; Yao et al., 1995; Yao et al., 1997). CD8^{+} T cells also express IL-17R (Lindemann et al., 2008; Yao et al., 1995), however, the

functional role of this receptor in $CD8^+$ T cell biology has not yet been recognized. To test if IL-17A may directly promote cytotoxic mediators expression in $CD8^+$ T cells, we treated $CD8^+$ T cells purified (~ 80 to 90% pure) from spleens of WNV-infected WT mice with the recombinant IL-17A (50 ng/ml) *ex vivo* for 24 h, then measured expression of cytotoxicity mediators by qPCR. IL-17A treatment significantly induced the expression of *perforin-1* (Fig. 4.15A), *granzyme A* (Fig. 4.15B), and *granzyme B* (Fig. 4.15C), but not *FasL* (Fig. 4.15D) in $CD8^+$ T cells purified from WNV-infected WT mice. Similar results were also obtained in $CD8^+$ T cell purified from WNV-infected *Il17a*$^{-/-}$ mice (Fig. 4.15A-D). In a separate experiment, we cultured splenocytes from WNV-infected *Il17a*$^{-/-}$ and WT mice with recombinant IL-17A (50 ng/ml) *ex vivo* for 24 h, separated $CD8^+$ T cells from $CD8^-$ cells, and then measured expression of cytotoxic mediators by qPCR. Consistently, IL-17A treatment induced the expression of cytotoxic mediators in $CD8^+$ T cells (data not shown), but not in $CD8^-$ cells (Fig. 4.15A-D).

To further confirm the role of IL-17A in promoting the expression of the cytotoxic mediators in brain $CD8^+$ T cells *in vivo*, we infected *Il17a*$^{-/-}$ mice with WNV (100 PFU), treated them with recombinant IL-17A (2.5 μg per mouse at day 6 p.i.), and performed flow cytometric analysis of brain leukocytes. The results showed that treatment of WNV-infected *Il17a*$^{-/-}$ mice with recombinant IL-17A significantly induced the production of perforin and granzyme A in brain infiltrating $CD8^+$ T cells (Fig. 4.15E). Taken together, these results suggest that IL-17A promotes the expression of cytotoxic mediator genes in $CD8^+$ T cells during WNV infection in mice.

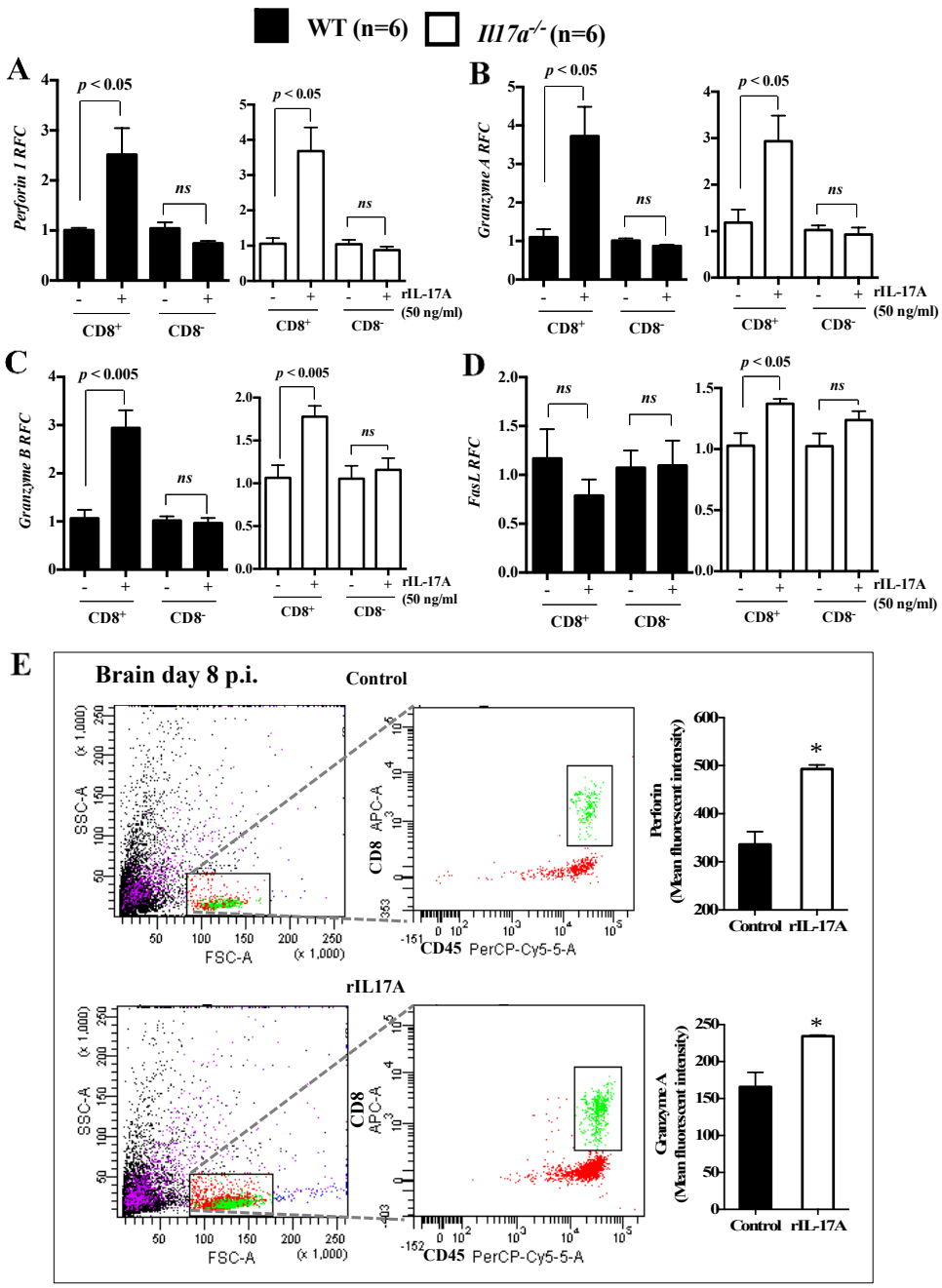

Figure 4.15 IL-17A directly induces cytotoxicity mediators in CD8$^+$ T cells

(A-D) Seven to nine weeks old WT (C57BL/6J) and *Il17a*$^{-/-}$ mice (n = 6) were infected with 100 PFU (i.p.) of WNV and sacrificed at 8 d.p.i. to collect spleens, followed by magnetic separation of CD8$^+$ T cells. Relative fold change (RFC) of the transcripts of *perforin-1* (A), *granzyme A* (B), *granzyme B* (C), and *FasL* (D) were measured in CD8$^+$ or CD8$^-$ T cells cultured *ex vivo* for 24 h with or without recombinant mouse IL-17A (50 pg/ml) by qPCR. (E) Eight week old *Il17a*$^{-/-}$ mice (n = 4 per group) infected with WNV (100 PFU, i.p.) were

treated with recombinant IL-17A (rIL17A, 2.5 µg/mouse) or PBS (control) via i.p. at day 6 p.i and sacrificed at 8 p.i. to characterize brain leukocytes by flow cytometry. Mean fluorescence intensities of perforin and granzyme A within the gated $CD45^{hi}$ $CD8^{+}$ cells (green) are shown in bar (right). All data (mean ± SEM) represent two independent experiments and compared by two-tailed student's t-test. All data from WT and *Il17a*$^{-/-}$ mice (shown in figures A to D) were normalized to respective mock treated controls. "*ns*" denotes no significant difference ($p > 0.05$).

4.16 IL-17A Treatment Increases Survival of WNV-infected Mice

Since $CD8^{+}$ T cells are essential to clear WNV from the CNS at a later phase of infection, we hypothesized that recombinant IL-17A treatment *in vivo* may offer protection from WNV infection by promoting cytotoxicity of $CD8^{+}$ T cells. To test if IL-17A may serve as a therapeutic reagent to treat WNV infection in mice, we infected WT female mice with WNV (100 PFU) via i.p. route. At day 6 p.i, mice were injected with the carrier-free mouse recombinant IL-17A (eBioscience) or PBS as control *via* i.p. route for survival analysis.

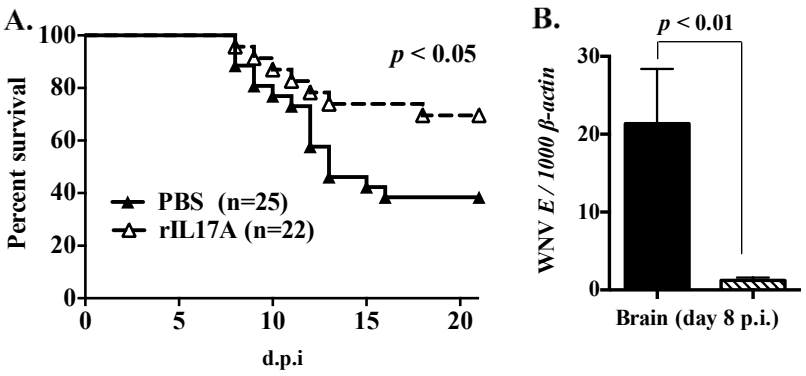

Figure 4.16 Recombinant IL-17A increases survival of WNV-infected mice

WT (C57BL/6J) mice (8-week old, female) were infected with 100 PFU of WNV via i.p. route. At day 6 p.i, mice were injected with i.p. administration of carrier-free mouse recombinant IL-17A (rIL17A, 2.5 µg/mouse) or PBS. (A) Percent (%) of survival was compared using the Kaplan-Meier survival and log-rank test. Viral burden in brain (B) was measured by qPCR and compared by two-tailed student's t-test; "*ns*" denotes no significant difference ($p > 0.05$).

Mice that received recombinant IL-17A showed significantly increased survival rate compared to the PBS-treated control mice (Fig. 4.16A). Consistent with the survival results, mice treated with recombinant IL-17A also showed lower viral burden in the brain compared to the PBS-treated control mice at day 8 p.i. (Fig. 4.16B).

4.17 IL-17A Induces Cytotoxicity Mediators in WNV-infected Mice

To test if the effect of the recombinant IL-17A treatment involves a promotion of cytotoxicity of $CD8^+$ T cells, we also measured the expression of the cytotoxicity mediators in splenic $CD8^+$ and $CD8^-$ T cells

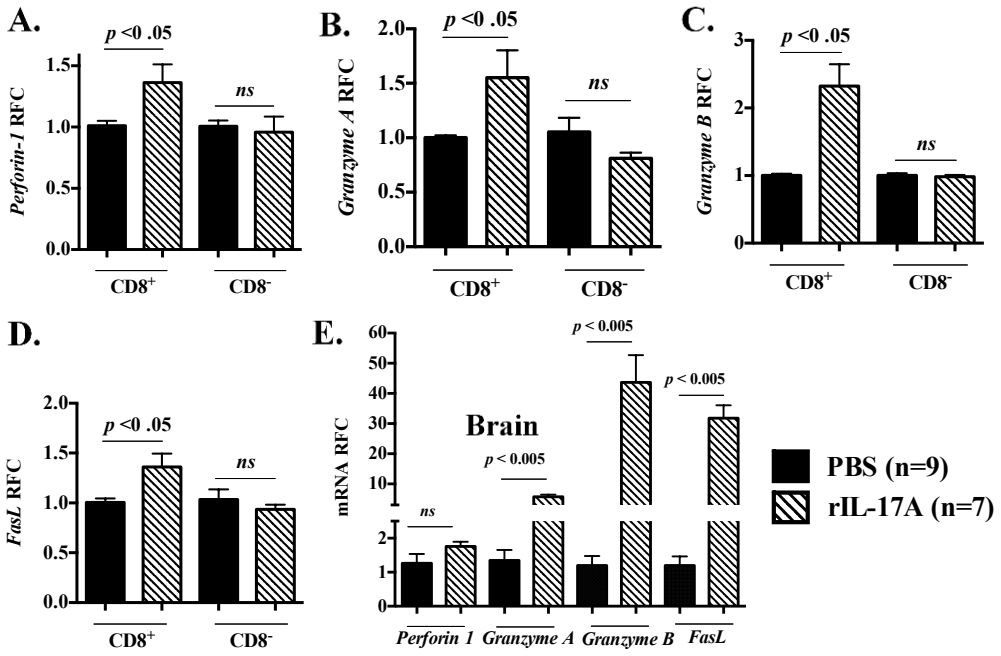

Figure 4.17 Recombinant IL-17A induces cytotoxicity in WNV-infected mice.

WT (C57BL/6J) mice (8-week old, female) were infected with 100 PFU of WNV via i.p. route. At day 6 p.i, mice were injected with i.p. administration of carrier-free mouse recombinant IL-17A (rIL17A, 2.5 μg/mouse) or PBS. (A to E) The transcripts of *perforin-1*, *granzyme A*, *granzyme B*, and *FasL* in splenic $CD8^+$ and $CD8^-$ T cells (A-D), and brain (E) were measured by qPCR and compared by two-tailed student's t-test; "*ns*" denotes no significant difference ($p > 0.05$).

. Consistent with reduced viral burden and increased survival, administration of recombinant IL-17A in WNV-infected mice induced the expression of the cytotoxic mediators in $CD8^+$ T cells, but not in $CD8^-$ T cells (Fig. 4.17A-D), which agreed with our *in vitro* results. In addition, we also detected increased expression of *granzyme A*, *granzyme B*, and *FasL* in brain tissues of WNV-infected mice after IL-17A treatment (Fig. 17E). Collectively, these results suggest a novel and promising therapeutic role of IL-17A in facilitating WNV clearance by promoting $CD8^+$ T cell cytotoxicity.

CHAPTER V – DISCUSSION

This study reveals a novel role of IL-17A in facilitating WNV clearance by inducing the expression of cytotoxic mediator genes and promoting $CD8^+$ T cell cytotoxicity. Specifically, we report here that 1) WNV induces IL-17A expression in both mice and humans; 2) *Il17a*$^{-/-}$ mice, generate a higher viral burden and are more susceptible to WNV infection; 3) $CD8^+$ T cells purified from *Il17a*$^{-/-}$ mice are less cytotoxic and express lower levels of cytotoxic mediators, i.e., *perforin-1*, *granzyme A*, *granzyme B* and *FasL*; and most importantly; and 4) *ex vivo* supply of recombinant IL-17A as late as day 6 post-infection significantly reduces viral burden in the brain and increases survival rate of WNV-infected mice, suggesting a therapeutic potential of IL-17A.

5.1 IL-17A Has a Protective Function in WNV Infection

It has been previously reported that WNV mediates production of IL-23, a cytokine that regulate IL-17A expression (Town et al., 2009). As expected, we found that WNV induced IL-17A production in human and mouse in an IL-23 dependent manner. We further found a protective function of IL-17A against WNV and its crucial role in clearance of WNV from brain, which was also consistent with function of IL-23 (Town et al., 2009). In support of our finding, it has been previously reported that mice depleted of IL-17A function by administration of anti-IL-17A antibody showed about 20% reduced survival rate compared to control mice after a lethal WNV challenge (Welte et al., 2011). However, no difference in WNV viral burden was observed between anti-IL-17A antibody treated and control mice (Welte et al., 2011). In contrast, our results

clearly demonstrate that *Il17a*$^{-/-}$ mice are more susceptible to WNV infection. The discrepancy between these two studies may be due to the transient effects of anti-IL-17A treatment when compared to complete genetic deficiency of IL-17A in *Il17a*$^{-/-}$ mice. More to this point, γδ-T cell produce IL-17A during early phage of WNV infection, whereas CD4^{+} Th-17 cells become the major IL-17A producer during the late phase of infection (Hamada et al., 2008). Thus, the administration of anti-IL-17A antibody at an early phase of WNV infection (0 and 5 d.p.i.) (Welte et al., 2011), may not have sufficiently blocked IL-17A production and its function during the later phase of infection. Indeed, we found that IL-17A was not essential for controlling the viremia on 2 d.p.i, but became critical for reducing viremia on 4 d.p.i and clearing the viruses from spleen and brain on 8 d.p.i.. Thus, it appears that IL-17A-mediated protective immunity against WNV infection predominantly occurs during the late phase of infection, which requires further investigation.

5.2 Role of IL-17A in IFN, Cytokines and Antibody Response

IL-17A is a pleiotropic cytokine that plays key roles during infection (Iwakura et al., 2008), inflammation (Park et al., 2005) and autoimmune diseases (Onishi and Gaffen, 2010). Further, it regulates the expression of a number of cytokines, including IL-1β, IFN-γ, and TNF-α (Maione et al., 2009; Song and Qian, 2013). Considering their critical roles in WNV pathogenesis (Ramos et al., 2012; Shrestha et al., 2006b; Shrestha et al., 2008b; Wang et al., 2004a), it is plausible that IL-17A may regulate the expression of these cytokines during WNV infection. However, we did not detect significant differences in the expression of

IL-1β, IFN-γ, and TNF-α in WNV-infected *Il17a*$^{-/-}$ mice when compared to WT controls. These results suggest the likelihood that IL-17A-mediated protective immunity against WNV infection may not be associated with functions controlled by these cytokines. Previous studies have suggested that type I IFNs (antiviral cytokines), which can potently suppress IL-17A expression (Curtis et al., 2009; Tilg et al., 2009), play a prominent role in controlling WNV infection (Lazear et al., 2011; Samuel and Diamond, 2005). Again, we did not detect any change in the expression of type I IFNs in *Il17a*$^{-/-}$ vs. WT control mice during WNV infection. Moreover, IL-17A has been suggested to regulate the humoral immune response (Grund et al., 2012; Tarlinton, 2008; Yuan et al., 2010), however, we did not see such effect during WNV infection in mice. Despite unaltered inflammatory response, type I IFN expression and humoral immune response, *Il17a*$^{-/-}$ mice generated higher viral burden, suggesting that IL-17A mediated protective immunity during WNV infection is independent of these immune responses.

5.3 Role of IL-17A in Leukocyte Homing to CNS

WNV invades the CNS and infects neurons and CNS-resident cells, such as microglia and astrocytes, which leads to production of cytokines and chemokines that recruit peripheral leukocytes into the brain (Glass et al., 2005; Hussmann and Fredericksen, 2014; Shrestha and Diamond, 2004; Town et al., 2009). We previously reported that IL-23 has an important role in recruiting CD11b^{+} monocytes and macrophages into the CNS to control WNV infection (Town et al., 2009). Considering the role of IL-23 in producing IL-17A, and the role of both of these cytokines in inducing and sustaining leukocyte recruitment to

infected sites (Roussel et al., 2010; Shahrara et al., 2009), we hypothesized that leukocyte migration into the CNS would be reduced in *Il17a*$^{-/-}$ mice. Surprisingly, we detected a modestly elevated leukocyte influx into WNV-infected *Il17a*$^{-/-}$ mouse brains by both flow cytometry and confocal microscopy.

In dissecting the mechanism, we found that the expression of CCL5 was elevated in both the CNS and the peripheral tissues of WNV-infected *Il17a*$^{-/-}$ mice compared to WT controls. CCL5 plays a protective role in WNV, influenza and parainfluenza infections by promoting leukocyte trafficking to infected tissues (Glass et al., 2005; Kohlmeier et al., 2008). Also, *Ccl5* expression sustains CD8^{+} T cell responses during influenza (Kohlmeier et al., 2008), parainfluenza (Kohlmeier et al., 2008) and chronic lymphocytic choriomeningitis virus (LCMV) (Crawford et al., 2011) infections. The up-regulation of *Ccl5* in *Il17a*$^{-/-}$ mice may be due to several reasons. First, the higher viral load in *Il17a*$^{-/-}$ mice could trigger a stronger *Ccl5* expression (Matsukura et al., 1998; Saito et al., 1997). Secondly, the presence of IL-17A in WT mice may suppress *Ccl5* expression, since IL-17A can down-regulate *Ccl5* expression (Andoh et al., 2002; Shen et al., 2005). Although increased *Ccl5* expression may account for modest elevation in brain infiltrating leukocytes in *Il17a*$^{-/-}$ mice, this did not result in protection of these mice from WNV infection, implying a possible functional defect in brain infiltrating effector leukocytes in *Il17a*$^{-/-}$ mice.

5.4 IL-17A Regulates CD8^{+} T Cell Cytotoxicity

CD8^{+} T cells play a critical role in clearance of viruses from the CNS (Binder and Griffin, 2001; Griffin, 2010; Shrestha and Diamond, 2004). CD8^{+} T

cells can control viral infection directly by inducing apoptosis of virus-infected cells *via* perforin, granzyme, or Fas-FasL interactions (Harty and Badovinac, 2002; Shresta et al., 1998), or indirectly by immune-mediated non-cytolytic clearance of viruses from neurons by producing cytokines, such as TNF-α and IFN-γ (Binder and Griffin, 2001; Griffin, 2010). Mice deficient in the expression of cytotoxic mediators such as *perforin*, *FasL,* or cytokines, such as TNF-α and IFN-γ, exhibit increased mortality and viral burden in the CNS and peripheral organs following WNV infection (Shrestha and Diamond, 2007; Shrestha et al., 2012; Shrestha et al., 2006a; Shrestha et al., 2006b; Shrestha et al., 2008b; Wang et al., 2004b). However, we did not detect any significant difference in the expression of the cytokines including TNF-α and IFN-γ between WNV-infected WT and *Il17a*$^{-/-}$ mice, suggesting that IL-17A-mediated control of WNV by CD8^{+} T cells may occur independently of the effects of these cytokines. We found that CD8^{+} T cells isolated from *Il17a*$^{-/-}$ mice have significantly reduced cytotoxicity compared to WT controls, which may explain higher viral burden and lower survival rates of *Il17a*$^{-/-}$ mice, despite elevated leukocyte infiltration (including CD8^{+} T cells) into the CNS. While we are not certain whether CD8^{+} T cells predominantly contribute to WNV clearance by causing cytolysis of WNV-infected neurons due to lower MHC I expression in these cells (Kimura and Griffin, 2000; Mucke and Oldstone, 1992), it is likely that CD8^{+} T cells can cause cytolysis of WNV-infected non-neural cells, such as infiltrating macrophages and neutrophils, and microglia in the CNS, thus facilitating the virus clearance (Bai et al., 2010; Shrestha and Diamond, 2004, 2007; Shrestha et al., 2006a). In line with reduced

cytotoxicity, $CD8^+$ T cells from *Il17a*$^{-/-}$ mice had significantly reduced expression of *perforin-1*, *granzyme A*, *granzyme B* and *FasL* in most of the tissue examined during WNV infection. Although NK cells (type of $CD8^-$ cells) also express these genes and mediate apoptosis of target cell, we did not detect difference in expression of cytotoxic mediators in CD8 negative ($CD8^-$) cell populations isolated from WNV-infected WT and *Il17a*$^{-/-}$ mouse spleens. This indicates that NK cells (type of $CD8^-$ cells) may not play a prominent role in IL-17A-mediated cytotoxicity during WNV infection. This notion is consistent with the previous reports that NK cells have a little or no role in controlling WNV infection (Shrestha et al., 2006a).

It is worthy to note that $CD8^+$ T cells have also been shown an immunopathological role when mice were infected intravenously with a high dose (10^8 PFU) of WNV (Sarafend strain), resulting in 100% mortality with a 6-day mean survival time (Wang et al., 2003). However, such an immunopathology role of $CD8^+$ T cells was likely not due to their cytotoxic functions because these cells are usually activated after one week post-infection. In support of this, that same study and many other reports also showed a recovery role of $CD8^+$ T cells when mice were infected with low doses (10^2-10^3 PFU) of WNV, which is similar to the current study (Brien et al., 2011; Brien et al., 2007; Shrestha et al., 2008a; Shrestha et al., 2012; Shrestha et al., 2006a; Wang et al., 2003). In addition, Th17 cells and IL-17A have been implicated in inflammation and immunopathology associated with autoimmune diseases (McFarland and Martin, 2007; van den Berg and Miossec, 2009; Zepp et al., 2011) and some virus-

induced chronic CNS diseases (Hou et al., 2014; Hou et al., 2009; Savarin et al., 2012), suggesting that Th-17/IL-17A axis may have different implications in such immunopathological and chronic inflammatory conditions.

5.5 IL-17A Has a Potential Therapeutic Role Against WNV

Our data demonstrates that IL-17A deficiency leads to an attenuated $CD8^+$ T cell cytotoxicity and deficient WNV clearance. Both $CD4^+$ Th-17 cells and **γδ** T cells, the major cells that produce IL-17A, have been previously shown to promote $CD8^+$ T cell cytotoxicity during infection (Hamada et al., 2009; Xu et al., 2010), autoimmune disease (Ankathatti Munegowda et al., 2011a), and cancers (Ankathatti Munegowda et al., 2011b; Martin-Orozco et al., 2009). Also, NK cells treated with IL-17A overexpress *perforin* and *granzymes,* and have better cytotoxic functions (Al Omar et al., 2013), which provides additional evidence that IL-17A signaling can induce the expression of cytotoxicity mediators. These reports support our finding and suggest that IL-17A may facilitate cytotoxicity of $CD8^+$ T cells in diverse disease conditions.

Various types of immune cells, such as $CD4^+$ Th-17 cells, **γδ**-T cells, NKT cells, and $CD8^+$ T cells can produce IL-17A (Gu et al., 2013; Welte et al., 2011). In addition, the IL-17A receptor (IL-17R) is expressed ubiquitously in virtually all cell types and tissues examined (Andoh et al., 2002; Moseley et al., 2003; Yao et al., 1997). Although $CD8^+$ T cells can produce IL-17A and also express its cognate receptor (Lindemann et al., 2008; Yao et al., 1995), the link between IL-17A and $CD8^+$ T cell cytotoxic function has not been previously recognized. In theory, it is possible that IL-17A may affect $CD8^+$ T cell development and/or

function either by directly acting on these cells or indirectly through other cell types. Since we found similar levels of $CD8^+$ T cells and $CD4^+/CD8^+$ ratios in splenocytes isolated from *Il17a*$^{-/-}$ and WT mice infected with WNV, it appears that $CD8^+$ T cell development remains unaltered in *Il17a*$^{-/-}$ mice and IL-17A may largely control $CD8^+$ T cell function during WNV infection. This hypothesis is confirmed by *ex vivo* treatment of IL-17A to splenocytes isolated from WNV-infected *Il17a*$^{-/-}$ and WT mice, which showed an up-regulation of *perforin-1*, *granzyme A*, and *granzyme B* expression in $CD8^+$ T cells by IL-17A, in a $CD4^+$ T cell-independent manner. These results suggest that IL-17A can induce the expression of cytotoxic mediators by directly acting on $CD8^+$ T cells. However, the induction of *FasL* by recombinant IL-17A was differentially affected in our experiments. For instance, *in vitro* IL-17A treatment did not induce the expression of *FasL* in $CD8^+$ T cells, while it was induced after *in vivo* administration of IL-17A. This discrepancy is likely due to the experimental conditions or may reflect differential role of IL-17A in regulating the expression of these cytotoxic mediators, which requires further investigation. We further demonstrated that the treatment of WNV-infected *Il17a*$^{-/-}$ mice with recombinant IL-17A significantly induced the production of perforin and granzyme A in brain infiltrating $CD8^+$ T cells, which confirmed the role of IL-17A in promoting the expression of the cytotoxic mediators in brain infiltrating $CD8^+$ T cells *in vivo.*

To extend upon our finding, we tested therapeutic potential of IL-17A in a mouse model of WNV infection. Importantly, administration of a single dose of recombinant IL-17A to WNV-infected mice, even as late as day 6 p.i., induced

the expression of cytotoxic mediators in $CD8^+$ T cells, dramatically reduced viral burden in the brain, and significantly increased survival rate, suggesting a promising therapeutic role of IL-17A against WNV infection.

CHAPTER VI – CONCLUSION AND FUTURE DIRECTIONS

6.1 Conclusion

IL-17A and $CD8^+$ T cells regulate diverse immune functions during microbial infections, malignancies, and autoimmune diseases. IL-17A is a proinflammatory cytokine produced by diverse cell types, while the $CD8^+$ T cells (known as cytotoxic T cells) are major cells that provide immunity against intracellular pathogens. Previous studies have demonstrated a crucial role of $CD8^+$ T cells in recovery from WNV infection. Here, we demonstrate that IL-17A protects mice from lethal WNV infection by promoting $CD8^+$ T-cell-mediated clearance of WNV. In addition, treatment of WNV-infected mice with recombinant IL-17A reduces viral burden and increases survival of mice, suggesting a potential therapeutic. In conclusion, this study uncovers a novel function of IL-17A in promoting $CD8^+$ T cell cytotoxicity during WNV infection in mice. This novel IL-17A-$CD8^+$ T cell axis may also have broad implications in immunity to other microbial infections and cancers, where $CD8^+$ T cell functions are crucial.

6.2 Future Directions

In this study, we found the production of IL-17A in WNV-infected human and mice, which we also confirmed in a mixed culture of human and mouse immune cells infected *in vitro* with WNV. We further demonstrated a novel function of IL-17A in promoting the cytotoxicity of $CD8^+$ T cell against WNV infection. In addition, a supply of recombinant IL-17A to a culture of $CD8^+$ T cells and WNV-infected mice clearly induced the expression of cytotoxicity mediator genes. The role of IL-17A in promoting the expression of cytotoxic mediators in

$CD8^+$ T cells has not been previously reported and the signaling mechanism by which IL-17A regulates the expression of cytotoxicity mediators is not currently understood. In addition, diverse cell types including immune cells, astrocytes, and microglia can produce IL-17A and express IL-17A receptor. Future studies are required for characterization of IL-17A producing and responding cells in both CNS and periphery during various stage of WNV infection. In addition, a better understanding of the regulation of IL-17A production and dissection of the signaling mechanism by which IL-17A induces expression of cytotoxicity mediators and promotes $CD8^+$ T cell cytotoxicity in the context of WNV and other diseases may help to exploit novel IL-17A-based therapeutic strategies.

THE UNIVERSITY OF
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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

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NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	15101601 (Replaces 12041201)
PROJECT TITLE:	**Immunotherapeutics Against Flaviviruses and Alphaviruses**
PROPOSED PROJECT DATES:	10/2015 - 09/2018
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Fengwei Bai**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**Wilson Research Foundation**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2018**

Frank Moore, PhD
IACUC Chair

10/01/2015
Date

The University of
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Institutional Animal Care and Use Committee

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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**12041201**
PROJECT TITLE:	**Immunotherapeutics against flaviviruses**
PROPOSED PROJECT DATES:	**08/01/2012-07/31/2015**
PROJECT TYPE:	**New**
PRINCIPAL INVESTIGATOR(S):	**Fengwei Bai**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	
IACUC COMMITTEE ACTION:	
PROTOCOL EXPIRATON DATE:	**7/31/2015**

Jodie M. Jawor, Ph.D.
IACUC Chair

10 September 2012
Date

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL REVIEW BOARD
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NOTICE OF COMMITTEE ACTION

The project has been reviewed by The University of Southern Mississippi Institutional Review Board in accordance with Federal Drug Administration regulations (21 CFR 26, 111), Department of Health and Human Services (45 CFR Part 46), and university guidelines to ensure adherence to the following criteria:

- The risks to subjects are minimized.
- The risks to subjects are reasonable in relation to the anticipated benefits.
- The selection of subjects is equitable.
- Informed consent is adequate and appropriately documented.
- Where appropriate, the research plan makes adequate provisions for monitoring the data collected to ensure the safety of the subjects.
- Where appropriate, there are adequate provisions to protect the privacy of subjects and to maintain the confidentiality of all data.
- Appropriate additional safeguards have been included to protect vulnerable subjects.
- Any unanticipated, serious, or continuing problems encountered regarding risks to subjects must be reported immediately, but not later than 10 days following the event. This should be reported to the IRB Office via the "Adverse Effect Report Form".
- If approved, the maximum period of approval is limited to twelve months.
  Projects that exceed this period must submit an application for renewal or continuation.

PROTOCOL NUMBER: **CH-R11120601**
PROJECT TITLE: **The Role of Neutrophic Virus Pathogenesis**
PROJECT TYPE: **Change to a Previously Approved Project**
RESEARCHER(S): **Fengwei Bai, Ph.D.**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR: **N/A**
IRB COMMITTEE ACTION: **Expedited Review Approval**
PERIOD OF APPROVAL: **04/21/2014 to 04/20/215**

Lawrence A. Hosman, Ph.D.
Institutional Review Board

REFERENCES

Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J., and Gurney, A.L. (2003). Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. The Journal of biological chemistry 278, 1910-1914.

Al Omar, S., Flanagan, B.F., Almehmadi, M., and Christmas, S.E. (2013). The effects of IL-17 upon human natural killer cells. Cytokine 62, 123-130.

Alpert, S.G., Fergerson, J., and Noel, L.P. (2003). Intrauterine West Nile virus: ocular and systemic findings. Am J Ophthalmol 136, 733-735.

Anderson, J.F., Andreadis, T.G., Vossbrinck, C.R., Tirrell, S., Wakem, E.M., French, R.A., Garmendia, A.E., and Van Kruiningen, H.J. (1999). Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science 286, 2331-2333.

Andoh, A., Fujino, S., Bamba, S., Araki, Y., Okuno, T., Bamba, T., and Fujiyama, Y. (2002). IL-17 selectively down-regulates TNF-alpha-induced RANTES gene expression in human colonic subepithelial myofibroblasts. Journal of immunology 169, 1683-1687.

Ankathatti Munegowda, M., Deng, Y., Chibbar, R., Xu, Q., Freywald, A., Mulligan, S.J., van Drunen Littel-van den Hurk, S., Sun, D., Xiong, S., and Xiang, J. (2011a). A distinct role of CD4+ Th17- and Th17-stimulated CD8+ CTL in the pathogenesis of type 1 diabetes and experimental autoimmune encephalomyelitis. J Clin Immunol 31, 811-826.

Ankathatti Munegowda, M., Deng, Y., Mulligan, S.J., and Xiang, J. (2011b). Th17 and Th17-stimulated CD8(+) T cells play a distinct role in Th17-induced preventive and therapeutic antitumor immunity. Cancer immunology, immunotherapy: CII 60, 1473-1484.

Arjona, A., Wang, P., Montgomery, R.R., and Fikrig, E. (2011). Innate immune control of West Nile virus infection. Cell Microbiol 13, 1648-1658.

Bai, F., Kong, K.F., Dai, J., Qian, F., Zhang, L., Brown, C.R., Fikrig, E., and Montgomery, R.R. (2010). A paradoxical role for neutrophils in the pathogenesis of West Nile virus. J Infect Dis 202, 1804-1812.

Bai, F., Town, T., Qian, F., Wang, P., Kamanaka, M., Connolly, T.M., Gate, D., Montgomery, R.R., Flavell, R.A., and Fikrig, E. (2009). IL-10 signaling blockade controls murine West Nile virus infection. PLoS Pathog 5, e1000610.

Bai, F., Wang, T., Pal, U., Bao, F., Gould, L.H., and Fikrig, E. (2005). Use of RNA interference to prevent lethal murine west nile virus infection. J Infect Dis 191, 1148-1154.

Benchetrit, F., Ciree, A., Vives, V., Warnier, G., Gey, A., Sautes-Fridman, C., Fossiez, F., Haicheur, N., Fridman, W.H., and Tartour, E. (2002). Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. Blood 99, 2114-2121.

Benjelloun, A., El Harrak, M., and Belkadi, B. (2015). West Nile Disease Epidemiology in North-West Africa: Bibliographical Review. Transboundary and emerging diseases.

Binder, G.K., and Griffin, D.E. (2001). Interferon-gamma-mediated site-specific clearance of alphavirus from CNS neurons. Science 293, 303-306.

Blazquez, A.B., and Saiz, J.C. (2010). West Nile virus (WNV) transmission routes in the murine model: intrauterine, by breastfeeding and after cannibal ingestion. Virus Res 151, 240-243.

Brien, J.D., Daffis, S., Lazear, H.M., Cho, H., Suthar, M.S., Gale, M., Jr., and Diamond, M.S. (2011). Interferon regulatory factor-1 (IRF-1) shapes both innate and CD8(+) T-cell immune responses against West Nile virus infection. PLoS pathogens 7, e1002230.

Brien, J.D., Uhrlaub, J.L., and Nikolich-Zugich, J. (2007). Protective capacity and epitope specificity of CD8(+) T cells responding to lethal West Nile virus infection. European journal of immunology 37, 1855-1863.

CDC (2015). West Nile virus.

Centers for Disease, C., and Prevention (2009). West Nile virus transmission via organ transplantation and blood transfusion - Louisiana, 2008. MMWR Morb Mortal Wkly Rep 58, 1263-1267.

Cernescu, C., Ruta, S.M., Tardei, G., Grancea, C., Moldoveanu, L., Spulbar, E., and Tsai, T. (1997). A high number of severe neurologic clinical forms

during an epidemic of West Nile virus infection. Romanian journal of virology 48, 13-25.

Chung, K.M., Liszewski, M.K., Nybakken, G., Davis, A.E., Townsend, R.R., Fremont, D.H., Atkinson, J.P., and Diamond, M.S. (2006). West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. Proc Natl Acad Sci U S A 103, 19111-19116.

Colpitts, T.M., Conway, M.J., Montgomery, R.R., and Fikrig, E. (2012). West Nile Virus: biology, transmission, and human infection. Clin Microbiol Rev 25, 635-648.

Conti, H.R., Shen, F., Nayyar, N., Stocum, E., Sun, J.N., Lindemann, M.J., Ho, A.W., Hai, J.H., Yu, J.J., Jung, J.W., et al. (2009). Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. The Journal of experimental medicine 206, 299-311.

Crawford, A., Angelosanto, J.M., Nadwodny, K.L., Blackburn, S.D., and Wherry, E.J. (2011). A role for the chemokine RANTES in regulating CD8 T cell responses during chronic viral infection. PLoS Pathog 7, e1002098.

Crowe, C.R., Chen, K., Pociask, D.A., Alcorn, J.F., Krivich, C., Enelow, R.I., Ross, T.M., Witztum, J.L., and Kolls, J.K. (2009). Critical role of IL-17RA in immunopathology of influenza infection. Journal of immunology 183, 5301-5310.

Curtis, M.M., Way, S.S., and Wilson, C.B. (2009). IL-23 promotes the production of IL-17 by antigen-specific CD8 T cells in the absence of IL-12 and type-I interferons. Journal of immunology 183, 381-387.

Das Sarma, J., Ciric, B., Marek, R., Sadhukhan, S., Caruso, M.L., Shafagh, J., Fitzgerald, D.C., Shindler, K.S., and Rostami, A. (2009). Functional interleukin-17 receptor A is expressed in central nervous system glia and upregulated in experimental autoimmune encephalomyelitis. Journal of neuroinflammation 6, 14.

Davis, C.T., Ebel, G.D., Lanciotti, R.S., Brault, A.C., Guzman, H., Siirin, M., Lambert, A., Parsons, R.E., Beasley, D.W., Novak, R.J., et al. (2005). Phylogenetic analysis of North American West Nile virus isolates 2001-2004: evidence for the emergence of a dominant genotype. Virology 342, 252-265.

de Almeida Nagata, D.E., Demoor, T., Ptaschinski, C., Ting, H.A., Jang, S., Reed, M., Mukherjee, S., and Lukacs, N.W. (2014). IL-27R-mediated regulation of IL-17 controls the development of respiratory syncytial virus-associated pathogenesis. The American journal of pathology 184, 1807-1818.

Diamond, M.S., Shrestha, B., Marri, A., Mahan, D., and Engle, M. (2003a). B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. Journal of virology 77, 2578-2586.

Diamond, M.S., Sitati, E.M., Friend, L.D., Higgs, S., Shrestha, B., and Engle, M. (2003b). A critical role for induced IgM in the protection against West Nile virus infection. The Journal of experimental medicine 198, 1853-1862.

Draganescu, N. (1979). Study of arboviruses in Romania (1958--1978). Results obtained in the "Stefan S. Nicolau" Institute of Virology. Virologie 30, 283-293.

Ebel, G.D., Carricaburu, J., Young, D., Bernard, K.A., and Kramer, L.D. (2004). Genetic and phenotypic variation of West Nile virus in New York, 2000-2003. Am J Trop Med Hyg 71, 493-500.

Gaffen, S.L. (2009). Structure and signalling in the IL-17 receptor family. Nature reviews Immunology 9, 556-567.

Glass, W.G., Lim, J.K., Cholera, R., Pletnev, A.G., Gao, J.L., and Murphy, P.M. (2005). Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. The Journal of experimental medicine 202, 1087-1098.

Griffin, D.E. (2010). Recovery from viral encephalomyelitis: immune-mediated noncytolytic virus clearance from neurons. Immunologic research 47, 123-133.

Griffin, G.K., Newton, G., Tarrio, M.L., Bu, D.X., Maganto-Garcia, E., Azcutia, V., Alcaide, P., Grabie, N., Luscinskas, F.W., Croce, K.J., et al. (2012). IL-17 and TNF-alpha sustain neutrophil recruitment during inflammation through synergistic effects on endothelial activation. Journal of immunology 188, 6287-6299.

Grund, L.Z., Komegae, E.N., Lopes-Ferreira, M., and Lima, C. (2012). IL-5 and IL-17A are critical for the chronic IgE response and differentiation of long-lived antibody-secreting cells in inflamed tissues. Cytokine 59, 335-351.

Gu, C., Wu, L., and Li, X. (2013). IL-17 family: cytokines, receptors and signaling. Cytokine 64, 477-485.

Guiton, R., Vasseur, V., Charron, S., Arias, M.T., Van Langendonck, N., Buzoni-Gatel, D., Ryffel, B., and Dimier-Poisson, I. (2010). Interleukin 17 receptor signaling is deleterious during Toxoplasma gondii infection in susceptible BL6 mice. J Infect Dis 202, 427-435.

Hamada, H., Garcia-Hernandez Mde, L., Reome, J.B., Misra, S.K., Strutt, T.M., McKinstry, K.K., Cooper, A.M., Swain, S.L., and Dutton, R.W. (2009). Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. Journal of immunology 182, 3469-3481.

Hamada, S., Umemura, M., Shiono, T., Tanaka, K., Yahagi, A., Begum, M.D., Oshiro, K., Okamoto, Y., Watanabe, H., Kawakami, K., et al. (2008). IL-17A produced by gammadelta T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver. Journal of immunology 181, 3456-3463.

Happel, K.I., Dubin, P.J., Zheng, M., Ghilardi, N., Lockhart, C., Quinton, L.J., Odden, A.R., Shellito, J.E., Bagby, G.J., Nelson, S., et al. (2005). Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. The Journal of experimental medicine 202, 761-769.

Harty, J.T., and Badovinac, V.P. (2002). Influence of effector molecules on the CD8(+) T cell response to infection. Curr Opin Immunol 14, 360-365.

Hayes, C.G. (2001). West Nile virus: Uganda, 1937, to New York City, 1999. Annals of the New York Academy of Sciences 951, 25-37.

Hayes, E.B., Sejvar, J.J., Zaki, S.R., Lanciotti, R.S., Bode, A.V., and Campbell, G.L. (2005). Virology, pathology, and clinical manifestations of West Nile virus disease. Emerg Infect Dis 11, 1174-1179.

Ho, A.W., and Gaffen, S.L. (2010). IL-17RC: a partner in IL-17 signaling and beyond. Seminars in immunopathology 32, 33-42.

Hou, W., Jin, Y.H., Kang, H.S., and Kim, B.S. (2014). Interleukin-6 (IL-6) and IL-17 synergistically promote viral persistence by inhibiting cellular apoptosis and cytotoxic T cell function. Journal of virology 88, 8479-8489.

Hou, W., Kang, H.S., and Kim, B.S. (2009). Th17 cells enhance viral persistence and inhibit T cell cytotoxicity in a model of chronic virus infection. The Journal of experimental medicine 206, 313-328.

Huang, F., Kao, C.Y., Wachi, S., Thai, P., Ryu, J., and Wu, R. (2007). Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-kappaB activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. Journal of immunology 179, 6504-6513.

Huang, W., Na, L., Fidel, P.L., and Schwarzenberger, P. (2004). Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. J Infect Dis 190, 624-631.

Hubalek, Z., and Halouzka, J. (1999). West Nile fever--a reemerging mosquito-borne viral disease in Europe. Emerging infectious diseases 5, 643-650.

Hussmann, K.L., and Fredericksen, B.L. (2014). Differential induction of CCL5 by pathogenic and non-pathogenic strains of West Nile virus in brain endothelial cells and astrocytes. The Journal of general virology 95, 862-867.

Iwakura, Y., Nakae, S., Saijo, S., and Ishigame, H. (2008). The roles of IL-17A in inflammatory immune responses and host defense against pathogens. Immunol Rev 226, 57-79.

Jie, Z., Liang, Y., Hou, L., Dong, C., Iwakura, Y., Soong, L., Cong, Y., and Sun, J. (2014). Intrahepatic innate lymphoid cells secrete IL-17A and IL-17F that are crucial for T cell priming in viral infection. Journal of immunology 192, 3289-3300.

Kebir, H., Kreymborg, K., Ifergan, I., Dodelet-Devillers, A., Cayrol, R., Bernard, M., Giuliani, F., Arbour, N., Becher, B., and Prat, A. (2007). Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. Nat Med 13, 1173-1175.

Kimura, T., and Griffin, D.E. (2000). The role of CD8(+) T cells and major histocompatibility complex class I expression in the central nervous system of

mice infected with neurovirulent Sindbis virus. Journal of virology 74, 6117-6125.

Kohlmeier, J.E., Miller, S.C., Smith, J., Lu, B., Gerard, C., Cookenham, T., Roberts, A.D., and Woodland, D.L. (2008). The chemokine receptor CCR5 plays a key role in the early memory CD8+ T cell response to respiratory virus infections. Immunity 29, 101-113.

Kohyama, S., Ohno, S., Isoda, A., Moriya, O., Belladonna, M.L., Hayashi, H., Iwakura, Y., Yoshimoto, T., Akatsuka, T., and Matsui, M. (2007). IL-23 enhances host defense against vaccinia virus infection via a mechanism partly involving IL-17. Journal of immunology 179, 3917-3925.

Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jager, A., Strom, T.B., Oukka, M., and Kuchroo, V.K. (2007). IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature 448, 484-487.

Kozaci, D.L., Chernajovsky, Y., and Chikanza, I.C. (2007). The differential expression of corticosteroid receptor isoforms in corticosteroid-resistant and -sensitive patients with rheumatoid arthritis. Rheumatology 46, 579-585.

Kramer, L.D., Styer, L.M., and Ebel, G.D. (2008). A global perspective on the epidemiology of West Nile virus. Annual review of entomology 53, 61-81.

Kuno, G., and Chang, G.J. (2005). Biological transmission of arboviruses: reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends. Clin Microbiol Rev 18, 608-637.

Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., and Cua, D.J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. The Journal of experimental medicine 201, 233-240.

Lazear, H.M., Pinto, A.K., Vogt, M.R., Gale, M., Jr., and Diamond, M.S. (2011). Beta interferon controls West Nile virus infection and pathogenesis in mice. Journal of virology 85, 7186-7194.

Le Guenno, B., Bougermouh, A., Azzam, T., and Bouakaz, R. (1996). West Nile: a deadly virus? Lancet 348, 1315.

Lim, P.Y., Behr, M.J., Chadwick, C.M., Shi, P.Y., and Bernard, K.A. (2011). Keratinocytes are cell targets of West Nile virus in vivo. Journal of virology 85, 5197-5201.

Lin, Y., Ritchea, S., Logar, A., Slight, S., Messmer, M., Rangel-Moreno, J., Guglani, L., Alcorn, J.F., Strawbridge, H., Park, S.M., et al. (2009). Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen Francisella tularensis. Immunity 31, 799-810.

Lindemann, M.J., Hu, Z., Benczik, M., Liu, K.D., and Gaffen, S.L. (2008). Differential regulation of the IL-17 receptor by gammac cytokines: inhibitory signaling by the phosphatidylinositol 3-kinase pathway. The Journal of biological chemistry 283, 14100-14108.

Liu, W.J., Wang, X.J., Clark, D.C., Lobigs, M., Hall, R.A., and Khromykh, A.A. (2006). A single amino acid substitution in the West Nile virus nonstructural

protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. J Virol 80, 2396-2404.

Maione, F., Paschalidis, N., Mascolo, N., Dufton, N., Perretti, M., and D'Acquisto, F. (2009). Interleukin 17 sustains rather than induces inflammation. Biochem Pharmacol 77, 878-887.

Martin-Orozco, N., and Dong, C. (2009). The IL-17/IL-23 axis of inflammation in cancer: friend or foe? Current opinion in investigational drugs 10, 543-549.

Martin-Orozco, N., Muranski, P., Chung, Y., Yang, X.O., Yamazaki, T., Lu, S., Hwu, P., Restifo, N.P., Overwijk, W.W., and Dong, C. (2009). T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity 31, 787-798.

Martina, B.E., Koraka, P., and Osterhaus, A.D. (2010). West Nile Virus: is a vaccine needed? Current opinion in investigational drugs 11, 139-146.

Matsukura, S., Kokubu, F., Kubo, H., Tomita, T., Tokunaga, H., Kadokura, M., Yamamoto, T., Kuroiwa, Y., Ohno, T., Suzaki, H., et al. (1998). Expression of RANTES by normal airway epithelial cells after influenza virus A infection. Am J Respir Cell Mol Biol 18, 255-264.

McFarland, H.F., and Martin, R. (2007). Multiple sclerosis: a complicated picture of autoimmunity. Nature immunology 8, 913-919.

McGeachy, M.J., Chen, Y., Tato, C.M., Laurence, A., Joyce-Shaikh, B., Blumenschein, W.M., McClanahan, T.K., O'Shea, J.J., and Cua, D.J. (2009).

The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. Nature immunology 10, 314-324.

Mehlhop, E., and Diamond, M.S. (2006). Protective immune responses against West Nile virus are primed by distinct complement activation pathways. The Journal of experimental medicine 203, 1371-1381.

Moore, T.C., Bush, K.L., Cody, L., Brown, D.M., and Petro, T.M. (2012). Control of early Theiler's murine encephalomyelitis virus replication in macrophages by interleukin-6 occurs in conjunction with STAT1 activation and nitric oxide production. Journal of virology 86, 10841-10851.

Moseley, T.A., Haudenschild, D.R., Rose, L., and Reddi, A.H. (2003). Interleukin-17 family and IL-17 receptors. Cytokine & growth factor reviews 14, 155-174.

Mostashari, F., Bunning, M.L., Kitsutani, P.T., Singer, D.A., Nash, D., Cooper, M.J., Katz, N., Liljebjelke, K.A., Biggerstaff, B.J., Fine, A.D., et al. (2001). Epidemic West Nile encephalitis, New York, 1999: results of a household-based seroepidemiological survey. Lancet 358, 261-264.

Moudy, R.M., Meola, M.A., Morin, L.L., Ebel, G.D., and Kramer, L.D. (2007). A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by Culex mosquitoes. Am J Trop Med Hyg 77, 365-370.

Mucke, L., and Oldstone, M.B. (1992). The expression of major histocompatibility complex (MHC) class I antigens in the brain differs

markedly in acute and persistent infections with lymphocytic choriomeningitis virus (LCMV). Journal of neuroimmunology 36, 193-198.

Mukherjee, S., Lin, T.Y., Dowd, K.A., Manhart, C.J., and Pierson, T.C. (2011a). The infectivity of prM-containing partially mature West Nile virus does not require the activity of cellular furin-like proteases. J Virol 85, 12067-12072.

Mukherjee, S., Lindell, D.M., Berlin, A.A., Morris, S.B., Shanley, T.P., Hershenson, M.B., and Lukacs, N.W. (2011b). IL-17-induced pulmonary pathogenesis during respiratory viral infection and exacerbation of allergic disease. The American journal of pathology 179, 248-258.

Mukhopadhyay, S., Kim, B.S., Chipman, P.R., Rossmann, M.G., and Kuhn, R.J. (2003). Structure of West Nile virus. Science 302, 248.

Murugaiyan, G., and Saha, B. (2009). Protumor vs antitumor functions of IL-17. Journal of immunology 183, 4169-4175.

Newcomb, D.C., and Peebles, R.S., Jr. (2013). Th17-mediated inflammation in asthma. Curr Opin Immunol 25, 755-760.

Nurieva, R., Yang, X.O., Martinez, G., Zhang, Y., Panopoulos, A.D., Ma, L., Schluns, K., Tian, Q., Watowich, S.S., Jetten, A.M., et al. (2007). Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature 448, 480-483.

Onishi, R.M., and Gaffen, S.L. (2010). Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology 129, 311-321.

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., et al. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nature immunology 6, 1133-1141.

Patera, A.C., Pesnicak, L., Bertin, J., and Cohen, J.I. (2002). Interleukin 17 modulates the immune response to vaccinia virus infection. Virology 299, 56-63.

Pealer, L.N., Marfin, A.A., Petersen, L.R., Lanciotti, R.S., Page, P.L., Stramer, S.L., Stobierski, M.G., Signs, K., Newman, B., Kapoor, H., et al. (2003). Transmission of West Nile virus through blood transfusion in the United States in 2002. N Engl J Med 349, 1236-1245.

Petersen, L.R., Carson, P.J., Biggerstaff, B.J., Custer, B., Borchardt, S.M., and Busch, M.P. (2013). Estimated cumulative incidence of West Nile virus infection in US adults, 1999-2010. Epidemiol Infect 141, 591-595.

Pinto, A.K., Daffis, S., Brien, J.D., Gainey, M.D., Yokoyama, W.M., Sheehan, K.C., Murphy, K.M., Schreiber, R.D., and Diamond, M.S. (2011). A temporal role of type I interferon signaling in CD8+ T cell maturation during acute West Nile virus infection. PLoS Pathog 7, e1002407.

Prow, N.A., Setoh, Y.X., Biron, R.M., Sester, D.P., Kim, K.S., Hobson-Peters, J., Hall, R.A., and Bielefeldt-Ohmann, H. (2014). The West Nile virus-like

flavivirus Koutango is highly virulent in mice due to delayed viral clearance and the induction of a poor neutralizing antibody response. J Virol 88, 9947-9962.

Ramos, H.J., Lanteri, M.C., Blahnik, G., Negash, A., Suthar, M.S., Brassil, M.M., Sodhi, K., Treuting, P.M., Busch, M.P., Norris, P.J., et al. (2012). IL-1beta signaling promotes CNS-intrinsic immune control of West Nile virus infection. PLoS Pathog 8, e1003039.

Raychaudhuri, S.P. (2013). Role of IL-17 in psoriasis and psoriatic arthritis. Clin Rev Allergy Immunol 44, 183-193.

Ribaux, P., Ehses, J.A., Lin-Marq, N., Carrozzino, F., Boni-Schnetzler, M., Hammar, E., Irminger, J.C., Donath, M.Y., and Halban, P.A. (2007). Induction of CXCL1 by extracellular matrix and autocrine enhancement by interleukin-1 in rat pancreatic beta-cells. Endocrinology 148, 5582-5590.

Roussel, L., Houle, F., Chan, C., Yao, Y., Berube, J., Olivenstein, R., Martin, J.G., Huot, J., Hamid, Q., Ferri, L., et al. (2010). IL-17 promotes p38 MAPK-dependent endothelial activation enhancing neutrophil recruitment to sites of inflammation. Journal of immunology 184, 4531-4537.

Rouvier, E., Luciani, M.F., Mattei, M.G., Denizot, F., and Golstein, P. (1993). CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. Journal of immunology 150, 5445-5456.

Rudner, X.L., Happel, K.I., Young, E.A., and Shellito, J.E. (2007). Interleukin-23 (IL-23)-IL-17 cytokine axis in murine Pneumocystis carinii infection. Infect Immun 75, 3055-3061.

Ruggiero, V., Antonelli, G., Gentile, M., Conciatori, G., and Dianzani, F. (1989). Comparative study on the antiviral activity of tumor necrosis factor (TNF)-alpha, lymphotoxin/TNF-beta, and IL-1 in WISH cells. Immunology letters 21, 165-169.

Saito, T., Deskin, R.W., Casola, A., Haeberle, H., Olszewska, B., Ernst, P.B., Alam, R., Ogra, P.L., and Garofalo, R. (1997). Respiratory syncytial virus induces selective production of the chemokine RANTES by upper airway epithelial cells. J Infect Dis 175, 497-504.

Samuel, M.A., and Diamond, M.S. (2005). Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. Journal of virology 79, 13350-13361.

Samuel, M.A., and Diamond, M.S. (2006). Pathogenesis of West Nile Virus infection: a balance between virulence, innate and adaptive immunity, and viral evasion. J Virol 80, 9349-9360.

Savarin, C., Stohlman, S.A., Hinton, D.R., Ransohoff, R.M., Cua, D.J., and Bergmann, C.C. (2012). IFN-gamma protects from lethal IL-17 mediated viral encephalomyelitis independent of neutrophils. Journal of neuroinflammation 9, 104.

Shahrara, S., Pickens, S.R., Dorfleutner, A., and Pope, R.M. (2009). IL-17 induces monocyte migration in rheumatoid arthritis. Journal of immunology 182, 3884-3891.

Shen, F., Ruddy, M.J., Plamondon, P., and Gaffen, S.L. (2005). Cytokines link osteoblasts and inflammation: microarray analysis of interleukin-17- and TNF-alpha-induced genes in bone cells. J Leukoc Biol 77, 388-399.

Shibata, K., Yamada, H., Hara, H., Kishihara, K., and Yoshikai, Y. (2007). Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. Journal of immunology 178, 4466-4472.

Shresta, S., Pham, C.T., Thomas, D.A., Graubert, T.A., and Ley, T.J. (1998). How do cytotoxic lymphocytes kill their targets? Curr Opin Immunol 10, 581-587.

Shrestha, B., and Diamond, M.S. (2004). Role of CD8+ T cells in control of West Nile virus infection. Journal of virology 78, 8312-8321.

Shrestha, B., and Diamond, M.S. (2007). Fas ligand interactions contribute to CD8+ T-cell-mediated control of West Nile virus infection in the central nervous system. Journal of virology 81, 11749-11757.

Shrestha, B., Ng, T., Chu, H.J., Noll, M., and Diamond, M.S. (2008a). The relative contribution of antibody and CD8+ T cells to vaccine immunity against West Nile encephalitis virus. Vaccine 26, 2020-2033.

Shrestha, B., Pinto, A.K., Green, S., Bosch, I., and Diamond, M.S. (2012). CD8+ T cells use TRAIL to restrict West Nile virus pathogenesis by controlling infection in neurons. Journal of virology 86, 8937-8948.

Shrestha, B., Samuel, M.A., and Diamond, M.S. (2006a). CD8+ T cells require perforin to clear West Nile virus from infected neurons. Journal of virology 80, 119-129.

Shrestha, B., Wang, T., Samuel, M.A., Whitby, K., Craft, J., Fikrig, E., and Diamond, M.S. (2006b). Gamma interferon plays a crucial early antiviral role in protection against West Nile virus infection. Journal of virology 80, 5338-5348.

Shrestha, B., Zhang, B., Purtha, W.E., Klein, R.S., and Diamond, M.S. (2008b). Tumor necrosis factor alpha protects against lethal West Nile virus infection by promoting trafficking of mononuclear leukocytes into the central nervous system. Journal of virology 82, 8956-8964.

Siakavellas, S.I., and Bamias, G. (2012). Role of the IL-23/IL-17 axis in Crohn's disease. Discov Med 14, 253-262.

Sitati, E.M., and Diamond, M.S. (2006). CD4+ T-cell responses are required for clearance of West Nile virus from the central nervous system. Journal of virology 80, 12060-12069.

Song, X., and Qian, Y. (2013). The activation and regulation of IL-17 receptor mediated signaling. Cytokine 62, 175-182.

Stramer, S.L., Fang, C.T., Foster, G.A., Wagner, A.G., Brodsky, J.P., and Dodd, R.Y. (2005). West Nile virus among blood donors in the United States, 2003 and 2004. N Engl J Med 353, 451-459.

Stritesky, G.L., Yeh, N., and Kaplan, M.H. (2008). IL-23 promotes maintenance but not commitment to the Th17 lineage. Journal of immunology 181, 5948-5955.

Suen, W.W., Prow, N.A., Hall, R.A., and Bielefeldt-Ohmann, H. (2014). Mechanism of West Nile virus neuroinvasion: a critical appraisal. Viruses 6, 2796-2825.

Suthar, M.S., Diamond, M.S., and Gale, M., Jr. (2013). West Nile virus infection and immunity. Nat Rev Microbiol 11, 115-128.

Sutton, C., Brereton, C., Keogh, B., Mills, K.H., and Lavelle, E.C. (2006). A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. The Journal of experimental medicine 203, 1685-1691.

Tarlinton, D. (2008). IL-17 drives germinal center B cells? Nature immunology 9, 124-126.

Tilg, H., Moschen, A.R., and Kaser, A. (2009). Suppression of interleukin-17 by type I interferons: a contributing factor in virus-induced immunosuppression? Eur Cytokine Netw 20, 1-6.

Town, T., Bai, F., Wang, T., Kaplan, A.T., Qian, F., Montgomery, R.R., Anderson, J.F., Flavell, R.A., and Fikrig, E. (2009). Toll-like receptor 7 mitigates lethal West Nile encephalitis via interleukin 23-dependent immune cell infiltration and homing. Immunity 30, 242-253.

van den Berg, W.B., and Miossec, P. (2009). IL-17 as a future therapeutic target for rheumatoid arthritis. Nat Rev Rheumatol 5, 549-553.

van den Hurk, A.F., Hall-Mendelin, S., Webb, C.E., Tan, C.S., Frentiu, F.D., Prow, N.A., and Hall, R.A. (2014). Role of enhanced vector transmission of a new West Nile virus strain in an outbreak of equine disease in Australia in 2011. Parasites & vectors 7, 586.

Wang, P., Bai, F., Zenewicz, L.A., Dai, J., Gate, D., Cheng, G., Yang, L., Qian, F., Yuan, X., Montgomery, R.R., et al. (2012). IL-22 signaling contributes to West Nile encephalitis pathogenesis. PLoS One 7, e44153.

Wang, Q., Zhou, J., Zhang, B., Tian, Z., Tang, J., Zheng, Y., Huang, Z., Tian, Y., Jia, Z., Tang, Y., et al. (2013). Hepatitis B virus induces IL-23 production in antigen presenting cells and causes liver damage via the IL-23/IL-17 axis. PLoS Pathog 9, e1003410.

Wang, T., Town, T., Alexopoulou, L., Anderson, J.F., Fikrig, E., and Flavell, R.A. (2004a). Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med 10, 1366-1373.

Wang, Y., Lobigs, M., Lee, E., and Mullbacher, A. (2003). CD8+ T cells mediate recovery and immunopathology in West Nile virus encephalitis. Journal of virology 77, 13323-13334.

Wang, Y., Lobigs, M., Lee, E., and Mullbacher, A. (2004b). Exocytosis and Fas mediated cytolytic mechanisms exert protection from West Nile virus induced encephalitis in mice. Immunol Cell Biol 82, 170-173.

Welte, T., Aronson, J., Gong, B., Rachamallu, A., Mendell, N., Tesh, R., Paessler, S., Born, W.K., O'Brien, R.L., and Wang, T. (2011). Vgamma4+ T cells regulate host immune response to West Nile virus infection. FEMS Immunol Med Microbiol 63, 183-192.

Welte, T., Lamb, J., Anderson, J.F., Born, W.K., O'Brien, R.L., and Wang, T. (2008). Role of two distinct gammadelta T cell subsets during West Nile virus infection. FEMS Immunol Med Microbiol 53, 275-283.

Witowski, J., Ksiazek, K., and Jorres, A. (2004). Interleukin-17: a mediator of inflammatory responses. Cell Mol Life Sci 61, 567-579.

Xu, S., Han, Y., Xu, X., Bao, Y., Zhang, M., and Cao, X. (2010). IL-17A-producing gammadeltaT cells promote CTL responses against Listeria monocytogenes infection by enhancing dendritic cell cross-presentation. Journal of immunology 185, 5879-5887.

Yao, Z., Fanslow, W.C., Seldin, M.F., Rousseau, A.M., Painter, S.L., Comeau, M.R., Cohen, J.I., and Spriggs, M.K. (1995). Herpesvirus Saimiri

encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity 3, 811-821.

Yao, Z., Spriggs, M.K., Derry, J.M., Strockbine, L., Park, L.S., VandenBos, T., Zappone, J.D., Painter, S.L., and Armitage, R.J. (1997). Molecular characterization of the human interleukin (IL)-17 receptor. Cytokine 9, 794-800.

Ye, C., Abraham, S., Wu, H., Shankar, P., and Manjunath, N. (2011). Silencing early viral replication in macrophages and dendritic cells effectively suppresses flavivirus encephalitis. PLoS One 6, e17889.

Ye, P., Rodriguez, F.H., Kanaly, S., Stocking, K.L., Schurr, J., Schwarzenberger, P., Oliver, P., Huang, W., Zhang, P., Zhang, J., et al. (2001). Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. The Journal of experimental medicine 194, 519-527.

Yuan, J., Cao, A.L., Yu, M., Lin, Q.W., Yu, X., Zhang, J.H., Wang, M., Guo, H.P., and Liao, Y.H. (2010). Th17 cells facilitate the humoral immune response in patients with acute viral myocarditis. J Clin Immunol 30, 226-234.

Zelante, T., De Luca, A., Bonifazi, P., Montagnoli, C., Bozza, S., Moretti, S., Belladonna, M.L., Vacca, C., Conte, C., Mosci, P., et al. (2007). IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. Eur J Immunol 37, 2695-2706.

Zepp, J., Wu, L., and Li, X. (2011). IL-17 receptor signaling and T helper 17-mediated autoimmune demyelinating disease. Trends Immunol 32, 232-239.

Zhang, X., Gao, L., Lei, L., Zhong, Y., Dube, P., Berton, M.T., Arulanandam, B., Zhang, J., and Zhong, G. (2009). A MyD88-dependent early IL-17 production protects mice against airway infection with the obligate intracellular pathogen Chlamydia muridarum. Journal of immunology 183, 1291-1300.

Zhou, L., Ivanov, II, Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D.E., Leonard, W.J., and Littman, D.R. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nature immunology 8, 967-974.