DEFINING THE ROLE OF ALPHA-MACROGLOBULINS IN THE PATHOGENESIS OF FLAVIVIRUS ENCEPHALITIS

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ABSTRACT

West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV) are positive sense, single-stranded RNA viruses belonging to the family *Flaviviridae*, where the primary mode of transmission occurs through arthropod vectors such as mosquitos. WNV and JEV are the leading etiological agents for arboviral encephalitis in humans, and sporadic outbreaks continue to occur over time resulting in symptomatic infections that account for thousands of deaths each year. Although a number of vaccines have been developed to prevent JEV infections in highly endemic regions, issues with efficacy exist and the vaccine often fails to protect at risk populations from the development of encephalitis, which can be fatal. For both WNV and JEV, no clinically approved therapies currently exist for treatment of the central nervous system (CNS) involvement, which can lead to encephalitis, the most severe, and highly fatal form of the disease. Alpha-macroglobulins, which are physiological proteinase inhibitors have been shown to bind to viral proteins and enhance viral infections *in vitro*. Moreover, in humans alpha-macroglobulins such as pregnancy zone protein (PZP) and alpha-2-macroglobulin (A2M) also serve as immune-modulatory proteins where their normal functions within the body include binding and shuttling of protease inhibitors, growth factors, cytokines, hormones, disease factors, and various small molecule nucleophilic ligands. Previous reports from our laboratory have shown the up-regulation of alpha-macroglobulins in wild type (WT) mice after a lethal subcutaneous WNV infection. Therefore, to define the role of α-macroglobulins during flavivirus infection *in vivo*, we investigated the susceptibility of mice deficient in α-macroglobulins (PZP and MUG-1 double knockout; DKO) against lethal subcutaneous infection with either WNV or JEV. Results of our study show that DKO mice are completely resilient to lethal flaviviral infections of WNV and JEV. Likewise, DKO mice had a significantly milder

clinical disease through the course of the study, and this outcome can be coupled with the finding of significantly reduced viral burden in the blood, peripheral organs (kidney and spleen), and brains of the DKO mice when compared to WT mice. The DKO mice had a significantly reduced inflammatory response which was characterized by lower concentrations of pro-inflammatory cytokines and chemokines in the blood, spleen, and brain of DKO mice when compared to WT. Consistent with the multiplex immunoassay data, DKO mice also displayed a significantly decreased level of mRNA corresponding to immune genes in response to WNV infection when compared to WT. Overall, the data from this study demonstrates the significant impact that alpha-macroglobulins have in the pathogenesis of flavivirus-associated encephalitis in mice.

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ABBREVIATIONS

A1M	alpha-1-macroglobulin
A2M	alpha- 2-macroglobulin
AD	Alzheimer's disease
AFP	acute flaccid paralysis
AT III	antithrombin III
C	capsid
CNS	central nervous system
DKO	double knockout
E	envelope
ER	endoplasmic reticulum
IBD	inflammatory bowel disease
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
LDL	low-density lipoprotein
LRP	low-density lipoprotein receptor related protein
M	membrane
MUG-1	murioglobulin-1
NS	nonstructural
PZP	pregnancy zone protein
RA	rheumatoid arthritis
RdRp	RNA-dependent RNA polymerase
RSV	respiratory syncytial virus

ROS	reactive oxygen species
SLEV	St. Louis encephalitis virus
US	United States
WML	white matter lesions
WNV	West Nile Virus
WNVND	West Nile Virus neuroinvasive disease
WT	wild type
ZIKV	Zika Virus

Chapter 1

Background

1.1 **West Nile Virus:**

1.1.1 History

West Nile Virus (WNV) is a neurotropic flavivirus that has spread across the globe and as a result has now become endemic to many regions of the world; however, the virus was originally isolated from a febrile 37-year-old woman participating in a malaria survey study in the West Nile district of Uganda in 1937.[1] Following the original isolation and identification of the virus, various outbreaks have occurred in humans infrequently, with some of the most notable outbreaks occurring in Israel (1951 – 1954 and 1957), as well as in South Africa (1974). More recently, other sporadic outbreaks have also occurred in Romania and Morocco (1996), Tunisia (1997), Italy (1998), Russia, Israel, and the United States (1999), and Israel, France, and the United States (2000).[2]

1.1.2 Epidemiology

Prior to the introduction of WNV in the United States (US), epidemic arbovirus outbreaks were relatively scarce with the exception of sporadic outbreaks of another closely related flavivirus, St. Louis encephalitis virus (SLEV), causing outbreaks in the mid to late 1970's. However, during 1999 in New York a cluster of human encephalitis cases occurred that was consistent with arboviral etiology, and the causative agent was later discovered to be WNV.[3] Only three years after its initial introduction to the east coast of the United States, WNV had spread throughout the majority of the contiguous US and even into the neighboring countries of Canada and Mexico.[4] Although symptomatic cases of WNV infection are not common in Central and South America, WNV has been detected during field surveillance efforts of vector specimens, which highlights the risk for a potential outbreak in human populations living in these regions as well.

To further highlight the spread of WNV, since its initial discovery in 1937 the virus has spread to the majority of the globe and is considered the most important causative agent for viral encephalitis worldwide.[4] *Figure 1* shows the global distribution of WNV by country, with the countries in red having human cases or human seropositivity, while the countries in blue show nonhuman or mosquito cases and seropositivity only. Countries in gray have no reported data, and the black lines represent the distribution of the mosquito vector (excluding the regions marked by dashed lines) and the circled numbers correspond to the number of lineages of WNV reported in that area.[4]

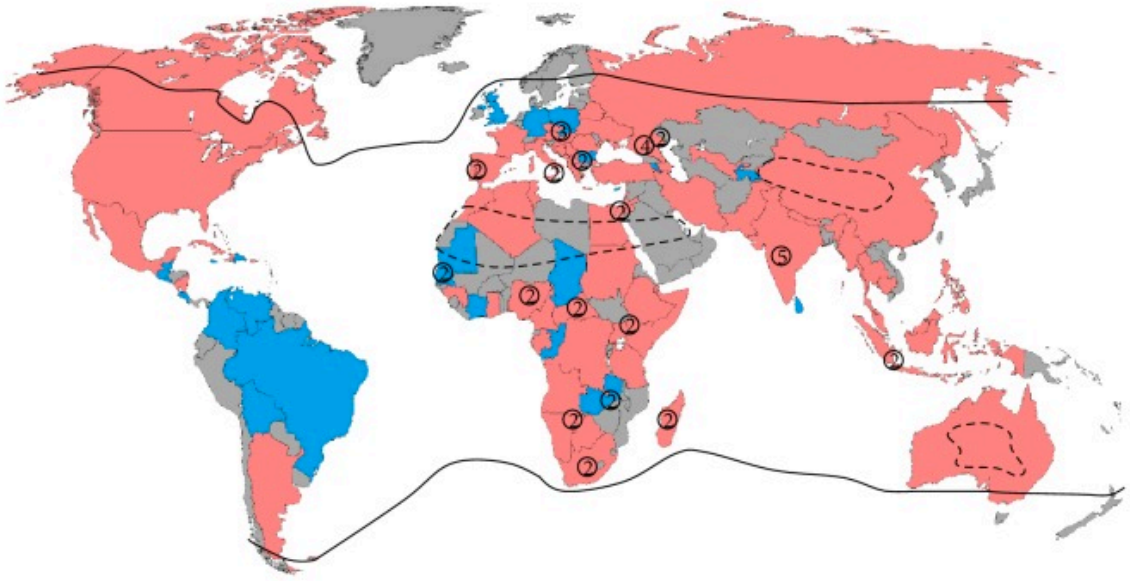


Figure 1: Global Distribution of WNV by Country[4]

1.1.3 Virology

WNV belongs to the genus *Flavivirus* which is within the family *Flaviviridae.* It has been classified as an arbovirus, meaning transmission occurs through arthropods, and in this case the female *Culex* mosquito is the culprit. WNV is an enveloped positive-sense single stranded RNA virus with a genome that is 11,029 nucleotides in length which code for ten viral proteins. Out of

the ten viral proteins three are considered structural proteins which have been termed the capsid (C), membrane (prM/M), and envelope (E); while the other seven proteins encoded by the WNV genome are considered nonstructural (NS) proteins and are numbered NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.[5] *Figure 2* shows a schematic diagram of the WNV genome, where one open reading frame is present and a distinction between structural and non-structural proteins is made on the figure. The resultant ten proteins are produced by proteolytic processing of the single polyprotein, and this is done so by the viral serine protease, or NS2B-NS3, as well as other host cellular proteases.[5]

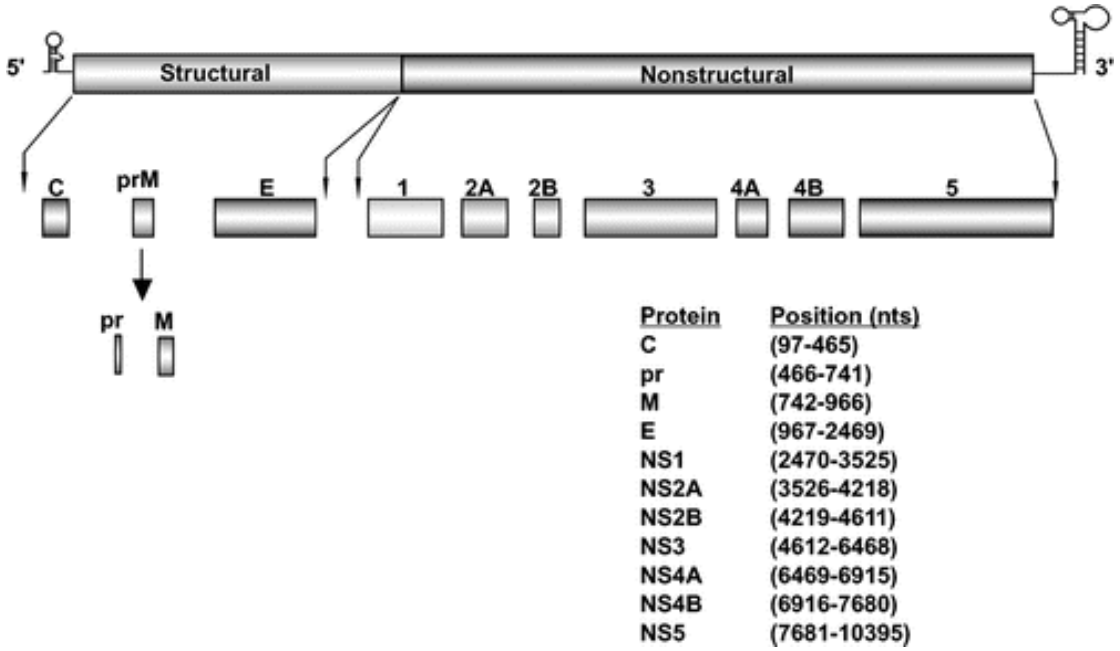


Figure 2: WNV Genome[5]

To further demonstrate the organization of the WNV virion and genome, a schematic diagram of the WNV virion is shown in *Figure 3*. The center of the particle contains the RNA genome (green) which is then directly surrounded by the capsid protein (blue), followed by the membrane (yellow), and finally the outer coating of the virus particle which is made up of the pre-membrane/membrane (prM/M) protein (gray) as well as the envelope protein dimers (red).[4]

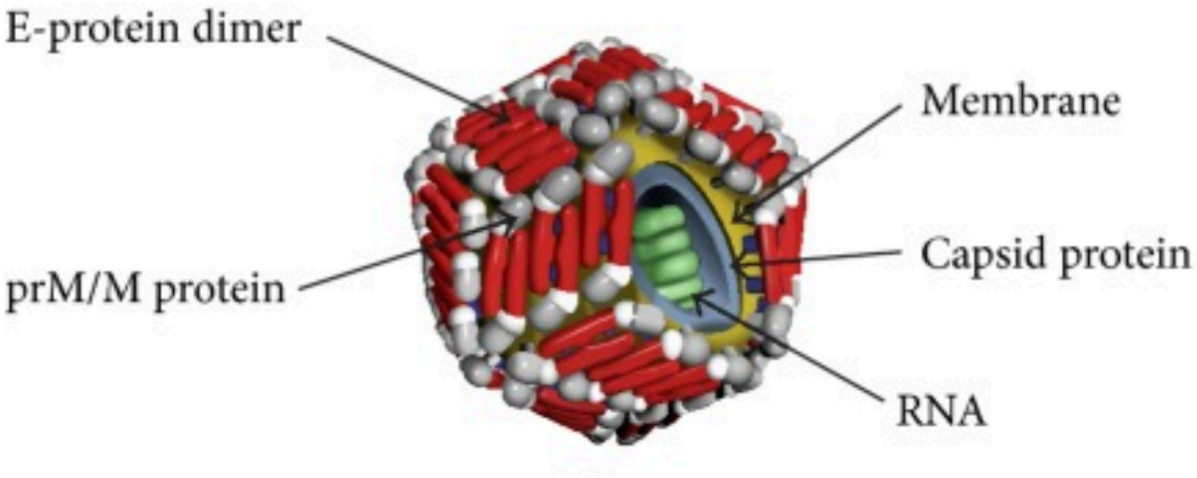


Figure 3: WNV Virion[4]

1.1.4 Lifecycle and Replication

The initial step in the lifecycle and replication of WNV is for the virus to bind to host cell receptors, which have yet to be fully elucidated, but this binding allows for the virus to enter host cells to hijack host cell machinery in order to replicate itself.[5] The viral replication cycle is shown in *Figure 4* where steps A through I correspond to important events that take place within the lifecycle of the virus upon infection of host cells. Upon entry into the cell via host cell receptor mediated endocytosis, the viral membrane goes through a low-pH fusion with the endosomal vesicle membrane which allows for the release of the viral genome into the cytoplasm of the cell, as shown in step A.[5] The viral genome is then translated into a single polyprotein (shown in B) as previously mentioned, which is subsequently cleaved by the serine protease NS2B-NS3 as well as other host cellular proteases leaving the ten mature viral proteins, shown in C.[5] A key step in the replication of WNV involves the RNA-dependent RNA polymerase (RdRp), NS5, as well as other proteins. In this step the enzyme RdRP as well as the other proteins copy complementary negative sense strands of RNA (shown in D) to be used subsequently as templates to synthesize new viral genetic materials (as shown in E and F). Virion

assembly occurs in the host rough endoplasmic reticulum (ER) membranes (as shown in G), then E and prM proteins on the surface of the virions are altered prior to the budding and release of the mature WNV particles (shown in H and I).[5]

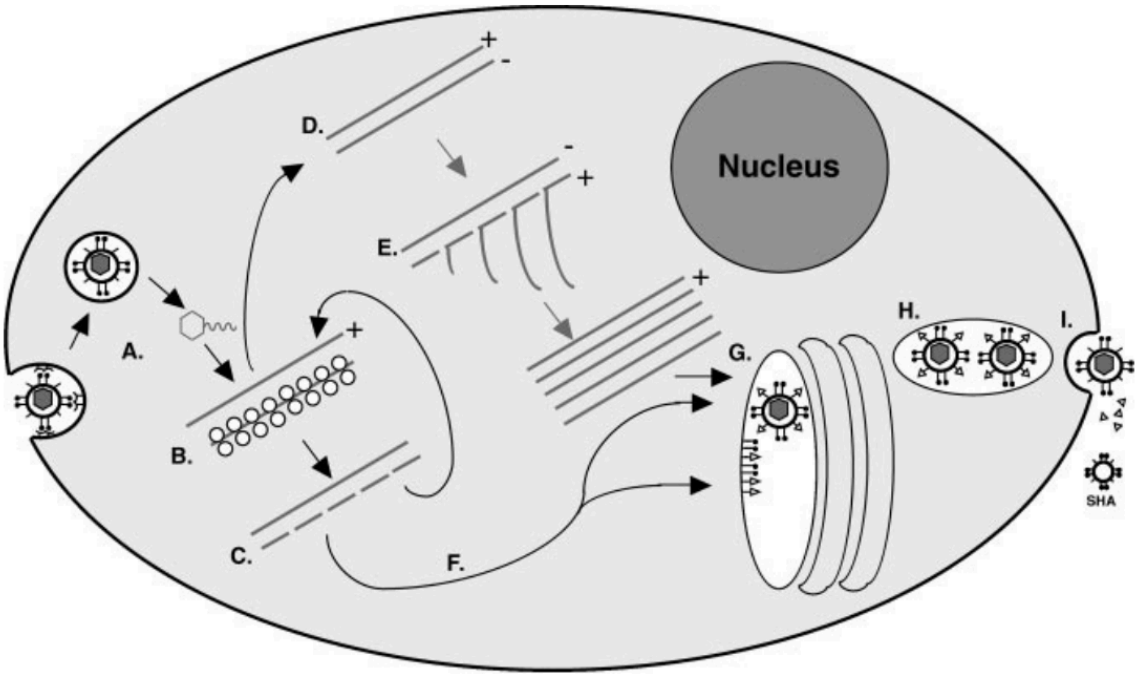


Figure 4: WNV Replication Cycle[5]

1.1.5 WNV Clinical Disease

Since its initial introduction to New York in 1999, there have been many cases of WNV infection in the United States resulting in consistent proportions of mortality in patients who develop West Nile Virus Neuroinvasive Disease (WNVND), ranging anywhere from 6% to 16%, or 2 to 276 total deaths annually, shown in *Table 1*.[6] Although WNVND overall is considered rare and occurs in approximately 1% of total WNV infected people, neurological complications can have long lasting clinical implications and can potentially be fatal. High risk groups for development of WNVND after infection with WNV include the elderly, immunocompromised, and child populations, which is shown in *Figure 5*.[7] Approximately 80% of total infections are

asymptomatic, meaning that the majority of individuals who become infected will likely be unaware; however, in the other 20% of infections symptomatic cases of WNV infection occur and the individual will experience a flu-like illness and show symptoms of malaise, eye pain, headache, myalgia, gastro-intestinal discomfort and rash.[8] In rare cases, approximately 1%, WNVND will develop and the individual may develop encephalitis, meningitis, or acute flaccid paralysis (AFP), and long-term neurological sequelae are common in more than 50% of these cases.[8]

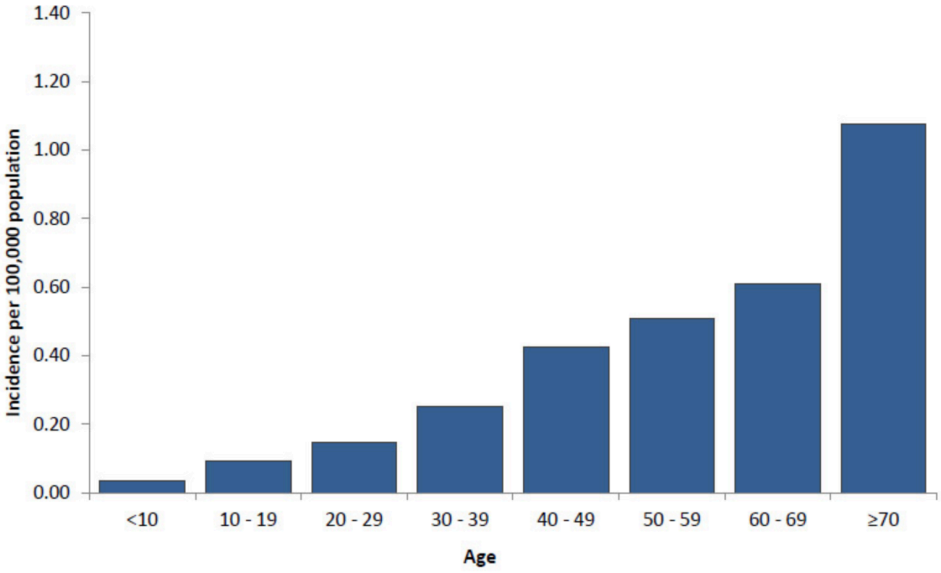
West Nile virus disease cases and deaths reported to CDC by year and clinical presentation, 1999-2016

	Neuroinvasive disease			Non-neuroinvasive disease			Total		
	Cases	Deaths		Cases	Deaths		Cases	Deaths	
Year	No.	No.	(%)	No.	No.	(%)	No.	No.	(%)
1999	59	7	(12)	3	0	(0)	62	7	(11)
2000	19	2	(11)	2	0	(0)	21	2	(10)
2001	64	10	(16)	2	0	(0)	66	10	(15)
2002	2,946	276	(9)	1,210	8	(1)	4,156	284	(7)
2003	2,866	232	(8)	6,996	32	(<1)	9,862	264	(3)
2004	1,148	94	(8)	1,391	6	(<1)	2,539	100	(4)
2005	1,309	104	(8)	1,691	15	(1)	3,000	119	(4)
2006	1,495	162	(11)	2,774	15	(1)	4,269	177	(4)
2007	1,227	117	(10)	2,403	7	(<1)	3,630	124	(3)
2008	689	41	(6)	667	3	(<1)	1,356	44	(3)
2009	386	32	(8)	334	0	(0)	720	32	(4)
2010	629	54	(9)	392	3	(1)	1,021	57	(6)
2011	486	42	(9)	226	1	(<1)	712	43	(6)
2012	2,873	270	(9)	2,801	16	(1)	5,674	286	(5)
2013	1,267	111	(9)	1,202	8	(<1)	2,469	119	(5)
2014	1,347	87	(6)	858	10	(1)	2,205	97	(4)
2015	1,455	142	(10)	720	4	(<1)	2,175	146	(7)
2016	1,309	105	(8)	840	1	(<1)	2,149	106	(5)
Total	**21,574**	**1,888**	**(9)**	**24,512**	**129**	**(<1)**	**46,086**	**2,017**	**(4)**

Source: ArboNET, Arboviral Diseases Branch, Centers for Disease Control and Prevention

Table 1: CDC reported WNV disease cases and deaths[6]

Average annual incidence of West Nile virus neuroinvasive disease reported to CDC by age group, 1999-2016



Source: ArboNET, Arboviral Diseases Branch, Centers for Disease Control and Prevention

Figure 5: CDC WNVND incidence[6]

1.1.6 Therapeutics and Vaccines

Unfortunately, to this date no clinically approved vaccines or treatment options for WNV disease are currently available for human use. There are, however, a number of ongoing clinical trials that are attempting to utilize treatment options such as interferon-alpha, corticosteroids, and WNV-specific immunoglobulin. Due to the fact that these clinical studies are ongoing, the overall efficacy of these treatment approaches has yet to be determined.[9] Ribavirin, which is a clinically approved drug used to treat other viral infections such as Respiratory Syncytial Virus (RSV) and Hepatitis C virus, was administered to patients with WNV disease during an outbreak in Israel in 2000, but unfortunately these treated patients did not show any improvement compared to untreated controls.[9] On the other hand, a vaccine approved for use in equine has been developed. Horses show a similar WNV disease process as that of humans in the sense that

severe neurological disease occurs in horses, which can ultimately lead to death or euthanasia.[10] Many vaccine platforms have been attempted for use in horses, however very limited data is available on the efficacy of these vaccines used in horses. Vaccine platforms such as inactivated whole virus vaccines, recombinant subunit vaccines, live attenuated or pseudo-infectious virion vaccines, live chimeric/recombinant virion vaccines, and DNA-vectored vaccines are currently being developed and tested in animals.[11] Conversely, a vaccination approach for the prevention of WNV disease may not be the most ideal solution to the problem given the previous clinical data showing that the majority of the population remains asymptomatic after infection (~80%) and the majority of symptomatic infections result in a mild-febrile illness without CNS involvement.[11] Due to these factors, a therapeutic treatment option that addresses the most severe forms of WNV disease rather than prophylactic vaccination seems to be the most logical approach in hopes of preventing mortality and long lasting neurological complications due to WNV infections.

1.2 **Japanese Encephalitis Virus:**

1.2.1 History

Similar to WNV, Japanese Encephalitis virus (JEV) is a neurotropic flavivirus that is the leading cause of viral encephalitis in Asia.[12] Japanese encephalitis (JE) outbreaks have been noted to occur as early as the 1800's in Japan, but the first confirmed JEV case was reported in Japan in 1924.[12] Other sporadic cases have also been confirmed in Korea (1933), China (1940), the Philippines (1950), India (1955), and a number of other Asian countries thereafter. Over the past few decades the incidence of JE has increased in countries such as Bangladesh, Cambodia, India, Indonesia, and Pakistan.[12] Currently, JEV affects approximately 25 Asian countries and

approximately 60% of the global population are at risk for infection with JEV. Another public health consideration for JEV is the absence of protective immunity for travelers who vacation to endemic regions where JEV transmission is ongoing through the mosquito vector.[12]

1.2.2 Epidemiology

Although JEV transmission and infection has been historically confined to its endemic regions in Asia, in the late 1990's JEV emerged in the Torres Strait Islands where subsequent spread to the Cape York Peninsula occurred. This event alerted public health officials to the potential risk of a serious health threat to the population of Australia, and demonstrated a possible spread of the virus throughout the world.[12] *Figure 6* shows the geographic distribution of the four members of the JE serological group, and from the data it is clear that these viruses have the potential to spread globally through arthropod vectors and zoonotic reservoirs such as birds.[12] In the early 1970's more than 100,000 cases of JE were reported each year with the vast majority of cases occurring in China.[13] However more recently due to vaccination efforts, increased urbanization, changes in agricultural practices, and mosquito control the annual reported cases of JE has declined significantly, but around 30,000 cases are reported each year. Although these numbers may not be entirely accurate due to poor diagnostic and surveillance capabilities in those countries where JEV infections are common.[13] JE can also be characterized as primarily a childhood disease because the vast majority of infections occur in children 15 years of age or less.[13]

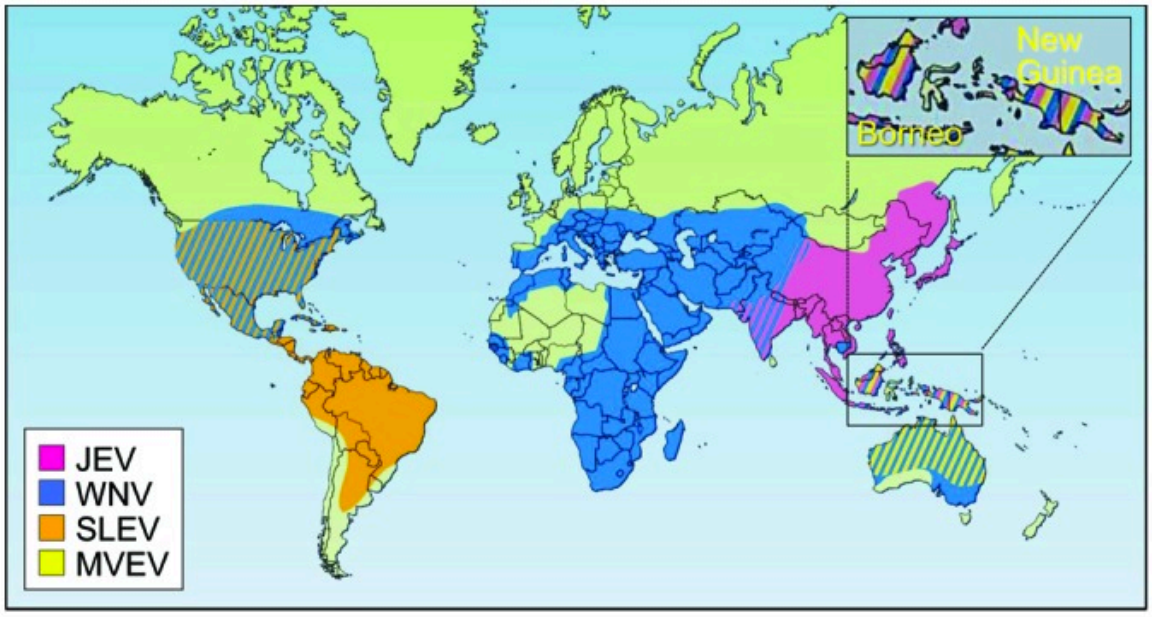


Figure 6: Geographic distribution of four members of the JE serological group[12]

1.2.3 Virology

JEV is a member of the genus *flavivirus* and belongs to the family *flaviviridae* where it is considered the prototype virus of the JE serological group which includes the previously discussed viruses such WNV and SLEV, among others. Similar to WNV, JEV is a positive-sense single-stranded RNA virus where its 11 kilo-base genome encodes for a total of ten viral proteins where three are considered structural proteins (capsid, pre-membrane/membrane, and envelope), and the remaining seven are considered non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). For the case of JEV, an initial single polyprotein is translated and subsequently cleaved into the ten viral proteins which is a similar process to that of WNV.[12] *Figure 7* represents a schematic diagram of the JEV genome and consequent gene expression.

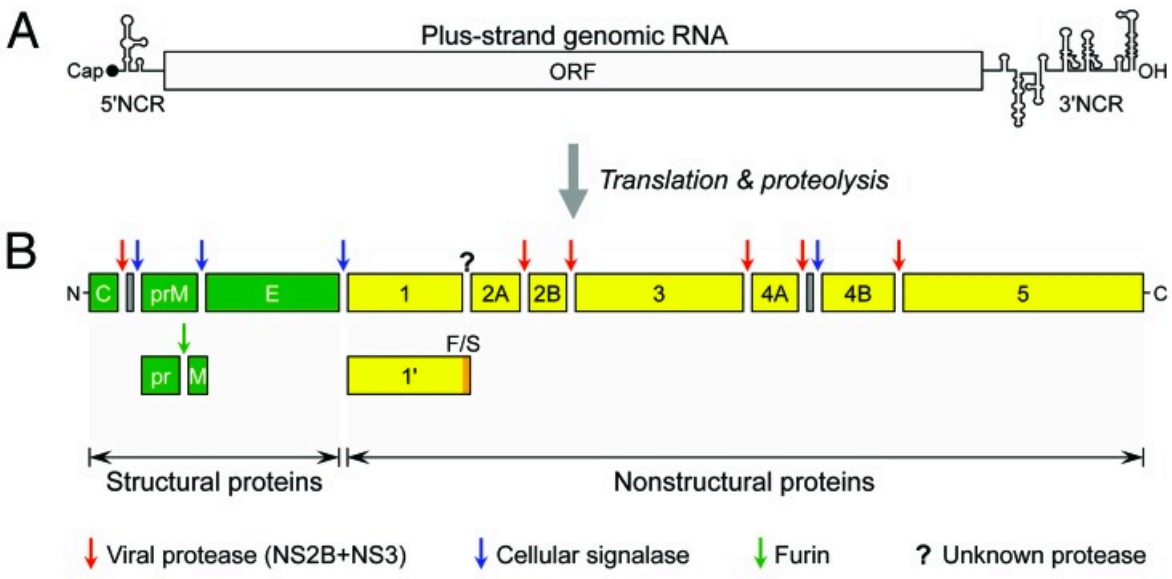


Figure 7: JEV genome structure and gene expression[12]

1.2.4 Lifecycle and Replication

The lifecycle and replication of JEV is first initiated by the binding or attachment of an infectious virus particle to host cell receptors located on the plasma membrane. After the initial binding, the virus is internalized via receptor mediated endocytosis. Due to a low pH within the host cell endosome, conformational changes occur in the viral E glycoprotein which then triggers the fusion of the viral membrane to the host endosome membrane. As a result of this binding the virus particle uncoats and the genetic material of the virus, or the viral RNA, is released into the cytoplasm of the host cell. Due to the fact that JEV is a positive-sense single-stranded RNA virus, the viral RNA is then directly translated through utilization of host cell rough ER, which results in the formation of the single polyprotein as previously described. This polyprotein is then processed into the ten mature viral proteins which allow for subsequent viral genomic RNA replication within the host cell, while virus particle assembly also occurs inside the virus-induced, ER-derived vesicles. Prior to budding, immature virion particles form a budding

complex where newly synthesized genomic RNA and C proteins associate in the lumen of the host ER. In the lumen of the host ER these immature virion particles then acquire the prM and E proteins on their membranes. The virions are still not yet considered mature and are transported to the host Golgi apparatus through the secretory pathway. Cleavage of the prM to M initiates the maturation of virus particles in the trans-Golgi network and the viral particles are then ready for budding, or release from the host cell. This process is achieved through exocytosis of the virus which results in progeny virus being released from the cell into the extracellular environment. *Figure 8* illustrates the replication and lifecycle of JEV as previously described.[12]

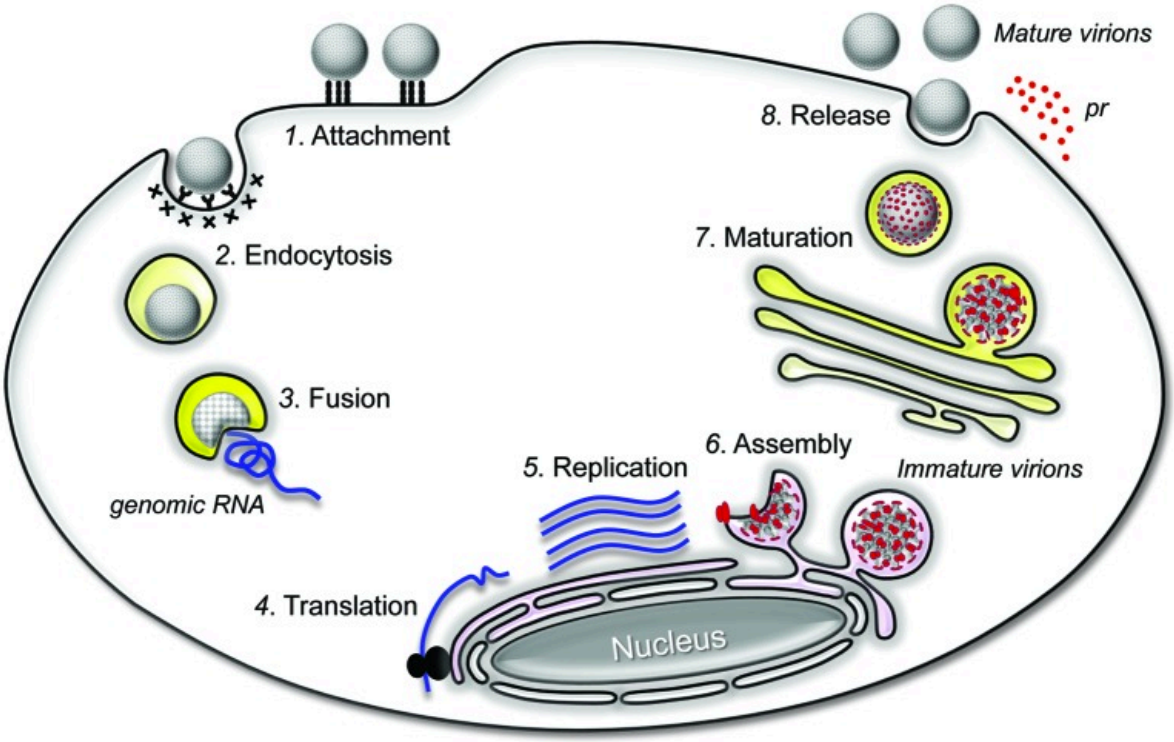


Figure 8: JEV replication cycle[12]

1.2.5 JEV Clinical Disease

With the majority of JEV infections being asymptomatic, an estimated <1% of people infected with the virus develop a clinical disease.[13] JEV has been characterized to show an incubation

period ranging anywhere from 5 to 15 days, meaning that clinical signs and symptoms of illness due to JEV infection will not be present until 5 to 15 days after the initial infection has occurred. JEV disease can show a wide array of clinical signs and symptoms through the course of disease, usually beginning with an acute fever, headache, and vomiting which can then progress to more severe symptoms that are more suggestive of CNS involvement; for example, mental status change, focal neurological deficits, generalized weakness, and movement disorders.[13] Among children infected with JEV seizures are common, however the classical description of JE includes a parkinsonian syndrome with mask-like facies, tremor, cogwheel rigidity, and choreoathetoid movements.[13] Another clinical feature includes acute flaccid paralysis resembling poliomyelitis, and the most common complications associated with poor outcome and death due to JEV infection include status epilepticus, brain hypoxia, increased intracranial pressure, brainstem herniation, and aspiration pneumonia.[13] Convulsions and abnormal behavior are common in children, whereas febrile illness and meningitis occur frequently in adults.[12] JE has a case fatality ratio estimated to be between 20% - 30% and although some motor deficits and movement disorders improve after the acute illness, approximately 30% to 50% of JE survivors have neurologic or psychiatric sequelae even years after their infections. These sequelae include seizures, upper and lower motor neuron weakness, cerebellar and extrapyramidal signs, flexion deformities of the arms, hyperextension of the legs, cognitive deficits, language impairment, learning difficulties, and behavioral problems.[13]

1.2.6 Therapeutics and Vaccines

Currently, there are four different types of JEV vaccines which are available for human use around the world. *Table 2* shows a summary for these four vaccines and illustrates the

differences between vaccine type, JEV strain used, and the vaccine name.[12] Of these four vaccines only two are approved for use in humans within the United States, the inactivated mouse brain--derived vaccine (JE-VAX [JE-MB]) and an inactivated Vero cell culture-derived vaccine (IXIARO [JE-VC]).[13] The JE-MB vaccine has been licensed for use in the United States since 1992 and is occasionally recommended for travelers aged ≥1 year who plan to travel to JEV endemic regions.[13] Due to the fact that several effective vaccines are available in Asia for human use, efficacy trials that randomized and controlled would not be ethically responsible and logistically difficult. The JE-VC vaccine was licensed based off of its ability to elicit JEV-specific neutralizing antibodies, which studies have shown to be a reliable evaluation of efficacy.[13] Although there are multiple vaccines available for human use, it is unlikely that the entire at-risk population will be vaccinated, and even though the vaccines have greatly reduced the JEV disease prevalence, only approximately 80% protective efficacy has been demonstrated. Therapeutic options for treatment of JE consists of only supportive care and management of clinical complications, and no specific antiviral medications have been developed to treat the severe forms of JEV disease.[13] In controlled clinical trials, corticosteroids, interferon-alpha-2a, and ribavirin were tested, but failed to improve the clinical outcome.[13] Due to these factors, it is clear that development of effective therapies against severe forms of JEV disease should be a research priority to address this public health concern.

Vaccine type	JEV strain	Vaccine name (Manufacturer)
Mouse brain-derived killed-inactivated	Nakayama/Beijing-1 (P1)	JE-VAX (BIKEN[a])
Cell culture-derived live-attenuated	SA_{14}-14-2	SA14-14-2 (CDIBP[b])
	Beijing-1 (P1)	JEBIK V (BIKEN); ENCEVAC, KD-287, or JEIMMUGEN INJ (Kaketsuken)
Cell culture-derived killed-inactivated	Beijing-3 (P3)	
	SA_{14}-14-2	IC51, IXIARO, JESPECT, or JEEV (Intercell AG)
Cell culture-derived live-attenuated chimeric	SA_{14}-14-2	ChimeriVax-JE, IMOJEV, JE-CV, or THAIJEV (Sanofi-Aventis)

[a]BIKEN, Research Foundation for Microbial Diseases of Osaka University. [b]CDIBP, Chengdu Institute of Biological Products.

Table 2: Summary of 4 different types of JEV vaccines[12]

1.3 Alpha-macroglobulins:

1.3.1 Human alpha-macroglobulins

Alpha-macroglobulins are an integral part of host innate immunity and for this reason these large glycoproteins have been evolutionarily conserved among invertebrates and vertebrates alike.[14] Proteins included in the alpha-macroglobulin family are the alpha-1-macroglobulin (A1M), alpha- 2-macroglobulin (A2M), complement components (C3, C4, and C5), and pregnancy zone protein.[14] For the case of A2M, it can function as an antiproteinase and is present in high abundance in human plasma, anywhere from 2 – 4 mg/mL, and factors such as the individuals diet, as well as other disease and immune status related factors such as diabetes can account for this variation.[14] Human A2M can be classified as a homotetramer, meaning the protein is made up of four identical subunits, which are bound together by a non-covalent association of two disulphide-bonded pairs of subunits.[14] Human A2M is unique in its function in that it can inhibit virtually any proteinase present in plasma, as well as from proteinases from other sources, but it also functions as the delivery vehicle of proteinases to an endocytic proteinase clearance pathway.[14] These alpha-macroglobulins are mainly synthesized within the liver; however, they are also locally synthesized by many important cell types in the context of WNV disease and

WNVND such as macrophages, fibroblasts, and cell populations within the brain tissue.[14] *Figure 9* shows a diagram of the mechanism that allows the A2M protein to entrap proteinases; up to two small proteinases per subunit or one large proteinase per subunit can be entrapped.[14] A2M can also bind to many other proteins, and overall functions as an immune-regulatory protein by binding and shuttling pro-inflammatory cytokines and chemokines during the host inflammatory response.[14] Again in the context of WNV disease, human A2M has been shown to bind to neurotrophic factors; and these neurotrophic factors are responsible for promoting neuron survival, maintenance and development of neurons, as well as the maintenance of neuronal homeostasis in response to injury.[14] It is clear that human alpha-macroglobulins have many function throughout the body in various tissues, and *Table 3 and Figure 10* highlight some of these functions of A2M.[15,16] A2M is known to bind to the low-density lipoprotein (LDL) receptor related protein, or LRP, and this receptor is present on many cell types throughout the body.[15] Due to the previously stated factors, examination of A2M in the context of WNV disease is warranted.

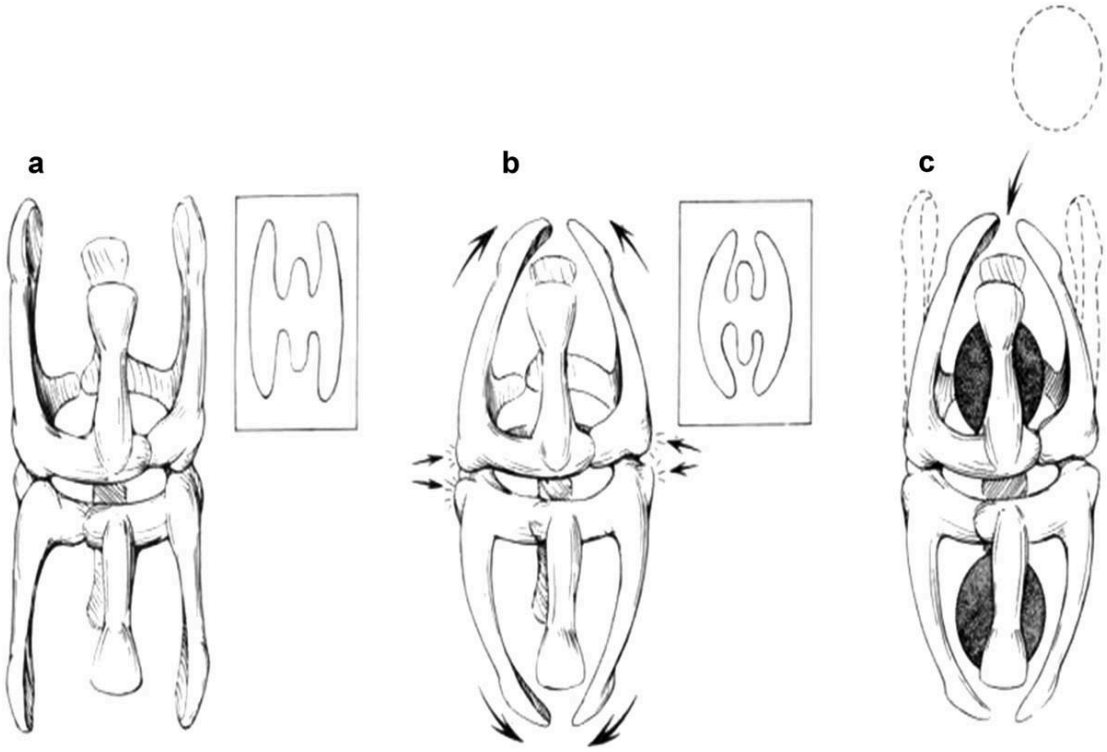


Figure 9: Model of Human A2M[14]

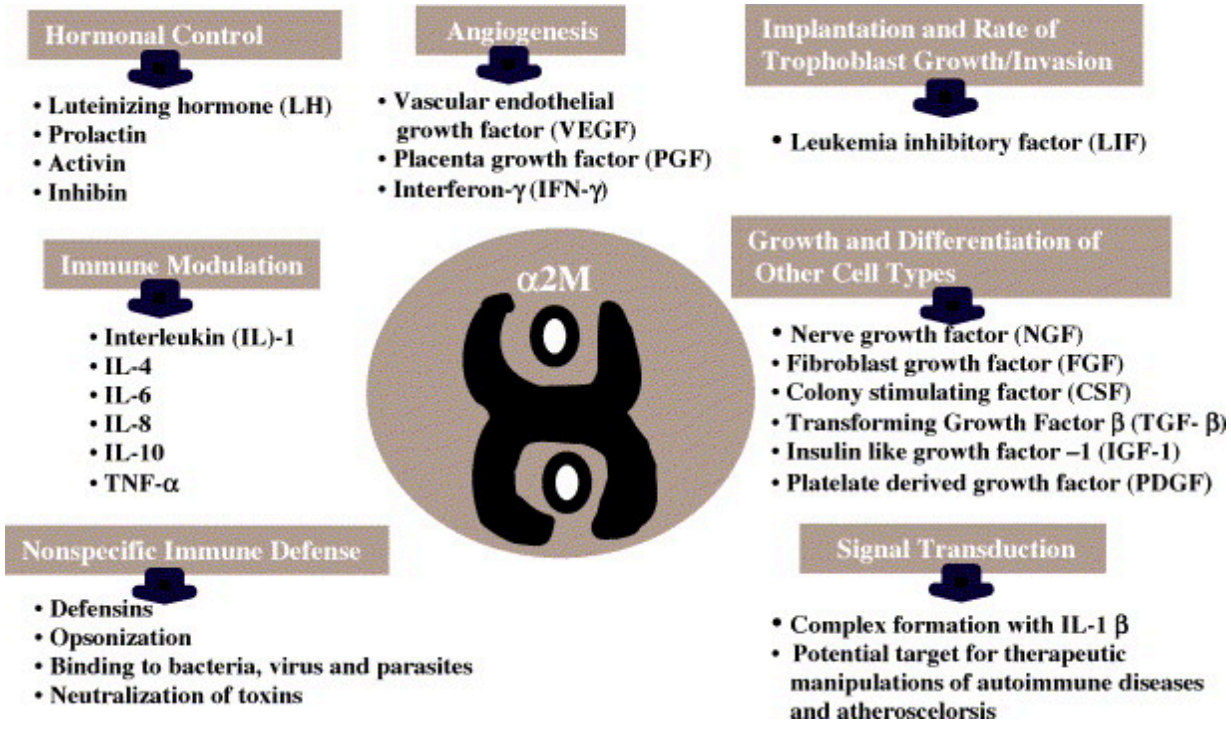


Figure 10: The heterogeneity of binding partners (white circles) and functions of α2M[16]

α_2-Macroglobulin	Active site inhibitors
Inhibits the proteolytic activity of proteases without inhibiting the hydrolysis of low molecular mass amide or ester substrates	Inhibits activity of target proteases against polypeptide and low molecular mass substrates
Reacts with endopeptidases of diverse catalytic mechanisms and substrate specificities	Reacts with a narrow spectrum of related proteases
Shields bound proteases from antibodies and high molecular mass active site inhibitors	Bound proteases remain reactive with antibodies
Presence of a unique internal reactive thiol ester group	Internal thiol ester is found only in proteins of the α_2-macroglobulin family

Table 3: Comparison of α2-macroglobulin with active site protease inhibitors[15]

1.3.2 Mouse alpha-macroglobulins

Overall much less is known about mouse alpha-macroglobulins; however, it is known that there are two main mouse alpha-macroglobulins present in the plasma of mice and these are known as PZP, formerly termed mouse alpha-2-macroglobulin (MA2M), and murioglobulin-1 (MUG-1).[14] PZP is a tetrameric protein and a close homologue of human A2M, and like human A2M the mouse PZP protein is highly expressed within the liver of adult mice.[17] On the other hand, MUG-1 is a single chain proteinase inhibitor and is primarily identified in the plasma of mice, and is similarly present at high concentrations from anywhere between 1 – 2 mg/mL.[14] Human A2M and mouse PZP are comparable in the sense that they share broad proteinase-inhibitory specificities and undergo equivalent proteinase-induced conformational changes.[17] An alpha-macroglobulin deficient mouse model has been created, and these alpha-macroglobulin mice

develop normally and are fertile. Interestingly, the alpha-macroglobulin deficient mice have been reported to respond differently than a comparable wild-type mouse when challenged with certain exogenous agents and have shown increased resistance to the lethal effects of endotoxins.[17] The inhibition of proteinases functionality of mouse PZP and MUG-1 have been shown to be partially redundant, however the growth factor carrier activities of these proteins are independent. During pregnancy an increased level of MUG-1 has been reported to be present in wild type mice, and this suggests that it plays a role in the immunological state during pregnancy.[17] In another mouse study it has been shown that treatment with mouse PZP via microparticles protected mice from sepsis, preserved neutrophil chemotactic responses in the presence of endotoxin, and stimulated bacterial phagocytosis as well as reactive oxygen species (ROS) production via its receptor LRP1.[18]

1.3.3 Alpha-macroglobulins and diseases

Alpha-macroglobulins have been shown to have important roles in the host immune regulation but have also been shown to play an important role in the context of certain infectious and immunological diseases. After parasitic infection with *Trypanosoma cruzi*, or *T. cruzi*, it has been shown that A2M can regulate cellular apoptosis that is triggered by the infection and can limit the generation of hydrogen peroxide (H_2O_2) from polymorphonuclear leukocytes, as well as clear unfolded and misfolded proteins in extracellular spaces as a result of the infection.[14] Likewise, A2M can bind endotoxins produced during bacterial infections and can protect tissues within the body from the harmful effects of the endotoxins.[14] A2M has been shown to bind viral particles and allow for uptake of the virus into host immune cells such as macrophages, as well as antigen presenting cells. In this sense A2M helps to bridge the gap between the innate and

adaptive responses of the host immune system in the acute phase of viral infections.[19] In the case of disease as a result of immunological dysregulation, A2M can be considered a diagnostic marker for several disease processes such as liver fibrosis, cardiac hypertrophy, differentiating myocardial infarcted diabetic patients from diabetic patients without myocardial infarction, and HIV with cardiac manifestations.[14] In the context of human neuronal diseases, which again highlights the important of the role of A2M in WNVND, A2M has also been characterized as a marker for preclinical Alzheimer's disease (AD) which reflects neuronal injury in these individuals.[20] On the other hand, A2M has been tested as a vaccine adjuvant and may be a beneficial antigen delivery system for the formulations of vaccines to promote a greater adaptive immune response, as well as a drug delivery system.[14]

Condition/activity	Disease/pathology
Downregulated	Rheumatoid arthritis in women
Upregulated	Chronic liver disease, inflammatory joint diseases, multiple sclerosis, Binswanger's disease, nephrotic syndrome
Accumulation in cells	Aging

Table 4: Role of α2M in various pathophysiological conditions[14]

Human A2M can be used as a marker in the diagnosis and prognosis in a number of disease processes, and in the case of liver fibrosis the use A2M as a clinical biomarker for disease may be a substitute for the standard risky and invasive procedure of a liver biopsy.[14] The serum concentration of A2M changes during the process of liver fibrosis and observation can be used to indicate the stage of damage and disease progression in the liver in Hepatitis C infection.[14] The cardiac isoform of human A2M in serum can be used as an early marker in cardiac hypertrophy and ventricular mass, and in diabetic patients this can also be used as a diagnostic marker for

myocardial infarction.[14] Likewise, the cardiac isoform of A2M in serum has also been shown to be a clinical biomarker for the onset and presence of heart disease such as ventricular septal defect, atrial septal defect, aortic regurgitation, aortic stenosis etc., resulting from abnormal left ventricular hypertrophy in HIV infected patients.[21] Patients who have undergone distal or total gastrectomy for the treatment of gastric cancer have been shown to have significantly higher concentrations of A2M in their blood.[14] In pancreatitis, the amount of complexed A2M has been shown to increase with the severity of the attack, and in the case of Inflammatory Bowel Disease (IBD) A2M is excreted in high concentrations and this also has the potential to be used as a clinical diagnostic biomarker.[14] The concentration of A2M, as well as myeloperoxidase and C-reactive protein, can be measured in urine and provides a non-invasive differential diagnostic tool for renal graft dysfunction.[14] A2M and A2M-proteinase complexes have also been employed in clinical use as a functionally relevant biomarker in the male genital tract secretion for prostate complications.[14] In the consideration of thrombosis for patients who have deficiency of antithrombin III (AT III), A2M is able to prevent thrombosis from occurring in children because they express A2M at higher levels than adults, which puts adults at a greater risk. Therefore A2M has the potential to be used as a clinical treatment in adult AT III deficient patients experiencing thrombosis.[14] A2M isolated from the synovial fluids in patients suffering from Rheumatoid Arthritis (RA) has been shown to be oxidized seven fold higher than healthy controls, and has a potential to be used as a clinical biomarker in the disease process of RA as well.[22] Other studies have shown that serum levels of A2M are significantly increased in patients who have suffered an ischemic stroke, and that there are strong correlations between concentrations of A2M in the blood and white matter lesions (WML).[23] WML are known to be associated with aging and ventricular risk factors, and the development of WML is known to be

a causative agent of cognitive impairment, dementia, and disability.[23] Along the same lines, A2M within the brain of Alzheimer's dementia (AD) affected patients has been identified as a genetic risk factor and it is thought that an increased amount of A2M within the brain of these AD affected patients leads to neurotoxicity and increased disease progression.[24]

Chapter 2

Thesis Scope

Objective, Hypothesis and Rationale:

The objective of this study is to define the function of α-macroglobulins in the replication and pathogenesis of WNV and JEV, using a mouse model. Our central hypothesis is that α-macroglobulins facilitate entry of flavivirus in myeloid cells, the first cells the virus encounter, leading to high viremia and virus entry in to the brain. The *overall rationale* is that high peripheral viremia during WNV and JEV infections results in high brain viral load, thereby leading to severe encephalitis. It is known that α-macroglobulins can bind and internalize virus protein into the cells. Moreover, gamma globulins (immunoglobulins) dependent enhancement of flavivirus infection is well known. In vivo loss of function experiments using $PZP^{-/-}/MUG1^{-/-}$ mice [hereafter, double knockout (DKO)] model will allow us to demonstrate the *in vivo* role of α-macroglobulins after virus infection. We expect that decrease in initial infection (viremia) will lead to a reduced virus entry in the brain, resulting in improved overall survival in the α-macroglobulins deficient mice when compared to wild-type mice.

Innovation and Significance:

Although α-macroglobulins are known to bind and internalize viral proteins and modulate the immune response, their *in vivo* function in viral infections has yet to be defined. The proposed research is *highly innovative* as this study will be the first to characterize the function of α-macroglobulins in the pathogenesis of WNV- and JEV-associated encephalitis. Our study supports a novel concept that α-macroglobulins enhance viral infection *in vivo*. Currently, α-macroglobulin immune therapy is used in the clinical setting for treatment of disease such as arthritis and α-2-macroglobulin is used as a disease marker for liver fibrosis. Overall, our study will provide new insight into a novel host factor for flavivirus replication and dissemination, and thus will have a

significant impact on the development of much-needed therapeutic interventions that will reduce virus spread and improve disease outcome.

Specific Aims:

Specific Aim 1: *Determine the role of α-macroglobulins in WNV and JEV disease progression and outcome in mice.*

1.1) Examine the survival pattern and clinical signs of wild-type (WT) and DKO mice after subcutaneous inoculation with WNV.

1.2) Examine the survival pattern and clinical signs of WT and DKO mice after subcutaneous inoculation with JEV.

Specific Aim 2: *Determine the role of α-macroglobulins in virus replication and anti-viral response in mice after WNV infection.*

2.1) Analyze the kinetics of WNV replication in the serum, peripheral organs (spleen, kidneys) and brain of WT and DKO mice.

2.2) Analyze the anti-viral responses, including interferon and antibody response, in WNV-infected WT and DKO mice.

Specific Aim 3: Examine WNV-induced peripheral- and central -nervous system inflammation in WT and DKO mice.

3.1) Analyze the protein levels of pro-inflammatory cytokines/chemokines in serum, spleen and brain of WNV-infected WT and DKO mice.

3.2) Analyze the mRNA levels of key CNS genes including anti-viral, inflammatory and apoptotic genes in the brains of WNV-infected WT and DKO mice.

Chapter 3

Alpha-macroglobulins deficient mice are resistant to lethal flavivirus encephalitis

Alpha-macroglobulins deficient mice are resistant to lethal flavivirus encephalitis

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Abstract

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are the leading causes of arboviral encephalitis in humans. No effective therapies exist for treating individuals with encephalitic flavivirus infections. α--macroglobulins are physiological proteinase inhibitors with important roles in inflammation and immune modulation. In mice, two main α-macroglobulins are present as plasma proteins, pregnancy zone protein (PZP) and murinoglobulin-1 (MUG). We have previously reported that WNV infection induced upregulation of α-macroglobulins in mice. To define the role of α-macroglobulins in flavivirus infection *in vivo*, we investigated the susceptibility of mice deficient in α-macroglobulins (PZP and MUG-1 double knockout; DKO) against lethal dose of WNV or JEV. We found that DKO mice were completely resistant to lethal WNV and JEV encephalitis, suggesting that α-macroglobulins play a deleterious role in flavivirus infection. Increased survival in WNV-infected DKO mice was associated with significantly reduced viral burden in serum, spleen, kidney and brain compared to wild-type (WT) mice. Moreover, levels of cytokines and chemokines in the serum, spleen and brain were significantly reduced in WNV-infected DKO mice compared to WT mice. Collectively our data demonstrate that α-macroglobulins contribute to the pathogenesis of flavivirus encephalitis in mice.

Background

Members of the Flavivirus genus are the leading causes of epidemic encephalitis worldwide and continue to spread globally. Since its initial introduction into New York in 1999, West Nile virus (WNV), a NIAID Category B Priority Pathogen, has become the leading cause of arboviral encephalitis in the United States, resulting in hundreds of deaths[5,9,25]. Similarly, Japanese encephalitis virus (JEV) is the primary cause of viral encephalitis in Asia, accounting for around 50,000 cases per year and an estimated 10,000 deaths, with long-term neurologic sequelae in about one-half of the survivors. JEV also affects U.S. travelers and U.S. military personnel deployed overseas[12]. WNV and JEV continue to spread and cause human disease in new areas of the world. Currently, no effective therapies exist for treating individuals with encephalitic flavivirus infections, and the disease pathogenesis is not completely understood[9,12].

α-macroglobulins are physiological proteinase inhibitors with important roles in inflammation and immune modulation[14]. α-macroglobulins are mainly produced by the liver, and also locally synthesized by macrophages, fibroblasts, and brain cells[14]. In human, two main α-macroglobulins are present as plasma proteins, α-2-macroglobulin (A2M) and pregnancy zone protein (PZP)[26]. A2M, a key member of the alpha macroglobulin superfamily, is a high-molecular weight homotetrameric glycoprotein. A2M is one of the most abundant protein in human plasma with a concentration of 2–4 mg/mL of plasma[14]. A2M is a major player involved in the binding and shuttling of protease inhibitors, growth factors, cytokines, hormones, disease factors, and various small molecule nucleophilic ligands[14,22,27]. Upregulation and/or downregulation of A2M in circulation and extravascular space have been linked to immunological abnormalities, tissue inflammation, Alzheimer disease, and numerous cytokine-

related diseases[14,22,24,28]. In several instances, A2M has proven exceedingly useful as a direct clinical biomarker for numerous disorders such as inflammatory bowel disease, myocardial infarction, stroke, and Hepatitis C virus associated-liver fibrosis[21,23,29-31]. In adult mice, two main α-macroglobulins are present as plasma proteins, PZP and murinoglobulin-1 (MUG-1)[14]. Like human A2M, mouse PZP is highly expressed in the liver of adult mice[32]. MUG-1 is a single chain proteinase inhibitor, which is also found at high concentrations in adult murine plasma (1–2 mg/mL)[27,32,33].

Human A2M is known to bind and enhance internalization of different toxins and pathogens such as bacteria or virus[34]. Viral proteins conjugated to A2M are taken up by antigen presenting cells more effectively than the free viral proteins[35]. It has been demonstrated that A2M binds to DENV virions of all four serotypes and enhances DENV-2 infectivity *in vitro*[36]. It has also been shown that HIV-1 envelope protein conjugated to A2M is effectively taken up by macrophages, which results in an increased production of specific antibodies against the peptide[34,37]. Similarly, A2M-antigen complexes of hepatitis B surface antigen also produce antibody responses in mice that are four to five orders of magnitude greater than those elicited by antigen alone[38]. In addition to antibody response, antigen delivery by A2M enhances the cytotoxic T lymphocyte response and production of inflammatory cytokines and chemokines[39,40]. Therefore, A2M packaging has been proposed as a valuable approach to develop a faster immune response with fewer doses of vaccine[35].

Although A2M is known to bind and internalize viral proteins and modulate immune response, and has been demonstrated to enhance virus infectivity *in vitro*, its *in vivo* function in viral

infection has yet to be defined. We have previously reported that WNV infection induced upregulation of α-macroglobulins in mice[41]. To define the *in vivo* role of α-macroglobulins in flavivirus infection, we investigated the susceptibility of mice deficient in α-macroglobulins (PZP and MUG-1 double knockout) against lethal dose of WNV or JEV. Our results demonstrate conclusively that α-macroglobulins promote virus replication, tissue tropism and inflammatory response, thereby enhancing disease severity.

Results

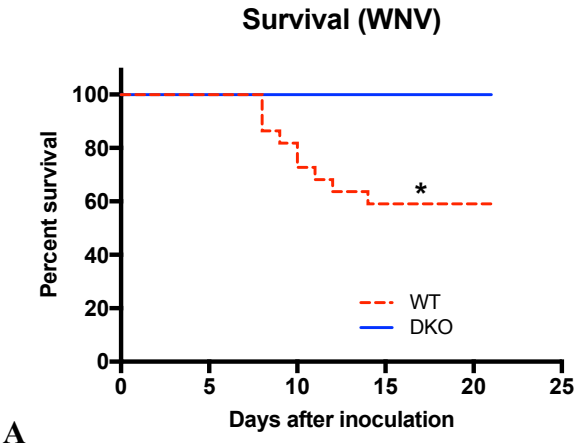
α-macroglobulins deficiency confers resistance to lethal WNV and JEV infection:

We inoculated C57BL/6J (WT) and mice deficient in PZP and MUG-1 (hereafter referred as; double knockout mice or DKO) with 100 plaque-forming units (PFU) of WNV via subcutaneous route. Animals were monitored daily for clinical signs and mortality. As expected, WT mice began to demonstrate clinical signs at day 6 after inoculation and exhibited high mortality (41%). In comparison, all DKO mice survived, suggesting a detrimental role for α-macroglobulins during WNV infection (Fig. 1A). As depicted in Fig. 1B, both WT and DKO mice demonstrated clinical evidence of infection characterized by ruffled fur and hunchbacked posture, however, severe neurological symptoms such as paresis, hind limb paralysis, tremors and ataxic gait were observed only in WT mice. The difference in morbidity and mortality between WT and DKO mice infected with was statistically significant. All surviving animals were positive for anti-WNV neutralizing antibodies, confirming virus infection.

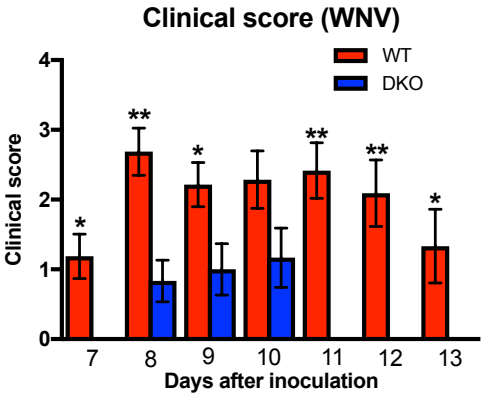
Next, we inoculated C57BL/6J (WT) and DKO mice with 10,000 PFU of JEV via subcutaneous route. Animals were monitored daily for clinical signs and mortality. As expected, WT mice

began to demonstrate clinical signs at day 7 after inoculation and exhibited high mortality (30%). In comparison, all DKO mice showed reduced sign of morbidity and no mortality, suggesting α-macroglobulins modulated the infectivity of JEV (Figs. 1C and 1D). The difference in morbidity and mortality between WT and DKO mice infected with JEV was statistically significant.

Figure 1



A



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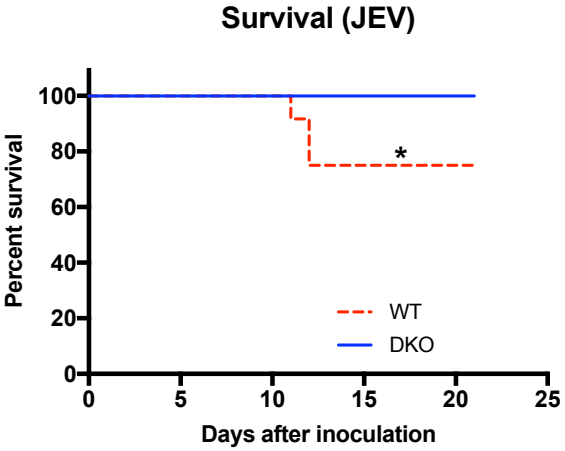

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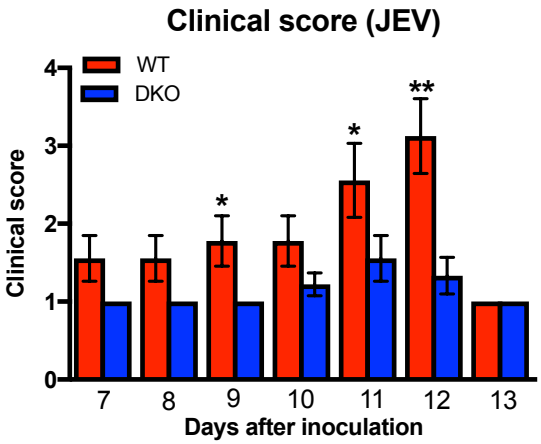


D

Figure 1. Survival analysis and clinical score of WT and DKO mice following WNV and JEV infection. (A) Eight-week old C57BL/6J WT and DKO mice were inoculated subcutaneously with 100 PFU of WNV. All mice were observed for 21 days. Data are combined from two independent experiments (n = 22 mice per group). The survival difference between WT and DKO mice was statistically significant. **(B)** Animals were monitored for clinical scores twice a day. The designation for the clinical scores is as follows: 1, ruffled fur/hunched back; 2, paresis/difficulty walking; 3, paralysis; 4, moribund/euthanized; and 5, dead. **(C)** Eight-week old C57BL/6J WT and DKO mice were inoculated subcutaneously with 10,000 PFU of JEV. All mice were observed for 21 days. Data are combined from two independent experiments (n = 18 mice per group). The survival difference between WT and DKO mice was statistically significant. **(D)** Animals were monitored for clinical scores twice a day as described above. Error bars represent SEM. *$p < 0.05$, **$p < 0.001$.

α-macroglobulins modulate WNV replication, tissue tropism, and neuroinvasion: To better understand how α-macroglobulins mediate WNV pathogenesis, we measured viral load in the serum, spleen, kidney and brain of the WT and DKO mice at various time points after WNV inoculation. Consistent with the survival result, WNV titers in the serum were significantly lower in DKO mice when compared to WT mice at both days 2 and 4 after inoculation (Fig. 2A). The virus was cleared from the serum of all infected mice by day 8 after inoculation. Virus titers assayed in the spleen also followed a similar trend, and DKO mice spleens had significantly lower virus titers than WT mice at both days 2 and 4 after inoculation (Fig. 2B). Virus replication kinetics observed in the kidneys of WT and DKO mice was similar to the spleen (Fig. 2C). We next measured virus titers in the brains at days 2, 6, and 8 after inoculation. WNV is typically detected in the CNS of mice between 4 and 6 days after WNV inoculation via the subcutaneous route. Consistent with this trend, WNV was not detected in the brains of both WT and DKO mice at day 2 after inoculation (Fig. 3A). High viral load was detected in the brains of 86% of the WT mice at day 6 after inoculation by plaque assay. In comparison, WNV was detected in only 50% of the DKO mice at day 6 after inoculation. Similarly, at day 8 after inoculation, high viral load was detected in all of the WT mice. However, WNV was detected in only 50% of the DKO mice at day 8 after inoculation. WNV titer was significantly higher in WT mice than in DKO mice at day 8 after inoculation (Fig. 3A). We also measured WNV RNA copies in the brains of WT and DKO mice. Similar to plaque assay, WNV RNA was significantly higher in WT mice than in DKO mice at days 6 and 8 after inoculation (Fig. 3B). These data indicate that α-macroglobulins facilitate WNV replication, tissue tropism and neuroinvasion.

Figure 2

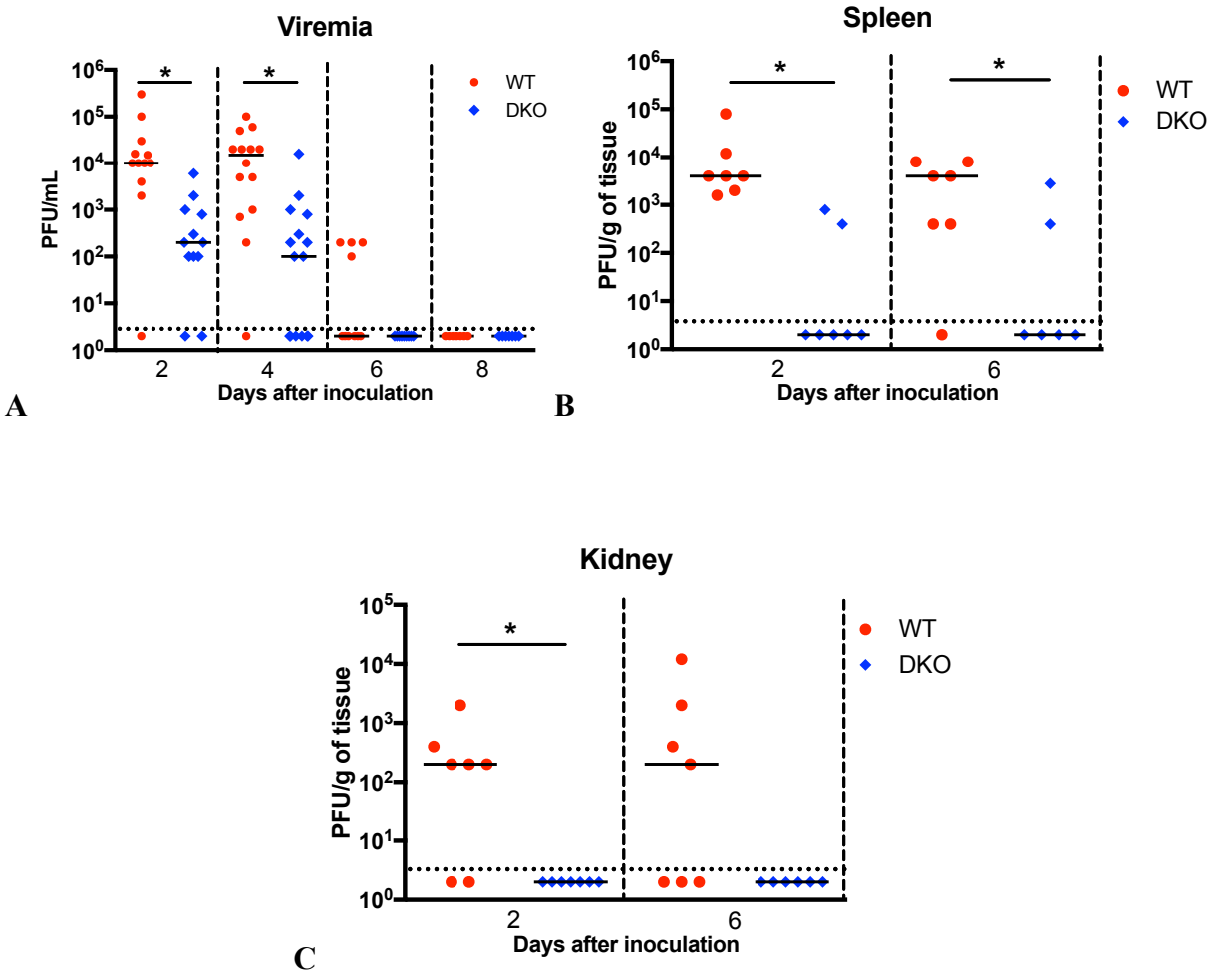


Figure 2. Viral burden in the serum and peripheral tissues of WT and DKO mice. Eight-week old C57BL/6J WT and DKO mice were inoculated subcutaneously with 100 PFU of WNV, and viral loads were measured in the **(A)** serum, **(B)** spleen, and **(C)** kidney at indicated time-points by plaque assay. The data are expressed as PFU/mL of serum and PFU/g of tissue. Each data point represents an individual mouse, and data from two independent experiments are depicted. The data for mock-inoculated mice were negative for WNV and are not depicted on the graphs. Data points below the horizontal dotted line are negative. The solid horizontal line signifies the median of 6 to 15 mice per group. *$p<0.05$.

Figure 3

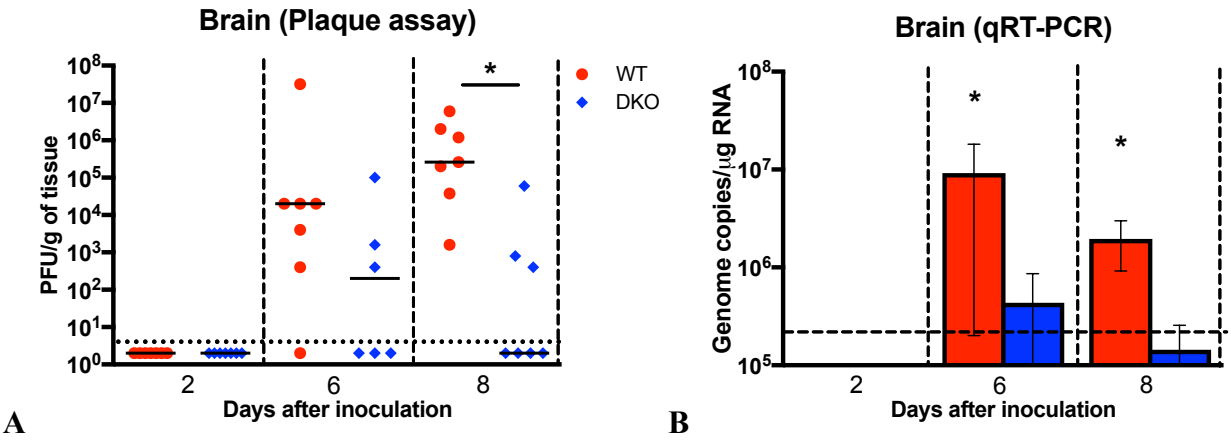


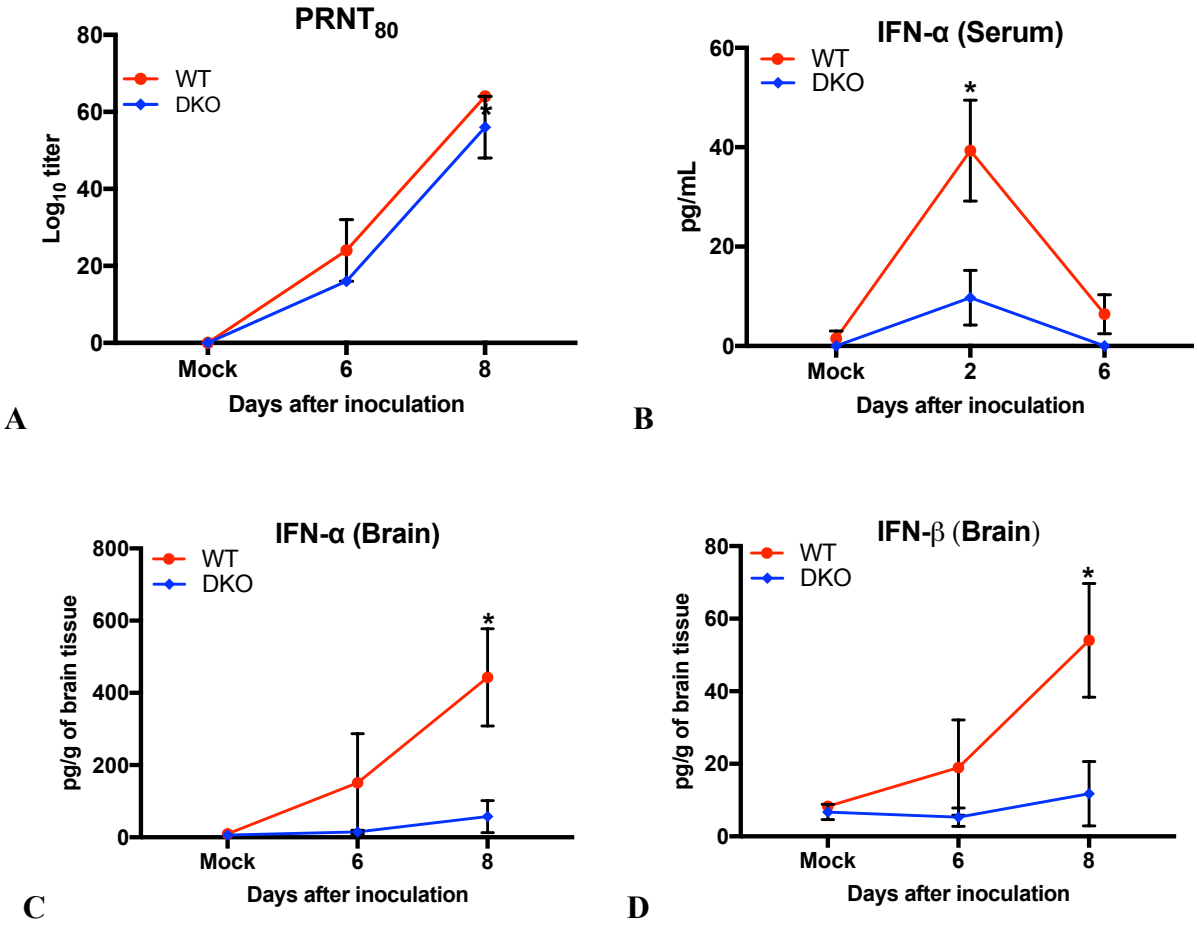
Figure 3. Brain viral load in WT and DKO mice. Eight-week old C57BL/6J WT and DKO mice were inoculated subcutaneously with 100 PFU of WNV. **(A)** Viral loads were measured in the brains at indicated time-points by plaque assay. The data are expressed as PFU/g of tissue. Each data point represents an individual mouse, and data from two independent experiments are depicted. The data for mock-inoculated mice were negative for WNV and are not depicted on the graphs. Data points below the horizontal dotted line are negative. The solid horizontal line signifies the median of 6 to 7 mice per group. *$p<0.05$. **(B)** WNV copy number in the brain was determined by qRT-PCR. The data are expressed as genome copies/µg of RNA. Error bars represent SEM (n = 6-7 mice per group). *$p<0.05$.

Antiviral immune responses in WT and DKO mice:

Humoral immunity is an essential component of the immune response to WNV infection. The induction of anti-WNV neutralizing antibody is essential for suppressing viremia and virus dissemination[1]. Therefore, we examined titers of anti-WNV neutralizing antibodies in the serum of WT and DKO mice using PRNT. WNV infection elicited robust neutralizing antibodies in both WT and DKO mice. Consistent with previous studies, anti-WNV neutralizing antibody was first detected by day 6 after inoculation and gradually increased at day 8 after inoculation (Fig. 4A). Titers of neutralizing antibodies were comparable in both WT and DKO mice.

Antiviral type I interferon (IFN-α and β) production is essential in suppressing viral titers in the periphery and the brain[1]. Therefore, we measured the levels of IFN-α in the serum and brain of WT and DKO mice. High levels of IFN-α were detected in the serum of WT mice at day 2 after inoculation, which then decreased at day 6 after inoculation. In contrast, DKO mice did not elicit strong IFN response at day 2 after inoculation, which was significantly low in comparison to WT mice (Fig. 4B). We next examined IFN-α and IFN-β levels in the brains at days 6 and 8 after inoculation. In the brain, WT mice developed a detectable interferon response at day 6 after inoculation, which increased further at day 8 after inoculation. Similar to serum, DKO mice did not elicit a strong IFN response in the brain and levels of IFN-α and IFN-β were significantly low in comparison to WT mice at day 8 after inoculation (Fig. 4C and 4D).

Figure 4


PRNT80
WT
DKO
Log10 titer
80
60
40
20
0
Mock
6
8
Days after inoculation
*
A
IFN-α (Serum)
WT
DKO
pg/mL
60
40
20
0
Mock
2
6
Days after inoculation
*
B
IFN-α (Brain)
WT
DKO
pg/g of brain tissue
800
600
400
200
0
Mock
6
8
Days after inoculation
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C
IFN-β (Brain)
WT
DKO
pg/g of brain tissue
80
60
40
20
0
Mock
6
8
Days after inoculation
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Figure 4. WNV-specific immune responses in WT and DKO mice. Serum was collected from WT and DKO mice at indicated time points after virus inoculation. **(A)** Serum was serially diluted from 1:40 to 1:5,000 and PRNT was conducted against WNV. Data are expressed as mean $\log_{10}$ titer ±SEM and is representative of two independent experiments (n = 6-7 mice per group). **(B)** IFN-α levels were measured in the serum using mouse IFN-α ELISA kit. The data are expressed as the mean concentration (pg/mL) ± SEM, representing two independent experiments (n = 6-7 mice per group). *$p<0.05$. **(C)** IFN-α, and **(D)** IFN-β levels were measured in the brain homogenates using ELISA. The data are expressed as the mean concentration (pg/g of tissue) ± SEM, representing two independent experiments (n = 6-7 mice per group). *$p<0.05$.

Peripheral cytokine responses are blunted in DKO mice:

WNV-induced expression of pro-inflammatory cytokines such as IL-1β and TNF modulate BBB permeability, facilitate leukocyte trafficking into the brain, activate glial cells, and mediate neuronal death after infection[1,42,43]. It has been demonstrated that WNV-induced expression of chemokines such as CCL2, CCL3, CCL4, CCL5, and CXCL10 are associated with enhanced trafficking of leukocytes into the brain[44-46]. Human and murine α-macroglobulins are also associated with an enhanced inflammatory response to various stimuli[14,33,40,47]. Therefore, we next assessed the protein levels of these cytokines and chemokines in the serum and spleen of WT and DKO mice using multiplex immunoassay. Very low levels of these cytokines and chemokines were detected in the serum of uninfected WT and DKO mice (Mock). We did not observe any differences in the basal cytokine and chemokine levels in the serum from uninfected WT and DKO mice. As expected, WNV infection resulted in a dramatic increase in protein levels of multiple cytokines and chemokines in the serum of WT mice at days 2 and 4 after inoculation (Fig. 5). However, unlike WT mice, infected DKO mice produced significantly lower levels of pro-inflammatory cytokines and chemokines. The levels of interleukin (IL)-6, -10, -13, and M-CSF were significantly higher in WT mice than DKO mice at day 4 after inoculation. The level of IL-5 was significantly higher in WT mice at day 2 after inoculation whereas, the level of G-CSF was significantly higher in WT mice at day 8 after inoculation (Fig. 5). A similar pattern was also observed for chemokine expression. The levels of CXCL2 and CXCL10 were significantly higher in WT mice than DKO mice at day 4 after inoculation. The level of CXCL1 was only increased significantly at day 2 after inoculation in WT mice when compared to DKO mice. However, the comparison of levels of serum cytokines and chemokines, such as IL-1α,

CCL4, and CXCL9 between both groups demonstrated no significant differences in their levels at any time point after inoculation (Fig. 5).

Similar to serum, we did not observe any differences in the basal cytokine and chemokine levels in the spleens from uninfected WT and DKO mice. WNV infection resulted in a dramatic increase in protein levels of multiple cytokines and chemokines in the spleens of WT mice at day 2 after inoculation (Fig. 6). However, unlike WT mice, cytokine responses were blunted in DKO mice. The levels of TNF-α, IFNγ, IL-6, IL-12, IL-13, IL-15, GM-CSF, LIF, CXCL10 and M-CSF were significantly higher in WT mice than DKO mice at day 2 after inoculation. No significant difference was observed in the levels of IL-1α, IL-10, CCL4, CXCL2, and CXCL9 between WT mice and DKO mice. These results demonstrate that the absence of α-macroglobulins result in reduced systemic cytokine response to WNV infection in the periphery.

Figure 5 (Cytokines)

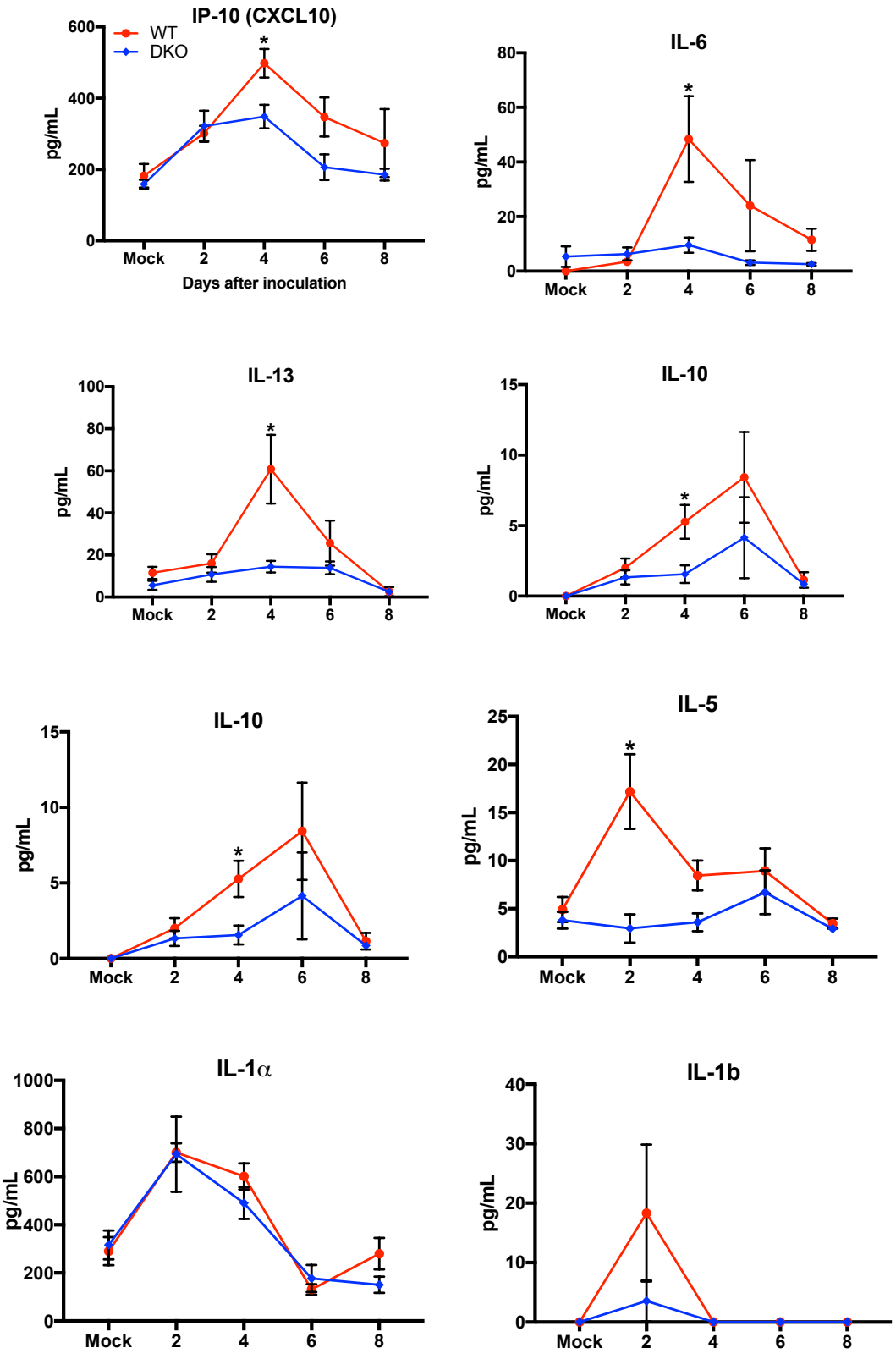


Figure 5 (Chemokines and Growth Factors)

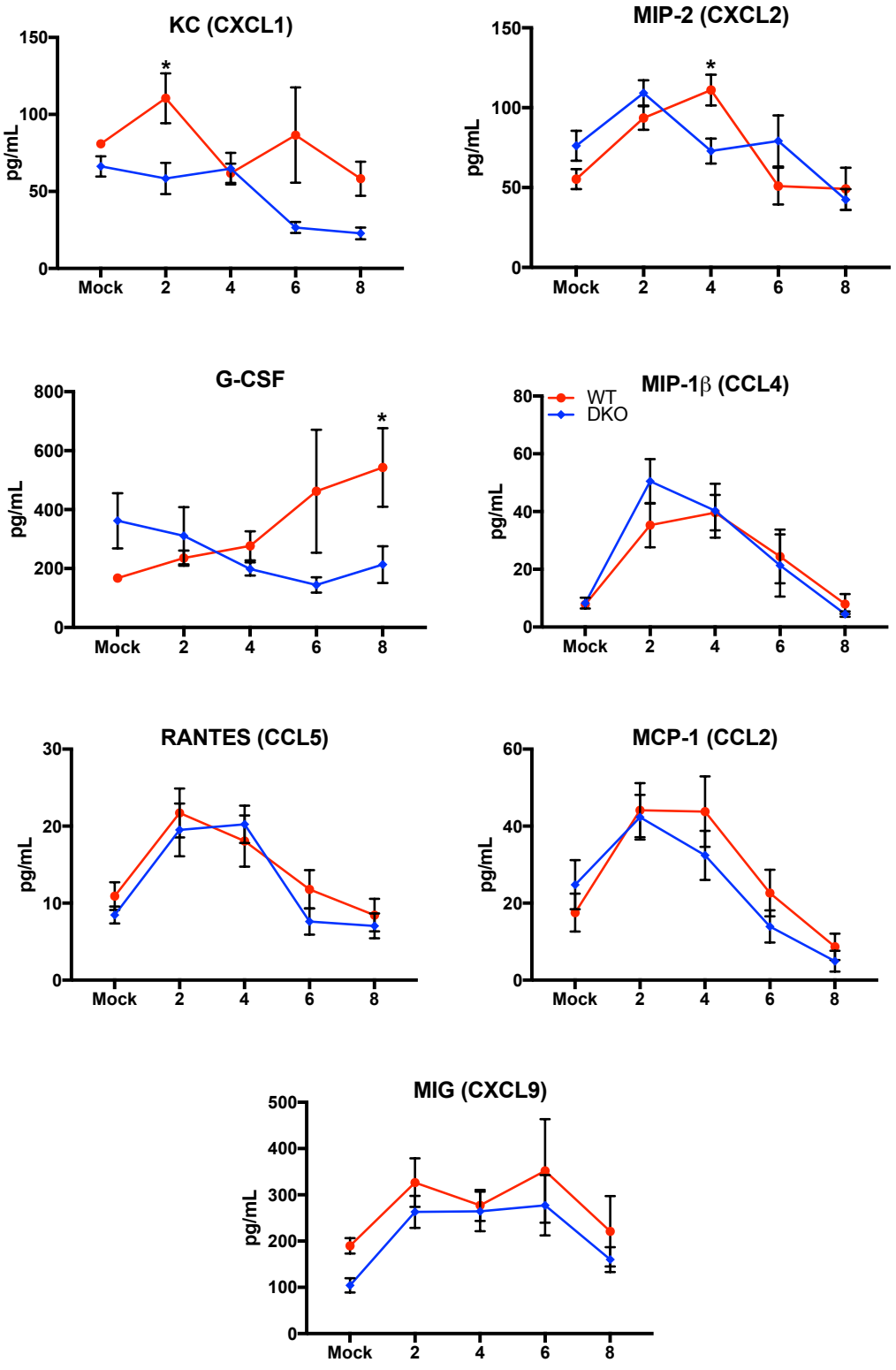


Figure 5. Cytokines and chemokines levels in the serum of WT and DKO mice. Serum was collected from WT and DKO mice at indicated time points after virus inoculation. Levels of chemokines and cytokines as noted in the figure were measured using multiplex immunoassay and are expressed as the mean concentration (pg/mL) ±SEM, representing two independent experiments (n = 7-16 mice per group). *$p<0.05$.

Figure 6 (Cytokines)

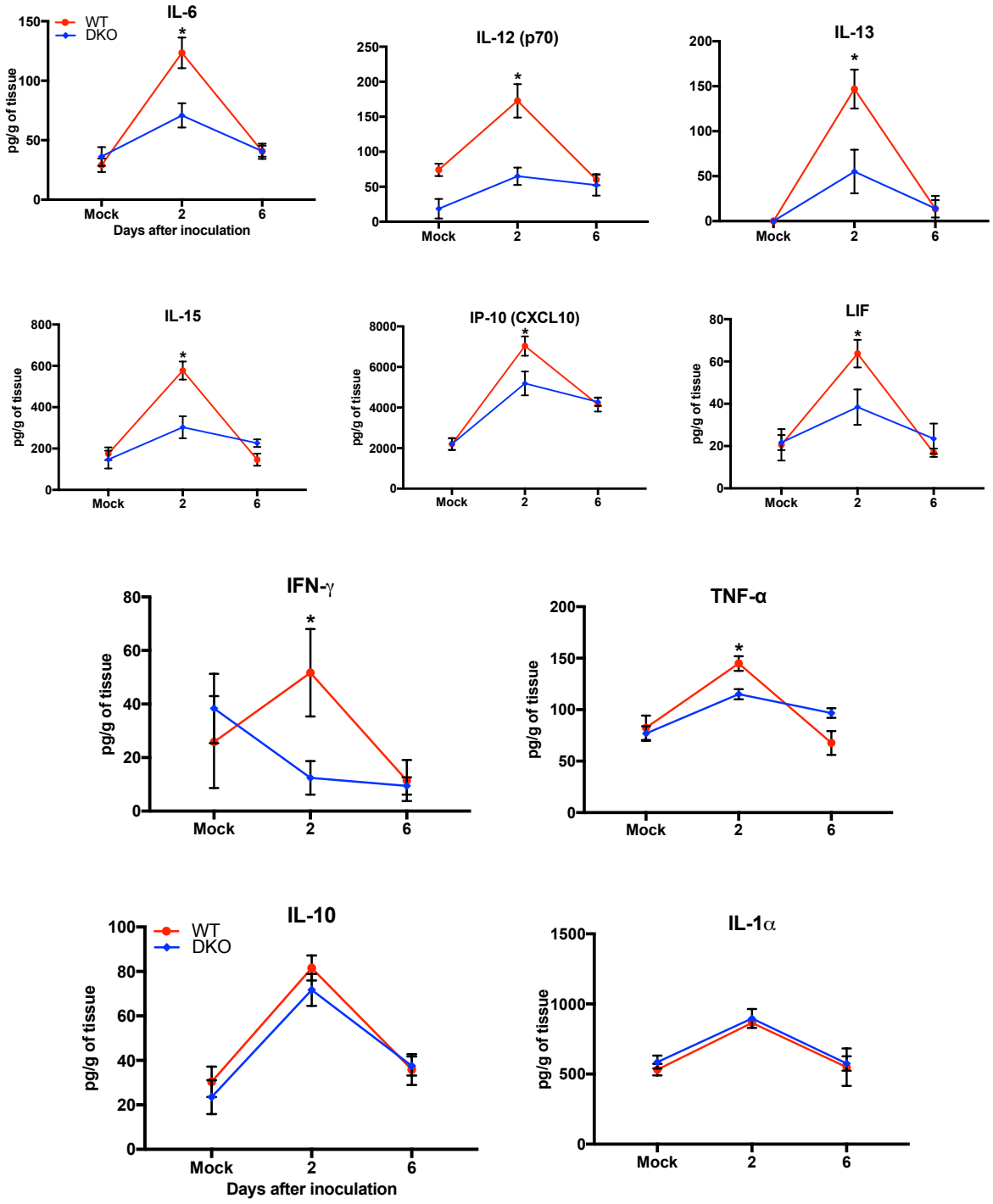


Figure 6 (Chemokines and Growth Factors)

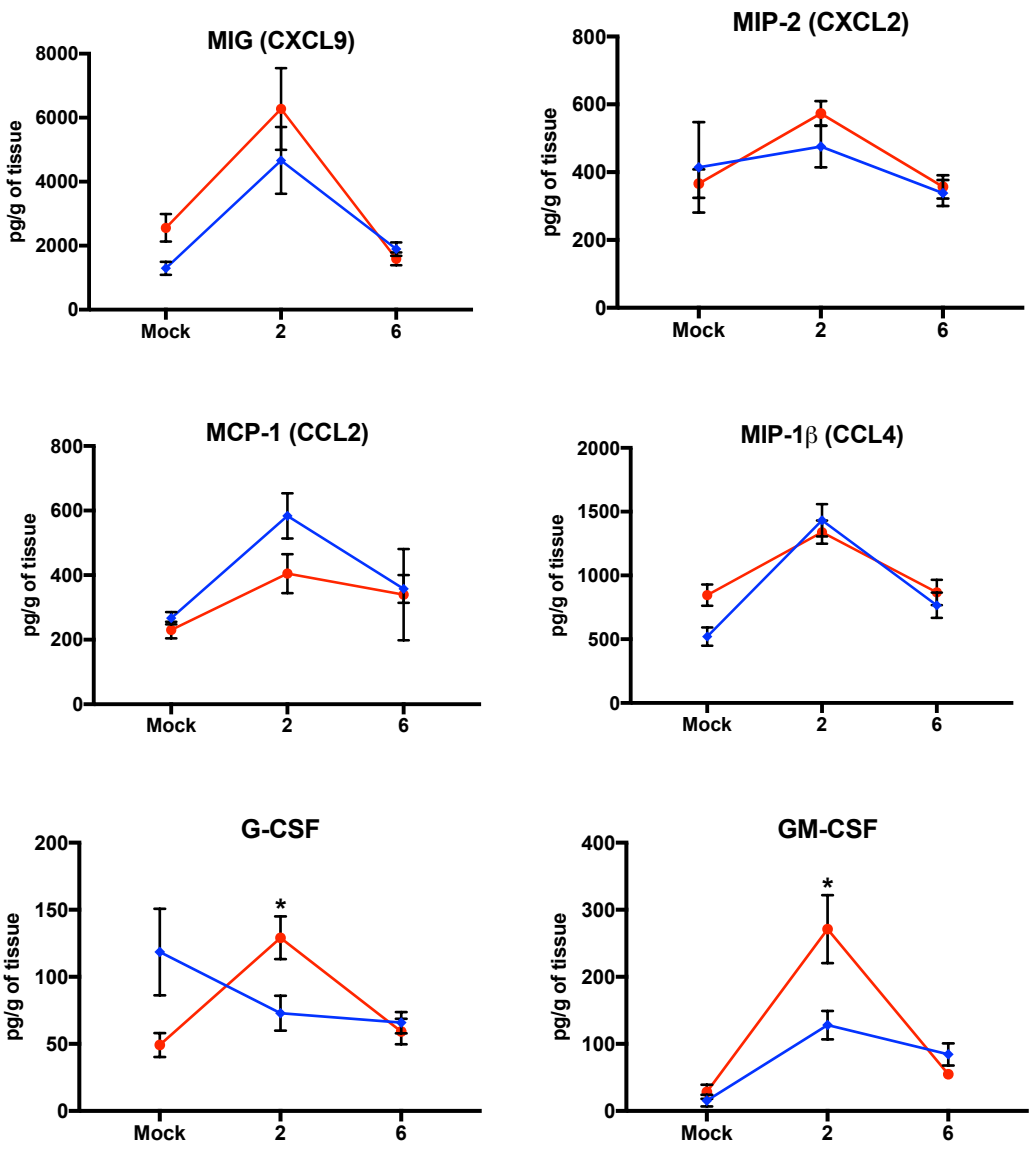


Figure 6. Cytokines and chemokines levels in the spleens of WT and DKO mice. Spleens were harvested from WT and DKO mice at indicated time points and homogenized as described in the materials and methods section. Levels of cytokines and chemokines as noted in the figure were measured using multiplex immunoassay and are expressed as the mean concentration (pg/g of tissue) ± SEM, representing two independent experiments (n = 6-7 mice per group). *$p<0.05$.

DKO mice exhibit reduced inflammation in the CNS:

We further examined the protein levels of multiple inflammatory cytokines and chemokines in the brain homogenates of WT and DKO mice. Very low levels of these cytokines and chemokines were detected in the brains of uninfected mice (mock). As expected, levels of multiple cytokines and chemokines increased dramatically in WT mice brains at day 8 after inoculation, but this increase was not observed in the DKO mice (Fig. 7). The levels of TNF-α, IFNγ, IL-1α, IL-1β, IL-6, IL-12 (p70), IL-13, G-CSF, and M-CSF were significantly higher in the brains of WT mice than DKO mice at day 8 after inoculation. Similarly, the levels of eotaxin, CCL2, CCL5, CXCL1, CXCL2, CXCL9, and CXCL10 were significantly higher in the brains of WT mice than DKO mice at day 8 after inoculation. However, the levels of IL-10 and IL-12 (p40) did not differ between both the groups (Fig. 7).

Next, we analyzed levels of mRNA from WT and DKO mouse brains at day 8 post WNV-infection. Analysis was performed for over 1,000 different genes linked to various inflammatory processes using Nanostring technology and nSolver software. The top 20 up-regulated genes in WT mice at 8 days post infection is shown in *table 1*. A consistent trend with our previous data is seen where mRNA levels in WT mice are drastically elevated at this time point post infection and comparing DKO mice to WT shows a significant decrease in mRNA levels in DKO animals. Overall mRNA fold changes are calculated compared to mock-infected controls.

Finally, further utilization of Nanostring technology and nSolver software allowed for the generation of multiple heatmaps corresponding to different immune pathways in response to WNV infection. *Figure 8* demonstrates the levels of mRNA present for genes correlating to

pathogen response in the brains of WT, DKO, and mock-infected control animals 8 days post infection.

Figure 7 (Cytokines)

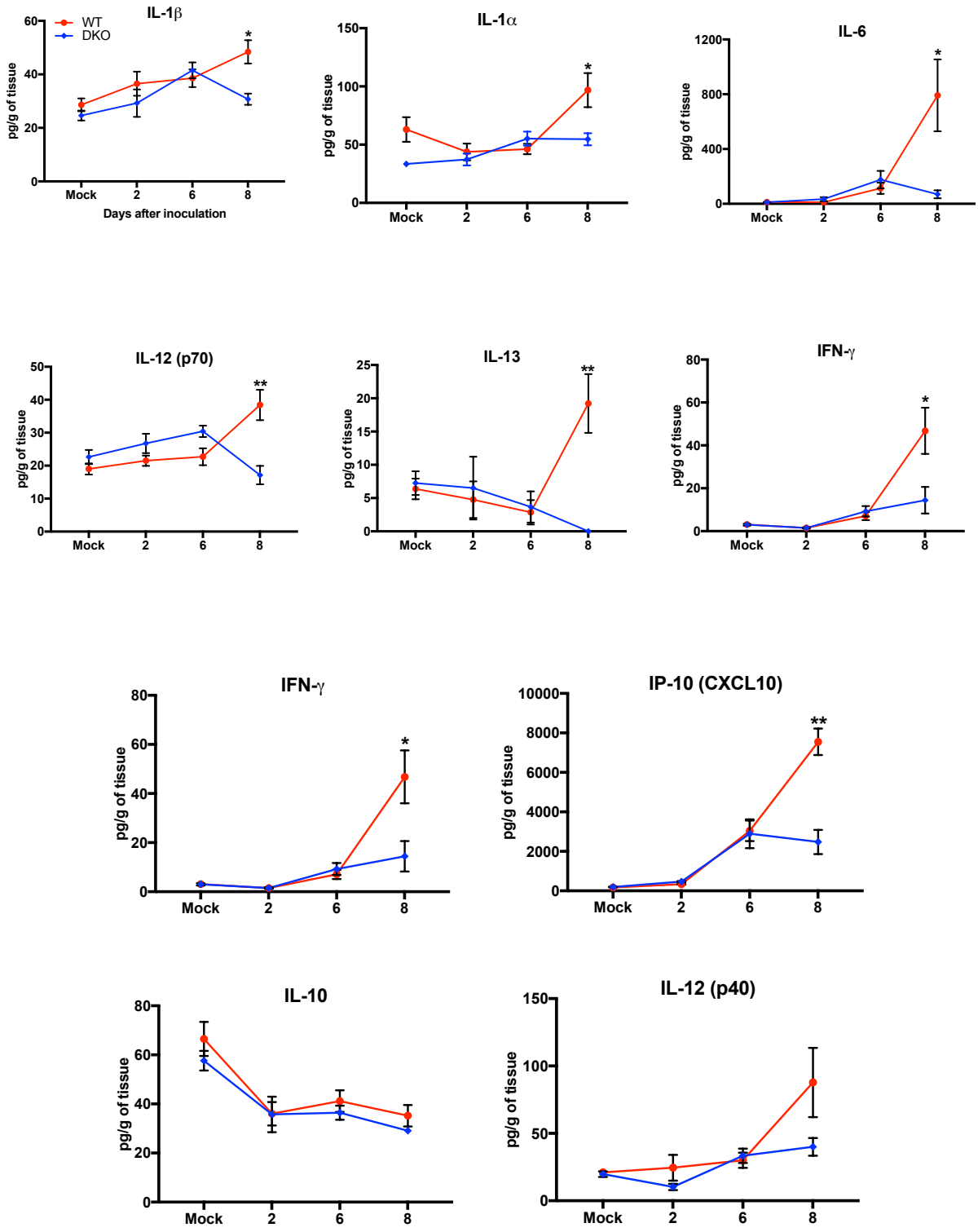


Figure 7 (Chemokines and Growth Factors)

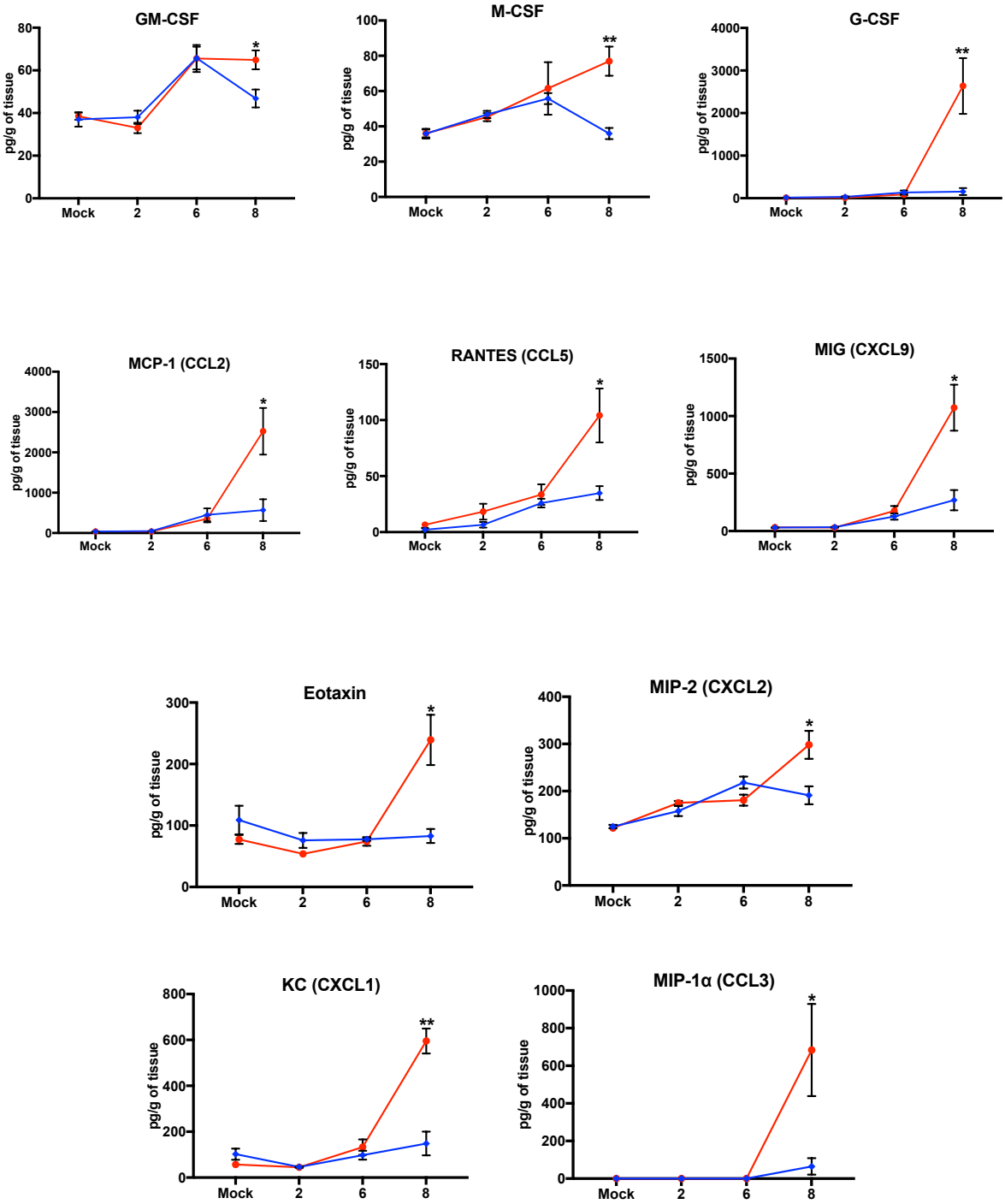


Figure 7. Cytokines and chemokines levels in the brains of WT and DKO mice. Brains were harvested from WT and DKO mice at indicated time points and homogenized as described in the materials and methods section. Levels of cytokines and chemokines as noted in the figure were measured using multiplex immunoassay and are expressed as the mean concentration (pg/g of tissue) ± SEM, representing two independent experiments (n = 6-7 mice per group). *$p<0.05$, **$p<0.0001$.

Table 1

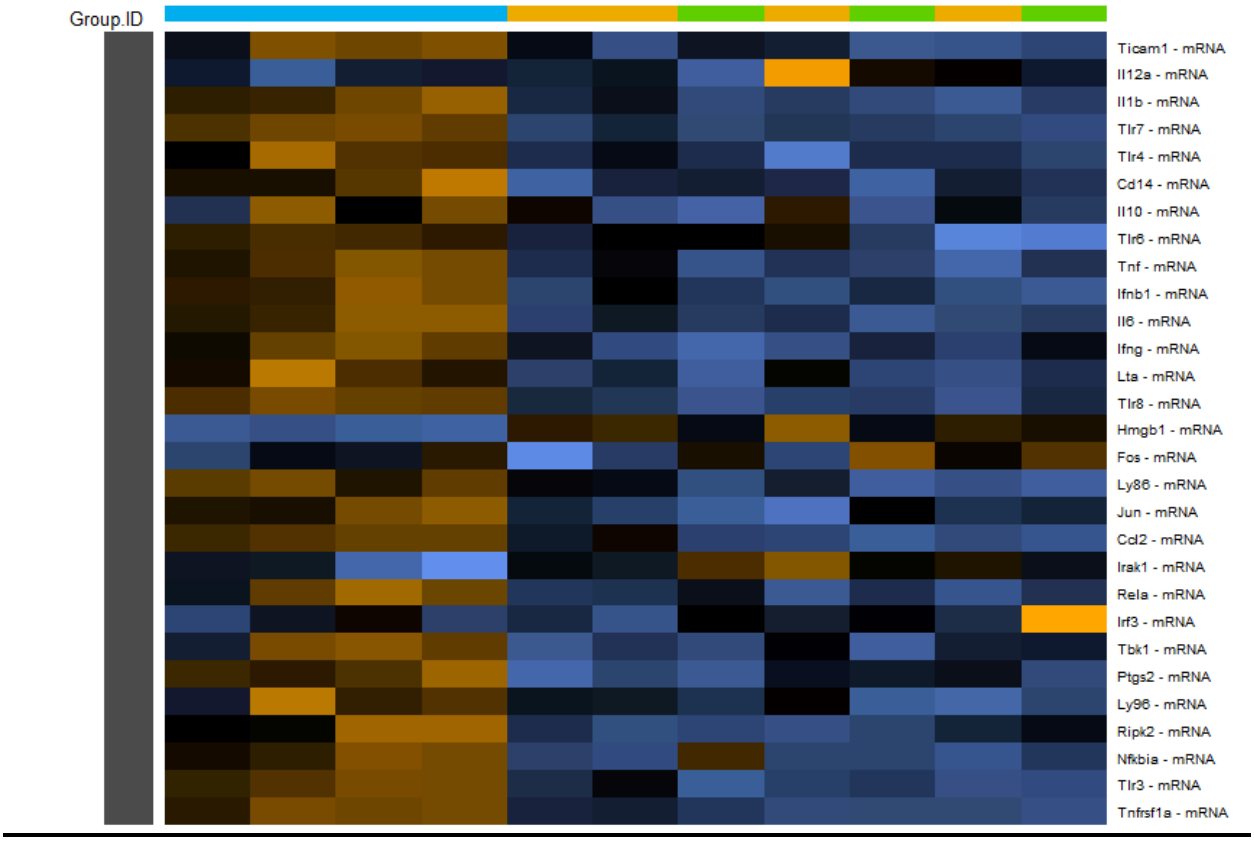
Gene Name	C57BL/6J	A2M -/-
Cxcl10	646.55	8.55
Il1rn	527.01	8.02
Cxcl9	289.1	12.92
Ifi44	242.1	5.06
Ccl2	241.92	5.57
Rsad2	170.64	4.92
Isg15	151.73	4.4
Usp18	144.77	5.09
Oasl1	139	4.44
Zbp1	136.25	4.89
Gzmb	132.16	4.87
Irf7	131.46	4.44
Ifit1	122.14	3.57
Ccl5	113.9	4.83
Cfb	101.36	3.56
Ifit3	89.83	3.73
Ccl12	88.13	5.18
Bst2	84.25	4.01
Ccl3	68.59	3.88
Nlrc5	63.42	2.98

Table 1. Top 20 up-regulated Immune-related Genes in WT mice

C57BL/6J WT mice and DKO mice were inoculated subcutaneously with 100 PFU of WNV and brains were harvested 8 days post infection. Brains were homogenized, and RNA extraction was performed on the brain homogenate as previously described. RNA was given to the UH Manoa Cancer Center for miRNA Nanostring analysis. Similarly, mock-infected controls were euthanized 8 days post mock-infection and brains were harvested and homogenized prior to RNA extraction.

*mRNA fold change relative to mock-infected controls

Figure 8



*Immune genes to pathogen response

Figure 8. Heatmap of Immune-related genes to Pathogen Response

C57BL/6J WT mice and DKO mice were inoculated subcutaneously with 100 PFU of WNV and brains were harvested 8 days post infection. Brains were homogenized, and RNA extraction was performed on the brain homogenate as previously described. RNA was given to the UH Manoa Cancer Center for miRNA Nanostring analysis. Similarly, mock-infected controls were euthanized 8 days post mock-infection and brains were harvested and homogenized prior to RNA extraction. Further statistical analysis and generation of heatmaps was performed using nSolver software. The above heatmap was generated for genes corresponding to pathogen response.

*mRNA fold change relative to mock-infected controls

Discussion

In this study, we demonstrate that α-macroglobulins enhance WNV and JEV infection *in vivo*. $PZP^{-/-}/MUG1^{-/-}$ mice (DKO mice) demonstrated complete resilience to subcutaneous lethal WNV and JEV infection and reduced viral burden in serum, spleen, kidney and brain. These observations were associated with significantly attenuated inflammatory responses in the periphery and the brain of DKO mice.

In adult mice, two main α-macroglobulins are present as plasma proteins, PZP (formerly termed 'mouse A2M' or MAM) and MUG-1[14,27,32]. Therefore, in this study we used mice deficient in both PZP and MUG-1. This mouse model has been previously used to understand the role of α-macroglobulins in acute pancreatitis, *Trypanosoma cruzi* infection, and drug pharmacokinetics[48-50]. In our study, DKO mice exhibited significantly lower levels of virus titers in the serum, which correlated with decreased tissue tropism as compared to WT mice. It is known that high viremia during WNV infection results in high brain viral load in mice, thereby leading to severe encephalitis[51,52]. WT mice displayed significant virus replication in peripheral tissues such as spleen and kidney compared to DKO mice. Similar to the periphery, there was reduced virus replication in the brains of DKO mice leading to increase survival. This is in agreement with previous observations showing that α-macroglobulins deficient mice had significantly lower parasitemia than in WT mice after infection with *T. Cruzi*[48]. It has also been demonstrated that α-macroglobulins deficient mice are resistant to the lethal effects of LPS[33,47]. Similarly, mice deficient in α-macroglobulins are also resistant to lethal infection by *Klebsiella pneumoniae* and had significantly less bacteria in the blood and in different organs than in WT mice[53].

Viral proteins conjugated to A2M are taken up by antigen presenting cells more effectively than the free viral proteins[34]. Therefore, A2M has been used as an adjuvant/delivery protein to enhance the weak immunogenicity of subunit vaccines[35,37]. A2M has been shown to interact with the envelope protein of dengue virus and enhances virus infectivity *in vitro*[36]. Similarly, it has been shown that A2M binds to HSV-1 particles and facilitates internalization of HSV resulting in increase in the synthesis of viral proteins in the neuronal cell line[54]. A2M binds to its specific receptor on the cell surface of many cell types[14,55]. It is possible that A2M facilitates internalization of WNV through the low-density lipoprotein (LDL) receptor related protein (LRP), a heterodimer glycoprotein and receptor of A2M[55]. HCV and other members of Flaviviridae are endocytosed via the LDL receptor[56]. It has been demonstrated that binding of HIV-1 transactivator (Tat) protein to LRP promotes efficient uptake of Tat into neurons[57]. Future studies are warranted to delineate the potential role of A2M as a shuttle for WNV to LRP as a receptor complex that may mediate virus endocytosis.

A2M binds to several important cytokines, including basic fibroblast growth factor, platelet-derived growth factor, interleukins, TNF and modifies their biological activity[14]. The exact role of the interaction with these non-proteolytic proteins remains unknown. WNV-induced pro-inflammatory cytokines and chemokines are known to modulate BBB permeability, activate glial cells, and mediate neuronal death, leading to the induction of lethal encephalitis[1,42,43]. In this study, we demonstrate significantly elevated levels of cytokines such as IL-1β, IL-6, TNF, IL-1α, and IFN-γ in the serum, spleen and brain of WT mice after WNV infection, which correlated with increased level of WNV observed in these mice. In contrast, absence of α-macroglobulins results in reduced cytokine response to WNV infection in the periphery and brain. This data is

consistent with previous observations that mice deficient in α-macroglobulins demonstrate an attenuated inflammatory response to various stimuli such as *T. cruzi*, in which a heightened inflammatory response was correlated with increased disease severity[47,48,50]. Murine α-macroglobulins are essential for the normal development of fever and inflammation in response to bacterial endotoxin[33,47]. It has been demonstrated that murine α-macroglobulin contributes to TNF-induced lethal inflammatory shock in mice[58]. Also, human and murine α-macroglobulins are able to induce prostaglandin E2 and nitric oxide synthesis[33]. Therefore, it is possible that increased cytokine and chemokine responses in the presence of α-macroglobulins may contribute to severe WNV disease severity.

In conclusion, our data for the first time demonstrate the critical role of α-macroglobulins during flavivirus infection. α-macroglobulins promote WNV replication, tissue tropism and inflammatory response, thereby enhancing WNV disease severity. Future studies are warranted to understand the mechanisms underlying the role of α-macroglobulins in flavivirus pathogenesis.

Materials and Methods

Animals: C57BL/6 J (WT) mice and $PZP^{-/-}/MUG1^{-/-}$ mice (DKO mice) on C57BL/6J genetic background were purchased from The Jackson Laboratory (Bar Harbor, Maine, United States). This study was approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC) (protocol number 15-2202), and conducted in strict accordance with guidelines established by the National Institutes of Health and the University of Hawaii IACUC. All animal experiments were conducted in consultation with veterinary and animal care staff at

the University of Hawaii in animal biosafety level-3 laboratory, and mice that exhibited severe disease were euthanized to limit suffering. The animal suite was maintained at 72^0F, 45% humidity and on 12/12-light/dark cycle. Sawdust bedding was provided along with paper towel.

WNV infection experiments and plaque assay: For survival studies, WT and DKO mice were inoculated subcutaneously via the footpad route with 100 plaque-forming units (PFU) of WNV or 10,000 PFU of JEV, and the disease symptoms and mortality were observed for 21 days as described previously[59,60]. Clinical symptoms were observed twice a day. These symptoms included ruffled fur, hunchbacked posture, difficulty walking, hind limb paralysis, tremors and ataxic gait. To limit suffering animals displaying severe symptoms such as tremors and ataxic gait, were euthanized immediately using CO2. On specific days after inoculation, 100 μL blood was collected from the tail vein, and serum was separated and frozen at −80°C for further analysis.

In a separate set of experiments, WT and DKO mice were inoculated with PBS or 100 PFU of WNV, and at days 2, 6, and 8 after inoculation, mice were anesthetized using isoflurane and perfused with PBS. Spleen, kidneys, and brain were harvested, and flash frozen in 2-methylbutane (Sigma). Tissues were weighed and homogenized in a bullet blender (Next Advance) using glass or zirconium beads. WNV and JEV titers in the serum and tissue homogenates were measured by plaque assay using Vero cells[61,62].

qRT-PCR: One half of the frozen brain tissues were powdered over dry ice to obtain a homogenous sampling and an aliquot of the frozen brain powder was used to extract total RNA.

Virus titers were analyzed in the brain by qRT-PCR as described previously. qRT-PCR was conducted using primers and 6-carboxyfluorescein (FAM)- and 6-carboxytetramethylrhodamine (TAMRA)-labeled probes specific for the WNV envelope region, and the standard curve was generated using RNA extracted from previously titrated WNV dilutions as described previously[59,63].

Plaque Reduction Neutralization Test (PRNT): The titers of anti-WNV neutralizing antibodies were measured in the serum using PRNT assay as described previously[51,60,64]. Serum collected from WT and DKO mice were serially diluted from 1:40 to 1:5000 and PRNT was conducted against WNV. The highest dilution of serum resulting in 80% reduction in the number of plaques compared to the growth of the virus control was determined.

Measurement of cytokines and chemokines: The levels of cytokines and chemokines were measured in the serum, spleen homogenates, and brain homogenates by multiplex immunoassay using MILLIPLEX MAP Mouse Cytokine/Chemokine kit (Millipore) as described previously[61,65,66].

Interferon ELISA: The levels of IFN-α and IFN-β were measured in the serum and brain homogenates using the VeriKine™ Mouse Interferon-α ELISA Kit and VeriKine™ Mouse Interferon-β ELISA Kit (PBL Interferon Source) as described previously[61].

Statistical analysis: Log-rank (Mantel-Cox) Test and Gehan-Breslow-Wilcoxon Test were used to analyze survival data. Mann-Whitney test and unpaired student t-test using GraphPad Prism

5.0 was used to calculate p values of difference between viral titers and immune responses, respectively. Differences of $p < 0.05$ were considered significant.

References

1 Brinton, M. A. The molecular biology of West Nile Virus: a new invader of the western hemisphere. *Annu Rev Microbiol* **56**, 371-402, doi:10.1146/annurev.micro.56.012302.160654 (2002).

2 Hayes, E. B. & Gubler, D. J. West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. *Annu Rev Med* **57**, 181-194 (2006).

3 O'Leary, D. R. *et al.* The epidemic of West Nile virus in the United States, 2002. *Vector Borne Zoonotic Dis* **4**, 61-70 (2004).

4 Yun, S. I. & Lee, Y. M. Japanese encephalitis: the virus and vaccines. *Hum Vaccin Immunother* **10**, 263-279, doi:10.4161/hv.26902 (2014).

5 Rehman, A. A., Ahsan, H. & Khan, F. H. alpha-2-Macroglobulin: a physiological guardian. *J Cell Physiol* **228**, 1665-1675, doi:10.1002/jcp.24266 (2013).

6 Petersen, C. M. Alpha 2-macroglobulin and pregnancy zone protein. Serum levels, alpha 2-macroglobulin receptors, cellular synthesis and aspects of function in relation to immunology. *Dan Med Bull* **40**, 409-446 (1993).

7 Wu, S. M., Patel, D. D. & Pizzo, S. V. Oxidized alpha2-macroglobulin (alpha2M) differentially regulates receptor binding by cytokines/growth factors: implications for tissue injury and repair mechanisms in inflammation. *J Immunol* **161**, 4356-4365 (1998).

8 Abe, K., Yamamoto, K. & Sinohara, H. Proteinase inhibitory spectrum of mouse murinoglobulin and alpha-macroglobulin. *J Biochem* **106**, 564-568 (1989).

9 Kovacs, D. M. alpha2-macroglobulin in late-onset Alzheimer's disease. *Exp Gerontol* **35**, 473-479 (2000).

10 Tiggelman, A. M., Linthorst, C., Boers, W., Brand, H. S. & Chamuleau, R. A. Transforming growth factor-beta-induced collagen synthesis by human liver myofibroblasts is inhibited by alpha2-macroglobulin. *J Hepatol* **26**, 1220-1228 (1997).

11 Becker, K., Niederau, C. & Frieling, T. Fecal excretion of alpha 2-macroglobulin: a novel marker for disease activity in patients with inflammatory bowel disease. *Z Gastroenterol* **37**, 597-605 (1999).

12 Ramasamy, S. *et al.* Cardiac isoform of alpha 2 macroglobulin, an early diagnostic marker for cardiac manifestations in AIDS patients. *AIDS* **20**, 1979-1981, doi:10.1097/01.aids.0000247122.97079.71 (2006).

13 Annapoorani, P. *et al.* Cardiac isoform of alpha-2 macroglobulin--a new biomarker for myocardial infarcted diabetic patients. *Atherosclerosis* **186**, 173-176, doi:10.1016/j.atherosclerosis.2005.07.004 (2006).

14 Ho, A. S. *et al.* Novel biomarkers predict liver fibrosis in hepatitis C patients: alpha 2 macroglobulin, vitamin D binding protein and apolipoprotein AI. *J Biomed Sci* **17**, 58, doi:10.1186/1423-0127-17-58 (2010).

15 Nezu, T. *et al.* Alpha2-macroglobulin as a promising biomarker for cerebral small vessel disease in acute ischemic stroke patients. *J Neurol* **260**, 2642-2649, doi:10.1007/s00415-013-7040-x (2013).

16 Bernhard, O. K., Kapp, E. A. & Simpson, R. J. Enhanced analysis of the mouse plasma proteome using cysteine-containing tryptic glycopeptides. *J Proteome Res* **6**, 987-995, doi:10.1021/pr0604559 (2007).

17 Webb, D. J. *et al.* Murine alpha-macroglobulins demonstrate divergent activities as neutralizers of transforming growth factor-beta and as inducers of nitric oxide synthesis.

A possible mechanism for the endotoxin insensitivity of the alpha2-macroglobulin gene knock-out mouse. *J Biol Chem* **271**, 24982-24988 (1996).

18 Liao, H. X. *et al.* Increased immunogenicity of HIV envelope subunit complexed with alpha2-macroglobulin when combined with monophosphoryl lipid A and GM-CSF. *Vaccine* **20**, 2396-2403 (2002).

19 Mitsuda, S., Nakagawa, T., Nakazato, H. & Ikai, A. Receptor-linked antigen delivery system. Importance of autologous alpha 2-macroglobulin in the development of peptide vaccine. *Biochem Biophys Res Commun* **216**, 399-405 (1995).

20 Huerta, V. *et al.* Receptor-activated human alpha2-macroglobulin interacts with the envelope protein of dengue virus and protects virions from temperature-induced inactivation through multivalent binding. *J Gen Virol* **95**, 2668-2676, doi:10.1099/vir.0.068544-0 (2014).

21 Mitsuda, S. *et al.* A receptor mediated delivery of an HIV-1 derived peptide vaccine. *Biochem Biophys Res Commun* **194**, 1155-1160, doi:10.1006/bbrc.1993.1943 (1993).

22 Cianciolo, G. J., Enghild, J. J. & Pizzo, S. V. Covalent complexes of antigen and alpha(2)-macroglobulin: evidence for dramatically-increased immunogenicity. *Vaccine* **20**, 554-562 (2001).

23 Bowers, E. V., Horvath, J. J., Bond, J. E., Cianciolo, G. J. & Pizzo, S. V. Antigen delivery by alpha(2)-macroglobulin enhances the cytotoxic T lymphocyte response. *J Leukoc Biol* **86**, 1259-1268, doi:10.1189/jlb.1008653 (2009).

24 Anderson, R. B., Cianciolo, G. J., Kennedy, M. N. & Pizzo, S. V. Alpha 2-macroglobulin binds CpG oligodeoxynucleotides and enhances their immunostimulatory properties by a receptor-dependent mechanism. *J Leukoc Biol* **83**, 381-392, doi:10.1189/jlb.0407236 (2008).

25 Kumar, M., Belcaid, M. & Nerurkar, V. R. Identification of host genes leading to West Nile virus encephalitis in mice brain using RNA-seq analysis. *Sci Rep* **6**, 26350, doi:10.1038/srep26350 (2016).

26 Suthar, M. S., Diamond, M. S. & Gale, M., Jr. West Nile virus infection and immunity. *Nat Rev Microbiol* **11**, 115-128, doi:10.1038/nrmicro2950 (2013).

27 Kumar, M., Verma, S. & Nerurkar, V. R. Pro-inflammatory cytokines derived from West Nile virus (WNV)-infected SK-N-SH cells mediate neuroinflammatory markers and neuronal death. *Journal of neuroinflammation* **7**, 73, doi:10.1186/1742-2094-7-73 (2010).

28 Shrestha, B., Zhang, B., Purtha, W. E., Klein, R. S. & Diamond, M. S. 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, doi:10.1128/JVI.01118-08 (2008).

29 Glass, W. G. *et al.* Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *J Exp Med* **202**, 1087-1098 (2005).

30 Lim, J. K. *et al.* Chemokine receptor Ccr2 is critical for monocyte accumulation and survival in West Nile virus encephalitis. *J Immunol* **186**, 471-478, doi:10.4049/jimmunol.1003003 (2011).

31 Klein, R. S. *et al.* Neuronal CXCL10 directs CD8+ T-cell recruitment and control of West Nile virus encephalitis. *Journal of virology* **79**, 11457-11466 (2005).

32 Gourine, A. V. *et al.* Role of alpha(2)-macroglobulin in fever and cytokine responses induced by lipopolysaccharide in mice. *Am J Physiol Regul Integr Comp Physiol* **283**, R218-226, doi:10.1152/ajpregu.00746.2001 (2002).

33 Waghabi, M. C. *et al.* Increased Trypanosoma cruzi invasion and heart fibrosis associated with high transforming growth factor beta levels in mice deficient in alpha(2)-macroglobulin. *Infect Immun* **70**, 5115-5123 (2002).
34 Umans, L., Serneels, L., Overbergh, L., Stas, L. & Van Leuven, F. alpha2-macroglobulin- and murinoglobulin-1- deficient mice. A mouse model for acute pancreatitis. *Am J Pathol* **155**, 983-993 (1999).
35 Shemesh, C. S. *et al.* Pharmacokinetic and Pharmacodynamic Investigations of ION-353382, a Model Antisense Oligonucleotide: Using Alpha-2-Macroglobulin and Murinoglobulin Double-Knockout Mice. *Nucleic Acid Ther* **26**, 223-235, doi:10.1089/nat.2016.0607 (2016).
36 Kumar, M., Roe, K., O'Connell, M. & Nerurkar, V. R. Induction of virus-specific effector immune cell response limits virus replication and severe disease in mice infected with non-lethal West Nile virus Eg101 strain. *Journal of neuroinflammation* **12**, 178, doi:10.1186/s12974-015-0400-y (2015).
37 Diamond, M. S. Virus and host determinants of West Nile virus pathogenesis. *PLoS pathogens* **5**, e1000452, doi:10.1371/journal.ppat.1000452 (2009).
38 Hochepied, T., Van Leuven, F. & Libert, C. Mice lacking alpha(2)-macroglobulin show an increased host defense against Gram-negative bacterial sepsis, but are more susceptible to endotoxic shock. *Eur Cytokine Netw* **13**, 86-91 (2002).
39 Alonso, M. *et al.* Interaction of alpha-2-macroglobulin and HSV-1 during infection of neuronal cells. *J Neurovirol* **7**, 556-563 (2001).
40 Kounnas, M. Z. *et al.* The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J Biol Chem* **267**, 12420-12423 (1992).
41 Agnello, V., Abel, G., Elfahal, M., Knight, G. B. & Zhang, Q. X. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci U S A* **96**, 12766-12771 (1999).
42 Liu, Y. *et al.* Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. *Nat Med* **6**, 1380-1387, doi:10.1038/82199 (2000).
43 Hochepied, T., Ameloot, P., Brouckaert, P., Van Leuven, F. & Libert, C. Differential response of a(2)-macroglobulin-deficient mice in models of lethal TNF-induced inflammation. *Eur Cytokine Netw* **11**, 597-601 (2000).
44 Kumar, M. *et al.* Inflammasome adaptor protein Apoptosis-associated speck-like protein containing CARD (ASC) is critical for the immune response and survival in west Nile virus encephalitis. *Journal of virology* **87**, 3655-3667, doi:10.1128/JVI.02667-12 (2013).
45 Kumar, M., O'Connell, M., Namekar, M. & Nerurkar, V. R. Infection with non-lethal West Nile virus Eg101 strain induces immunity that protects mice against the lethal West Nile virus NY99 strain. *Viruses* **6**, 2328-2339, doi:10.3390/v6062328 (2014).
46 Kumar, M. *et al.* Impaired virus clearance, compromised immune response and increased mortality in type 2 diabetic mice infected with West Nile virus. *PLoS One* **7**, e44682, doi:10.1371/journal.pone.0044682 (2012).
47 Verma, S. *et al.* West Nile virus infection modulates human brain microvascular endothelial cells tight junction proteins and cell adhesion molecules: Transmigration across the in vitro blood-brain barrier. *Virology* **385**, 425-433, doi:10.1016/j.virol.2008.11.047 (2009).

48 Roe, K. *et al.* West Nile virus-induced disruption of the blood-brain barrier in mice is characterized by the degradation of the junctional complex proteins and increase in multiple matrix metalloproteinases. *J Gen Virol* **93**, 1193-1203, doi:10.1099/vir.0.040899-0 (2012).
49 Kumar, M., Krause, K. K., Azouz, F., Nakano, E. & Nerurkar, V. R. A guinea pig model of Zika virus infection. *Virol J* **14**, 75, doi:10.1186/s12985-017-0750-4 (2017).
50 Kumar, M. *et al.* Reduced immune cell infiltration and increased pro-inflammatory mediators in the brain of Type 2 diabetic mouse model infected with West Nile virus. *Journal of neuroinflammation* **11**, 80, doi:10.1186/1742-2094-11-80 (2014).
51 Kumar, M. & Nerurkar, V. R. Integrated analysis of microRNAs and their disease related targets in the brain of mice infected with West Nile virus. *Virology* **452-453**, 143-151, doi:10.1016/j.virol.2014.01.004 (2014).

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Author Contributions

M.K. and V.R.N. designed, analyzed results, and wrote the manuscript. K.K., F.A., E.N., and M.K. conducted the experiments. K.K., F.A., V.R.N., and M.K. analyzed data. All authors have read and approved the final version of the manuscript.

Competing Financial Interests

Authors have no competing interests.

Chapter 4

Future Directions

4.1 **Summary:**

West Nile virus (WNV) and Japanese Encephalitis virus (JEV) are leading causes of arboviral encephalitis worldwide, and WNV accounts for hundreds of deaths each year in the US alone.[4,6] The epidemiological data compiled shows that although these viruses are closely related flaviviuses, they are circulating within different geographical regions and the highest at risk populations for severe complications due to infection differ between WNV and JEV as well.[6,12] Currently, there are no clinically approved therapies for the most severe forms of the diseases associated with WNV and JEV, therefore studies that provide novel insights in the host factors that play a role in viral pathogenesis and disease progression are of utmost importance. Results of our studies have demonstrated for the first time the crucial role that alpha-macroglobuins play in WNV and JEV infections and disease progression *in vivo*. This was demonstrated in alpha-macroglobulin deficient double knockout mice which have been genetically altered to allow for the absence of alpha-macroglobulins. The overall summary and trend of our study shows that WT mice demonstrated a significant amount of mortality and clinical disease after lethal subcutaneous WNV and JEV infections. When compared to WT, the DKO mice demonstrated complete resilience to these infections (100% survival) over multiple independent experiments and in a similar trend showed a significantly milder clinical disease as well. Viral load and inflammatory responses were analyzed in the periphery and multiple tissues, including the brain, and similar to the survival and clinical disease outcomes data the WT mice show a high viral burden and elevated inflammatory responses in these tissues. On the contrary, the DKO mice had a significantly decreased viral burden in multiple tissues, and significantly dampened inflammatory responses as well. Due to the fact that in humans A2M has been examined in the clinical settings as a maker for several diseases, it is clear that this protein is a crucial immune-

regulatory protein for many diseases processes, and subsequently its role(s) in viral infections and associated disease warrants further investigations. Studies that seek to elucidate the mechanisms behind alpha-macroglobulins and their role in flaviviral infections are necessary to take the next step towards developing clinically applicable therapeutic options for infected individuals.

4.2 **Future Directions:**

Future studies are necessary to develop a further understanding of the functional role of alpha-macroglobulins during flavivirus infections. Due to the fact that it has been previously shown that alpha-macroglobulins can bind to closely related flaviviruses such as Dengue virus (DENV) and enhance their infections *in vitro*, similar *in vitro* experiments should also be performed with WNV and JEV.[36] Infection studies performed *in vitro* on cell populations isolated from WT and alpha-macroglobulin deficient (DKO) mice would allow us to determine whether the absence of alpha-macroglobulins has an effect on infections with WNV or JEV *in vitro* and which cell types show a greater enhancement of infection. Following the initial experiments and data analysis, similar *in vitro* infection studies with human cell types would be of interest to compare with mouse cell infection experiments. In this approach we can determine how the presence of alpha-macroglobulins translates from mouse tissue to human tissue. On the other hand, because we know that A2M binds to the LRP receptor in human cells, *in vitro* receptor blockade experiments could be performed on cell types where an enhanced infection occurs in the presence of alpha-macroglobulins in mice. Using this approach, we can determine which cell types are most critical for enhancement of flaviviral infections in human cell types in the presence of A2M *in vitro*, and subsequently attempt to block enhancement of the infection by inhibiting the LRP receptor. If the

receptor blocking approach is unsuccessful, an alternate approach would be to perform a co-immunoprecipitation assays to determine first if WNV binds to the A2M protein, allowing for enhancement of infection, and if so determine the binding site of the virus to the protein. With this approach, certain sites on the A2M protein could be blocked which have been shown to bind the virus, and resultantly prevent binding of the virus to A2M. Once either receptor blocking, or the binding site for virus on the A2M protein has been tested and established *in vitro*, *in vivo* studies could be performed as well once a blocking technique has been established. Furthermore, these experiments can be performed with other closely related flaviviruses for a comparative analysis on viral pathogenesis as it relates to the presence and absence of alpha-macroglobulins.

Bibliography

1 Suthar, M. S., Diamond, M. S. & Gale, M., Jr. West Nile virus infection and immunity. *Nat Rev Microbiol* **11**, 115-128, doi:10.1038/nrmicro2950 (2013).

2 Petersen, L. R. & Roehrig, J. T. West Nile virus: a reemerging global pathogen. *Emerg Infect Dis* **7**, 611-614, doi:10.3201/eid0704.010401 (2001).

3 Roehrig, J. T. West nile virus in the United States - a historical perspective. *Viruses* **5**, 3088-3108, doi:10.3390/v5123088 (2013).

4 Chancey, C., Grinev, A., Volkova, E. & Rios, M. The global ecology and epidemiology of West Nile virus. *Biomed Res Int* **2015**, 376230, doi:10.1155/2015/376230 (2015).

5 Brinton, M. A. The molecular biology of West Nile Virus: a new invader of the western hemisphere. *Annu Rev Microbiol* **56**, 371-402, doi:10.1146/annurev.micro.56.012302.160654 (2002).

6 CDC. *West Nile virus disease cases and deaths reported to CDC by year and clinical presentation, 1999-2016.*
Average annual incidence of West Nile virus neuroinvasive disease reported to CDC by age group, 1999-2016., <https://www.cdc.gov/westnile/statsmaps/cumMapsData.html#> (2016).

7 Meek, J. West Nile virus in the United States. *Curr Opin Pediatr* **14**, 72-77 (2002).

8 Lim, S. M., Koraka, P., Osterhaus, A. D. & Martina, B. E. West Nile virus: immunity and pathogenesis. *Viruses* **3**, 811-828, doi:10.3390/v3060811 (2011).

9 Hayes, E. B. & Gubler, D. J. West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. *Annu Rev Med* **57**, 181-194, doi:10.1146/annurev.med.57.121304.131418 (2006).

10 Michael C. Pearce, e. a. Serum neutralising antibody response of seronegative horses against lineage 1 and lineage 2 West Nile virus following vaccination with an inactivated lineage 1 West Nile virus vaccine. *Journal of the South African Vetrinary Association* **84** (2013).

11 Amanna, I. J. & Slifka, M. K. Current trends in West Nile virus vaccine development. *Expert Rev Vaccines* **13**, 589-608, doi:10.1586/14760584.2014.906309 (2014).

12 Yun, S. I. & Lee, Y. M. Japanese encephalitis: the virus and vaccines. *Hum Vaccin Immunother* **10**, 263-279, doi:10.4161/hv.26902 (2014).

13 Fischer, M. *et al.* Japanese encephalitis vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* **59**, 1-27 (2010).

14 Rehman, A. A., Ahsan, H. & Khan, F. H. alpha-2-Macroglobulin: a physiological guardian. *J Cell Physiol* **228**, 1665-1675, doi:10.1002/jcp.24266 (2013).

15 Armstrong, P. B. & Quigley, J. P. Alpha2-macroglobulin: an evolutionarily conserved arm of the innate immune system. *Dev Comp Immunol* **23**, 375-390 (1999).

16 Tayade, C., Esadeg, S., Fang, Y. & Croy, B. A. Functions of alpha 2 macroglobulins in pregnancy. *Mol Cell Endocrinol* **245**, 60-66, doi:10.1016/j.mce.2005.10.004 (2005).

17 Steven L. Gonias, e. a. Murine α-Macroglobulins Demonstrate Divergent Activities as Neutralizers of Transforming Growth Factor-β and as Inducers of Nitric Oxide Synthesis. *Journal of Biological Chemistry* **271**, 24982-24988 (1996).

18 Dalli, J. *et al.* Microparticle alpha-2-macroglobulin enhances pro-resolving responses and promotes survival in sepsis. *EMBO Mol Med* **6**, 27-42, doi:10.1002/emmm.201303503 (2014).

19 Barton F. Hayes, e. a. Increased immunogenicity of HIV envelope subunit complexed with α2-macroglobulin when combined with monophosphoryl lipid A and GM-CSF. *Elsevier* **20**, 2396-2403 (2002).
20 M.Thambisetty, e. a. Alpha-2 macroglobulin in Alzheimer's disease: a marker of neuronal injury through the RCAN1 pathway. *Nature: Molecular Psychiatry* **22**, 13-23 (2017).
21 Ramasamy, S. *et al.* Cardiac isoform of alpha 2 macroglobulin, an early diagnostic marker for cardiac manifestations in AIDS patients. *AIDS* **20**, 1979-1981, doi:10.1097/01.aids.0000247122.97079.71 (2006).
22 Wu, S. M., Patel, D. D. & Pizzo, S. V. Oxidized alpha2-macroglobulin (alpha2M) differentially regulates receptor binding by cytokines/growth factors: implications for tissue injury and repair mechanisms in inflammation. *J Immunol* **161**, 4356-4365 (1998).
23 Nezu, T. *et al.* Alpha2-macroglobulin as a promising biomarker for cerebral small vessel disease in acute ischemic stroke patients. *J Neurol* **260**, 2642-2649, doi:10.1007/s00415-013-7040-x (2013).
24 Kovacs, D. M. alpha2-macroglobulin in late-onset Alzheimer's disease. *Exp Gerontol* **35**, 473-479 (2000).
25 O'Leary, D. R. *et al.* The epidemic of West Nile virus in the United States, 2002. *Vector Borne Zoonotic Dis* **4**, 61-70 (2004).
26 Petersen, C. M. Alpha 2-macroglobulin and pregnancy zone protein. Serum levels, alpha 2-macroglobulin receptors, cellular synthesis and aspects of function in relation to immunology. *Dan Med Bull* **40**, 409-446 (1993).
27 Abe, K., Yamamoto, K. & Sinohara, H. Proteinase inhibitory spectrum of mouse murinoglobulin and alpha-macroglobulin. *J Biochem* **106**, 564-568 (1989).
28 Tiggelman, A. M., Linthorst, C., Boers, W., Brand, H. S. & Chamuleau, R. A. Transforming growth factor-beta-induced collagen synthesis by human liver myofibroblasts is inhibited by alpha2-macroglobulin. *J Hepatol* **26**, 1220-1228 (1997).
29 Becker, K., Niederau, C. & Frieling, T. Fecal excretion of alpha 2-macroglobulin: a novel marker for disease activity in patients with inflammatory bowel disease. *Z Gastroenterol* **37**, 597-605 (1999).
30 Annapoorani, P. *et al.* Cardiac isoform of alpha-2 macroglobulin--a new biomarker for myocardial infarcted diabetic patients. *Atherosclerosis* **186**, 173-176, doi:10.1016/j.atherosclerosis.2005.07.004 (2006).
31 Ho, A. S. *et al.* Novel biomarkers predict liver fibrosis in hepatitis C patients: alpha 2 macroglobulin, vitamin D binding protein and apolipoprotein AI. *J Biomed Sci* **17**, 58, doi:10.1186/1423-0127-17-58 (2010).
32 Bernhard, O. K., Kapp, E. A. & Simpson, R. J. Enhanced analysis of the mouse plasma proteome using cysteine-containing tryptic glycopeptides. *J Proteome Res* **6**, 987-995, doi:10.1021/pr0604559 (2007).
33 Webb, D. J. *et al.* Murine alpha-macroglobulins demonstrate divergent activities as neutralizers of transforming growth factor-beta and as inducers of nitric oxide synthesis. A possible mechanism for the endotoxin insensitivity of the alpha2-macroglobulin gene knock-out mouse. *J Biol Chem* **271**, 24982-24988 (1996).
34 Liao, H. X. *et al.* Increased immunogenicity of HIV envelope subunit complexed with alpha2-macroglobulin when combined with monophosphoryl lipid A and GM-CSF. *Vaccine* **20**, 2396-2403 (2002).

35 Mitsuda, S., Nakagawa, T., Nakazato, H. & Ikai, A. Receptor-linked antigen delivery system. Importance of autologous alpha 2-macroglobulin in the development of peptide vaccine. *Biochem Biophys Res Commun* **216**, 399-405 (1995).
36 Huerta, V. *et al.* Receptor-activated human alpha2-macroglobulin interacts with the envelope protein of dengue virus and protects virions from temperature-induced inactivation through multivalent binding. *J Gen Virol* **95**, 2668-2676, doi:10.1099/vir.0.068544-0 (2014).
37 Mitsuda, S. *et al.* A receptor mediated delivery of an HIV-1 derived peptide vaccine. *Biochem Biophys Res Commun* **194**, 1155-1160, doi:10.1006/bbrc.1993.1943 (1993).
38 Cianciolo, G. J., Enghild, J. J. & Pizzo, S. V. Covalent complexes of antigen and alpha(2)-macroglobulin: evidence for dramatically-increased immunogenicity. *Vaccine* **20**, 554-562 (2001).
39 Bowers, E. V., Horvath, J. J., Bond, J. E., Cianciolo, G. J. & Pizzo, S. V. Antigen delivery by alpha(2)-macroglobulin enhances the cytotoxic T lymphocyte response. *J Leukoc Biol* **86**, 1259-1268, doi:10.1189/jlb.1008653 (2009).
40 Anderson, R. B., Cianciolo, G. J., Kennedy, M. N. & Pizzo, S. V. Alpha 2-macroglobulin binds CpG oligodeoxynucleotides and enhances their immunostimulatory properties by a receptor-dependent mechanism. *J Leukoc Biol* **83**, 381-392, doi:10.1189/jlb.0407236 (2008).
41 Kumar, M., Belcaid, M. & Nerurkar, V. R. Identification of host genes leading to West Nile virus encephalitis in mice brain using RNA-seq analysis. *Sci Rep* **6**, 26350, doi:10.1038/srep26350 (2016).
42 Kumar, M., Verma, S. & Nerurkar, V. R. Pro-inflammatory cytokines derived from West Nile virus (WNV)-infected SK-N-SH cells mediate neuroinflammatory markers and neuronal death. *Journal of neuroinflammation* **7**, 73, doi:10.1186/1742-2094-7-73 (2010).
43 Shrestha, B., Zhang, B., Purtha, W. E., Klein, R. S. & Diamond, M. S. 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, doi:10.1128/JVI.01118-08 (2008).
44 Glass, W. G. *et al.* Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *J Exp Med* **202**, 1087-1098 (2005).
45 Lim, J. K. *et al.* Chemokine receptor Ccr2 is critical for monocyte accumulation and survival in West Nile virus encephalitis. *J Immunol* **186**, 471-478, doi:10.4049/jimmunol.1003003 (2011).
46 Klein, R. S. *et al.* Neuronal CXCL10 directs CD8+ T-cell recruitment and control of West Nile virus encephalitis. *Journal of virology* **79**, 11457-11466 (2005).
47 Gourine, A. V. *et al.* Role of alpha(2)-macroglobulin in fever and cytokine responses induced by lipopolysaccharide in mice. *Am J Physiol Regul Integr Comp Physiol* **283**, R218-226, doi:10.1152/ajpregu.00746.2001 (2002).
48 Waghabi, M. C. *et al.* Increased Trypanosoma cruzi invasion and heart fibrosis associated with high transforming growth factor beta levels in mice deficient in alpha(2)-macroglobulin. *Infect Immun* **70**, 5115-5123 (2002).
49 Umans, L., Serneels, L., Overbergh, L., Stas, L. & Van Leuven, F. alpha2-macroglobulin- and murinoglobulin-1- deficient mice. A mouse model for acute pancreatitis. *Am J Pathol* **155**, 983-993 (1999).

50 Shemesh, C. S. *et al.* Pharmacokinetic and Pharmacodynamic Investigations of ION-353382, a Model Antisense Oligonucleotide: Using Alpha-2-Macroglobulin and Murinoglobulin Double-Knockout Mice. *Nucleic Acid Ther* **26**, 223-235, doi:10.1089/nat.2016.0607 (2016).
51 Kumar, M., Roe, K., O'Connell, M. & Nerurkar, V. R. Induction of virus-specific effector immune cell response limits virus replication and severe disease in mice infected with non-lethal West Nile virus Eg101 strain. *Journal of neuroinflammation* **12**, 178, doi:10.1186/s12974-015-0400-y (2015).
52 Diamond, M. S. Virus and host determinants of West Nile virus pathogenesis. *PLoS pathogens* **5**, e1000452, doi:10.1371/journal.ppat.1000452 (2009).
53 Hochepied, T., Van Leuven, F. & Libert, C. Mice lacking alpha(2)-macroglobulin show an increased host defense against Gram-negative bacterial sepsis, but are more susceptible to endotoxic shock. *Eur Cytokine Netw* **13**, 86-91 (2002).
54 Alonso, M. *et al.* Interaction of alpha-2-macroglobulin and HSV-1 during infection of neuronal cells. *J Neurovirol* **7**, 556-563 (2001).
55 Kounnas, M. Z. *et al.* The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J Biol Chem* **267**, 12420-12423 (1992).
56 Agnello, V., Abel, G., Elfahal, M., Knight, G. B. & Zhang, Q. X. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci U S A* **96**, 12766-12771 (1999).
57 Liu, Y. *et al.* Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. *Nat Med* **6**, 1380-1387, doi:10.1038/82199 (2000).
58 Hochepied, T., Ameloot, P., Brouckaert, P., Van Leuven, F. & Libert, C. Differential response of a(2)-macroglobulin-deficient mice in models of lethal TNF-induced inflammation. *Eur Cytokine Netw* **11**, 597-601 (2000).
59 Kumar, M. *et al.* Inflammasome adaptor protein Apoptosis-associated speck-like protein containing CARD (ASC) is critical for the immune response and survival in west Nile virus encephalitis. *Journal of virology* **87**, 3655-3667, doi:10.1128/JVI.02667-12 (2013).
60 Kumar, M., O'Connell, M., Namekar, M. & Nerurkar, V. R. Infection with non-lethal West Nile virus Eg101 strain induces immunity that protects mice against the lethal West Nile virus NY99 strain. *Viruses* **6**, 2328-2339, doi:10.3390/v6062328 (2014).
61 Kumar, M. *et al.* Impaired virus clearance, compromised immune response and increased mortality in type 2 diabetic mice infected with West Nile virus. *PLoS One* **7**, e44682, doi:10.1371/journal.pone.0044682 (2012).
62 Verma, S. *et al.* West Nile virus infection modulates human brain microvascular endothelial cells tight junction proteins and cell adhesion molecules: Transmigration across the in vitro blood-brain barrier. *Virology* **385**, 425-433, doi:10.1016/j.virol.2008.11.047 (2009).
63 Roe, K. *et al.* West Nile virus-induced disruption of the blood-brain barrier in mice is characterized by the degradation of the junctional complex proteins and increase in multiple matrix metalloproteinases. *J Gen Virol* **93**, 1193-1203, doi:10.1099/vir.0.040899-0 (2012).
64 Kumar, M., Krause, K. K., Azouz, F., Nakano, E. & Nerurkar, V. R. A guinea pig model of Zika virus infection. *Virol J* **14**, 75, doi:10.1186/s12985-017-0750-4 (2017).

65 Kumar, M. *et al.* Reduced immune cell infiltration and increased pro-inflammatory mediators in the brain of Type 2 diabetic mouse model infected with West Nile virus. *Journal of neuroinflammation* **11**, 80, doi:10.1186/1742-2094-11-80 (2014).
66 Kumar, M. & Nerurkar, V. R. Integrated analysis of microRNAs and their disease related targets in the brain of mice infected with West Nile virus. *Virology* **452-453**, 143-151, doi:10.1016/j.virol.2014.01.004 (2014).