January 2019

Avian IgY As An Immunotherapy For Flaviviral Infections

Kyle O'donnell

AVIAN IGY AS AN IMMUNOTHERAPY FOR FLAVIVIRAL INFECTIONS

By

Kyle Lee O'Donnell

A Dissertation

Submitted to the Graduate Faculty

of the

University of North Dakota

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota

May 2019

This dissertation, submitted by Kyle O'Donnell in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Dr. David S. Bradley

Dr. Matthew L. Nilles

Dr. Colin K. Combs

Dr. Jyotika Sharma

Dr. Jefferson Vaughan

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Chris Nelson
Associate Dean of the School of Graduate Studies

4/25/19
Date

PERMISSION

Title: Avian IgY as an Immunotherapy for Flaviviral Infections

Department: Biomedical Sciences

Degree: Doctor of Philosophy

In presenting this dissertation in partial fulfillment of the requirements for a graduate degree from the University of North Dakota, I agree that the library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by the professor who supervised my dissertation work or, in his absence, by the Chairperson of the department or the dean of the School of Graduate Studies. It is understood that any copying or publication or other use of this dissertation or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of North Dakota in any scholarly use which may be made of any material in my dissertation.

Kyle O'Donnell
April 24th, 2019

Acknowledgments

I would like to express my deepest gratitude to everyone who has provided guidance and support throughout my graduate research career. To top that list is my advisor Dr. Bradley, for allowing me the freedom to develop my own scientific ideas and to develop into an independent scientist while still continually providing me with encouragement and support when needed. His genuine commitment to the professional development of young scientist and continued progression of scientific knowledge makes me honored to have learned under him and I hope to one day carry that same standard when I establish my own lab. I would also like to thank Dr. Vaughan for broadening my scientific perspective from not only focusing on the clinical aspect, but the pathogenic process as a whole. I believe this has not only allowed me to grow as a more complete virologist, but given me a unique insight that I hope to apply to new projects in the future. I would also like to thank the remaining members of my dissertation committee Dr. Nilles, Dr. Sharma, and Dr. Combs for always having your doors open to me whenever I had a question I needed insight to and for furthering my career with either asking questions at seminars, letters of recommendation, to casual scientific talks in the hallways after invited speakers. I valued each of your thoughts and suggestions highly and they greatly impacted my graduate career development. I would also like to thank the rest of the Biomedical Sciences faculty for numerous discussions and instructions provided. I would like to thank members of the Bradley lab former and present for riveting scientific discussions and keeping me sane after all nighters. I would like to

thank Steve Adkins for assisting in the microinjections of mosquitoes and your unique insights. I would like to thank Dr. Peter Knopick and Dr. Travis Alvine for their mentorship and friendship. Both of you challenged me intellectually and gave important insights into my work as well as your own. I am a far better scientist for working with both of you. I would like to thank the numerous undergraduate students whom worked in the Bradley lab and assisted with my projects. Science can never be done completely alone and their help was greatly appreciated. Finally, I would like to thank my family for not only putting up with me constantly talking about virology at family functions, but for providing me the support when needed and for instilling in me that hard work will pay off in the end.

Table of Contents

List of Figures

List of Tables

Abstract

Flaviviruses compose a group of positive single strand RNA viruses. This group possess 70 individual viruses that cause disease in humans and animals. This group contains the most prevalent arbovirus dengue virus (DENV) and a recently emerging arbovirus zika virus (ZIKV). Both DENV and ZIKV represent significant world health threats and both viruses, at the moment, are contained to tropical and sub tropical regions due to vector habitat restrictions. DENV can cause severe hemorrhagic fever termed dengue hemorrhagic fever and dengue shock syndrome depending on the extent of vascular permeability. The disease burden attributed to dengue infection is approximately 390 million infections per year. Of these infections, 96 million will result in clinical disease and 500,000 patients require hospitalization resulting in 25,000 deaths a year. ZIKV presents a less severe disease pathology, with a majority of infections in healthy adults being asymptomatic. The more severe infections in adults result in an autoimmune disease called Guillain-Barre syndrome. What caused the world health organization to declare ZIKV a world health emergency in 2016 is the viruses' ability to transverse the placenta barrier and infect a developing fetus. The most severe symptom associated with *in utero* infection is the development of microcephaly, which leads to severe cognitive impairment. As case studies expand and the disease pathology of ZIKV is more fully understood a class of symptoms termed congenital zika syndrome fully classifies the extent of cognitive abnormalities induced by this virus. A commonality of these two viruses is that there are no approved treatments for either of

these viral infections. A vaccine for DENV was recently introduced, but due to immunological complications it was withdrawn from distribution. A significant issue to combat with the development of therapies for dengue and zika viral infection is the induction of antibody dependent enhancement (ADE). ADE is mediated when cross-reactive low affinity antibodies bind to the virus and are internalized via the FcγR on myeloid cells, but do not neutralize the virus resulting in an induction of pro-inflammatory cytokines and increased viral titer. In the studies presented here we hypothesized that the utilization of avian IgY, which does not interact with mammalian Fc receptors, would provide a viable therapy for ZIKV and DENV viral infection. Polyvalent anti-ZIKV IgY was purified from eggs of ZIKV immunized geese. The purified anti-ZIKV IgY preparation was assessed for it's ability to neutralize ZIKV infection *in vitro* and *in vivo*. We also assessed for the ability of polyvalent anti-ZIKV IgY to enhance viral infection *in vitro*. Our data suggests that anti-ZIKV IgY is able to neutralize ZIKV infection *in vitro* and *in vivo* without inducing ADE. Our data also demonstrates novel viral epitopes recognized in our polyvalent anti-ZIKV IgY preparation. Previously our lab had established that polyvalent anti-DENV IgY was able to neutralize ZIKV infection *in vitro* and *in vivo* without inducing ADE. Novel non-structural protein 1 (NS1) epitopes were recognized and determined by microarray analysis. In our study we expand upon this work and determine if the novel NS1 IgY have the ability to neutralize DENV infection *in vitro* and *in vivo.* Our data suggests that anti-NS1 IgY has the ability to neutralize DENV infection *in vitro* and *in vivo*. This is highly atypically of anti-NS1 DENV

antibodies, which typically only exhibit efficacy *in vivo*. Thus our data suggests not only a novel epitope of neutralization, but a unique neutralization mechanism distinctive to IgY. Due to the emergence of zika virus as a global health threat a greater vector competency profile was needed to be conducted to estimate the true pathogenic range and which populations could be potentially at risk. In collaboration with the Biology department at UND we conducted vector competency studies of mosquito species native to the region. To our surprise our common pest mosquito Aedes vexans was not only able to establish a midgut infection, but also developed disseminated infection in a small percentage of cases. We then tested the ability for the mosquito to transmit the virus under laboratory settings and Aedes vexans were able to transmit the virus at a much higher rate than the positive control population Aedes aegypti. This study greatly expands the potential geographical area that zika virus could potentially spread to, as Aedes vexans is common in many temperate climates across North America and Europe.

Chapter I

Introduction Flaviviruses

Flaviviruses compose a group of positive single strand RNA viruses. This group possesses 70 individual viruses that cause disease in humans and animals. All viruses in this group are named for the location in which they were discovered. This is divergent from many other viral groups which rely on genetic characteristics for nomenclature. Many of these pathogenic flaviviruses are arboviruses which indicates they are spread by mosquitoes or ticks. Of the 70 viruses that cause diseases 5 of them are of great public health concern for humans they are; Dengue virus, West Nile virus, Japanese encephalitis virus, Yellow fever virus, and Zika virus. Flaviviruses encode 10 genes three structural genes and seven non-structural genes. The structural genes are the lipid envelope (E) the pre-membrane (prM) and the internal capsid (C). These proteins are involved in composing the extra-RNA protective matrix, receptor fusion of an infectious virion, and immune evasion. The seven non-structural proteins are non-structural protein 1 (NS1), non-structural protein 2a/b (NS2a, NS2b), non-structural protein 3 (NS3), non-structural protein 4a/b (NS4a, NS4b), and non-structural protein 5 (NS5). The primary function of these proteins in developing the viral replication complex and copying the viral RNA for the production of mature virions. Many non-structural proteins also possess immune evasive properties particularly against intracellular innate pattern recognition receptors. Initially the viral RNA encodes a polyprotein precursor which is then enzymatically cleaved to individual viral proteins.

In further detail a model virion to describe viral replication of this group is DENV. The 50 nm viral particle attaches to an extracellular receptor on susceptible host cells. The primary host receptor has not been fully elucidated, but many receptors have been demonstrated experimentally. The primary candidate is DC-SIGN/L-SIGN expressed on dendritic cells and macrophages (1, 2, 3). Heparan sulfate receptor is also a prime candidate as it has been identified as a receptor in multiple in vitro models of dengue virus infection including Vero cells, BHK-21 cells, and SW-13 cells (4, 5). Mannose receptor may be a target for viral attachment on macrophages (6). These three receptors are the only receptors identified that are involved in the internalization of all four DENV serotypes. The virion is then internalized by clathrin mediated endocytosis once the endosomal pH starts to lower a conformational change of the viral envelope exposes the DIII fusion loop that allow for the viral envelop to fuse with the endosomal membrane and release the viral ribonucleocpasid into the cytoplasm (7). Viral non-structural proteins and host factors co-integrate and form a membrane associated replication complex that allows for viral RNA replication to take place. The viral replication complex is formed inside a vesicular compartment of the endoplasmic reticulum (ER) lumen. NS4A and B anchor the complex on the cytoplasmic side and a NS1 dimer anchors the ER lumen side. Protein-protein interactions between NS1 and NS4A and B has been demonstrated (8). NS4B is also in direct contact with NS3 which functions as the RNA helicase (9). This interaction is not complete without NS2B which is a co-factor for NS3 helicase activity (10). NS5 is the RNA dependent RNA

polymerase and methyltranserase which is important for RNA capping of the new viral genome. NS2A anchors the viral RNA to the ER lumen to allow for complete replication (11). The newly synthesized RNA is packaged by the capsid protein to start the formation of a new immature viral particle. The prM and E proteins form heterodimers in the ER lumen. Once integrated together into trimers a curved lattice structure is formed which directs virion budding (12, 13). The immature virion then travels through the trans-Golgi network. The moderately acidic pH of the trans-Golgi network induces a conformational change of the prM/E trimers to lie flat on the surface of the particle. This restructuring is critical to allow for the cellular endoprotease furin to cleave prM to generate the mature M and dissociate the “pr” particle that is then secreted from infected cells and act to evade host immune responses (14, 15, 16). The now mature virion is exocytosed from the cell preferentially through lipid rafts harboring NS1 dimers. As stated previously not only is the mature virion secreted but also pr and NS1 hexamer which as involved in immune evasion and increase in disease pathology.

Flaviviral pathogenesis can be separated into two separate clades based on the type of clinical manifestations presented in patients. The first is clade are the viruses that induce hemorrhaging for the purpose of this dissertation Dengue virus will be used as an example as it is applicable to future studies in combating the pathogenesis. The second clade are those that induce neurological symptoms, Zika virus will be used as

an example in this dissertation even though it possesses unique characteristics and is not a stereotypical example of this clade.

Infection with any of the four serotypes of DENV in a majority of cases result in an asymptomatic infection. Infections may progress into a wide variety of clinical manifestations ranging from mild flu like symptoms to more severe symptoms including vascular permeability and shock. The World Health Organization has four grades of dengue hemorrhagic fever (DHF). Grades I and II are less severe and do not induce shock. Grades III and IV cases are more severe and contain hypovolemic shock as a symptom. Pathology of DENV can be broken down into three major organs that play a role: immune system, the liver, and endothelial cell linings of blood vessels. Initial introduction of DENV to the host via the mosquito proboscis into the skin, which allows for the infection of Langerhans cells (17). These residential dendritic cells then migrate to local lymph nodes which exposes infiltrating monocytes and macrophages for infection. Once these cells have become infected the virus is then able to spread throughout the limbic system continually encountering susceptible cells for continued viral infection and replication (18-20). Secondary infection with a heterotypic serotype of DENV can result in antibody mediated infection of monocytes, but this will be explained in greater detail in the section on antibody dependent enhancement (ADE). Mononuclear cells primary die via apoptosis which in turns stimulates inflammatory mediators which contributes to the disease pathology (20, 21, 22). Organ pathology traditionally has been a challenge due to the lack of resources in endemic areas of this

disease and cultural traditions that inhibit autopsies from being performed. Virus has been identified in patients who died within 36 hour of developing shock in the skin, liver, spleen, lymph nodes, kidney, bone marrow, lung, thymus, and brain (23). Infectious virions could only be cultured from the liver and peripheral blood mononuclear cells (PBMCs), which could indicate that the virus in the other organs was degraded or in an immature state. Liver is typically associated with DENV infection in humans and mice. A unique attribute of this area of pathology is that little inflammation has been detected in the liver and the tissue disruption is directly viral related as compared to a secondary immune scaring event. Extensive work form the Eva Harris lab has demonstrated the pathogenic role of NS1 on the endothelial glycocalyx. This occurs when circulating pentameric NS1 molecules bind to the surface of endothelial cells in capillary beds. The interaction between the endothelial cell surface and NS1 results in the up regulation of sialadases Neu1-3, which leads to the cleavage of sialic acid on the surface of the endothelial cells. The binding of NS1 also induces an increased expression of cathepsin L, which in turns over expresses heperanase and results in the cleavage of hepran sulfate and hepran sulfate proteoglycans. When all of these processes occur simultaneously they result in loss of endothelial glycocalyx layer and barrier integrity (24).

Taking a closer look at the immune response elicited by DENV not all are favorable to the host. Four immunological events can contribute to DENV pathology: complement activation, ADE, cross reactive T-cells, and soluble factors. The

complement system is an integral part of the humoral innate response and can act rapidly when induced by a pathogen antigen. It has been previously demonstrated that high levels of complement activation subunits C3a and C5a are found circulating in patient blood whom develop DHF and dengue shock syndrome (DSS) (25, 26). NS1 can induce antibodies that can target the dimeric NS1 expressed on infected cells. In other cases, these antibodies can target cells that NS1 is binding to inducing antibody mediated complement lyses of uninfected cells as well. (27). The generation of less than ideal antibodies in DENV infection is a common theme throughout the infection process. DENV utilizes a number of factors to evade or miss lead the antibody response to make it as ineffective as possible, such mechanism have previously been described as in NS1 over activating the complement system, pr is a potent B cell antigen that has no neutralization properties to a mature virion effectively DENV uses it as an antigenic decoy. One of the critical follies in the antibody repertoire of DENV infection is the production of high affinity and sub neutralizing antibodies. These antibodies have high affinity to the primary serotype the patient is infected with but on a secondary heterotypic infection they will bind, but not neutralize the virus enabling the virus to utilize FcγR's as an additional route of infecting cells. This mechanism will be discussed later in a separate section. T cells like antibodies can be a double edge sword in DENV pathogenesis. Memory cross reactive CD8 T cells can provide robust protection (28), but they can also contribute to pathogenesis leading to the "original antigenic sin hypothesis." The basis for this hypothesis is that low-avidity cross reactive CD8 T cells

would preferentially expand compared to high-avidity cross reactive CD8 T cells. The high-avidity cross reactive CD8 T cells produce robust pro- and anti-inflammatory cytokine response resulting in a balanced immune response that has the ability to lyse virally infected cells and then mitigate the immunological scaring due to the lysis process. Due to the robust activation of these cells they rapidly undergo apoptosis. This leaves a window for the low avidity CD8 T cells to preferentially expand in the place of the now expired high avidity counter parts. The low avidity CD8 T cells have the ability to produce high levels of pro-inflammatory cytokines, but lack effective cytolytic activity and produce minimal levels of anti-inflammatory cytokines to reduce immunological scaring (29, 30). This has lead to the development of a cytokine storm in severe dengue patients. Through multiple patient screenings a panel of cytokines has been complied that are consistently higher in plasma levels with patients with severe dengue disease these are; IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, TGF-1β, TNFα and IFNγ (31,32,33,34,35,36,37,38,39,40,41). The issue for researchers is to determine which cytokine or combination of cytokines are actually inducing the pathology and which are still protective. One of the key hallmarks of severe dengue disease is vascular permeability which can partially be attributed to the over production of TNFα and IL-6 (42).The original antigenic sin hypothesis has also been observed in LCMV, Influenza, and MCMV (43, 44, 29). It is likely that not only factor contributes wholly to DENV pathogenesis. Some factors such as host genetic background are still being researched and are not fully understood how anomalies interact with the pathogenesis of the virus.

By placing the subsequent sections together an integrated mechanism of pathogenesis can be composed.

Pathogenesis of Zika virus early in infection is extremely similar to Dengue virus. During a mosquito blood meal, the virus is able to invade the body and infect keratinocytes, fibroblasts, and Langerhans cells (45,46,47). Once initially viral replication is successful viremia ensues and allows the virus to infect monocytes, which allows for the virus to spread throughout the body (48). Utilizing a “Trojan horse” methodology the infected monocytes are able to infiltrate immune privileged sites such as the brain, testes, and placenta (49). Presently, there is no physiologically established receptor for Zika viral infection. An attractive target that has shown promising results *in vitro* is the AXL receptor. This receptor is a member of the TAM family which interacts with Gas6 and Protein S which bind to the viral envelope (50). This receptor is expressed on the early cells in which the virus infects such as keratinocytes and endothelial cells (51, 52, 53). Even more promising is that AXL is also expressed on neural progenitor cells and target cells within the testes (54, 55). These two tissue tropism causes unique clinical manifestations. The first is the ability for the virus to infect neural progenitor cells, once the virus is within the neuron viral replication ensues and eventually induces apoptosis of the neuron. Continued viral replication results in major tissue damage resulting in major neuronal developmental abnormalities. Describing the clinical features of these outcomes is much more extensive. A large percentage (80%) of Zika infections are asymptomatic. The mild form of the disease with clinical

manifestations presents as a self-limiting febrile illness that lasts from 4-7 days. The more severe form of Zika infection results primarily when the virus is transmitted *in utero.* Microcephaly and Gullian-Barre syndrome rates were determined by Meta analysis of 2.3% and 1.23% of all pregnancies respectively (56,57). Many more neurological manifestations have been reported in Zika viral infections, these include craniofacial disproportion, spasticity, seizures, brainstem dysfunction, and feeding difficulties. More commonly than Microcephaly ocular abnormalities occur as a result of Zika viral infection, these include focal pigment mottling, chorioretinal macular atrophy, optic nerve abnormalities, cataract, intra-ocular calcifications, optic disk cupping, foveal reflex loss, macular hypoplasia, and scarring (58). To combat Zika viral infection a patients' body first will induce an innate response. The early innate response is centralized around pattern recognition receptors recognizing viral pathogen associated molecular patterns. Most important of the early innate response is the stimulation of type I and type III interferons. Type-I interferons will induce the JAK/STAT pathway which allows for IRF9 to translocate to the nucleus and induce the expression of interferon stimulated genes. Type-III interferons are critical to combating the virus at the maternal fetal interface. The Adaptive immune response to Zika virus is very similar to Dengue viral infection and poses the same immunological issues.

A critical event in both Dengue and Zika viral infection is the induction of antibody dependent enhancement (ADE). The first description of ADE was by Halstead et al. describing Murray Valley encephalitis virus where higher concentrations of antibody

neutralized the virus on chicken embryo monolayer, but at lower concentrations the viral titer increased to levels much greater than the viral control. Initially the type of Fc receptor to which the antibody binds influences the effectiveness of the phagocytosis. FcγRIIA is more highly expressed and has been most associated with ADE (59). Once the antibody and receptor complex is formed a signaling cascade of ITAM molecules are phosphorylated which recruit Syk/ZAP-70 to initiate phagocytosis (60). The amount of antibody bound to the complex directly influences the amount of virus and the method in which the complex is internalized. A smaller amount of antibody will utilize clathrin mediated endocytosis to bring in a single viral particle. Larger amounts of antibodies will induce aggregates and phagocytize via the mechanism previously stated bringing in a larger amount of virus at one time (61). Once internalized the viral complex has the ability to evade RIG-1 and MDA-5 both critical intracellular PRRs to induce type-I interferons (62). The inhibition of these PRRs also dramatically alters the cytokine profile of the infected cell, shifting from a pro inflammatory state to an anti-inflammatory state (63, 64, 65, 66). Down stream of the RIG-1 and MDA-5 type-I interferons utilize the Jak/STAT pathway to stimulate interferon stimulated genes that are potent antiviral inhibitors. To overcome this the viruses, utilize NS5 to target the IFNAR for proteasomal degradation (67) or to target STAT2 for degradation stopping the signaling cascade (68,69). Cohort studies in Cuba and Taiwan indicate that patients with secondary DENV infection had higher levels of serum IL-10 levels (70,71). In contrast to early in infection which induces high levels of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6

secondary heterologous infection has demonstrated a slightly lower inflammatory phenotype, which could be attributed to the induction of IL-10 (72). The cell type in which the virus is infecting has significant effects on the amount of IL-10 induced. Monocytes have been shown to induce high levels of IL-10 while macrophages are poor inducers of IL-10 (66, 73). Once IL-10 is produced it decreases the production of IL-12 and IFNγ both of which are potent inducers of activating adaptive immune cells with antiviral properties, must notably TH1 T cells and NK cells. Intracellular production of IL-10 has been shown to increase the production of SOCS-3 which has been shown to be a biological marker for severe dengue viral infection (62). In conjunction with the immune modulation of the cells the viruses also influence other cellular functions including autophagy. DENV and ZIKV have both demonstrated the ability to increase autophagy and autophagasome formation in a Atg5 dependent manner (74, 75). This process may involve NS4a up regulating autophagy utilizing the PI3 kinase pathway (76).

Chapter II

Introduction Avian IgY

Utilizing avian derived antibodies as alternative immunotherapies has developed into an attractive alternative for the treatment of animals and humans. Unique characteristics of avian antibodies can partially be contributed to the way in which the avian immune

system differs from the mammalian system. Like the mammalian immune system, the avian immune system possesses two major branches the innate and the adaptive systems. The innate system is the earliest system activated in response to a pathogen or antigenic stimuli. The innate response is made up of physical and chemical barriers to initially halt invading pathogens. These barriers also include biochemical responses such as defensins, complement, and lysozyme to name but a few. The innate system also harbors coordinated recruitment of specialized cells such as macrophages, granulocytes, thrombocytes, and natural killer cells. A distinct difference between mammalian innate system and avian system is the avian system lacks neutrophils, basophils, and eosinophils (77,78). Heterophils are the avian counterparts to the mammalian neutrophils. Heterophils, like neutrophils, are the fast acting cells of the innate system and are for the large part the first cell to the site of infection. Like neutrophils heterophils have the ability to phagocytize invading pathogens. Heterophils do not have the capabilities to produce an oxidative burst to destroy phagocytized microbes, and the components of the granules contained within the cell differ from mammalian neutrophils. Cellular receptors are also responsible for the innate immune response. These class of receptors are term pattern recognition receptors (PRR) because they interact with conserved pathogen-associated molecular patterns (PAMPs). There are four primary classes of PRR, C-type lectins, TLRs, NLRs, and RLRs. C-type lectins are responsible for recognize extracellular PAMPs, while NLRs and RLRs are responsible for recognizing intracellular PAMPs. TLRs can recognize

both classes of PAMPs and are one of the most widely studied PRR within the field of avian immunology. Avian species possess TLR1/6/10, 2,3,4,5,7, and 8 which act essentially as homologs to the mammalian counter parts (79-90). It is apparent that chickens respond to the PAMP unmethylated CpG motif the classical TLR9 agonist, but no evidence has been found for TLR9 expression (91-93). Chickens also possess two unique TLRs 15 and 21 (94, 95). Studies have shown that *S. enterica* challenge increased expression of chTLR15 specifically in the gut (95). Surprisingly chTLR21 has been shown to respond to unmethylated DNA containing CpG motifs, which could be the reason there has been no evidence of TLR9 in chickens because the function is already dominate by chTLR21 (96,97).

Adaptive Immune system

To assist in the maturation and development of the adaptive immune system the avian system possesses primary and secondary lymphoid organs. The primary lymphoid organs include the thymus, bone marrow, and an organ unique to avian species the Bursa of Fabricus (BF). The secondary organs include spleen, Harderian gland, germinal centers and other lymphoid tissues. Avian T cells have many of the structure and adaptive functions as mammalian T cells. Each T cell have a heterodimeric surface receptor (TCR) that is composed of two immunoglobulin domains that undergo extensive recombination to produce antigenic diversity (98). There are two heterodimer clusters the TCR$\alpha\beta$ or TCR $\gamma\Delta$. A distinction of avian maturation is that unlike mammalian gene TCR clustering which is in one location avian species have two

locations. The second location most resembles IgH V segments of mammals (99). A second structural abnormality in the avian TCR development is the difference in CD3 expression. Mammals possess four CD3 subgroups while the avian species only have two (100). To date TCR signaling of the avian system has no dissimilarities to the mammalian system indicating that the lack of CD3 diversity and increased TCR diversity does not influence downstream signaling drastically. The development of T cells spatially within the avian host is remarkably similar to that of mammals. Naïve T cells undergo development in the thymus. Differential expression in the periphery is dictated by the rate in which the T cells enter they thymus for development. The $\gamma\Delta$T cells are first to exit the thymus and circulate the periphery followed by the traditional $\alpha\beta$T cells. The development of avian B-cells is drastically different from that of the mammalian system due to the presence of the BF. A common lymphoid progenitor cell expressing CCRA+ undergo Ig rearrangement which are then transported by the blood to the BF specially the mesenchyme (101, 102). Further Ig rearrangement and proliferation occurs in the Bursal mesenchyme and it is suggested that surface Ig expression is needed for the precursor cells to migrate to the Bursal follicles (103, 104). In order to remain in the bursal follicles, the precursor B-cells must express AID and expression of CXCR5 to bind to CXCL13. Gene conversion and a further round of proliferation occurs within the follicles that is driven by BAFF (105, 106). Upon further maturation the bursal follicle separates into the medulla and cortex. Upon antigen expression by a dendritic cell the cells specific for that antigen then migrate to the follicular cortex (85,86). Once within

the follicular cortex the antigen specific b cells undergo another round of gene conversion and proliferation. At this point the B-cells are able to leave the bursa and migrate to a secondary lymphoid organ such as the spleen. The B-cell can become activated by exposure to the antigen in which they are specific for along with T-cell help (107). After this point the antibody secretion process and plasma cell development is indistinguishable from mammalian development (108, 109).

Immunoglobulin Maturation

The maturation in which antibodies of the avian adaptive immune system undergo diverges significantly from their mammalian counter parts. There are three antibody isotypes, IgM, IgA, and IgY present in the avian immune system. These isotypes are mammalian homologs to IgM, IgA, and IgG (110). Antibody homologous to mammalian IgE and IgD have been proposed, but the data is inconclusive on identification (111). Avian IgY is distinguished from mammalian IgG due to the decay chain of the molecule is larger and antigenically different from IgG, and at the genomic level IgY is more similar to mammalian IgE than IgG (112). The structure of IgY is similar to IgG with two light (L) and two heavy (H) chains. The molecular mass of IgY is slightly larger than IgG 167-250 kDa compared to 160 kDa respectively. The H chain has one variable (V) region and four constant (C) regions. The L chain is composed of one (V) and one (C) regions (113). Antibody diversity in the avian immune systems develops differently than in the mammalian system. Loci rearrangements contribute slightly to diversity as both

the H and L chain have only one V gene. A major mechanism of diversity of antibody repertoire in the avian system is due to increased instances of somatic hyper conversion (114, 115). The avian immune system has developed three additional mechanisms to expand the antibody diversity: gene hyper conversion (115,116), V-J flexible joining (117), and somatic point mutations (118). Gene hyper conversion occurs in the BF, during which pseudo-V genes are processed and recombined into new variable regions resulting in increased immunoglobulin diversity depending on where the block of DNA from the V gene is spliced (119). This process is also applied to pseudo-V(D) genes (120). These genes are considered pseudo genes because they lack in promoter sequences necessary for transcription. (Reyanud CA). This unique feature allows for antibody diversity equivalent to the VDJ & VJ rearrangement of the mammalian system.

Biochemical Properties of IgY

Biochemically IgY is distinct from IgG partially due to the increased rigidity because of the lack of hinge region in the C domain of the H chain. This changes the the precipitation curve of IgY under high salt conditions (121). IgY is also more stable under acidic conditions maintaining it's functionality between pH 4.0-11.0 (122, 123). IgY thermo satiability is comparable to IgG retaining functionality between 60-65ºC (122, 123). IgY is also more resistant to proteases which may allow it to retain functionality as it passes through the stomach if orally administered (123, 124).

Advantages of IgY Immunotherapy

There are distinct advantages to using IgY as an immunotherapy. The first is the phylogenetic distance between avian and mammalian species. This distance means that there is no cross-reactivity between IgY and mammalian IgG (125). This allows for the production of antibodies generated against conserved mammalian proteins (126). The phylogenetic distance also allows for greater antigenic recognition and affinity of IgY than mammalian IgG. It has been demonstrated that chicken IgY will bind to porcine antibodies with 3-5 times greater affinity than rabbit antibodies (126, 127). In a study antibodies generated against E7 oncoprotein of human papillomavirus type 16 IgY reacted against all of the peptides while IgG from rabbits recognized only two of the eight (128). The use of avian egg yolk as a source for antibody production greatly reduces the number of animals and strain on the animals. A single chicken yolk can produce 100-200 mg of total IgY (129), with 2-10% of total IgY being antigenic specific upon vaccination (130). IgY does not interact with mammalian complement systems, which in the case of DENV infection is a positive attribute. A study demonstrated that patients with Dengue hemorrhagic fever have elevated levels of activated complement components specifically C3a and C5a (131). Another critical characteristic of IgY is the lack of interaction with mammalian or known bacterial FcγR or Fc binding receptors. The lack of interaction with Fc receptors leads to premise that IgY will not induce ADE when encountering subsequent serotypes of Dengue or cross reactivity with ZIKV (134-137). Finally, IgY does not react with rheumatoid factor or human anti-mouse IgG antibodies (132, 133). This is important, because if the antibody reacts with the Fc part of

mammalian IgG, which is typical of patients with rheumatoid arthritis, the amount of antibody needed to neutralize the target antigen will be greatly increased.

General uses of IgY in virology

Due to the broader range of antigenic recognition and increased affinity to mammalian antigens IgY has been implemented in the development of a number of diagnostic tests and employed as immunotherapeutics. An enzyme-linked immunosorbent assay (ELISA) was developed to detect severe acute respiratory syndrome-associated coronavirus. This ELISA utilized IgY against the nucleocapsid protein as the detection antibody to increase the sensitivity of the assay. When the nucleocapsid protein IgY was compared directly against an IgG monoclonal antibody the detection limit of the assay was increased dramatically from 20-200 pg/mL to 10 pg/mL (138). To increase the power of IgY technology as a diagnostic tool for severe acute respiratory syndrome-associated coronavirus phage display generated monoclonal IgY antibodies with a single-chain variable fragment. Using this phage display system monoclonal IgY antibodies were generated against the spike protein which can be applied for rapid diagnostics and can be applied therapeutically (139). The diagnostic capabilities of IgY is also extended to hepatitis viruses. An indirect immunofluorescence assays was developed using IgY as the primary antibody to recognize hepatitis A virus in frozen liver sections (140). This technique is advantages because it is able to detect minute amount of virus where IgM levels are too low to detect in the serum and the virus is

unable to be detected by molecular means (141,142). The same anti-hepatitis IgY is used is a immunoenzymatic assay to measure the total anti-hepatitis antibody levels. This immunoenzymatic assay demonstrated increased sensitivity and specificity compared to the commercially available kit. This test could potentially then be used in place of the commercial kit or an alternative to the traditional IgG immunoassays. (143). This same principal was extended to hepatitis B virus as well. The purification process of the anti-hepatitis B virus was more extensive, but the resulting product could be used as a secondary antibody in a rapid detection ELISA (144, 145). Another promising area of assay development is utilizing IgY in immunochromatographic assays (ICAs). ICAs are also called lateral flow dipstick immunoassays which are typically used for rapid identification of a pathogen or substrate. Due to the short incubation time for these tests they are commonly referred to as point of care tests as well. A ICA was developed for canine parvovirus, which utilized a oligoclonal IgY preparation generated against canine parvovirus virus-like particle. When the IgY ICA was compared with PCR identification the ICA exhibited 94.17% conformity to the PCR results (146). Similar ICAs were also developed against soft-shell turtle systemic septicemia spherical virus and swimming crab reovirus (147, 148).

IgY as an Immunotherapeutic

Avian IgY has attracted considerable attention as an alternative to antibiotic therapy due to the increasing rise of antibiotic resistant pathogens, specifically those which infect the gastrointestinal tract. This trend hence has extended to viral pathogens of the

gastrointestinal tract as well as systemic viral infections. One such example is the production of anti-norovirus P particle to combat norovirus infection. The authors demonstrated efficacy of their IgY preparation *in vitro* and suggested this could be used as a passive immunotherapy for high risks groups such as young children and the elderly (149). Building upon that study the production of a dual IgY preparation was created to not only combat the P particle of norovirus, but also the VP8 spike protein of rotavirus. The dual IgY preparation also demonstrated *in vitro* efficacy with NT_{50} of 0.25mg/ml for rotavirus and a blocking titer 50 (BT_{50}) of 1: 1,300 for norovirus A and 1: 1,200 for norovirus B (150). Anti-rotavirus IgY has been utilized in newborn cattle which dramatically reduced the viral shedding and severity of diarrhea compared to controls (151, 152). A randomized double-blind clinical trial with 79 children with a history of rotavirus induced diarrhea were treated with freeze dried egg yolk IgY or placebo, and both groups received oral rehydration therapy. The group receiving IgY had significantly reduced viral shedding and the stool output frequency was reduced, only 1 day after treatment however (153). These results were mirrored in another clinical trial testing Rotamix IgY, this trial the severity of diarrhea was also reduced in the treated group compared to the placebo group. Viral shedding and oral hydration fluid administration needed was decreased in the treated group (154). Goose derived IgY has demonstrated efficacy against systemic viral infections. Goose derived IgY generated against the DNA vaccine of hantavirus produced a robust humoral immune response and the purified anti-hantavirus was able to protect Syrian hamsters from a lethal

challenge of hantavirus (155). In another study geese were immunized with inactivated DENV2 and the humoral immune response was monitored. The subsequent IgY preparation was then tested for it's neutralization capacity *in vitro* and *in vivo.* The NT_{50} of the anti-DENV2 IgY was 2.5 µg/ml. Mice treated with anti-DENV2 IgY survived a lethal challenge of DENV2 in a dose dependent manner with 1mg having 100% survival, 500µg 66%, and 50µg 33% survival (156). In each of these studies epitope mapping was done on the newly developed IgY preparations and in each instance novel antigenic epitopes were found in both viruses. This demonstrates the epitope diversity that can be achieved utilizing the IgY platform. Upon further study enriching the IgY preparations with novel epitopes could bring light to new neutralizing epitopes. It will also allow for enrichment of IgY antibodies of known neutralization epitopes from previously described monoclonal IgG panels. The previous studies demonstrate the versatile and robust nature of utilizing IgY as not only a diagnostic tool, but also as an alternative immunotherapeutic.

Chapter III

Zika Virus Vector Competency

Abstract

Zika virus is an emerging arbovirus of humans in the western hemisphere. With its potential spread into new geographical areas, it is important to define the vector competence of native mosquito species. We tested the vector competency of Aedes vexans (Meigen) from the Lake Agassiz Plain of northwestern Minnesota and northeastern North Dakota. Aedes aegypti (L.) was used as a positive control for comparison. Mosquitoes were fed blood containing Zika virus and 2 weeks later were tested for viral infection and dissemination. Aedes vexans (n=60) were susceptible to midgut infection (28% infection rate) but displayed a fairly restrictive midgut escape barrier (3% dissemination rate). Co-fed Ae. aegypti (n=22) displayed significantly higher rates of midgut infection (61%) and dissemination (22%). To test virus transmission, mosquitoes were inoculated with virus and 16–17 d later, tested for their ability to transmit virus into fluid-filled capillary tubes. Unexpectedly, the transmission rate was significantly higher for Ae. vexans (34%, n=47) than for Ae. aegypti (5%, n=22). The overall transmission potential for Ae. vexans to transmit Zika virus was 1%. Because of its wide geographic distribution, often extreme abundance, and aggressive human biting activity, Ae. vexans could serve as a potential vector for Zika virus in northern latitudes where the conventional vectors, Ae. aegypti and Ae. albopictus Skuse, cannot survive.

However, Zika virus is a primate virus and humans are the only amplifying host species in northern latitudes. To serve as a vector of Zika virus, Ae. vexans must feed repeatedly on humans. Defining the propensity of Ae. vexans to feed repeatedly on humans will be key to understanding its role as a potential vector of Zika virus.

Introduction

Zika virus (family Flaviviridae) is a mosquito-borne virus of primates in sub-Saharan Africa that has spread rapidly within the past decade to cause serious epidemics throughout the western Pacific and Latin America (157). Several features of Zika virus make its spread particularly troubling. In addition to being transmitted by infective mosquitoes, Zika virus can also be transmitted sexually (158). No other arbovirus is known to be sexually transmitted. In most cases, Zika virus infections in humans do not produce life-threatening illness, but in some instances, Zika virus infections can lead to a neuropathic condition known as Guillain-Barre´ syndrome, and in pregnant women, Zika viral infection can infect the fetus causing a brain abnormality known as microcephaly in the unborn child (159). Sylvatic circulation of Zika virus in Africa involves primarily monkeys and several species of tree hole and container-breeding Aedes spp., including Aedes africanus (Theobold) (160), Aedes luteocephalus (Newstead), and Aedes vittatus Bigot (161) Urban circulation involves a human–mosquito–human cycle with Aedes aegypti (L.) as the primary vector (162). Other mosquito species have been implicated as competent vectors of Zika virus, most

notably Aedes albopictus (Skuse) (163), and to a lesser extent, Aedes hensilli Farner (164), and some (165) but not allstrains within the Culex pipiens L./Culex quinquefasciatus Say complex (166-170). In this report, we tested Aedes vexans (Meigen) from the upper Great Plains for their ability to transmit Zika virus. Figure 1 demonstrates the multi facet transmission cycles and how they are interconnected

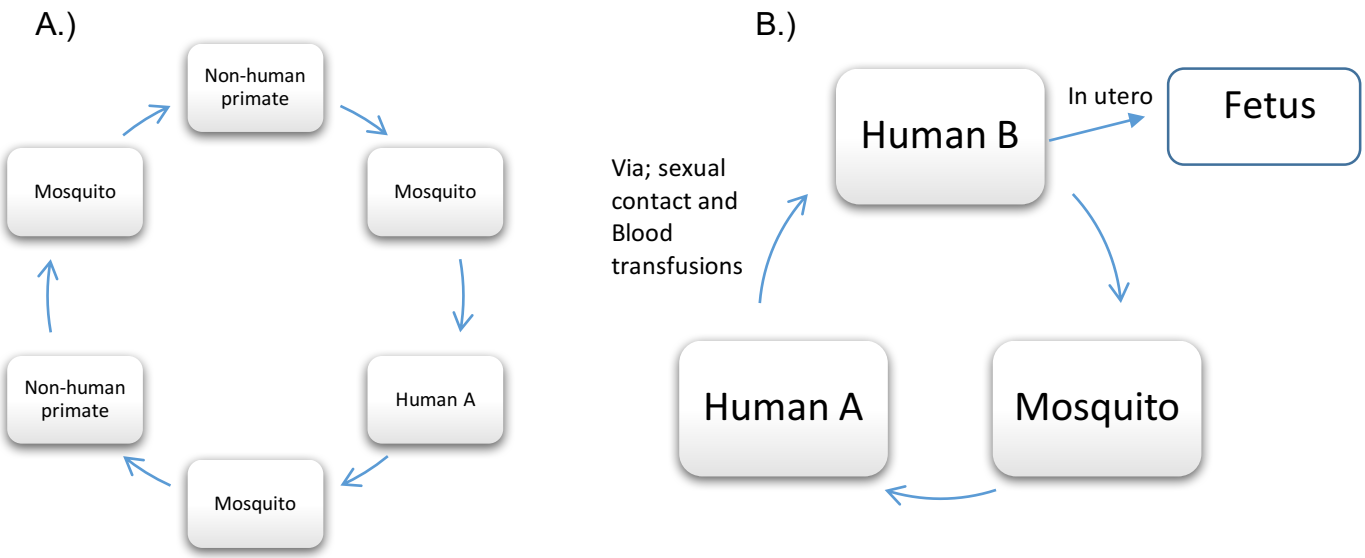


Figure 1: **A.)** Enzootic transmission cycle of Zika virus. **B.)** Epidemic transmission cycle of Zika virus

Materials and Methods

Mosquitoes

Host-seeking mosquitoes were collected using a Mosquito Magnet X trap (kind gift of Metropolitan Mosquito Control District, St. Paul, MN) baited with bottled CO2 released at a flow rate of ca. 500 ml per minute. The trap was operated overnight at rural residences in Polk County, MN (31 July 206) and Grand Forks County, ND (22 September 2016). Both sites are located within the Lake Agassiz Plain eco-region of northwestern Minnesota and northeastern North Dakota. The next morning, the trap was transported to the laboratory and mosquitoes were released into a large, cubic-meter screened cage. Mosquitoes were maintained for several days on cotton pads soaked in 10% sugar water and 0.05% antibiotic solution. Antibiotics were given to eliminate variation in bacterial loads within the alimentary tracts of wild-caught mosquitoes and thus minimize any potential confounding effects that midgut bacteria may exert on the infectivity of experimentally administered virus (171). As a positive control for vector competence, an Aedes aegypti colony (Costa Rica strain, F39) was established from eggs obtained from BEI Resources. Female Ae. aegypti mosquitoes were likewise maintained for several days on the glucose-antibiotic solution prior to testing.

Virus

The strain of Zika virus used in this study was originally isolated from a patient in Puerto Rico in 2016 (PRVABC59). Viral stocks were prepared after two passages of the isolation onto Vero cells maintained at 37°C. Viral titer was estimated via a plaque assay, 10-fold serial dilutions were inoculated onto Vero cells overlaid with 1% methylcellulose and complete DMEM, which were then incubated for 7 days. The overlay was then removed and the monolayer was fixed with 10% formaldehyde and stained with crystal violet. Infection was measured by the observance of cytopathic effect and the resulting titer was expressed as plaque-forming units per milliliter (PFU/ml). The viral stock was diluted 1:1 with heat inactivated fetal bovine serum then divided into 1 ml aliquots and stored at -80°C.

Oral infection of mosquitoes

Prior to infection, mosquitoes were transferred to 3.8-liter cylindrical cardboard cages (ca. 50 per cage) each fitted with a screened top secured with tape and a single high-security double-layered dental dam access portal. Infectious blood consisted of Zika virus culture media mixed 1:1 with de-fibrinated cow blood (Pel-Freez Biologicals, Rodgers, AK USA). The first trial (Minnesota mosquitoes plus Ae. aegypti) used thawed virus fed to mosquitoes at an estimated blood meal concentration 9.2×10^6 PFU/ml. The second trial (North Dakota Ae. vexans) used fresh virus grown on Vero cells prior to mosquito feeding and with an estimated blood meal concentration of 2.0×10^5 PFU/ml. Blood meals were

administered via water-jacketed membrane feeders (circulating water at ca. 38°C) fitted with de-salted pork sausage casing. Mosquitoes were given 1 hour to feed, after which unfed mosquitoes were removed. Cages containing engorged mosquitoes were placed within transparent plastic tubs and maintained on glucose-antibiotic solution for 14 days in a biosafety level-2 insectary with restricted access and environmental settings at 28°C and 16:8 L:D light cycle. At 14 days, mosquitoes were killed by freezing at -20°C for > 2 hours. For each mosquito, the legs were pulled off and the bodies (=infection) and mosquito legs (=disseminated infection) were ground separately in 200 µl grinding solution (M-199 + 5% calf serum + 0.5% antibiotics).

Parenteral infection and salivary transmission

Virus was inoculated into mosquitoes to test their ability to transmit virus orally. Mosquitoes were immobilized by chilling (1 minute in -20°C freezer) in batches of 5 to 15 each and placed on a chill table (BioQuip, Rancho Dominguez, CA). Mosquitoes were injected intrathoracically with 0.2 µl of media containing 1.8 x 10^7 PFU/mL using a glass needle powered by a microinjection pump (TriTech Research Inc., Los Angeles CA). Following injection, mosquitoes were placed into 0.5 liter cylindrical cardboard cages and maintained as described above. On 16 to 17 days after injection, mosquitoes were tested for their ability to secrete virus in their saliva (172). To do this, 3 to 5 mosquitoes at a time were chilled, legs amputated and then placed on a strip of double-stick tape running

along the edge of a glass plate. Mosquito were carefully positioned on the plate so that their proboscises were free and hanging over the edge. Immediately after mosquitoes were in position, a small amount of malathion insecticide (0.4 µl of 0.14%AI in acetone) was applied to the thorax of each mosquito to stimulate salivation (173). After 10 to 15 minutes, this plate was abutted against a second glass plate to which capillary tubes containing ca. 20 µl of M-199 media plus 10% calf serum were affixed. Each mosquito proboscis was carefully inserted into a liquid-filled capillary tube and mosquitoes were given 20 to 30 minutes to salivate during which they were examined periodically under a stereoscope for probing behavior, salivation and ingestion of capillary tube contents. After the allotted time, capillary tubes were collected and the contents expelled into individual microfuge tubes containing 50 µl of media. Likewise, the mosquitoes were removed and placed into individual microfuge tubes for processing. Tubes were labeled so that expectorate samples could be matched to the individual mosquitoes from which they had been collected.

Zika virus detection

Viral RNA was detected using reverse transcriptase polymerase chain reaction (RT-PCR) techniques. Frozen triturates of mosquito bodies and legs were thawed, centrifuged at 14,500 rpm for 5 minutes and 140µL of supernatant was extracted for RNA using Qiagen QIAamp Viral RNA Mini Kits according to manufacturer's instructions. Real time PCR

was conducted using Qiagen one-step RT-PCR kit with primers specific for the envelope gene. Probe sequence:5'-56FAM/ACGCCTAAT/ZEN/TCACCAAGAGCGGAA/3IABkFQ-3' Primer1: 5'-TCCTAAGCTTCCAAAGCCTCCCAA-3' and Primer2: 5'-TATCAGTGCATGGCTCCCAGCATA-3'. Cycle parameters were (i) 30 min at 50°C, (ii) 15 min at 95°C, (iii) 40 cycles of; 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, and 10 min 72°C. Reactions were performed using CFX96™ IVD Real-Time PCR Systems and accompanying software to determine cut-off values based on a minimum of 2 negative controls (water only) and two positive controls (Zika viral culture extracts) per assay.

Results

Susceptibility to Oral Infection and Viral Dissemination

Membrane feeding success was higher for colonized Ae. aegypti (69%, n=39) than for wild-caught Ae. vexans (43%, n=168; χ^2 =8.82, P=0.003). Aedes aegypti had significantly higher rates of viral infection and dissemination than Ae. vexans (Fisher exact tests, P<0.024). Seventeen of 60 Ae. vexans tested (28%) became infected and two (3%) developed disseminated infections, indicating a moderately severe midgut escape barrier for Zika virus in this species. Eleven of 18 Ae. aegypti tested (61%)

became infected and four (22%) developed disseminated infections (Figure 2). However, the proportion of infected mosquitoes having disseminated infections did not differ statistically between Ae. aegypti (4 of 11) and Ae. vexans (2 of 17; Figure 2; Fisher exact test, P.0.14). Interestingly, there were no differences in infection or dissemination rates between Ae. vexans fed fresh versus thawed virus (P's>0.59, Fisher exact tests), even though thawed virus had nearly 100-fold higher titer (Table 1). This supports earlier findings that freshly cultured Zika virus is more infective to mosquitoes than frozen virus that is thawed just prior to feeding to mosquitoes (174).

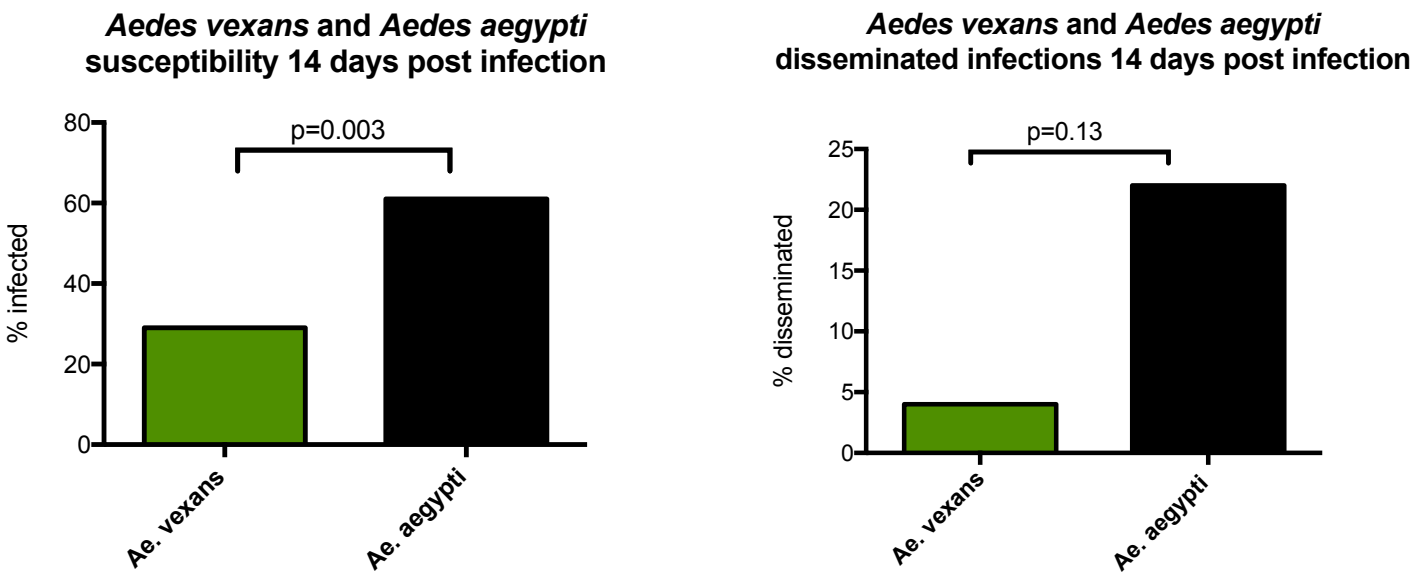


Figure 2: Midgut and disseminated infection of Ae. vexans and Ae. aegypti. A.) Percent midgut infection of Ae. vexans and Ae. aegypti mosquitoes exposed to a blood meal containing Zika virus. B.) Percent disseminated infections from the midgut of Ae. vexans and Ae. aegypti with midgut infections of Zika virus.

Mosquito Species	Mosquito Origin	Virus and Blood meal Concentration	% Infection[a]	% Dissemination[b]	% Dissemination / Infection[c]
Aedes aegypti	Colonized; Costa Rica strain (BEI Resources)	Thawed 9.2×10^6 PFU/ml	61 (11/18)	22 (4/18)	36 (4/11)
Aedes vexans	Wild-caught; Grand Forks Co., ND	Fresh 2.0×10^5 PFU/ml	28 (9/32)	3 (1/32)	11 (1/9)
	Wild-caught; Polk Co., MN	Thawed 9.2×10^6 PFU/ml	29 (8/28)	4 (1/28)	12 (1/8)
Aedes trivittatus	Wild-caught; Polk Co., MN	Thawed 9.2×10^6 PFU/ml	0 (0/6)	0 (0/6)	0 (0/0)
Culex tarsalis	Wild-caught; Polk Co., MN	Thawed 9.2×10^6 PFU/ml	0 (0/3)	0 (0/3)	0 (0/0)
Culiseta inornata	Wild-caught; Polk Co., MN	Thawed 9.2×10^6 PFU/ml	0 (0/1)	0 (0/1)	0 (0/0)

Table 1. Infection and dissemination of Zika virus in wild-caught and colonized mosquitoes 14 days after ingesting defibrinated blood containing virus.

[a]Percentage of mosquitoes containing virus in their bodies (number positive / number tested).
[b]Percentage of mosquitoes containing virus in their legs (number positive / number tested).
[c]Percentage of infected mosquitoes containing virus in their legs (number positive / number tested).

Virus Transmission by Innoculated Mosquitoes

To test for salivary gland barriers, *Ae. vexans* and *Ae. aegypti* were inoculated with virus and, after 16-17 days incubation, were stimulated to salivate into capillary tubes. When the bodies of inoculated mosquitoes were assayed, all 47 inoculated *Ae. vexans* were positive (100%) and 22 of 23 inoculated *Ae. aegypti* (96%) were positive. Saliva samples were tested for virus from body-positive mosquitoes. Thirty-three of 47 body-positive *Ae. vexans* (70%) were observed to either salivate or imbibe fluid during the salivation period, whereas only 5 of the 22 body-positive *Ae. aegypti* (23%) were observed to do so. Sixteen of the 47 *Ae. vexans* (34%) transmitted virus, whereas only one of the 22 *Ae. aegypti* (5%) transmitted virus (Table 2) (Fisher exact test, p=0.06). Whether or not a mosquito was observed to salivate into and/or imbibe fluid from its capillary tubes had no bearing on whether or not virus was transmitted. Of the 16 *Ae. vexans* that transmitted virus, 12 (75%) were observed to salivate and/or imbibe and 4 (25%) were not. Conversely, of the 31 *Ae. vexans* that did not transmit virus, 21 (68%) were observed to

salivate and/or imbibe and 10 (28%) were not (χ^2=0.27, p=0.61). The single *Ae. aegypti* that transmitted virus was not observed to salivate nor imbibe fluid. Thus stereoscopic observation of mosquitoes during *in vitro* salivation tests was not useful in detecting actual expectoration of saliva.

Mosquito Species	Mosquito Origin	% Transmission[a]
Aedes aegypti	Colonized; Costa Rica strain (BEI Resources)	5 (1/22)
Aedes vexans	Wild-caught; Grand Forks Co., ND	34 (16/47)

Table 2. Transmission of Zika virus by mosquitoes 16 to 17 days after being inoculated intrathoracically with 0.3 μl of media containing 1.8 x 10^7 plaque-forming units per milliliter of virus.

[a]Percentage of mosquitoes with a disseminated viral infection that expectorated virus into fluid-filled capillary tubes following a topical application of 0.4 μl acetone containing malathion (0.14% AI) to induce salivation.

Discussion

This study indicates that Ae. vexans from the upper Great Plains is physiologically capable of becoming orally infected and transmitting Zika virus. Aedes vexans displayed moderate to severe midgut and midgut escape barriers to virus infection and dissemination, respectively. However, once disseminated infections were established, the transmission rate of Zika virus by Ae. vexans (34%, n.47) was comparable to transmission rates reported at similar incubation periods (i.e., 14 d) for some (but not all) strain combinations of Zika virus and Ae. aegypti (i.e., 175 [33%, n.12], 176 [21%, n.14], 177 [42%, n.33], 178 [5%, n.189], 179 [31%, n.36]). Aedes vexans is the first indigenous North American mosquito species found capable of transmitting Zika virus under laboratory conditions. Several other mosquito species have been shown in laboratory studies to be refractory to Zika virus, including Aedes triseriatus (Say) (Aliota et al. 2016b), Aedes taeniorhynchus (Wiedemann) (170), and Culex tarsalis (Coq.) (174) from North America, Aedes polynesiensis Marks from French Polynesia (177), and Aedes notoscriptus (Skuse), Aedes procax (Skuse), Aedes vigilax (Skuse), Culex annulirostris Skuse, and Culex sitiens Wiedemann from Australia (180). The primary urban vectors of Zika are Ae. aegypti and Ae. albopictus. As Zika virus spread from Africa into Asia, Oceania, and the Western Hemisphere, the virus encountered novel populations and

strains of these vector species. It is now clear from recent vector competency studies that the efficiencies by which Ae. aegypti and Ae. albopictus transmit Zika virus varies

greatly depending on the strains of mosquito and virus examined. In our study, the rates of viral infection (61%) and dissemination (22%) for Zika virus in our Ae. aegypti strain were comparable to other strain combinations examined, yet the transmission rate (5%, Table 2) was uncharacteristically low. We had purposely selected our “positive control system”—i.e., a Latin American strain of Ae. aegypti (Costa Rica) and a Caribbean isolate of Zika virus (Puerto Rico)—based on the assumption that the close geographic proximity of origin between mosquito strain and virus isolate would result in optimal virus transmission. This proved not to be the case. But this is not the first example of incompatibility between geographically proximate strains. Interestingly, two separate strains of Ae. aegypti from Senegal have been reported to have severely limiting midgut escape barriers and insurmountable salivary gland barriers to the native Sengalese strain of Zika virus (161). Thus, Ae. aegypti is probably not the vector for Zika virus in Senegal. Conversely, a strain of Ae. aegypti from Mexico produced a significantly higher rate of disseminated infection with a Zika virus strain from Senegal (60%, n.48) than with Zika virus isolated from a patient in Puerto Rico (42%, n.48;
174). These studies indicate that the combination of mosquito and virus strains in the Ae. aegypti–Zika system greatly influences vector competency although the outcome cannot be predicted based on geographic origins of strains and isolates. Aedes aegypti is widely distributed throughout tropical latitudes and analogously, Ae. vexans is widely distributed throughout temperate and subarctic latitudes. Thus, it may be anticipated that Ae. vexans may also display geographic heterogeneity in vector competence to

Zika virus. Such heterogeneity has been shown to occur with Ae. vexans and Rift Valley fever virus, where southern populations of North American Ae. vexans are susceptible but northern populations are not
(181-183). Beyond its ability to transmit the virus, there are other characteristics of Ae. vexans that could contribute to its role as a potential vector of Zika virus in the Northern Hemisphere. First, Ae. vexans is an aggressive, nearly cosmopolitan Holarctic mosquito species with a long flight range (184-186). It feeds primarily on large mammals and readily attacks humans during both day and night (187). Second, Ae. vexans is extremely prolific and often cited as the most abundant species found in mosquito surveys conducted throughout the northern hemisphere, including central Canada (188), most of the United States (189), France (190), Germany (191), Czech Republic (192), Slovakia (193), Hungary (194), Croatia (195), Russia (196), Iran (197), South Korea (198), and China (199). Third, Ae. vexans has been shown in laboratory studies to be a competent vector for several arboviruses, including West Nile virus (200), eastern equine encephalomyelitis virus (201), Saint Louis encephalitis virus (202), Rift Valley fever virus (203), Tahyna virus (204), and Geta virus (205). Thus, it would seem that Ae. vexans could potentially play a role in transmitting Zika virus if the virus were to be introduced into more northerly latitudes of North America or Eurasia where the primary vectors, Ae. aegypti and Ae. albopictus, are absent. On the other hand, Zika virus is a virus of primates. Outside the tropics, humans are essentially the only amplifying host. Thus, for Ae. vexans to be a vector of Zika virus in northern latitudes, it

would have to feed on a viremic human, survive the extrinsic incubation period of the virus, and then feed again on a human. Using mark–release–recapture techniques, Jensen and Washino (1994) estimated the field survivorship of Ae. vexans in California to be fairly low (daily survival.0.70) compared to typical values reported for Ae. aegypti (e.g., 0.80 to 0.95). Low field survival would reduce the overall vectorial capacity of Ae. vexans. Nevertheless, densities of host-seeking Ae. vexans can be extreme, even in residential areas (206). Prodigious numbers may compensate for low daily survival and weak vector competence, and thus elevate the importance of Ae. vexans as a vector for zoonotic arboviruses in some regions (172). But again, the transmission pattern of Zika virus in the north would undoubtedly be human-to mosquito-to-human. Thus, information regarding the propensity of Ae. vexans to feed repeatedly on humans is key in evaluating its potential as a vector of Zika virus. In the rural Midwest, Ae. vexans feed primarily on deer (207). But the primary bloodmeal sources for Ae. vexans are less well-defined in suburban and urban areas where human density, and hence human availability as blood sources, typically exceeds that of deer and other large mammals. To help define the actual vector potential of Ae. vexans for Zika virus in North America, mosquito surveillance programs could include pools of field-collected Ae. vexans as part of their Zika virus testing— particularly in the southeast USA where Ae. vexans and Ae. aegypti co-occur and the threat of local virus amplification exists. This may reveal whether or not Ae. vexans mosquitoes are being naturally exposed to Zika virus. Additional vector competence studies with Ae. vexans (e.g., comparing geographic

strains, defining extrinsic incubation period, transovarial transmission, etc.) are warranted because of this species' expansive distribution, considerable dispersal capability, often excessive abundance, and propensity to attack humans. Other Holarctic anthropophagic mosquito species should also be examined.

Chapter IV

Avian IgY as an Immunotherapy for Zika Virus

Abstract

Zika virus (ZIKV) is a newly emerged pathogen in the Western hemisphere. It was declared a global health emergency by the World Health Organization in 2016. There have been 223,477 confirmed cases including 3,720 congenital syndrome cases since 2015. ZIKV infection symptoms range from asymptomatic to Gullain-Barré syndrome and extensive neuropathology in infected fetuses. Passive and active vaccines have been unsuccessful in protecting from or treatment for ZIKV infection due to antibody dependent enhancement (ADE). ADE causes an increased viral load due to increased monocyte opsonization by non-neutralizing low avidity antibodies from a previous dengue virus (DENV) infection or previous exposure to ZIKV. We have previously demonstrated polyclonal avian IgY generated against whole-killed DENV-2 ameliorates DENV infection in mice while not inducing ADE. This is likely due to the inability of the Fc portion of IgY to bind to mammalian Fc receptors. We have shown here that ZIKV oligoclonal IgY is able to neutralize the virus *in vitro* and in IFNAR$^{-/-}$ mice. The concentration of ZIKV-specific IgY yielding 50% neutralization (NT_{50}) was 25μg/mL. Exposure of the ZIKV, prior to culture with ZIKV-specific IgY or 4G2 flavivirus envelope IgG demonstrated that the ZIKV-specific IgY does not induce ADE. ZIKV IgY was protective *in vivo* when administered following a lethal ZIKV challenge in a 3 week old

IFNAR$^{-/-}$ mice. We propose polyclonal ZIKV-specific IgY may provide a viable passive immunotherapeutic for ZIKV infection without inducing ADE.

Introduction

Flaviviruses are a major health concern throughout the world. A newly emerged flavivirus that has major health implications is Zika virus (ZIKV). A majority of ZIKV infections are asymptomatic in the host. Severe ZIKV symptoms extend to neurological diseases including Gullian-Barre Syndrome (GBS) (208,209) and congenital Zika syndrome (CZS). CZS can be further differentiated into microcephaly, brain abnormalities, and other severe birth defects (210-214). In 2016 the world health organization (WHO) declared ZIKV a world health threat and pushed for the development of vaccines and antivirals to combat ZIKV infection (215).

ZIKV is a member of the *Flaviviridae* family which also includes dengue virus (DENV), west nile virus (WENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV). ZIKV is primarily spread by *Aedes* species of mosquito (160). Recently our lab has identified that *Aedes vexans* is also a potential competent vector which extends the geographical range of infection from tropical to temperate climates as well (216). In the absence of quality vector control in the countries affected by ZIKV the development of new antivirals and vaccine candidates is required to control the spread of ZIKV.

Severe flavivirus infection can be attributed to cross reactive inflammatory T cells and non-neutralizing antibodies that induce antibody dependent enhancement (ADE) (217,218). ADE occurs via antibodies from a primary infection binding to a heterotypic flavivirus. The viral immune-complex is then recognized by the FcγR on a dendritic cell or macrophage and binds via the Fc portion of the antibody. This complex is then internalized in the endosome where the pH decreases as the endosome matures. This drop in pH lowers the affinity of the antibody and virus allowing the virus to dissociate from the antibody. Once the virus has dissociated from the antibody normal viral replication is commenced by the fusion of the envelope to the membrane of the endosome releasing the viral nucleic acid (219).

To date there is an unmet need for an effective antiviral therapeutic for ZIKV infection. Recently, one area of research that is showing promising results is the utilization of truncated antibodies that lack the Fc portion. Settler *et al*. demonstrated that truncated monoclonal antibodies were able to neutralize primary ZIKV infection and a secondary DENV infection without inducing ADE. They further go on to demonstrate that the truncated monoclonal antibodies were able to protect $IFNAR^{-/-}$ mice challenged with a lethal dose of ZIKV (219). We hypothesize that avian IgY, the avian homolog of IgG, will be an effective therapeutic against flavivirus infections based on the unique characteristic that full length IgY does not bind to mammalian FcγR. Previously we have demonstrated that Dengue specific IgY was effective at neutralizing lethal infections with DENV2 without inducing ADE (156).

IgY is the avian homologue of mammalian IgG and shares characteristics with mammalian IgG and IgE. IgY is the predominant isotype in sera after the initial production of IgM, and is the primary antibody produced upon a secondary response (110,111). IgY is found in two isoforms, in the serum of water fowl, full-length IgY that contains two constant regions and an alternatively spliced IgY that lacks these two constant regions (110,115). The alternatively spliced IgY would be the avian structural equivalent of the truncated IgG proposed by Settler *et al*. Previous studies from our laboratory have demonstrated the efficacy of the utilization of IgY in viral infections. We first demonstrated that Andes virus-specific IgY provided protection from Hantavirus pulmonary syndrome in a hamster model (155). Most recently we have demonstrated that DENV specific IgY was able to protect AG129 mice from a lethal dose of DENV2 (156).

There are many benefits for the utilization of IgY as an immunotherapy in mammals, first the phylogenic distance between the avian species producing the antibody and the recipient mammalian host allows for a much broader range of antigenic recognition. This is due to the increased avidity for mammalian antigens and the ability to recognize antigens that normally would not be immunogenic in a mammalian host. Increased antigenic recognition and avidity of IgY is attributed to the unique antibody maturation process. During IgY maturation gene hyper conversion allows for pseudo-V genes to recombine with existing variable regions of the Ig genes to produce mature B-cells. The second reason is V-J flexible joining; this is when partial

conversions of rearranged segments are replaced by pseudo-V(D) genes. The third mechanism to produce diversity is the large amount of somatic hyper mutations that is undergone (110-116). Another critical characteristic of IgY is the lack of interaction with mammalian or known bacterial FcγR or Fc binding receptors. The lack of interaction with Fc receptors leads to premise that IgY will not induce ADE when encountering subsequent serotypes of Dengue or cross reactivity with ZIKV. Further, IgY does not interact with mammalian complement systems, which in the case of DENV infection is a positive attribute. A study demonstrated that patients with Dengue hemorrhagic fever have elevated levels of activated complement components specifically C3a and C5a (220). Not only are the immune characteristics of IgY attractive but the industrial production potential also makes IgY an attractive therapeutic candidate. Due to the fact that IgY can be isolated from the eggs of laying geese or hens, this allows for a non-invasive way of harvesting an immunotherapy compared to production in a mammalian host such as rabbits. The antibody yield is also much greater per month due to this non-invasive aspect, 1,300 to 1,500 mg of IgY can be produced from one bird per month compared to 200 mg from a mammalian source (221). There are currently large-scale production methods and sites functional at this time making IgY an economically feasible therapeutic.

In this study we demonstrate the efficacy of goose derived anti-ZIKV IgY *in vitro* and *in vivo*. Specifically, ZIKV IgY produce protection greater than 50% reduction in a plaque reduction neutralization test, and ZIKV IgY did not produce ADE *in vitro*. Finally,

we demonstrate that ZIKV specific IgY can protect 3 week old IFNAR$^{-/-}$ mice from a lethal ZIKV challenge.

Materials and Methods

Ethics Statement

Research was conducted in compliance with the Animal Welfare Act and adheres to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of North Dakota and the Institutional Biosafety Committee.

Geese

The geese (*Anser domesticus* 25 months old) used to produce IgY are an inbred hybrid of a combination of German Embden, the Royal Chinese, and the Royal English breeds. The animals were housed at the Specific Pathogen Free facility on the Schiltz Goose Farm, Inc. The barn also housed a clean room for ISO egg production which was equipped with HEPA filtration to remove circulating particles. Only authorized personnel were allowed into the facility.

Mice

B6.129S2-Ifnar1^{tm1Agt}/Mmjax (IFNAR$^{-/-}$) were purchased from Jackson Laboratory MMRRC stock #32045 (222). Mice were maintained in clean rooms in the Center for Biomedical Research Center, UND.

Viruses and Cell Lines

Zika virus PRVABC59 was propagated in Vero cells (ATCC). Briefly Vero cells at 80% confluency in 6 well plates were infected at an MOI of 0.1 and allowed to adhere for one hour at 37ºC with 5% CO_2. The cells were washed with DMEM containing 10% fetal bovine serum (FBS) (Atlanta biologicals, Atlanta, GA), Penicillin/Streptomycin (Corning, Corning, NY), and 2.5mM HEPES, (cDMEM). 3ml of cDMEM was replaced in the wells

the plates were incubated at 37ºC 5% CO_2 for five days. The cell supernatant was centrifuged at 4,000x *g* for 5 min at 4 ºC to remove cell debris. The clarified cell supernatant was diluted 1:2 in heat inactivated FBS and frozen at -80ºC. The resulting viral stocks were quantified via plaque assay.

Vaccination

The vaccination schedule to produce DENV specific IgY was previously described. Zika virus PRVABC59 was propagated in Vero cells to a titer of $3.8x10^5$ PFU/ml. The cell supernatant was centrifuged 4,000x *g* 10 min at 4 ºC to remove cell debris. The resulting supernatant was exposed to 5.23 Mrad of gamma irradiation**.** The resulting viral stock was shown to be inactivated via plaque assay quantification. Geese were vaccinated with $1.52x10^5$ PFU/ml of irradiated Zika virus. Dose was based on pre-irradiated viral titer. Immunizations consisted of 2 x 200 µl subcutaneous injections at the back of the neck in two different injection spots. Eggs were collected starting from week 6 after the first vaccination and stored at 4ºC until further use.

Purification of IgY from Goose Egg Yolk

Zika virus IgY was purified using polyethylene glycol. Briefly the egg yolk homogenate was thawed and centrifuged at 10,000 x *g* for 30 min 4 ºC. The supernatant was passed through a 0.2 µm sterile filter and concentrated using a 100 kDa (MilliporeSigma,

Burlington, MA) tangential flow unit. 12% (weight/volume) of 6,000 MW PEG was added to the concentrated supernatant that was then stirred for 30 min at room temperature. The PEG mixture was then centrifuged at 10,000 x *g* for 30 min 4 ºC. The supernatant was discarded and the pellet was resuspended to the original volume in 1x PBS. 12% 6,000 MW PEG was then added to the resuspended pellet and mixed for 30 min at room temperature. The mixture was centrifuged at 10,000 x *g* for 30 min 4 ºC and the pellet was saved. The pellet was resuspended in the original volume of 1x PBS (pH 7.4) and concentrated to 50ml using 100 kDa cassette (MilliporeSigma) the final sample was then diluted with 450ml of 1x PBS. Samples were additionally dialyzed in 1x PBS to remove residual PEG.

Antibody Detection

The binding activity of flavivirus IgY was determined by ELISA as previously described (155). Briefly 100 µl of antigen was coated onto a 96 well micro plate and stored overnight at 4ºC. The following day the plate was washed 3x and then blocked with 400µl of blocking buffer and incubated at room temperature for 30 min. The wells were washed three times and incubated with 100µl of IgY serially diluted 1:2 across the wells of the plate. The plate was incubated at 37ºC for 30 min. The plate was washed 3x and blocked at room temperature for 10 min. 100µl of biotinylated rabbit anti-goose IgY antibody in blocking buffer was added to the plate and incubated at 37ºC for 30 min. Following this, 100 µl of diluted strepavidin-HRP antibody in blocking buffer was added

to each well, and the plates were incubated at 37°C for 30 min. Finally, the plate was washed 3x and 100µl of o-phenylenediamine dihydrochloride color substrate was added to each well and developed at room temperature protected from light for 15 min. The reaction was stopped by adding 50µl of 1N H_2SO_4, and the absorbance was read in a BioTek plate reader at A_{490}. Data are represented as endpoint titers.

Plaque Assay

Vero cells were seeded into 6 well plates at $2x10^5$ cells/well and allowed to come to confluency. Viral stocks were thawed in a 37ºC water bath and diluted from 10^{-1} to 10^{-7} in cDMEM. Triplicate wells were infected with 250µl of viral dilutions whereas cell controls were inoculated with media alone. Plates were placed at 37ºC, 5% CO_2 for 1 hour lightly shaking every 15 min. The viral inoculum was removed, and the wells were washed with 1x PBS. Next, 3ml of 1:1 of 2% methyl cellulose and cDMEM mixture was added to each well. Plates were incubated for 7 days (ZIKV) and 10 days (DENV). At the end of the incubation, the overlay was removed and the cells were fixed with 400 µl of 10% formalin for 30 min at room temperature Formalin was removed and crystal violet solution was added to each well for 10 min to stain the cell layer. The crystal violet was removed and the wells were washed 3-5x with 1x PBS until plaques were clearly visible.

Plaque Reduction Neutralization

The plaque reduction neutralization assay (PRNT) is similar to the plaque assay noted above with the following exceptions. The virus is diluted to 40-60 PFUs that is then treated with IgY ranging in concentrations from 250 µg/ml to 3.125 µg/ml. Plaques are then counted and a 50% or greater reduction of plaques was consider a significant reduction (in accordance with WHO standards) (223).

Antibody Dependent Enhancement

The presence of ADE was assessed using the protocol in Diamond, et al. with minor modifications (224). We used a MOI of 2 for ZIKV and 10 for DENV, due to the difference in binding kinetics between ZIKV and DENV noted in Charles and Christofferson (225). THP-1 cells were seeded at 1.76 x 10^4 cells/well for DENV infection and 1.2 x 10^5 cells/well for ZIKV to accommodate for differences in titer of the viral stocks. Antibody treatment was done with 300 ng of mAb 4G2 (Anti-flavivirus group antigen antibody, Novus) 1 µg and 100 µg of anti-ZIKV IgY were incubated at 37ºC, 5% CO_2 for 1 hour prior to infection. Cells were infected for 90 min at 37ºC, 5% CO_2. After infection cells were centrifuged at 900 x *g* for 5 min and washed 3x. Cells were suspended in cDMEM. Identical concentrations of the treatment antibody were added to cells and incubated for four days at 37ºC, 5% CO_2. Cells were centrifuged again and the supernatant was collected and viral titer was determined by plaque assay.

In vivo ZIKV-Specific IgY Protection

The therapeutic potential of ZIKV-specific IgY was tested using conditions that cause 100% mortality in IFNAR$^{-/-}$ mice. 3 week old IFNRA$^{-/-}$ mice were challenged with a lethal dose of ZIKV PRVABC59 (1.0x10^4 PFUs) intravenously (i.v.) via the retro-orbital sinus. 24 and 48 hours after infection, mice were injected intraperitoneally with the indicated amounts of ZIKV-specific IgY or control naïve IgY in a volume of 100 µL, or 100 µL of PBS as a negative control. Mice were observed twice daily for 14 days for signs of morbidity and mortality.

Viral Load Quantification

At the mortality end point or conclusion of the study tissue samples (brain, spleen, and liver) were processed for viral load. Briefly the tissues were collected in 1 mL of cDMEM, and homogenated using a bullet blender. The supernatant was then centrifuged at 4,500 x *g* for 10 min. The clarified supernatant was then diluted in cDMEM in order to allow for 20-50 plaques per well in a 6 well plate. The plaque assay protocol described above was then used to quantify viral load in the respective organs. Viral copy number in the respective organs was also quantified via RT-qPCR. Viral RNA was extracted from the clarified tissue supernatant with the Viral RNA mini kit (Qiagen, Hilden, Germany). We then utilized Zika virus Genesig Easy kit (Genesig, Plymouth Meeting, MA) to quantify the viral copy number of the infected organs.

Epitope Mapping

Anti-ZIKV IgY epitopes were mapped using peptide arrays for E, prM, C, NS1, NS2a, NS3, and NS5 proteins. 11 amino acid overlapping 15mer peptides were covalently linked to a microarray slide (JPT Innovative Peptide Solutions, Mainz, Germany), according to the manufacturer's protocol. Briefly an uncoated slide and the slide containing the microarray were gently laid upon one another separated by spacers. 50 µg/ml of control naïve IgY or anti-ZIKV specific IgY was incubated on the slides overnight at 4ºC in a moist environment. Slides were then rinsed 5x with tris-buffered saline and Tween20 (T-TBS) for 4 min, then 5x for 4 min with 18.5-ohm water. The slides were then incubated with 1 µg/ml Cy5 goat anti-chicken IgY secondary antibody (Abcam, Cambridge, UK) for 45 min protected from light. Slides were washed 5x with T-TBS and 5x ultra-pure water, and dried with dust-free, oil free, canned air. A Genepix 4000 microarray reader was used to measure the fluorescent signal at a pixel size of 10 µm. The signal intensity mean values were calculated for each of the 3 sub-arrays and corrected for background.

Statistical Analysis

Differences in PFU/ml and copy number between the antibody groups and controls as well as the comparison of organ weights between groups were analyzed using ANOVA with a Bonferroni post-test for multiple comparisons. Survival was measured using Kaplan Meir and Mantel-Cox tests. The Mann-Whitney test was used to compare the

difference in body weight percentages. GraphPad Prism (Version 7.0a, GraphPad Sofware inc., CA) was used for statistical analysis with p values of < 0.05 considered significant.

Results

Antibody characterization

The characterization of polyclonal anti-DENV2 has been demonstrated previously by our lab [14]. Following the polyethylene glycol purification of anti-ZIKV IgY, SDS-PAGE was used to verify the presence and purity of full length IgY and alternatively spliced IgY. We were able to detect both full length and alternatively spliced forms of anti-ZIKV IgY from the goose egg yolk homogenate (Fig. 3). An ELISA was then performed to detect the presence of ZIKV specific IgY and to determine the endpoint titer of our IgY preparation. The mean endpoint titer of the samples tested was 1:160,000.

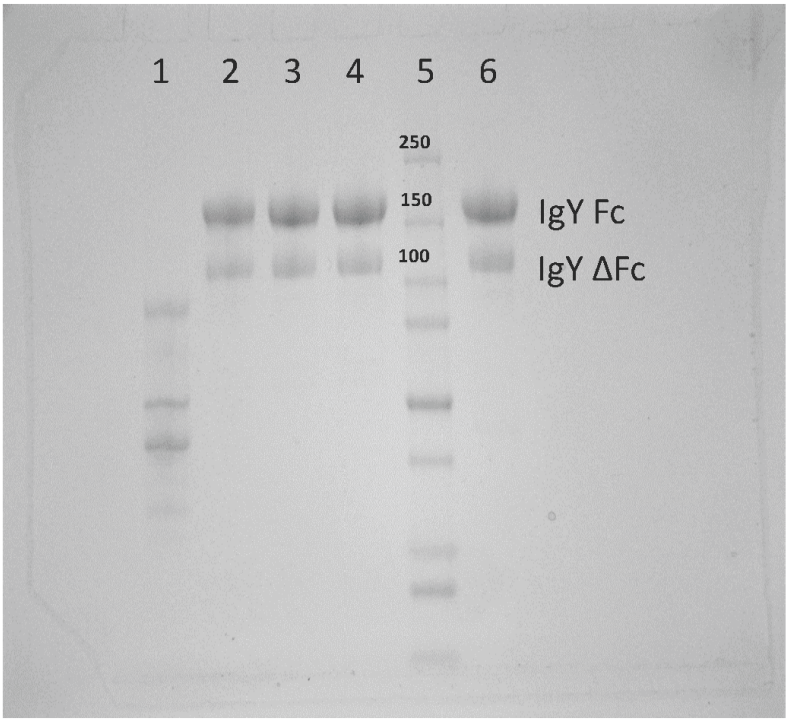


Fig. 3: Anti-ZIKV IgY purification: Purity of anti-ZIKV IgY after the second PEG precipitation. Lane 1, supernatant from the PEG precipitation, lane 2, resuspended PEG pellet. Lanes 3 and 4, molecular weight concentration and diafiltration. Lane 5, molecular weight marker. Lane 6, control naïve IgY that was previously purified via mercapto-ethyl pyridine HyperCel Hydrophobic Charged Induced Chromatograph for comparison.

In vitro viral neutralization and enhancement of Zika-specific IgY

Polyvalent anti-ZIKV IgY was purified from goose egg yolks that were tested for neutralization capacity. Concentrations ranging from 250 µg/mL to 3.125 µg/mL were utilized to determine the neutralization capacity of anti-ZIKV IgY. 40-50 PFUs of Zika virus PRVABC59 was treated with anti-ZIKV IgY and then inoculated onto Vero cells to determine the NT_{50}. The concentration that yielded the NT_{50} was 25 µg/mL (Fig. 4a). To determine if anti-ZIKV IgY induces ADE the experimental positive control Pan-flavivirus IgG 4G2 antibody produced a productive viral replication cycle in THP-1 cells yielding a significantly higher viral load compared to the untreated viral infection. The ZIKV-specific IgY did not enhance viral burden at any of the concentrations tested (Fig. 4b). Cross reactivity and potential enhancement of a secondary Dengue virus infection was also assessed as described above and no viral enhancement was demonstrated in a DENV-2 infection of THP-1 cells.

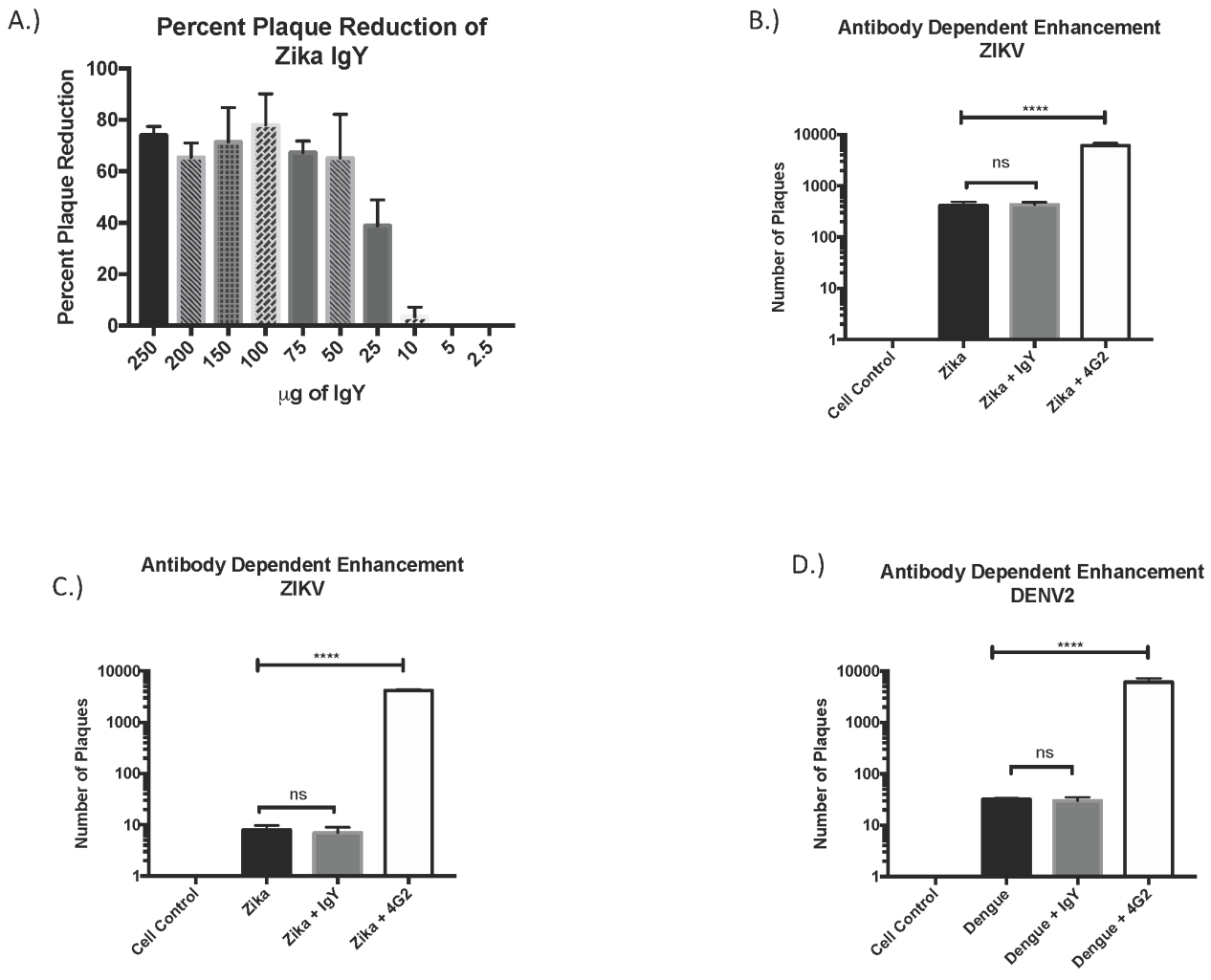


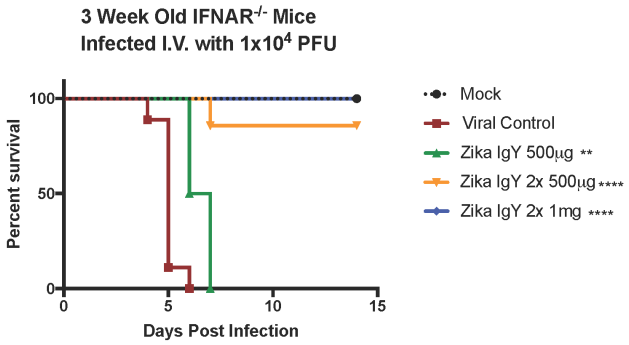
Fig. 4: Anti-ZIKV IgY neutralizes ZIKV *in vitro* without antibody-dependent enhancement. A.) Anti-ZIKV IgY neutralized ZIKV infection *in vitro* with a NT_{50} of 25 µg/mL. **B.)** Purified anti-ZIKV IgY did not enhance infection *in vitro* when virus was treated with 1µg/mL of IgY, whereas control 4G2 flavivirus IgG resulted in a significant increase of infection. **C.)** Purified anti-ZIKV IgY did not enhance ZIKV infection *in vitro* at

100 µg/mL. **D.)** Purified anti-ZIKV IgY did not enhance DENV2 infection *in vitro* at 100 µg/mL, where 4G2 flavivirus IgG significantly enhanced the viral load. ****p=<0.0001, ns=not significant.

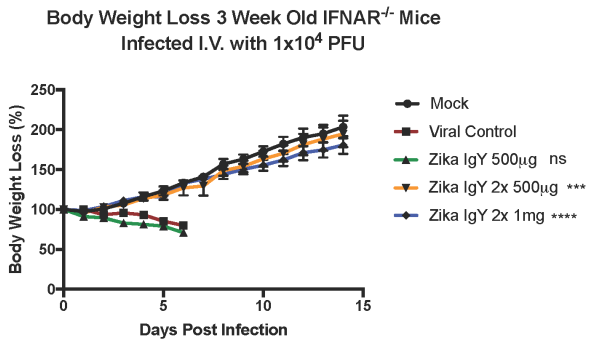
In vivo efficacy of ZIKV-specific IgY

IFNAR$^{-/-}$ mice were challenged with a lethal dose of ZIKV PRVABC59 (1x10^4 PFUs) and the therapeutic potential of ZIKV-specific IgY was determined (Fig. 5). The results of four independent experiments using a lethal dose of 1x10^4 PFUs were combined. Mice receiving only PBS as a negative control succumbed to the infection by 6 days post infection. An initial dose of 500 µg of ZIKV-specific IgY (n=6) 24 hours post infection significantly extended the lifespan but did not allow for recovery from infection and all mice succumbed to infection by day 7. 500 µg of ZIKV-specific IgY(n=7) administered 24 and 48 hours post infection lead to 85% therapeutic efficacy. This dose significantly reduced infectious viral load and genomic viral copies in the brain, liver, and spleen (Fig. 6). We achieved 100% therapeutic efficacy with 1 mg of ZIKV-specific IgY (n=6) administered 24 and 48 hours post infection. This regimen resulted in sterilizing immunity in all mice measured by infectious viral load and viral genomic copies (Fig. 6). Naïve IgY was used as a control, two doses of 500 µg and 1 mg did extend the mice's lifespan but no subjects survived the challenge.

A.)


3 Week Old IFNAR-/- Mice
Infected I.V. with 1x10^4 PFU
Percent survival
100
50
0
0
5
10
15
Days Post Infection
Mock
Viral Control
Zika IgY 500µg **
Zika IgY 2x 500µg ****
Zika IgY 2x 1mg ****

B.)


Body Weight Loss 3 Week Old IFNAR-/- Mice
Infected I.V. with 1x10^4 PFU
Body Weight Loss (%)
250
200
150
100
50
0
0
5
10
15
Days Post Infection
Mock
Viral Control
Zika IgY 500µg ns
Zika IgY 2x 500µg ***
Zika IgY 2x 1mg ****

C.)

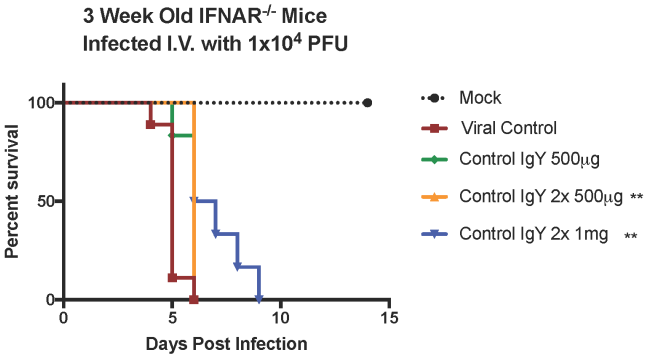

3 Week Old IFNAR-/- Mice
Infected I.V. with 1x10^4 PFU
Percent survival
100
50
0
0
5
10
15
Days Post Infection
Mock
Viral Control
Control IgY 500µg
Control IgY 2x 500µg **
Control IgY 2x 1mg **

Fig. 5: Therapeutic efficacy of anti-ZIKV IgY *in vivo*. 3 week old IFNAR-/- mice were administered a lethal dose of ZIKV i.v. in 100 µL total volume. At 24 and 48 hour post infection mice were immunized i.p. with the indicated amount of antibody in a volume of 100µL. Control mice were treated with 100 uL of PBS. Panels **A** and **C** demonstrate the survival outcome of the treatments. **B.)** Weight was monitored daily until day 14 or until morbid. Weights are reported as percent of starting weight, p-values are denoted when comparing groups to viral control. **p<0.01, ***p<0.005, ****p<0.0001

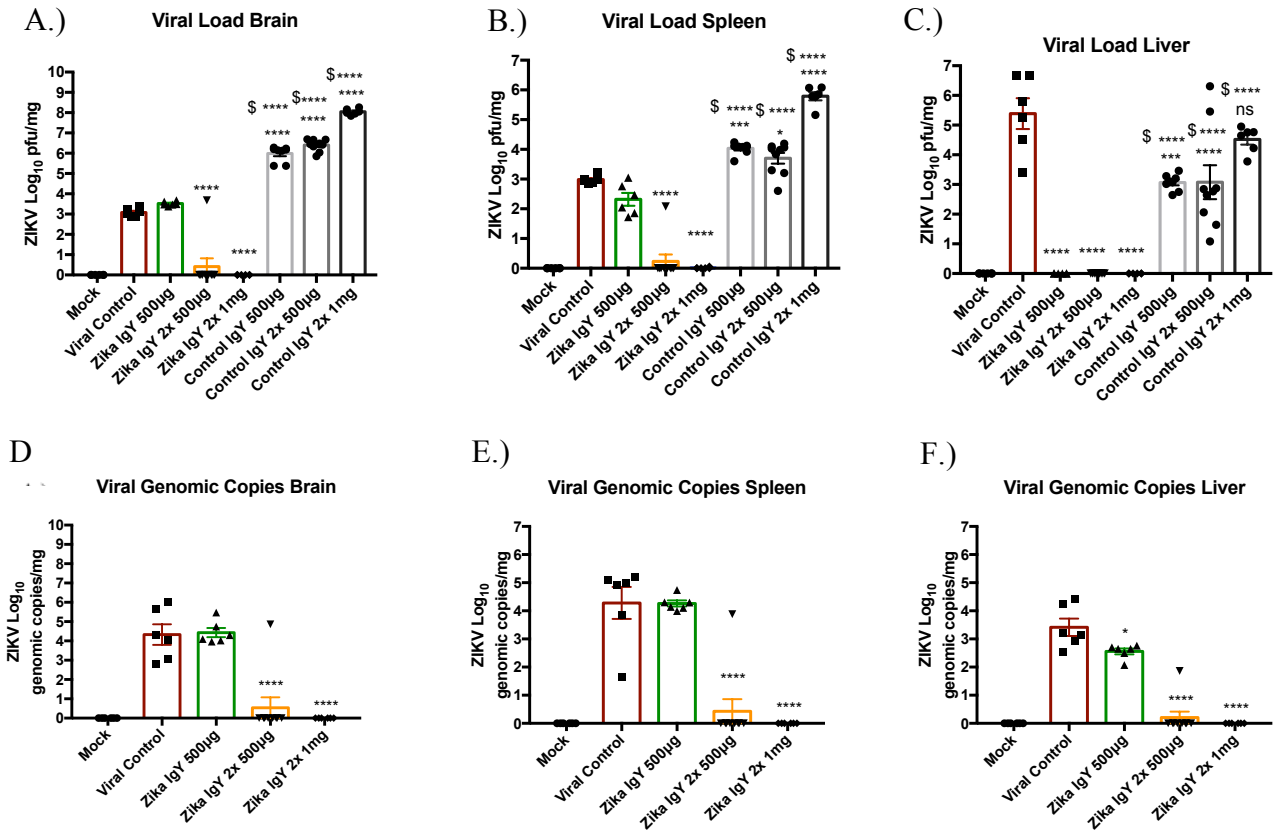


Fig. 6: Viral load reduction upon treatment with anti-ZIKV IgY. The viral load reduction upon treatment with anti-ZIKV IgY: (A–C) The infectious viral load was quantified in the brain (A), spleen (B), and liver (C) via a plaque assay. (D–F) The viral genomic copy number was quantified in the brain (D), spleen (E), and liver (F) via RT-qPCR. The viral load of the treated mice was compared to the viral control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.0001$, and $ is the comparison to the matching ZIKV-specific IgY group.

ZIKV-IgY Epitope Mapping

Epitope mapping was implemented to determine the specificity of the ZIKV specific IgY. A pan-flavivirus microarray displaying overlapping linear epitopes of envelope (E), membrane (M), peptide pr (PR) capsid protein (C), Non-structural proteins 1, 2, 3, and 5 (NS1, NS2A, NS3, NS5) reactivity was compared to negative control peptides MFI average. Unbiased heat maps were generated in order to compare ZIKV-specific IgY to control naïve IgY (Fig. 7). A majority of epitopes recognized by ZIKV-specific IgY were centralized on the M and NS5 protein. ZIKV-specific IgY did recognize a known neutralizing epitope on the E protein and a novel highly conserved NS1 epitope. We

measured the potential cross reactivity of the polyclonal Zika specific IgY with other flaviviruses via microarray analysis. We found significant cross reactivity with Dengue virus, Yellow Fever virus, and West Nile virus (Fig. 8). The epitopes corresponding to the number on the microarray are displayed in Table 2. The potential for cross neutralization of these three viruses will be tested in the future.

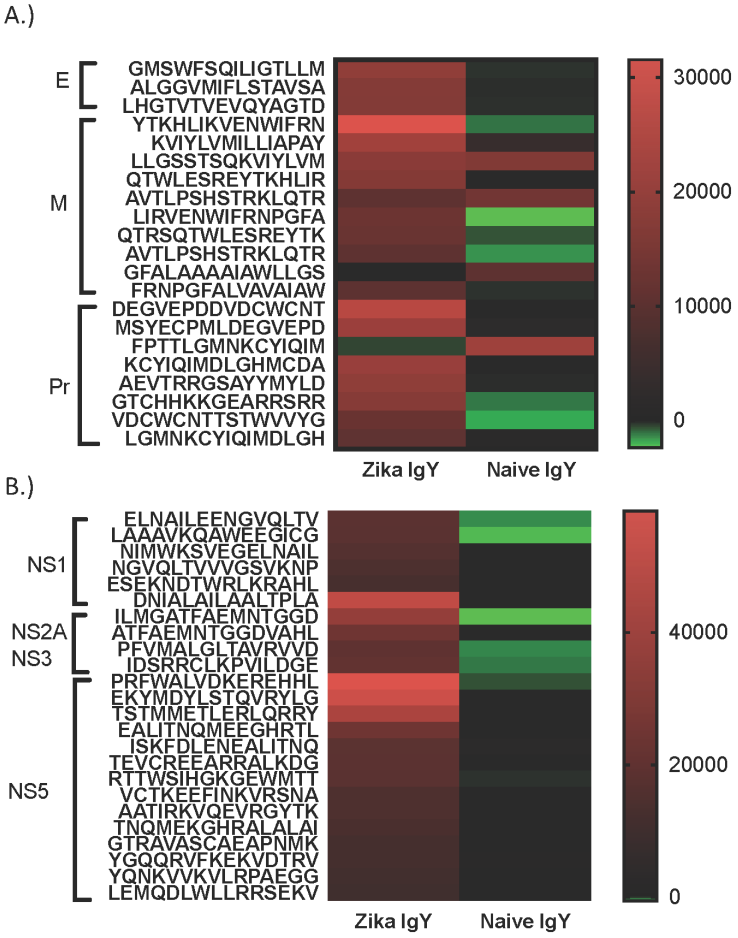


Fig. 7: Epitope mapping of structural and non-structural genes. A.) A heat map of structural epitopes of Zika virus recognized by Zika specific IgY and naïve IgY. **B.)** A heat map of non-structural epitopes of Zika virus recognized by Zika specific IgY and naïve IgY. The strength of binding is indicated on a colorimetric scale with red being strong binding affinity and green being no binding.

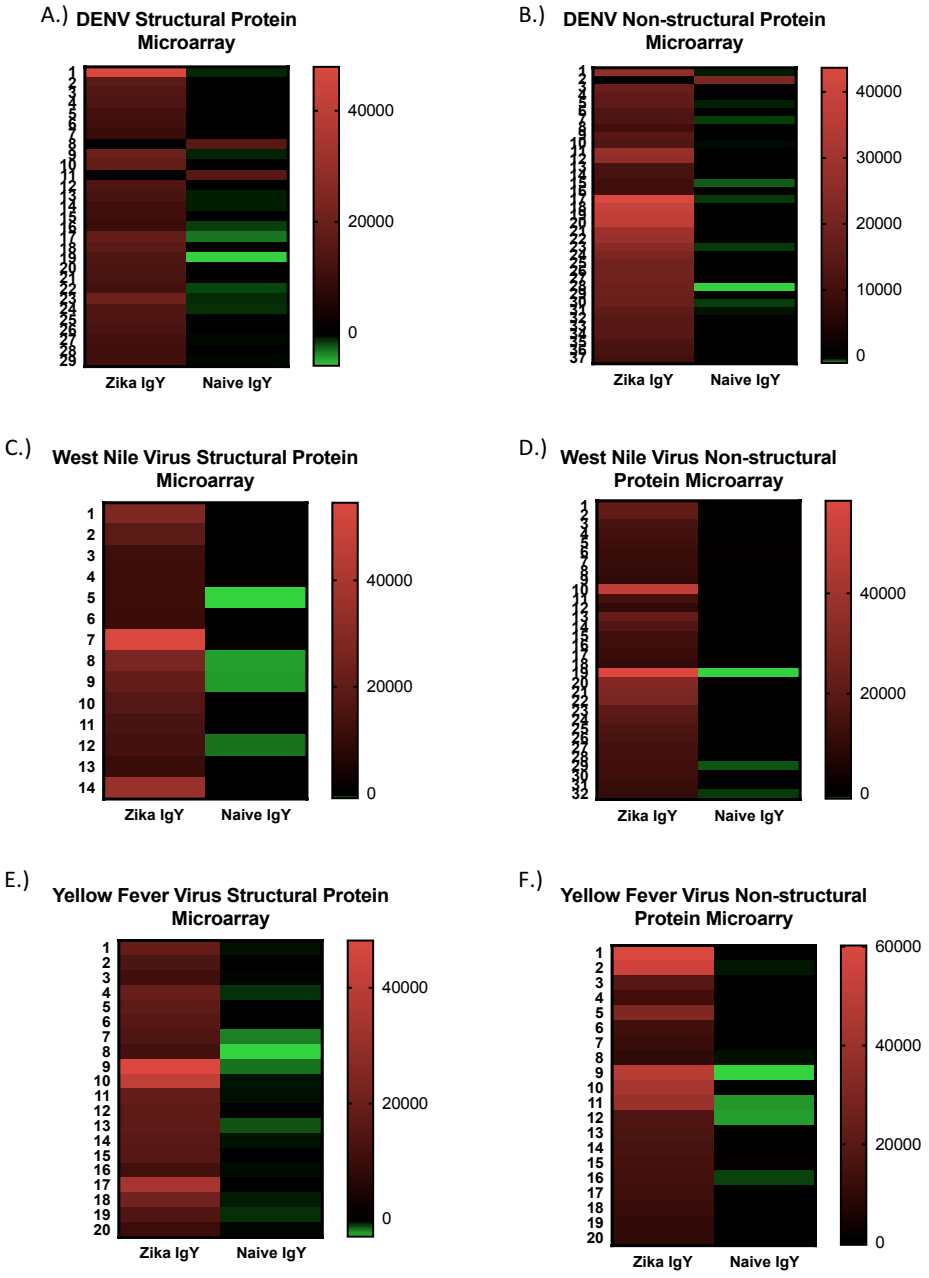


Fig. 8: **Epitope mapping of structural and non-structural genes. A.)** A heat map of structural epitopes of DENV recognized by Zika specific IgY and naïve IgY. **B.)** A heat map of non-structural epitopes of DENV recognized by Zika specific IgY and

naïve IgY. **C.)** A heat map of structural epitopes of WENV recognized by Zika specific IgY and naïve IgY. **D.)** A heat map of non-structural epitopes of WENV recognized by Zika specific IgY and naïve IgY. **E.)** A heat map of structural epitopes of YFV recognized by Zika specific IgY and naïve IgY. **F.)** A heat map of non-structural epitopes of YFV recognized by Zika specific IgY and naïve IgY The strength of binding is indicated on a colorimetric scale with red being strong binding affinity and green being no binding.

Position	Dengue Structural Proteins		Dengue Non-Structural Proteins	
1	LEHGSCVTTMAKNKP	E	GTTVVVDEHCGNRGP	NS1
2	TAWDFGSLGGVFTSI	E	KAKIIGADVQNTTFI	NS1
3	QGEPSLNEEQDKRFV	E	ASAIQKAHEEGICGI	NS1
4	PLPWLPGADTQGSNW	E	FIIDGPNTPECPSAS	NS1
5	TTESRCPTQGEPSLN	E	MEIRPLSEKEENMVK	NS1
6	QKETLVTFKNPHAKK	E	GTTVVITENCGTRGP	NS1
7	LTLDCEPRSGIDFNE	E	MEIRPLSEKEENMVK	NS1
8	NIEAEPPFGESYIVI	E	LENLMWKQITPELNH	NS1
9	MAAILAYTIGTTHFQ	M	EKYQLAVTIMAILCV	NS2A
10	MAYMIGQTGIQRTVF	M	IAGVFFTFVLLLSGQ	NS2A
11	VEKVETWALRHPGFT	M	NQILEENVEVEIWTK	NS3
12	SVALVPHVGMGLETR	M	FRKRKLTIMDLHPGA	NS3
13	LFLAHAIGTSITQKG	M	TDGPERVILAGPIPV	NS3
14	IGQTGIQRTVFFVLM	M	PGSRDPFPQSNAPIM	NS3
15	SSEGAWKHAQRIETW	M	DAFPQSNAPIQDEER	NS3
16	VPHVGMGLETRTETW	M	TATPPGATDPFPQSN	NS3
17	FKTEDGVNMCTLMAM	Peptide_pr	EDMLTVWNRVWIEDN	NS5
18	IDCWCNLTSTWVMYG	Peptide_pr	GEGLHRLGYILEEID	NS5
19	EPRMIVGKNERGKSL	Peptide_pr	DVFYLPPEKCDTLLC	NS5
20	CNLTSTWVMYGTCTQ	Peptide_pr	LGEKWKSRLNALGKS	NS5
21	CPLLVNTEPEDIDCW	Peptide_pr	YFHRRDLRLASNAIC	NS5
22	FKTTEGINKCTLIAM	Peptide_pr	GDDCVVKPLDERFGT	NS5
23	RWGSFKKNGAIKVLR	C	HGSYEVKATGSASSM	NS5
24	AFITFLRVLSIPPTA	C	KKVTEVKGYTKGGPG	NS5
25	RKEIGRMLNILNGRK	C	DVFFIPPEKCDTLLC	NS5
26	PTAGILKRWGQLKKN	C	YILRDVSKKEGGAMY	NS5
27	LIGFRKEIGRMLNIL	C	YFHRRDLRLASMAIC	NS5
28	LAKRFSKGLLSGQGP	C	QVRSLIGNEEYTDYM	NS5
29	MNQRKKVVRPPFNML	C	QEEQGWTSASEAVND	NS5

30			YILEEIDKKDGDLMY	NS5
31			KPTEQVDTLLCDIGE	NS5
32			DLENPHLPEKKITQW	NS5
33			LVKLHSGKDVFFIPP	NS5
34			CVVKPLDDRFASALT	NS5
35			SVWNRVWIEENPWME	NS5
36			SRKEFDLYKKSGITE	NS5
37			DVSKKEGGAMYADDT	NS5

Table 3: Corresponding epitopes to the heat maps of structural and non-structural proteins of DENV recognized by anti-ZIKV IgY and naïve IgY.

Position	West Nile Structural Proteins		West Nile Non-Structural Proteins	
1	VVFAILLLLVAPAYS	E	ECPTQNRAWNSLEVE	NS1
2	IDVKMMNMEAANLAE	E	NTLLKENGVDLSVVV	NS1
3	EFEEPHATKQSVIAL	E	TRTTTESGKLITDWC	NS1
4	SLTVQTHGESTLANK	M	CDSKIIGTAVKNNMA	NS1
5	QTHGESTLANKKGAW	M	IIPITLAGPRSNHNR	NS1
6	ESTLANKKGAWMDST	M	TPQGLAKIIQKAHKE	NS1
7	KVMMTVNATDVTDVI	Peptide_pr	VSRLEHQMWEAVKDE	NS1
8	CEDTITYECPVLAAG	Peptide_pr	VVDGPETKECPTQNR	NS1
9	ITYECPVLAAGNDPE	Peptide_pr	EIDFDYCPGTTVTIS	NS1
10	DVITIPTAAGKNLCI	Peptide_pr	ASTGVFNPMILAAGL	NS2A
11	ITYECPVLSAGNDPE	Peptide_pr	LLVVFLATQEVLRKR	NS2A
12	CEDTITYECPVLAAG	Peptide_pr	KEKRSSAAKKKGACL	NS2A
13	PEDIDCWCTKSSVYV	Peptide_pr	FLELLRTADLPVWLA	NS3
14	CPVLAAGNDPEDIDC	Peptide_pr	PGAGKTRRILPQIIK	NS3
15	KSRAVNMLKRGMPRV	C	NPSQVGDEYCYGGHT	NS3
16			QRRGRIGRNPSQVGD	NS3
17			WLAYKVAAAGISYHD	NS3
18			SAIVQGERMEEPAPA	NS3
19			GRTTWSIHAGGEWMT	NS5
20			VVPCRGQDELIGRAR	NS5
21			RTWNYHGSYDVKPTG	NS5
22			KDIQEWKPSTGWYDW	NS5

23			AENIHVAINQVRSVI	NS5
24			ENEWMEDKTPVERWS	NS5
25			STGWYDWQQVPFCSN	NS5
26			VSRASGNVVHSVNMT	NS5
27			EEQNQWRSAREAVED	NS5
28			KNSGGGVEGLGLQKL	NS5
29			GKREDIWCGSLIGTR	NS5
30			HRRLARAIIELTYRH	NS5
31			IERLRREYSSTWHHD	NS5
32			YGWNIVTMKSGVDVF	NS5

Table 4: Corresponding epitopes to the heat maps of structural and non-structural proteins of WENV recognized by anti-ZIKV IgY and naïve IgY.

Position	Yellow Fever Structural Proteins		Yellow Fever Non-Structural Proteins	
1	KGTTYGVCSKAFKFL	E	WCCRSCTMPPVSFHG	NS1
2	KVIMGVVLIWVGINT	E	AAVNGKKSAHGSPTF	NS1
3	FGGLSWITKVIMGVV	E	AINFSKRELKCGDGI	NS1
4	TALTIAYLVGSNMTQ	M	NLVFSPGRKNGSFII	NS1
5	ERWLVRNPFFAVTAL	M	WKLEGRWDGEEEVQL	NS3
6	ERWLVRNPFFAATAL	M	GKKLVPSWASVKEDL	NS3
7	VRNPFFAVTALTIAY	M	WQVAKAGLKTNDRKW	NS3
8	LQKIERWLVRNPFFA	M	EPTRVVNWEVIIMDE	NS3
9	VTLVRRNRWLLLNVT	Peptide_pr	VTRMAMTDTTPFGQQ	NS5
10	YGKCDSAGRSRRSRR	Peptide_pr	WHYCGSYVTRTSGSA	NS5
11	YGKCDSAGRSRRSRR	Peptide_pr	KVDTRAKDPPAGTRK	NS5
12	CWCYGVENVRVAYGK	Peptide_pr	RTTWSIHGKGEWMTT	NS5
13	REEPDDIDCWCYGVE	Peptide_pr	WRDVPYLTKRQDKLC	NS5
14	VTLVRKNRWLLLNVT	Peptide_pr	VSPGNGWMIKETACL	NS5
15	TLVRRNRWLLLNVTS	Peptide_pr	GGVEGIGLQYLGYVI	NS5
16	FSVGTGNCTTNILEA	Peptide_pr	KGPLDRAAIEERVER	NS5

17	GLAVLRKVKRVVASL	C	CVVRPIDDRFGLALS	NS5
18	QKTKQIGNRPGPSRG	C	QDKTMVKEWRDVPYL	NS5
19	WKMLDPRQGLAVLRK	C	GKVDTGVAVSRGTAK	NS5
20	WKMLDPRQGLAVLRK	C	GKAYMDVISRRDQRG	NS5
21	FNILTGKKITAHLKR	C		

Table 5: Corresponding epitopes to the heat maps of structural and non-structural proteins of YFV recognized by anti-ZIKV IgY and naïve IgY.

Discussion

The results reported herein show that anti-ZIKV IgY purified from goose egg yolk has the ability to neutralize ZIKV infection *in vitro* and *in vivo* without inducing ADE. We observed therapeutic efficacy with 1 mg of anti-ZIKV IgY administered 24 and 48 hours post infection in a lethal IFNAR$^{-/-}$ mouse model. Our results indicated that naïve IgY was able to partially delay death in a lethally infected mouse but all mice immunized with naïve IgY succumbed to infection. This is in contrast to previous data from our lab demonstrating non-specific protection in a lethal DENV2 mouse infection model (156). This may be due to unique structural features of ZIKV, the mature virion is more thermally stable allowing for less dynamic rearrangement making the fusion loop on the envelope protein more difficult to access (226,227). As indicated in Figure 1 there is a full length and alternatively spliced form of IgY. All experiments performed here utilized both the full length and alternatively spliced forms of IgY in our purified product. In the future these distinct forms will be separated and the neutralization capacity of each will be assessed.

Epitope mapping was implemented to determine the specificity of the ZIKV specific IgY and identify potential broad neutralizing epitopes. A pan-flavivirus microarray displaying overlapping linear epitopes of envelope (E), membrane (M), peptide pr (PR) capsid protein (C), Non structural proteins 1, 2, 3, and 5 (NS1, NS2A, NS3, NS5) was compared to negative control peptides. The mean fluorescent intensity of the negative control peptides was used to remove fluorescent signal from non-specific binding. Unbiased heat maps were generated in order to compare ZIKV-specific IgY to control naïve IgY (Figure 5). We found that the peptide LHGTVTVEVQYAGTD of the IgG III like domain of the E protein was strongly recognized which is critical for cell receptor binding as well as endosomal fusion. A NS1 epitope was a highly recognized peptide, ESEKNDTWRLKRAHL, that is conserved across multiple strains of ZIKV. This epitope has not been previously identified in other antibody screening studies. Future studies are planned to isolate these epitope specific IgY populations and determine their ability to neutralize ZIKV. We measured the potential cross reactivity of the polyclonal Zika specific IgY with other flaviviruses via microarray analysis. We found significant cross reactivity with Dengue virus, Yellow Fever virus, and West Nile virus (data not shown). The potential for cross neutralization will be tested in the future.

Since the production of antivirals and vaccines may be difficult, alternative therapies must be explored for emerging pathogens such as ZIKV. Avian IgY and egg powder has been declared by the United States Code of Federal Regulation as generally recognized as safe (GRAS) (228). The therapeutic potential of polyclonal

avian antibodies has been demonstrated for a number of pathogens. Gastrointestinal infections such as enterotoxic *Escherichia coli*, *Salmonellal* spp, Rotavirus, and *Helicobacter pylori* have been successfully treated in mice and humans utilizing IgY (229). IgY has also been implemented in Sweden as an orphan drug for the prevention of *Pseudomonas aeruginosa* in cystic fibrosis patients (228). We recognize that translating anti-ZIKV IgY to a human therapy will encounter challenges. It has been previously demonstrated that pigs and mice will develop a humoral response to avian IgY, notably the subclass IgG (229-231). Unpublished data from our lab demonstrated weekly IgY injections begin to produce anti-IgY IgG in the third to fourth week of immunizations. Purified IgY is unknown to have the ability to induce an allergic reaction in humans. What has been demonstrated is that raw egg yolk, purified IgY, and alternatively spliced IgY did not induce an IgE response in mice. Egg allergies are important to consider when implementing IgY as a therapy in case residual egg yolk lipids still remain in the purified product. Future allergenicity studies will need to be conducted to definitively determine if purified IgY induces an allergic response or not.

At the present there are no FDA approved vaccines or therapies for ZIKV infection. Multiple platforms; live attenuated, nucleic acid, vector, and peptide based, have been employed to develop a safe efficacious vaccine for ZIKV (232-234). A major conundrum for the development of a ZIKV vaccine is the risk of inducing ADE by a subsequent DENV infection. Settler et al demonstrated that cross-reactive ZIKV antibodies have the ability to enhance DENV infection (219). The severity of developing

a therapy without properly assessing its ability to induce ADE was demonstrated by the deployment of the tetravalent dengue vaccine that resulted in increased hospitalization in younger and serum naïve individuals (235). This study emphasized the need to produce vaccine and therapies for flaviviruses that do not induce ADE such as the case of anti-ZIKV IgY demonstrated here. IgY is similar to aglycosylated IgG as IgY does not bind to FcγR. IgY is advantageous because it does not require any genetic modification or post production processing to prevent enhancement.

Teratogenic pathology induced by ZIKV when a mother is infected and the infection spreads to the fetus is a major concern in the recent outbreak. Due to the causal relationship between ZIKV and the development of microcephaly lead the WHO to declare ZIKV a public health emergence and to expedite therapies needed to combat ZIKV infection. Taking this statement into account future studies will examine the therapeutic efficacy of immunizing dams with anti-ZIKV IgY and determining the prevention of viremia of the fetus.

In summary, our studies comprehensively characterized the therapeutic potential of anti-ZIKV IgY and demonstrated that IgY does not induce ADE. Our results indicate that utilizing a polyethylene glycol extraction method obtained a highly pure IgY product. Our purified anti-ZIKV IgY was able to inhibit ZIKV infection *in vitro* and did not induce ADE at multiple concentrations. Anti-ZIKV IgY therapeutically administered to mice protected against lethal ZIKV challenge. Epitope mapping demonstrated the recognition of known neutralizing E epitopes as well as novel NS1 epitopes unique to avians. Thus,

our study has addressed an important aspect of ZIKV infection, i.e., a safe and effective immunotherapy that may be utilized in an epidemic outbreak of ZIKV.

Chapter V

Avian antibodies targeting dengue virus NS1 neutralize infection *in vitro* and confer protection against lethal dengue disease in a mouse model

Abstract

Dengue fever is caused by four dengue virus (DENV) serotypes (DENV1-4) and is a continually increasing in global public health burden. A foremost challenge in dengue vaccine and immunotherapy development is that cross reactive anti-DENV antibodies can induce increased disease severity, via antibody dependent enhancement (ADE). DENV nonstructural protein 1 (NS1) is an attractive immunotherapy target, as antibody responses against this protein should not induce ADE. In this study, we evaluated the ability of avian antibodies (IgY) targeting epitopes in NS1 to neutralize DENV infection *in vitro* and *in vivo.* We characterized two unique anti-NS1 IgY preparations which were capable of neutralizing DENV infection *in vitro*, similar to an oligoclonal anti-DENV IgY preparation. Additionally, using ELISA we characterized the binding specificity of these two anti-NS1 IgY isolates across all four dengue serotypes, and established that they do not induce ADE in an *in vitro* system. We also demonstrated that anti-NS1 IgY isolates did not block binding of soluble NS1 to

endothelial cells to prevent the disruption of endothelial monolayers. Finally, we showed that prophylactic treatment of mice with anti-NS1 IgY conferred significant protection against lethal challenge with DENV2. Taken together, our findings highlight the potential of anti-NS1 IgY antibodies as a promising prophylactic approach to prevent lethal DENV infection.

Introduction

Dengue virus (DENV) is a mosquito-borne positive single strand mosquito-borne RNA virus which is widespread in tropical and subtropical climates, and a significant global public health burden. DENV causes approximately 390 million infections per year, with 96 million will resultig in clinical disease and 500,000 patients requiring hospitalization (236). The majority of dengue cases present as flu-like symptoms and joint pain, known as dengue fever (DF). However, a subset of individuals develop more severe forms of the disease, including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Severe DENV infection is characterized by increased vascular leakage, which causes hypotension and circulatory collapse leading to shock and potentially death (237).

The DENV nonstructural protein 1 (NS1) is a 48-55 kDa protein which induces endothelial hyperpermeability *in vitro* and vascular leakage *in vivo.* NS1 is secreted from DENV-infected cells and high levels of circulating NS1 in patients with severe dengue disease has been observed (238). In mice, administration of NS1 and a sublethal dose of DENV resulted in the production of pro-inflammatory cytokines TNFα and IL-6, as

well as in lethal vascular leakage syndrome (239). Circulating NS1 is capable of binding to the surface of endothelial cells in capillary beds (240). Further, interactions between the endothelial glycocalyx layer (EGL) and NS1 result in the up regulation of sialidases Neu1-3, which leads to the cleavage of sialic acid on the surface of the endothelial cells. Binding of NS1 to the EGL also induces an increased expression of cathepsin L, which in turn, over expresses heperanase and results in the cleavage of heparan sulfate and heparan sulfate proteoglycans. Together, these processes result in EGL degradation and loss of barrier integrity (241). Anti-NS1 antibodies capable of recognizing NS1 on the surface of DENV-infected cells can trigger complement activation and complement dependent cellular lysis (240). However, NS1 bound to uninfected cells can induce similar mechanisms and result in increased vascular disruption (242,243). Intracellular expression of NS1 is critical in the formation of viral replication complexes in the endoplasmic reticulum (ER) and deletion of NS1 halts viral replication (244-246). Indeed, NS1 co-localizes with viral RNA, as well as other non-structural proteins, to help stabilize the viral replication complex within the ER lumen. It has also been demonstrated that NS1 co-localizes with the structural E and prM proteins, which indicates that NS1 is involved in trafficking of mature virions to the cell membrane for efficient viral budding (247). Therefore, given the role of NS1 in the pathogenesis of severe dengue disease, and its functions in immune evasion and viral replication, it is reasoned that NS1 blockade could result in an effective therapeutic.

Due to the challenges of developing an effective and safe vaccination for DENV alternative antiviral therapies need to be explored. IgY is the avian homologue of mammalian IgG and shares characteristics with mammalian IgG and IgE. IgY is the predominant isotype in sera after the initial production of IgM and is the primary antibody produced upon a secondary response (110,111). IgY is found in two isoforms in the serum of water-fowl; full-length IgY, which contains two constant regions and an alternatively spliced IgY, which lacks these two constant regions (125). Due to the phylogenetic distance between avian and mammalian species, a much broader range of epitope recognition and higher binding affinities are achieved, which allows for novel epitopes to be recognized (126,129). IgY comprises 75% of all immunoglobulins in avian hosts with concentrations of 5 mg/ml in serum (129). IgY has been utilized to treat human pathogens such as *H. pylori*, *S. mutans*, *S. sobrinus*, *P. gingivalis*, Rotavirus, and *P. aeruginosa* in Cystic fibrosis patients (227-230). An attractive characteristic of IgY for flavivirus infection is that IgY does not interact with mammalian FcγR, which eliminates the risk of ADE. This is critical for developing immunotherapies for DENV infection. Another unique characteristic of IgY is that it does not interact with the mammalian complement system, thus preventing off-target effects of anti-NS1 antibodies by triggering lysis of non-infected cells (131). We employed the neutralization capabilities of IgY to non-structural protein 1 of DENV2.

We previously demonstrated that avian IgY is effective at neutralizing DENV2 infection *in vitro* and *in vivo* without inducing antibody dependent enhancement (ADE)

(156). Epitope mapping of a polyclonal anti-DENV IgY preparation revealed the presence of antibodies specific against NS1 epitopes. We hypothesized that these antibodies could neutralize NS1-induced pathology and be protective in a mouse DENV challenge model. We found that 2 IgY antibody preparations, denoted NS1-1 and NS1-8, were effective at neutralizing the virus *in vitro*. However, these avian antibodies did not prevent soluble NS1 from binding to endothelial cells or limit endothelial permeability *in vitro*. Finally, passive transfer of NS1-specific IgY antibodies into interferon-α/β receptor-deficient C57BL/6 ($Ifnar^{-/-}$) mice conferred significant protection against DENV-induced morbidity and mortality.

Materials and Methods

Ethics statement

Experimental procedures involving the use of animals were conducted in compliance with the Animal Welfare Act and in adherence to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of California, Berkeley.

Epitope specific IgY isolation

Peptides 15 amino acids in length were produced by JPT Innovative Peptide Solutions for NS1 of DENV2. Utilizing AminoLink Plus Immobilization Kit (Thermo Scientific) IgY was isolated for specific epitopes. The protein was coupled to the AminoLink column according to the manufacture's instructions. Briefly, the AminoLink Plus resin was resuspended and washed twice with pH 10 Coupling Buffer. Two ml of a 1.5 mg/ml of peptide solution specific for the epitope of interest were added to the column and was rocked end over end overnight. The column was centrifuged and washed with 2ml of Quenching Buffer twice. Two ml of Quenching Buffer were added and column were rocked for 30 minutes. The Quenching Buffer was then removed and the column washed four times with 1X PBS. Two ml of 6.9 mg/ml of DENV2 oligoclonal IgY were added to the column with 200 µl of Binding Buffer. The column was rocked end over end for one hour. The sample was removed and 1 ml of Binding Buffer was added and eluted into the same tube as the sample. Column was washed three times with 1x PBS and antibodies were eluted with 2 ml of Elution Buffer three times. A Coomassie Plus Assay Kit (Thermo Scientific Waltham, MA) was used to quantify the protein after elution. The microplate procedure was followed according to manufacture's instructions.

Viruses and cell lines

DENV-2 VR-1584 and Zika virus PRVABC59 were propagated in Vero cells [American Type Culture Collection (ATCC)]. Briefly, Vero cells at 80% confluency in 6 well plates

were infected at an MOI of 0.1 and allowed to adhere for one hour at 37ºC 5% CO_2. The cells were washed with DMEM containing 10% fetal bovine serum (FBS) (Atlanta biologicals, Atlanta, GA), Penicillin/Streptomycin (Corning, Corning, NY), and 2.5mM HEPES, (cDMEM). Three ml of cDMEM were replaced in the wells and the plates were incubated at 37ºC 5% CO2 for 5 days. The cell supernatant was centrifuged at 4,000 x g for 5 minutes to remove cell debris. The clarified cell supernatant was diluted 1:2 in heat inactivated fetal bovine serum and frozen at -80ºC. DENV2 D220 was generated from the parental strain DENV2 PL046 (247). The virus was propagated in *Aedes albopictus* C6/36 cell line (ATCC) and titered by plaque assays using baby hamster kidney (BHK) cells.

Mice

Interferon-α/β receptor–deficient C57BL/6 (*Ifnar*$^{-/-}$) mice were bred and housed in specific pathogen-free conditions at the University of California, Berkeley Animal Facility. Five- to 8-week-old male and female mice were used for all experimental procedures.

Lactate dehydrogenase assay

Vero cells were seeded into 6 well plates at $2x10^5$ cells/well. DENV2 was treated with 25 µg of anti-NS1 IgY, control naïve IgY, and oligoclonal DENV IgY, and incubated for 1 hour at 37ºC 5% CO_2. Cells were then infected with 250 µl (MOI 0.1) and incubated for 1

hour at 37ºC 5% CO_2. Viral inoculum was removed and cells were refed with cDMEM and incubated for 5 days at 37ºC 5% CO_2. Cell supernatants were removed and a Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, Rockford, IL) was utilized to determine cytotoxicity per the manufacture's instructions.

Plaque assay

Vero cells were seeded into 6 well plates at $2x10^5$ cells/well. The cells were incubated 37ºC 5% CO_2 and were allowed to come to confluency. Viral stocks were thawed in a 37ºC water bath and diluted from 10^{-1} to 10^{-7} in cDMEM. Wells were infected with 250 µl of viral dilutions in triplicate also cell controls were inoculated with media alone. Plates were placed at 37ºC 5% CO_2 for 1 hour lightly shaking every 15 minutes. Viral inoculum was removed and the wells were washed with 1X PBS. Next, 3ml of 1:1 of 2% methyl cellulose and cDMEM were added to each well and the plates were incubated for 10 days (DENV) and 7 days (ZIKV). At the end of the incubation the overlay was removed and 400µl of 10% formalin were added to each well and incubated for 30 minutes at room temperature to fix the samples. Finally, the formalin was removed and crystal violet solution was added to each well for 10 minutes to stain the cell layer. The crystal violet was removed and the wells were washed 3-5 times with 1x PBS until plaques were clearly visible.

Neutralization assays

Neutralization assays were used to evaluate the ability of anti-NS1 IgY antibodies to block DENV infection *in vitro.* In brief, IgY antibodies were diluted to a starting concentration of 0.1 mg/mL. Ten 4-fold dilutions were mixed with equivalent volumes of DENV2 D220 for 1 hour before infecting U937 DC-SIGN cells, a DENV-permissive human monocytic cell line (248). Two hours following infection, cells were washed, and then fixed and stained for DENV E protein using 4G2 mAb-Alexa 488 24 hours later. The percentage of infected cells was calculated using a Guava flow cytometer (EMD Millipore) by gating Alexa 488-positive cells. The antibody dilution that reduced DENV infection by 50% (50% neutralizing antibody titer [NT50]) was calculated using Prism software (GraphPad).

Antibody dependent enhancement

ADE was assessed by co-incubating virus (MOI of 1) and antibody solutions for 1 hour at 37ºC 5% CO_2 then added to $2x10^5$ K562 cells/well and incubated for 1 hour under same conditions. Cells were then washed with 100 µl of cDMEM and maintained for 2 days at 37ºC 5% CO_2. Cells were fixed with 10% formalin for 30 min at room temperature. Cells were subsequently washed with FACS buffer and with flow cytometry perm buffer (TONBO, San Diego, CA) and stained with 4G2 mAb-Alexa 488 conjugated antibody (Anti-flavivirus group antigen antibody, Novus Centennial, CO). Once stained and washed, the cells were analyzed via FACS and the number of Alexa 488 positive cells were calculated (225).

ELISA

Binding of IgY antibodies to the 4 DENV NS1 serotypes (DENV1, Nauru/Western Pacific/1974; DENV2, Thailand/16681/84; DENV3, Sri Lanka D3/H/IMTSSA-SRI/2000/1266); and DENV4, Dominica/814669/1981) was evaluated by ELISA. Briefly, MaxiSorp® ELISA plates (Thermo Scientific Nunc) were coated with 50 µl of NS1 (The Native Antigen Company) (0.5 µg/ml) and incubated overnight at room temperature. After blocking with 1% BSA in PBS (1% BSA-PBS), wells were incubated for 1 hour at room temperature with anti-NS1 IgY antibodies. Plates were then washed and incubated for 1 hour at room temperature with horse radish peroxidase-labeled goat anti-chicken IgY (abcam) at 0.5 µg/ml in 1% BSA-PBS. The assay was developed using an ABTS-HRP substrate kit (SeraCare), according to the manufacturer's specifications.

NS1 binding assay

A recently developed NS1 binding assay were used to determine if anti-NS1 IgY antibodies could prevent binding of NS1 to FreeStyle 293F suspension cells (Thermo Fisher Scientific) [Puerta-Guardo et al. 2019 Cell Rep.]. Briefly, eight 3-fold IgY antibody dilutions were mixed in 96-well tissue culture plates with recombinant His-tagged DENV2 NS1 (10 ug/ml) and incubated for 30 min using gentle oscillation. A suspension of 5 x 10^4 293F cells was then added to each well and incubated for 45 min at 37°C, 5%$C0_2$. Finally, an AlexaFluor 647-conjugated anti-His-tag antibody (mAb-A647) was

used to detect NS1 bound to the surface of 293F cells, and samples were analyzed by flow cytometry.

Trans-endothelial electrical resistance (TEER) assay

To determine if anti-NS1 igY antibodies could prevent NS1-induced endothelial hyperpermeability, human pulmonary microvascular endothelial cell (HPMEC) monolayers grown on a 24-well Transwell polycarbonate membrane system (Transwell permeable support, 0.4 mM, 6.5-mm insert; Corning Inc.) were incubated with NS1 (5 μg/ml) and IgY antibodies. Endothelial permeability was evaluated by measuring TEER in ohms at sequential 2-h time points. Measurements were performed using an epithelial voltohmmeter with “chopstick” electrodes (World Precision Instruments). Transwell inserts containing untreated HPMEC cells were used as a negative control and inserts with medium alone were used for blank resistance measurements. Relative TEER was expressed as the ratio of resistance value as follows: [ohm(experimental condition) − ohm (medium alone)]/[ohm (nontreated endothelial cells) − ohm (medium alone)]. After 24 h of treatment, 50%of upper and lower chamber media was replaced by fresh endothelial cell medium.

Dengue virus challenge experiments

To evaluate the protective capacity of anti-NS1 IgY antibodies, mice were injected intraperitoneally (i.p.) with 5 μg of 4G2 mAb and 150 μg of IgY antibodies. Twenty-four

hours later, mice were infected with 3 x10^5 PFU of DENV2 D220 by intravenous (i.v.) tail vein injection. Mice were monitored for 10 days and scored for morbidity and mortality using a standardized 5-point system (249).

Results

Neutralization capacity of anti-DENV2 NS1 in vitro.

Epitope anti-DENV2 NS1 IgY was column purified from the oligoclonal anti-DENV IgY stock. Each epitope was given a number ranging from 1-8 to denote a particular amino acid sequence. To assess potential candidates for further neutralization analysis each anti-DENV2 NS1 preparation was tested for inhibition of DENV-induced cellular cytotoxicity (Fig. 9A). Candidates that demonstrated a significant reduction in cellular cytotoxicity were then assayed for neutralization capacity via plaque reduction neutralization tests. Concentrations ranging from 100 µg/mL to 3.125 µg/mL were administered to determine the 50% neutralization titer (NT_{50}). In these experiments, the NT_{50} of NS1-1 estimated at was 3.125 µg/mL while NS1-8 was 6.25 µg/mL (Fig. 9B-C). In adition, using a flow cytometry-based neutralization assay the NT_{50} of NS1-1 and NS1-1 were 1.2 µg/mL and 1.6 µg/mL, respectively (Fig. 9D).

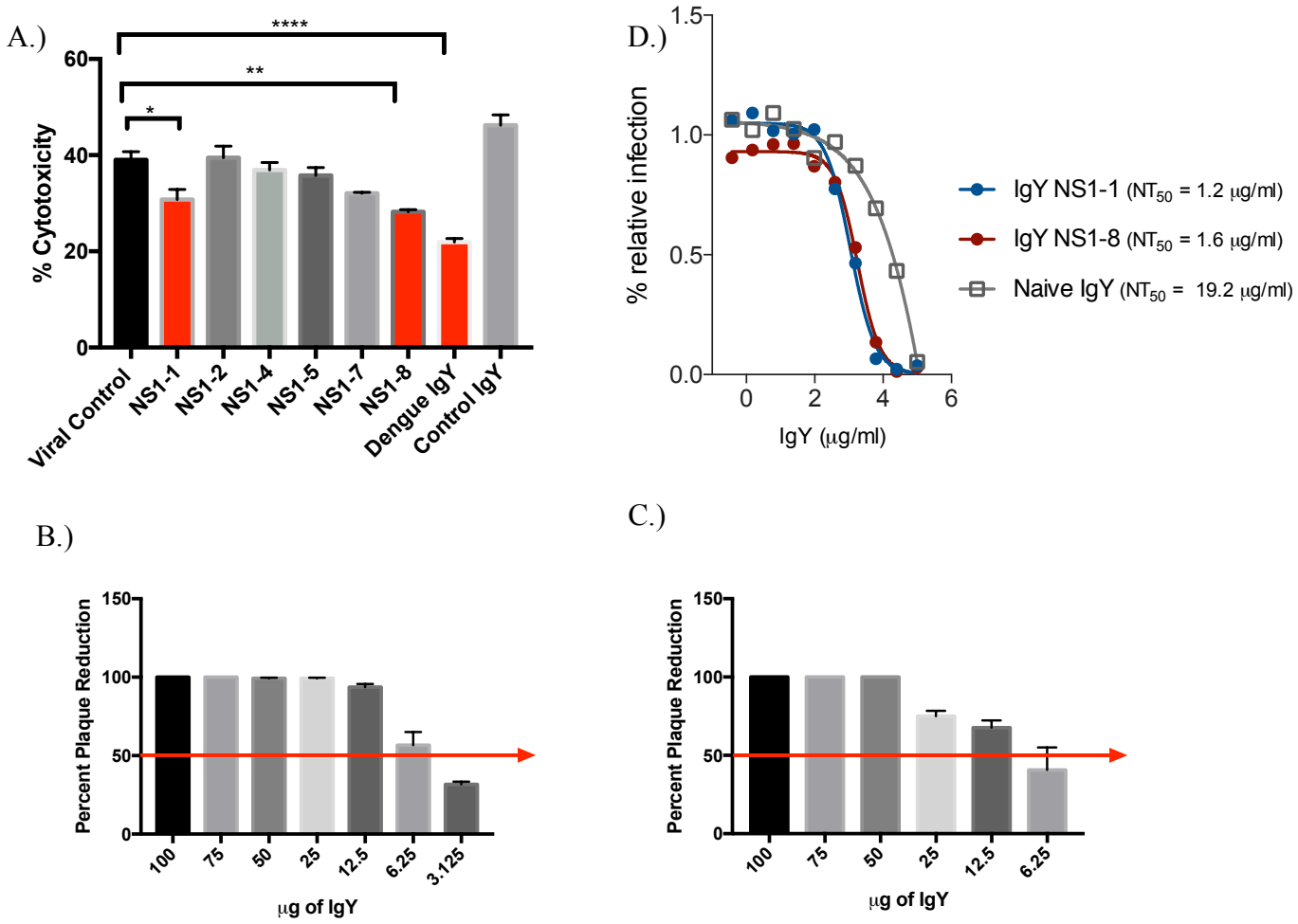


Fig. 9: Anti-DENV2 NS1 IgY neutralizes DENV2 *in vitro*.

A.) Multiple unique NS1 epitope IgY preparations assayed for the reduction of cytotoxicity. **B.)** Purified anti-NS1-1 IgY PRNT NT50 3.125 μg/mL. **C.)** Purified anti-NS1-8 IgY PRNT NT50 6.25 μg/mL. **D.)** Serial dilutions of IgY antibodies were tested for neutralization of DENV2 D220. Data were fitted with a four-parameter sigmoidal dose-response curve to estimate the 50% neutralizing antibody titer (NT50). Data are representative of 2 independent experiments.

Anti-DENV2 NS1 IgY binds NS1 from multiple DENV serotypes

Each of the anti-DENV2 NS1 IgY preparations that demonstrated significant neutralization were assessed by ELISA for their ability to bind to NS1 from the 4 DENV serotypes. NS1-1 was capable of binding to the 4 DENV NS1 serotypes at higher concentrations, but showed a higher affinity for DENV2 NS1 and DENV 3 (Fig. 10A). A similar trend was observed for NS1-8 (Fig. 10B). Higher concentrations of naïve IgY bound non-specifically to serotypes DENV2 NS1, DENV3 NS1, and DENV4 (Fig. 10C). This data demonstrates a broad recognition of multiple serotypes of DENV NS1 by anti-NS1 IgY antibodies with a preference DENV2 NS1, the serotype used to immunize geese for IgY production.

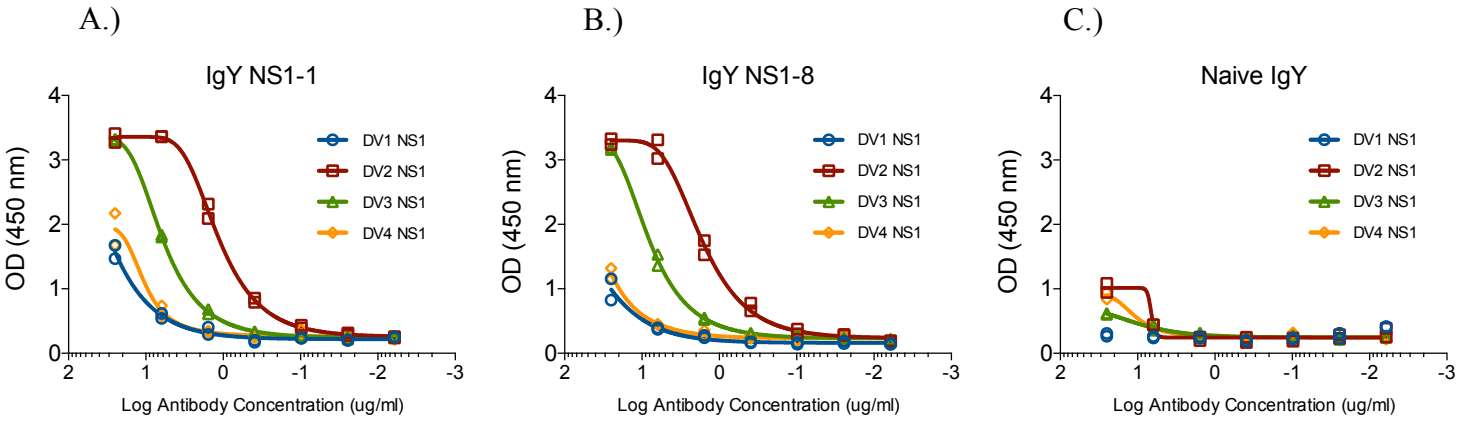
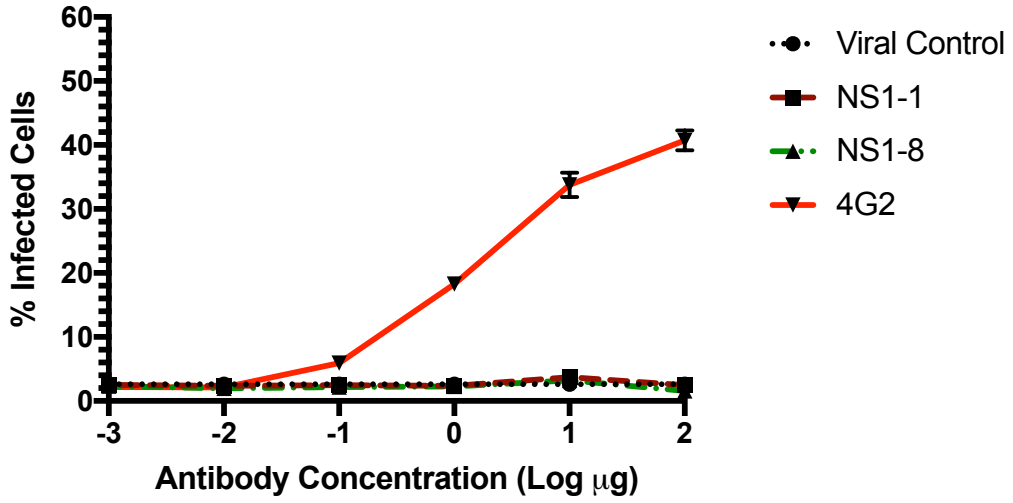


Fig. 10: Anti-NS1 binding to DENV NS1. NS1-1 (A.) NS1-8 (B) and naïve (C) IgY antibodies were serially diluted and binding to NS1 protein from each of the four serotypes was measured by ELISA.

Anti-DENV2 NS1 IgY does not induce ADE of DENV or ZIKV

As NS1 is not a structural protein, anti-NS1 should not facilitate ADE of mature virions. To test this, we utilized the bone marrow lymphoblast cell line K562 which are not permissive to flaviviral infection except via the FcR, to determine if anti-NS1 Igy antibodies could mediate ADE. As measured via FACs. NS1-1 and NS1-8 did not enhance either DENV infection or ZIKV infection of K562 cells (Fig. 11A-B).

A.`



B.)

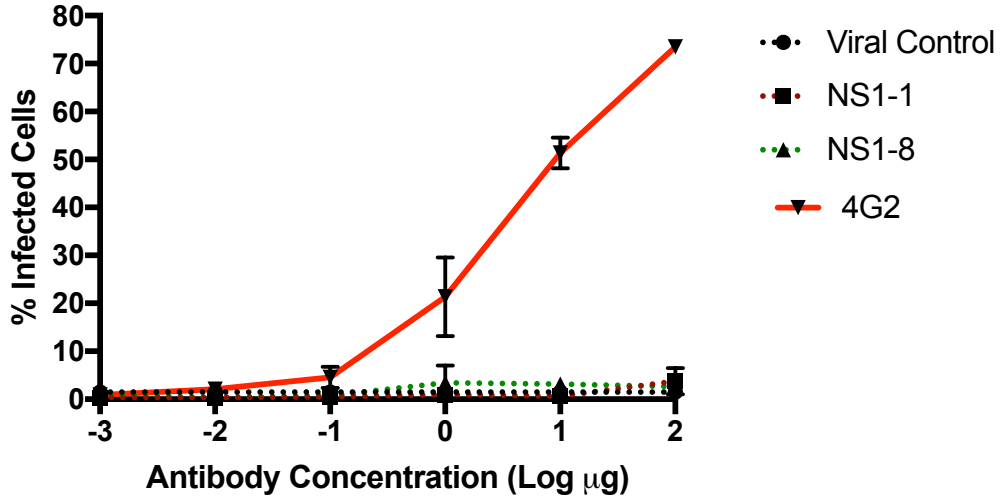


Fig. 11: Anti-NS1 IgY antibodies do not enhance *in vitro* DENV or ZIKV infection.

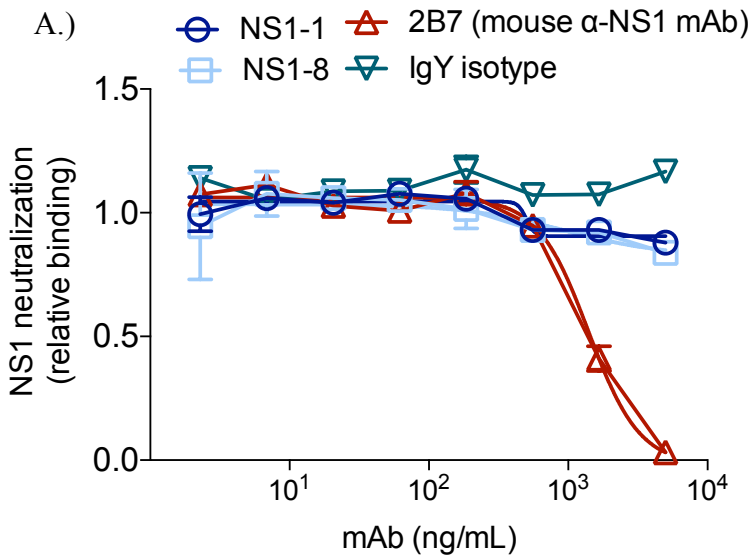
DENV or ZIKV were incubated with serially diluted anti-NS1 IgY antibodies and used to

infect K562 cells. The 4G2 mAb, specific for the flavivirus envelope protein, was used as a positive control.

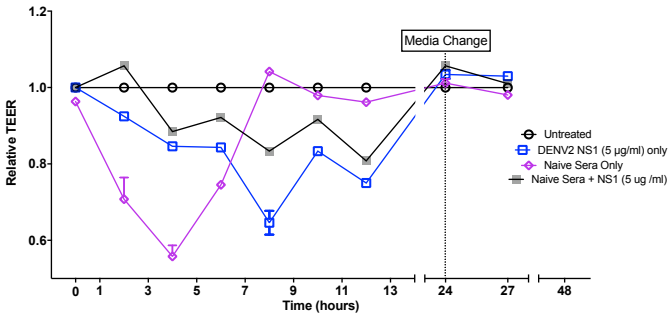
Anti-DENV NS1 IgY antibodies do not inhibit NS1 cell binding or NS1-induced endothelial permeability

To address if our anti-DENV NS1 IgY antibodies can prevent NS1 cell binding, each of the anti-IgY preparations were incubated with soluble NS1 and then added to 293F cells. At the tested concentartions, NS1-1 and NS1-8 were unable to inhibit NS1 binding to 293F cells (Fig. 12A). Further, we used a TEER assay to evaluate the ability of anti-NS1 IgY antibodies to prevent NS1-induced endothelial barrier dysfunction of HPMECs. In these experiments, NS1-1 and NS1-8 were unable to prevent the loss of electrical resistance due to increased endothelial permeability (Fig. 12B-E).

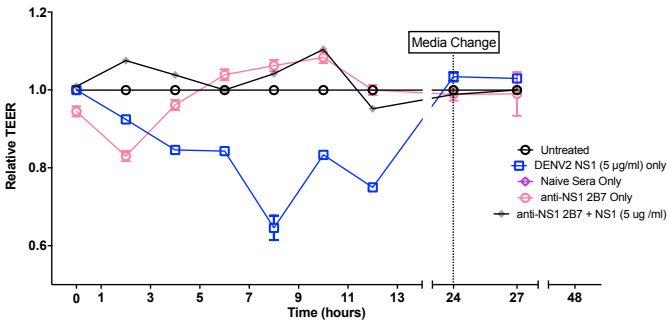
A.)


NS1-1
2B7 (mouse α-NS1 mAb)
NS1-8
IgY isotype
NS1 neutralization (relative binding)
1.5
1.0
0.5
0.0
10^1
10^2
10^3
10^4
mAb (ng/mL)

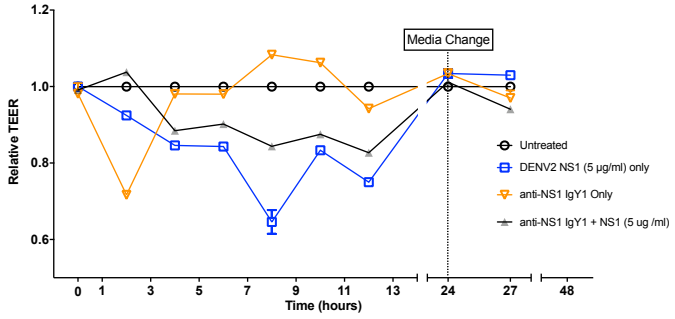
B.)


Media Change
Relative TEER
Time (hours)
Untreated
DENV2 NS1 (5 µg/ml) only
Naive Sera Only
Naive Sera + NS1 (5 ug /ml)

C.)


Media Change
Relative TEER
Time (hours)
Untreated
DENV2 NS1 (5 µg/ml) only
Naive Sera Only
anti-NS1 2B7 Only
anti-NS1 2B7 + NS1 (5 ug /ml)

D.)


Media Change
Relative TEER
Time (hours)
Untreated
DENV2 NS1 (5 µg/ml) only
anti-NS1 IgY1 Only
anti-NS1 IgY1 + NS1 (5 ug /ml)

E.)

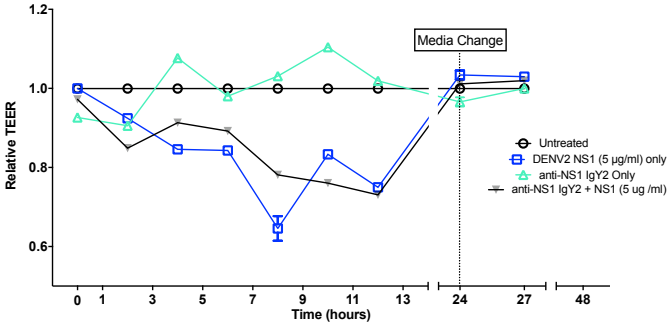

Media Change
Relative TEER
Time (hours)
Untreated
DENV2 NS1 (5 µg/ml) only
anti-NS1 IgY2 Only
anti-NS1 IgY2 + NS1 (5 ug /ml)

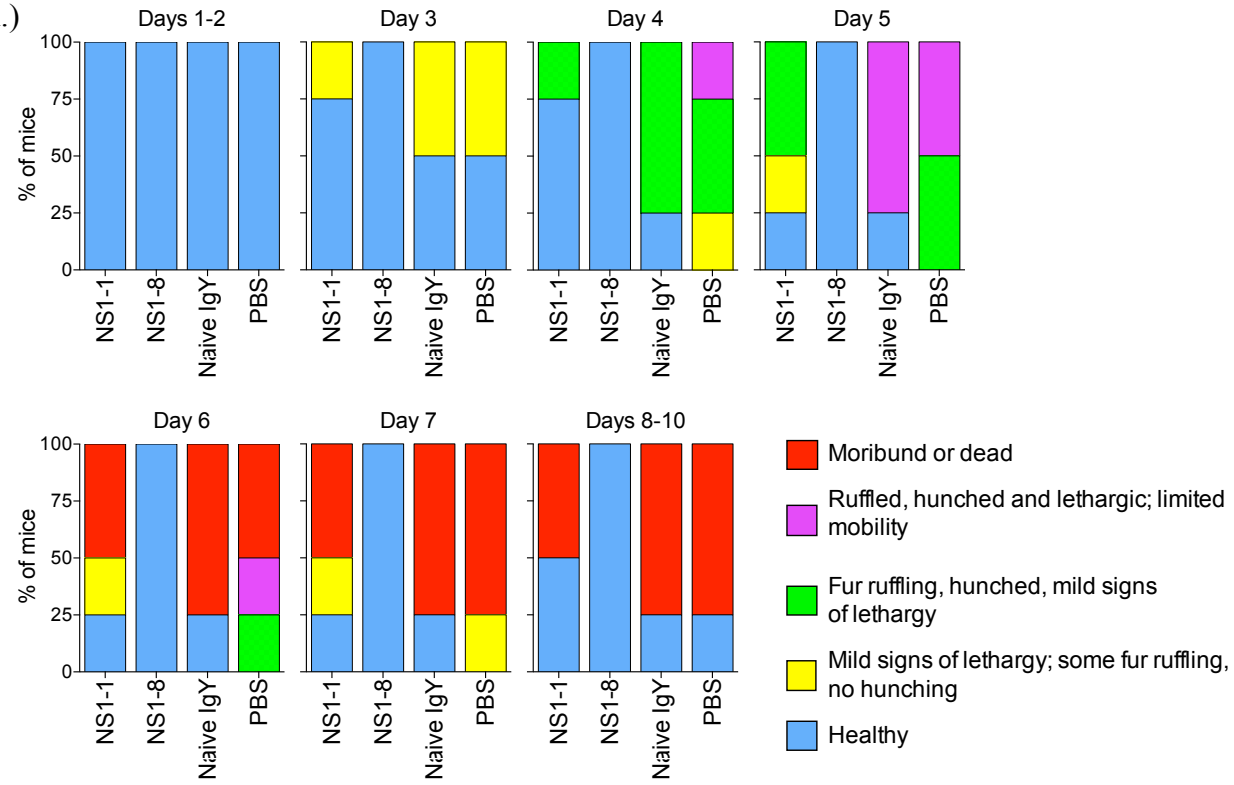
Fig. 12: Soluble NS1 binding and TEER assays: A.) NS1 binding to the surface of 293F cells in the presence of anti-NS1 IgY antibodies was measured by flow cytometry. An anti-His-tag antibody (mAb-A647) was used to detect NS1 bound to the surface of 293F cells. The mouse monoclonal antibody (mAb) 2B7, specific for NS1, was used as a positive control. **B.)** HPMECs were grown on Transwell semi-permeable membranes (0.4 mm pore size), and DENV2 NS1 protein (5 µg/mL, 1.5 ug total protein) in combination with IgY antibodies or controls were added to the apical chamber. A TEER assay was used to assess the effect of IgY antibodies on NS1-induced endothelial permeability at indicated time-points over 48 h. Naïve IgY was unable to block soluble NS1 from inducing loss of electrical resistance in the endothelial monolayer. **C.)** 2B7 was able to inhibit the decrease in electrical resistance of the endothelial monolayer caused by soluble NS1. **D.)** NS1-1 IgY was unable to block soluble NS1 from inducing loss of electrical resistance in the endothelial monolayer. **E.)** NS1-8 IgY was unable to block soluble NS1 from inducing loss of electrical resistance in the endothelial monolayer.

In vivo efficacy of Anti-DENV NS1 IgY

To determine if anti-NS1 IgY antibodies could protect against DENV-induced morbidity and mortality, Ifnar$^{-/-}$ mice were prophylactically treated with 150 µg of anti-DENV2 NS1 IgY antibodies or naïve IgY. Twenty-four hours post treatment, mice were challenged

with 3 x 10^5 PFU of DENV2 D220 using ADE conditions and morbidity and mortality were monitored for 10 days. Notably, none of the mice treated with NS1-8 IgY showed signs of morbidity or succumbed due to DENV infection (Fig 13 A). However, survival among Ns1-1 IgY-treated mice was only 50%, while only 25% of the animals treated with naïve IgY or PBS did not succumb upon DENV challenge (Fig 13 B).

A.)


Days 1-2
Day 3
Day 4
Day 5
Day 6
Day 7
Days 8-10
% of mice
100
75
50
25
0
NS1-1
NS1-8
Naive IgY
PBS
Moribund or dead
Ruffled, hunched and lethargic; limited mobility
Fur ruffling, hunched, mild signs of lethargy
Mild signs of lethargy; some fur ruffling, no hunching
Healthy

B.)

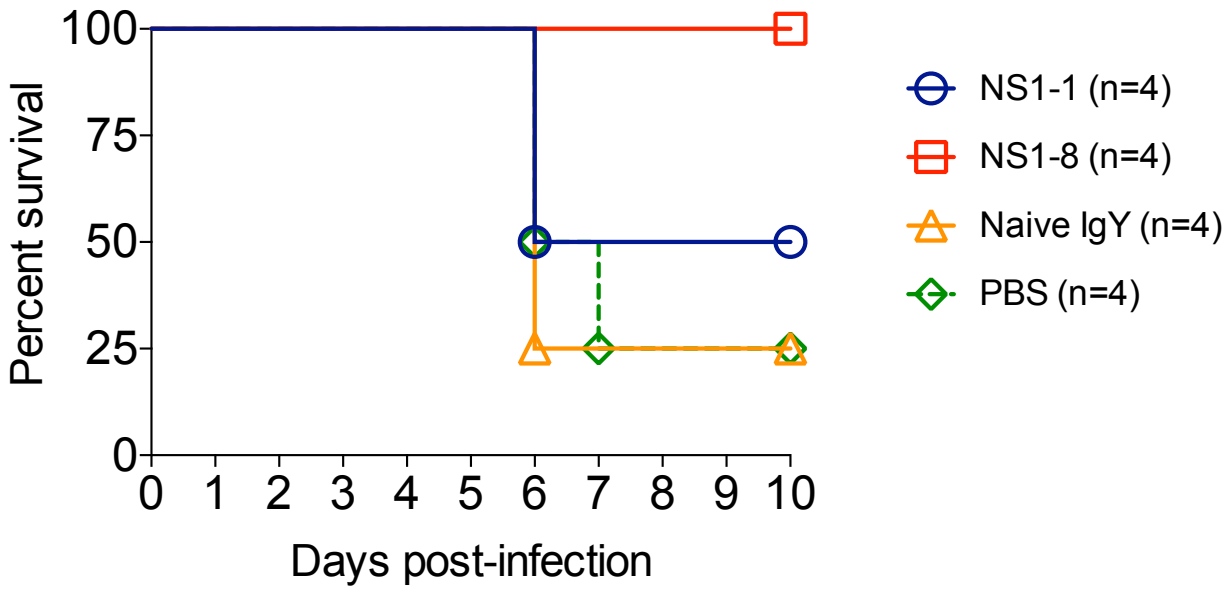

Percent survival
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10
Days post-infection
NS1-1 (n=4)
NS1-8 (n=4)
Naive IgY (n=4)
PBS (n=4)

Fig. 13: Passive transfer of anti-NS1 IgY antibodies decreases DENV-induced morbidity and mortality in *Ifnar*$^{-/-}$ mice. IgY antibodies (150 μg) were passively transferred into *Ifnar*$^{-/-}$ mice and, 24 hours later, challenged i.v. with lethal antibody-enhanced DENV2 D220 infection. **(A)** Signs of DENV-induced morbidity were recorded daily for 10 days. The proportion of each group of mice displaying the indicated signs is shown. **(B)** A Kaplan–Meier survival curve is shown. Animals treated with anti-NS1 IgY antibodies were significantly protected compared to Naïve IgY or PBS-treated controls.

Discussion

Several groups have created monoclonal antibodies to DENV NS1 protein. A majority of these antibodies target the wing domain, which enables the antibody to bind to the hexameric NS1 lipid complex structure preventing the pathology induced by soluble NS1 binding to endothelial cells (250-254). Due to the unique maturation steps of the colonial expansion of avian antibodies, we were able to generate two anti-NS1 IgY molecules that target novel epitopes on NS1. The anti-NS1 IgY preparations demonstrated *in vitro* neutralization capacity similar to that of the oliogoclonal anti-DENV IgY our lab has previously tested. To our knowledge this is the first anti-NS1 antibody able to inhibit infection *in vitro*. We went on to further characterize the binding affinity to multiple serotypes of DENV NS1 and saw that at high concentrations anti-NS1

IgY is able to bind multiple serotypes of DENV NS1, but at lower concentrations a preference for DENV2 was seen in both anti-NS1-1 and NS1-8 IgY. This can be attributed to the serotype of DENV that was originally used to make the oligoclonal anti-DENV IgY, which was used to isolate the epitope specific NS1 IgY was DENV2. We then tested the ability of anti-NS1 IgY to block soluble NS1 from binding endothelial cells, which is the typical neutralization mechanism of anti-NS1 antibodies. We determined that anti-NS1-1 and NS1-8 IgY were unable to block soluble NS1 from binding to endothelial cells or inhibit vascular permeability measured by relative TEER. This may be due to the fact that the epitopes that NS1-1 and NS1-8 target are on the inside of the hexamer lipid complex making them inaccessible in that form. A secondary explanation could be that the epitopes recognized by NS1-1 and NS1-8 are not required for NS1-endothelial cell interactions. We next assessed the ability of anti-NS1-1 and NS1-8 IgY to protect mice from lethal DENV infection. Intriguingly, NS1-8 conferred stronger protection against DENV-induced morbidity and mortality than NS1-1.

Due to the challenges of developing immunotherapeutics for flaviviral infections alternative therapies that do not induce ADE are critical in combating increasingly prevalent disease burden. Avian IgY and egg powder have already been utilized to treat veterinary and human infections and have been recognized as safe agent by the United States Code of Federal Regulation (228). To translate anti-NS1 IgY to a human therapy there are still challenges associated with IgY treatment that must be overcome. One challenge that has been previously shown is that porcine and mice develop a humoral

response to avian IgY, notably subclass IgG (230,231). Unpublished data from our lab demonstrates that weekly immunizations with IgY in a mouse model will induce an anti-IgY humoral response on the third or fourth weekly of vaccination. It is unknown if the hosts humoral immune system recognizes IgY, which is bound to the target antigen if the target antigen is still cleared by traditional antibody mechanisms. This would be especially important for flaviviral treatment to ensure that the high affinity IgY remains bound to the virus and the IgG molecule recognizing IgY would act as a trap antibody and utilize traditional clearance mechanisms. Being that IgY is isolated directly from the egg possible allergic reactions are a concern. It has not been demonstrated that purified IgY can induce allergic reaction in humans. What has been demonstrated is that purified IgY does not induce an IgE response in piglets (231). Future studies will need to be conducted to determine any risk of harmful allergic reaction due to purified IgY.

At the moment the only licensed vaccine for Dengue infection is Dengvaxia® Sanofi Pasteur (255). Due to apparent short comings in naïve patients which resulted in an increased risk of developing severe symptoms the vaccine was disbanded and the age range and serostatus criteria of patients receiving the vaccine were altered (256). The complications associated with Dengvaxia® has lead researchers to explore other targets for vaccine candidacy one of which is including NS1 in chimeric vaccine strategies. This will allow for a safer vaccine profile because of the decreased risk of ADE it will also directly target the pathogenic effects of the soluble NS1 protein that contributes to severe pathology. Two live attenuated vaccines from NIH and Takeda

included NS1 in their vaccine platforms (257). Human trials demonstrated that CD4+ and CD8+ T cells were induced by the vaccine NS1 protein, which solidifies the potency and immunogenicity of including it in additional vaccines in the future (258,259).

In summary, our studies comprehensively characterized the protective capacity of anti-DENV NS1 IgY and demonstrated that anti-DENV NS1 IgY does not induce ADE. Our purified anti-DENV NS1 IgY preparations were able to inhibit DENV infection *in vitro* and did not induce ADE at multiple concentrations. Anti-DENV NS1 IgY antibodies were able to bind NS1 proteins from multiple serotypes, although they did not prevent soluble NS1 from binding endothelial cells nor did they inhibit NS1-induced hyperpermeability of endothelial cells. However, anti-DENV NS1 IgY prophylactically administered to mice protected against lethal DENV challenge. Taken together, our data indicate that the avian IgY platform has a promising prophylactic applications against DENV infection, which warrants further investigation.

Chapter VI

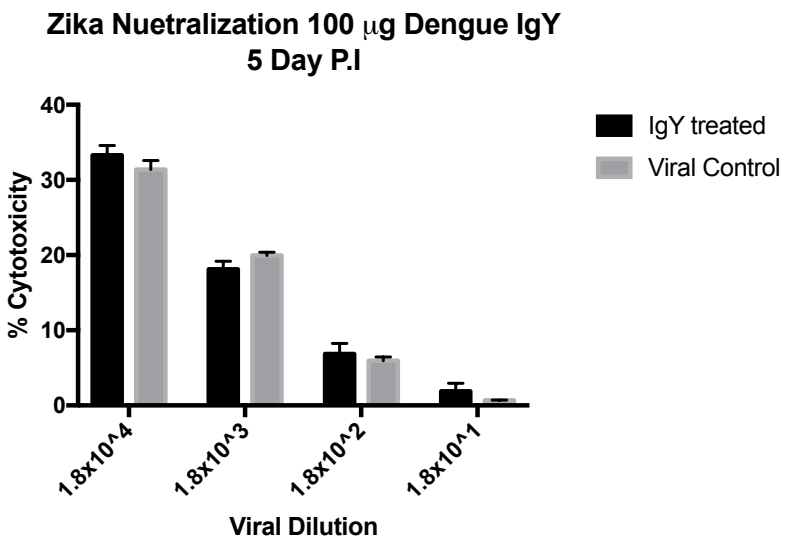
Discussion

The development of safe and effective anti-viral therapies is the corner stone and highest priority to combating emerging viral pathogens around the world. Vector competency studies should also be highly regard to know the extent in which the pathogen could spread and what areas should be considered for the implementation of vaccines or therapies. Our study on the vector competency of Aedes vexans was the first demonstration of a moderate climate mosquito able to be infected with and have the ability to transmit ZIKV. The relevance of this study impacted not only the addition of the mosquito species to the list of possible vectors, but it greatly expanded the geographical area in which this virus may potentially spread. Up until that study the known vectors were the traditional tropical mosquito vectors Aedes aegypti and albopictus both are traditional vectors of DENV as well. These two species of mosquito are confined to tropical regions and are slowly spreading to moderate climates because of changes in regional temperatures. The addition of Aedes vexans however to the competent vector list instantly makes the entire moderate climate a possible location for the spread of ZIKV. Aedes vexans is a flood water mosquito, and also one of the most common pest mosquitoes across most of the central United States and much of Europe. This is an excellent example of how complete vector competency profiles are necessary to determine the extent in which a pathogen can spread. The safety and efficacy of antibody therapy for flaviviral infections is of paramount concern. This can be extended

to not only the development of therapeutic monoclonal antibody therapies, but also the antibody responses elicited by a vaccine candidate. A recent cohort study in Nicaragua determined that after primary exposure to a flavivirus there is a specific antibody titer range in which the patient is more susceptible to ADE. This was demonstrated with not only different serotypes of DENV but also with ZIKV. A secondary example of this is in the recently recalled DENV vaccine Dengivax. This vaccine was developed by the Institute of Pasteur and showed strong efficacy against DENV serotypes 1, 3, and 4 and moderate protection against serotype 2. This was a high achievement due to traditional antigenic bias of previous vaccines that protected strongly against one serotype and moderate to weakly against the others. Once implemented into a high risk population however a critical characteristic had been over looked. In patients that were serologically naïve for DENV infection the vaccine actually increased their chance of developing sever dengue fever on secondary naturally acquired infection. It is hypothesized that the vaccine acted as the primary infection for these patients and because the circulating viruses were not genomically identical to the vaccine strain the primary antibody response held high titers of low affinity antibodies (256). Our lab's reports on the safety profile and advantages of anti-flavivirus IgY demonstrates that alternative immunotherapeutics are not only effective, but preferable to the traditional human or mouse derived antibodies because they eliminate problems such as ADE. To expand upon the studies detailed herein our lab plans to explore the cross neutralization capabilities of different IgY preparations between flaviviruses. Preliminary studies

demonstrating that there is little *in vitro* efficacy of the oligoclonal anti-ZIKV or anti-DENV2 IgY to cross reactive with the opposite virus (Fig. 14). This suggests a closer look at the epitope mapping is needed to determine optimal cross neutralizing antibodies to enrich future IgY preparations to enable the production of a “anti-flavivirus” IgY stock.

A.)



B.)

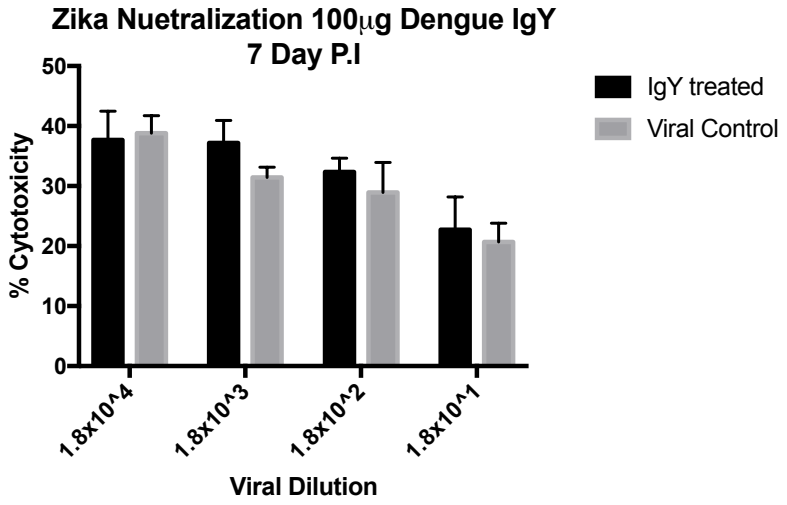


Fig. 14: Cytotoxicity of Vero cells treated with anti-DENV2 IgY infected with ZIKV. A.) Cytotoxicity five days' post infection of cells infected with ZIKV and ZIKV pretreated with anti-DENV2 IgY. **B.)** Cytotoxicity seven days' post infection of cells infected with ZIKV and ZIKV pretreated with anti-DENV2 IgY.

Another future direction of our lab is to isolate epitope specific IgY preparations from the polyclonal anti-ZIKV IgY stock to determine novel neutralization epitopes. Due to our prior success with anti-NS1 IgY with DENV, four stocks of anti-NS1 Zika IgY and a highly conserved NS5 epitope were isolated and PRNT was done to determine the extent of *in vitro* efficacy (Fig 15). Unlike the anti-NS1 for DENV the epitopes chosen did not exhibit any *in vitro* neutralization capabilities. What is yet to be determined is if there is efficacy of these epitopes *in vivo*, which is traditionally where you would see efficacy of an anti-NS1 antibody. To further expand upon the anti-ZIKV IgY project it is yet to be determined if IgY can cross the placental barrier to a developing fetus. This as well as functionality of the transferred IgY would need to be determined. Once that is done the protective efficacy of anti-ZIKV IgY to protect against trans-placental infection could be conducted.

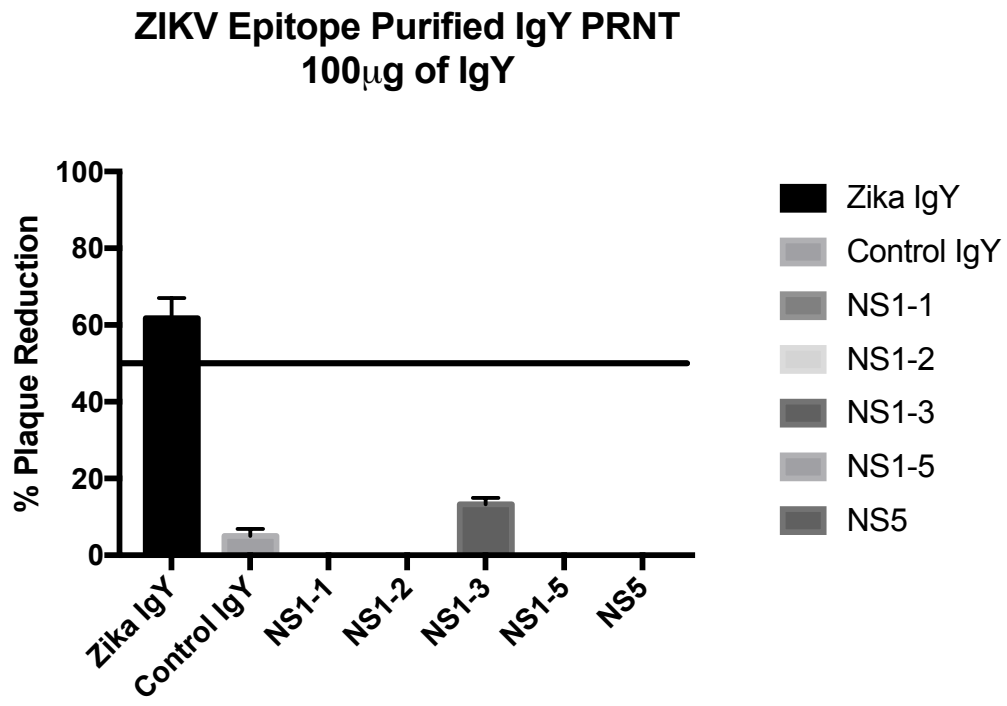


Fig. 15: Anti-NS1 Zika IgY and anti-NS5 Zika IgY does not neutralize ZIKV infection *in vitro* at 100 µg/ml.

To continue our collaboration with the Biology department our lab plans to assist in studies determining if microfilariae infection of the midgut of mosquitoes can increase the dissemination rate of flaviviral infections. Previous work by Dr. Vaughan demonstrated that alphaviruses were able to attach to the microfilariae while they exit the midgut essentially utilizing them as a conductor to evade the midgut barrier. It was previously thought that the virus escaped as a result of the hole in the lining of the midgut tissue caused by the microfilariae exiting. After multiple years of working on avian antibodies there are areas of research in which I think the lab should pursue. The first is the production of dual specific antibodies. This has already been demonstrated and discussed previously with rotavirus and norovirus (50). This technique could easily be expanded to the two flaviviruses currently being studied in our lab. It would be my recommendation to target known highly neutralizing epitopes on the envelope of the viruses and construct a fusion protein for immunizations. It is known that the three dimensional orientation of this epitopes can greatly impact the potency of the antibodies being produced. As a result, extensive protein analysis would have to been done in order to determine the conformation of the two epitopes once fused together. Not only could this greatly expand the industrial potential of flaviviral IgY, but it could also increase the potency of it's immunotherapeutic value. Upon the same lines IgY hybridoma production is necessary for IgY to take the next step into acceptance in modern medicine. The continual production of a specific antigen immunoglobulin would greatly cut down on quality control and quality assurance costs and variability. This is not to say that the polyclonal IgY stocks do not have their place as therapeutics, but in an age that is moving closer to personalized medicine specificity and targeting of therapies is becoming increasingly valued. A final thought for the future direction of the lab is to continue the virology aspect, but to start the process of manipulating viral vectors for the production of antigen specific immunization. This will allow for the production of site specific interactions while still allowing for the avian system to recognize novel epitopes within the target protein being expressed. This could decrease

the screening time necessary to the discovery of novel epitopes as well as increasing the antigenicity within the avian host itself resulting in the increased production of antigen specific IgY titers. Recently a paper described utilizing a viral vector for not only antigenic epitope display it also encoded the genomic sequence to express a highly potent monoclonal antibody (260). A technique similar to this could be employed to immunize subjects against DENV NS1 which has been shown to be protective in mice (250-254), but also to produce anti-DENV2 IgY specific for the envelope protein. This would allow for the targeting of the traditional structural epitopes that have been shown to have high potency, but can induce ADE and natural host immunity to a viral antigen with protective properties devoid of ADE thus targeting the invading virus structurally and the secondary proteins used to induce pathology.

References

1. Pokidysheva, E. *et al.* Cryo-EM Reconstruction of Dengue Virus in Complex with the Carbohydrate Recognition Domain of DC-SIGN. *Cell* **124**, 485–493 (2006).
2. Tassaneetrithep, B. *et al.* DC-SIGN (CD209) Mediates Dengue Virus Infection of Human Dendritic Cells. *The Journal of Experimental Medicine* **197**, 823–829 (2003).
3. Navarro-Sanchez, E. *et al.* Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. *EMBO Reports* **4**, 723–728 (2003).
4. Jo-Mei Chen *et al.* Bovine Lactoferrin Inhibits Dengue Virus Infectivity by Interacting with Heparan Sulfate, Low-Density Lipoprotein Receptor, and DC-SIGN. *International Journal of Molecular Sciences* **18**, 1957 (2017).
5. Kato, D. *et al.* Antiviral activity of chondroitin sulphate E targeting dengue virus envelope protein. *Antiviral Research* **88**, 236–243 (2010).
6. Miller, J. L. *et al.* The Mannose Receptor Mediates Dengue Virus Infection of Macrophages. *PLoS Pathogens* **4**, e17 (2008).
7. Caillet-Saguy, C., Lim, S. P., Shi, P.-Y., Lescar, J. & Bressanelli, S. Polymerases of hepatitis C viruses and flaviviruses: Structural and mechanistic insights and drug development. *Antiviral Research* **105**, 8–16 (2014).

8. Akey, D. L. *et al.* Flavivirus NS1 Structures Reveal Surfaces for Associations with Membranes and the Immune System. *Science* **343**, 881–885 (2014).
9. Zou J, Lee LT, Wang QY, Xie X, Lu S, Yau YH, Yuan Z, Geifman Shochat S, Kang C, Lescar J, Shi P-Y (2015) Mapping the interactions between the NS4B and NS3 proteins of dengue virus. J Virol 89: 3471– 3483
10. Umareddy I, Chao A, Sampath A, Gu F, Vasudevan SG (2006) Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. J Gen Virol 87(Pt 9): 2605– 2614
11. Xie X, Gayen S, Kang C, Yuan Z, Shi PY (2013) Membrane topology and function of dengue virus NS2A protein. J Virol 87(8): 4609– 4622
12. Falgout B, Chanock R, Lai CJ (1989) Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J Virol 63(5): 1852– 1860
13. Chambers TJ, Hahn CS, Galler R et al (1990) Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44: 649– 688
14. Yamashita, T. *et al.* Crystal structure of the catalytic domain of Japanese encephalitis virus NS3 helicase/nucleoside triphosphatase at a resolution of 1.8 Å. *Virology* **373**, 426–436 (2008).

15. Xu, T. *et al.* Structure of the Dengue Virus Helicase/Nucleoside Triphosphatase Catalytic Domain at a Resolution of 2.4 A. *Journal of Virology* **79**, 10278–10288 (2005).
16. Jiang L, Yao H, Duan X, Lu X, Liu Y (2009) Polypyrimidine tract-binding protein influences negative strand RNA synthesis of dengue virus. Biochem Biophys Res Commun 385(2): 187– 192.
17. Limon-Flores, A. Y. *et al.* Dengue virus inoculation to human skin explants: an effective approach to assess in situ the early infection and the effects on cutaneous dendritic cells. *International journal of experimental pathology* **86**, 323–334 (2005).
18. Boonnak, K. *et al.* Role of Dendritic Cells in Antibody-Dependent Enhancement of Dengue Virus Infection. *Journal of Virology* **82**, 3939–3951 (2008).
19. Ho, L.-J. *et al.* Infection of Human Dendritic Cells by Dengue Virus Causes Cell Maturation and Cytokine Production. *The Journal of Immunology* **166**, 1499–1506 (2001).
20. Ho, L.-J. *et al.* Infection of human Dendritic Cells by Dengue Virus activates and primes T cells towards Th0-like phenotype producing both Th1 and Th2 cytokines. *The Journal of Immunological Investigations* **33**(4), 423–437 (2004).
21. Bosch I *et al.* Heart and skeletal muscle are targets of dengue virus infection. *Pediatr Infect Dis J* 29(3): 238– 242. (2010)

22. Libraty, D. H., Pichyangkul, S., Ajariyakhajorn, C., Endy, T. P. & Ennis, F. A. Human Dendritic Cells Are Activated by Dengue Virus Infection: Enhancement by Gamma Interferon and Implications for Disease Pathogenesis. *Journal of Virology* **75**, 3501–3508 (2001).

23. Balsitis S. J *et al.* Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3-specific immunostaining*. Journal of Tropical Medicine and Hygiene* 80(3): 416-424. (2009).

24. Glasner, D. R., Puerta-Guardo, H., Beatty, P. R. & Harris, E. The Good, the Bad, and the Shocking: The Multiple Roles of Dengue Virus Nonstructural Protein 1 in Protection and Pathogenesis. *Annual review of virology* **5**, 227–253 (2018).

25. Churdboonchart, V., N. Bhamarapravati, and P. Futrakul. 1983. Crossed immunoelectrophoresis for the detection of split products of the third complement in dengue hemorrhagic fever. I. Observations in patients' plasma. Am. J. Trop. Med. Hyg. 32:569–576.

26. Shaio, M. F., F. Y. Chang, and S. C. Hou. 1992. Complement pathway activity in serum from patients with classical dengue fever. Trans. R. Soc. Trop. Med. Hyg. 86:672–675.

27. Avirutnan, P., P. Malasit, B. Seliger, S. Bhakdi, and M. Husmann. 1998. Dengue virus infection of human endothelial cells leads to chemokine

production, complement activation, and apoptosis. J. Immunol. 161:6338–6346.

28. An, J., D. S. Zhou, J. L. Zhang, H. Morida, J. L. Wang, and K. Yasui. 2004. Dengue-specific CD8_ T cells have both protective and pathogenic roles in dengue virus infection. Immunol. Lett. 95:167–174.

29. Klenerman, P., and R. M. Zinkernagel. 1998. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. Nature 394:482–485.

30. Mongkolsapaya, J., W. Dejnirattisai, X. N. Xu, S. Vasanawathana, N. Tangthawornchaikul, A. Chairunsri, S. Sawasdivorn, T. Duangchinda, T. Dong, S. Rowland-Jones, P. T. Yenchitsomanus, A. McMichael, P. Malasit, and G. Screaton. 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat. Med. 9:921–927.

31. Azeredo, E. L., S. M. Zagne, M. A. Santiago, A. S. Gouvea, A. A. Santana, P. C. Neves-Souza, R. M. Nogueira, M. P. Miagostovich, and C. F. Kubelka. 2001. Characterisation of lymphocyte response and cytokine patterns in patients with dengue fever. Immunobiology 204:494–507.

32. Bozza, F. A., O. G. Cruz, S. M. Zagne, E. L. Azeredo, R. M. Nogueira, E. F.

Assis, P. T. Bozza, and C. F. Kubelka. 2008. Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC. Infect. Dis. 8:86.

33. Chakravarti, A., and R. Kumaria. 2006. Circulating levels of tumour necrosis factor-alpha & interferon-gamma in patients with dengue & dengue haemorrhagic fever during an outbreak. Indian J. Med. Res. 123:25–30.

34. Juffrie, M., G. M. Meer, C. E. Hack, K. Haasnoot, Sutaryo, A. J. Veerman, and L. G. Thijs. 2001. Inflammatory mediators in dengue virus infection in children: interleukin-6 and its relation to C-reactive protein and secretory phospholipase A2. Am. J. Trop. Med. Hyg. 65:70–75.

35. Laur, F., B. Murgue, X. Deparis, C. Roche, O. Cassar, and E. Chungue. 1998. Plasma levels of tumour necrosis factor alpha and transforming growth factor beta-1 in children with dengue 2 virus infection in French Polynesia. Trans. R. Soc. Trop. Med. Hyg. 92:654–656.

36. Mustafa, A. S., E. A. Elbishbishi, R. Agarwal, and U. C. Chaturvedi. 2001. Elevated levels of interleukin-13 and IL-18 in patients with dengue hemorrhagic fever. FEMS Immunol. Med. Microbiol. 30:229–233.

37. Nguyen, T. H., H. Y. Lei, T. L. Nguyen, Y. S. Lin, K. J. Huang, B. L. Le, C. F.

Lin, T. M. Yeh, Q. H. Do, T. Q. Vu, L. C. Chen, J. H. Huang, T. M. Lam, C. C. Liu, and S. B. Halstead. 2004. Dengue hemorrhagic fever in infants: a study of clinical and cytokine profiles. J. Infect. Dis. 189:221–232.

38. Perez, A. B., G. Garcia, B. Sierra, M. Alvarez, S. Vazquez, M. V. Cabrera, R. Rodriguez, D. Rosario, E. Martinez, T. Denny, and M. G. Guzman. 2004. IL-10 levels in dengue patients: some findings from the exceptional epidemiological conditions in Cuba. J. Med. Virol. 73:230–234.

39. Raghupathy, R., U. C. Chaturvedi, H. Al-Sayer, E. A. Elbishbishi, R. Agarwal, R. Nagar, S. Kapoor, A. Misra, A. Mathur, H. Nusrat, F. Azizieh, M. A. Khan, and A. S. Mustafa. 1998. Elevated levels of IL-8 in dengue hemorrhagic fever. J. Med. Virol. 56:280–285.

40. Restrepo, B. N., D. M. Isaza, C. L. Salazar, R. Ramirez, M. Ospina, and L. G. Alvarez. 2008. Serum levels of interleukin-6, tumor necrosis factoralpha and interferon-gamma in infants with and without dengue. Rev. Soc. Bras. Med. Trop. 41:6–10.

41. Suharti, C., E. C. van Gorp, T. E. Setiati, W. M. Dolmans, R. J. Djokomoeljanto, C. E. Hack, C. H. ten, and J. W. van der Meer. 2002. The role of cytokines in activation of coagulation and fibrinolysis in dengue shock syndrome. Thromb. Haemost. 87:42–46.

42. Beatty PR, Puerta-Guardo H, Killingbeck SS, Glasner DR, Hopkins K, Harris E. Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. Sci Transl Med. 2015;7(304):304ra141. pmid:2635503

43. Selin, L. K., M. A. Brehm, Y. N. Naumov, M. Cornberg, S. K. Kim, S. C. Clute, and R. M. Welsh. 2006. Memory of mice and men: CD8_ T-cell cross-reactivity and heterologous immunity. Immunol. Rev. 211:164–181.

44. Selin, L. K., S. M. Varga, I. C. Wong, and R. M. Welsh. 1998. Protective heterologous antiviral immunity and enhanced immunopathogenesis mediated by memory T cell populations. J. Exp. Med. 188:1705–1715.

45. Culshaw, A., Mongkolsapaya, J. & Screaton, G. R. The immunopathology of dengue and Zika virus infections. Curr. Opin. Immunol. 48, 1–6 (2017).

46. Hirsch, A. J. et al. Zika virus infection of rhesus macaques leads to viral persistence in multiple tissues. PLoS Pathog. 13, e1006219 (2017).

47. Govero, J. et al. Zika virus infection damages the testes in mice. Nature 540, 438–442 (2016).

48. Ma, W. et al. Zika virus causes testis damage and leads to male infertility in mice. Cell 167, 1511–1524 (2016).

49. Joguet, G. et al. Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. Lancet Infect. Dis.

17, 1200–1208 (2017).

50. Meertens, L. *et al.* Axl Mediates ZIKA Virus Entry in Human Glial Cells and Modulates Innate Immune Responses. *Cell Reports* **18**, 324–333 (2017).

51. Hamel, R. *et al.* Biology of Zika Virus Infection in Human Skin Cells. *Journal of Virology* **89**, 8880–8896 (2015).

52. Retallack, H. *et al.* Zika virus cell tropism in the developing human brain and inhibition by azithromycin. *Proceedings of the National Academy of Sciences* **113**, 14408–14413 (2016).

53. Savidis, G. *et al.* The IFITMs Inhibit Zika Virus Replication. *Cell Reports* **15**, 2323–2330 (2016).

54. Nowakowski, T. J. *et al.* Expression Analysis Highlights AXL as a Candidate Zika Virus Entry Receptor in Neural Stem Cells. *Cell Stem Cell* **18**, 591–596 (2016).

55. Wells, M. F. *et al.* Genetic Ablation of AXL Does Not Protect Human Neural Progenitor Cells and Cerebral Organoids from Zika Virus Infection. *Cell Stem Cell* **19**, 703–708 (2016).

56. Platt, D. J. et al. Zika virus-related neurotropic flaviviruses infect human placental explants and cause fetal demise in mice. Sci. Transl. Med. 10, eaao7090 (2018).

57. Delaney, A. et al. Population-Based surveillance of birth defects potentially related to Zika virus infection — 15 States and U.S. Territories, 2016. MMWR Morb. Mortal. Wkly Rep. 67, 91–96 (2018).

58. Tang, H. et al. Zika virus infects human cortical neural progenitors and attenuates their growth. Cell Stem Cell 18, 587–590 (2016).

59. Taylor, A. *et al.* Fc receptors in antibody-dependent enhancement of viral infections. *Immunological reviews* **268**, 340–364 (2015).

60. Mero, P. *et al.* Phosphorylation-independent Ubiquitylation and Endocytosis of FcγRIIA. *Journal of Biological Chemistry* **281**, 33242–33249 (2006).

61. Gollins SW, Porterfield JS. Flavivirus infection enhancement in macrophages: an elctron microscopic study of viral cellular entry. *J Gen Virol* (1985) 66(Pt9): 1969-1982. Doi:10.1099/0022-1317-66-9-1969

62. Ubol, S., Phuklia, W., Kalayanarooj, S. & Modhiran, N. Mechanisms of Immune Evasion Induced by a Complex of Dengue Virus and Preexisting Enhancing Antibodies. *The Journal of Infectious Diseases* **201**, 923–935 (2010).

63. Lin, J.-C. *et al.* Dengue Viral Protease Interaction with NF- B Inhibitor / Results in Endothelial Cell Apoptosis and Hemorrhage Development. *The Journal of Immunology* **193**, 1258–1267 (2014).

64. Huang, X. *et al.* Antibody-dependent enhancement of dengue virus infection inhibits RLR-mediated Type-I IFN-independent signalling through upregulation of cellular autophagy. *Scientific Reports* **6**, (2016).

65. Nguyen TH, Lei HY, Nguyen TL, Lin YS, Huang KJ, Le BL, et al. Dengue hemorrhagic fever in infants: a study of clinical and cytokine profiles. *J infect Dis* (2004) 189:221-32.

66. Chareonsirisuthigul, T., Kalayanarooj, S. & Ubol, S. Dengue virus (DENV) antibody-dependent enhancement of infection upregulates the production of anti-inflammatory cytokines, but suppresses anti-DENV free radical and pro-inflammatory cytokine production, in THP-1 cells. *Journal of General Virology* **88**, 365–375 (2007).

67. Lubick, K. J. *et al.* Flavivirus Antagonism of Type I Interferon Signaling Reveals Prolidase as a Regulator of IFNAR1 Surface Expression. *Cell Host & Microbe* **18**, 61–74 (2015).

68. Morrison, J. *et al.* Dengue Virus Co-opts UBR4 to Degrade STAT2 and Antagonize Type I Interferon Signaling. *PLoS Pathogens* **9**, e1003265 (2013).

69. Grant, A. *et al.* Zika Virus Targets Human STAT2 to Inhibit Type I Interferon Signaling. *Cell Host & Microbe* **19**, 882–890 (2016).

70. Pérez, A. B. *et al.* IL-10 levels in Dengue patients: Some findings from the exceptional epidemiological conditions in Cuba: Increased IL-10 During Secondary Dengue Infection. *Journal of Medical Virology* **73**, 230–234 (2004).

71. Yeh, W.-T. *et al.* Implications of previous subclinical dengue infection but not virus load in dengue hemorrhagic fever. *FEMS Immunology & Medical Microbiology* **48**, 84–90 (2006).

72. Tang, Y. *et al.* Both Viremia and Cytokine Levels Associate with the Lack of Severe Disease in Secondary Dengue 1 Infection among Adult Chinese Patients. *PLoS ONE* **5**, e15631 (2010).

73. Michael S. Rolph, Ali Zaid, Nestor E. Rulli, Suresh Mahalingam; Downregulation of

Interferon-β in Antibody-Dependent Enhancement of Dengue Viral Infections of Human Macrophages Is Dependent on Interleukin-6, *The Journal of Infectious Diseases*, Volume 204, Issue 3, 1 August 2011, Pages 489–491, https://doi.org/10.1093/infdis/jir271

74. Lee, Y.-R. *et al.* Autophagic machinery activated by dengue virus enhances virus replication. *Virology* **374**, 240–248 (2008).
75. Cao, Bin et al. "Inhibition of autophagy limits vertical transmission of Zika virus in pregnant mice" *Journal of experimental medicine* vol. 214,8 (2017): 2303-2313.
76. McLean, Jeffrey E et al. "Flavivirus NS4A-induced autophagy protects cells against death and enhances virus replication" *Journal of biological chemistry*vol. 286,25 (2011): 22147-59.
77. Montali, R.J et al. "Comparative pathology of inflammation in the higher vertebrates (reptiles, birds, and mammals). *Journal Computational Pathology.* 99, 1-26 (1988).
78. Harmon, B. G. "Avian heterophils in inflammation and disease resistance." *Poultry Science.* 77, 972-977 (1998).
79. Ozinsky, A. *et al.* The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proceedings of the National Academy of Sciences* **97**, 13766–13771 (2000).
80. Takeuchi, O. *et al.* Cutting Edge: Role of Toll-Like Receptor 1 in Mediating Immune Response to Microbial Lipoproteins. *The Journal of Immunology* **169**, 10–14 (2002).
81. Iqbal, M., Philbin, V. J. & Smith, A. L. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. *Veterinary Immunology and*

Immunopathology **104**, 117–127 (2005).

82. Almedia, I. C. et al. "Highly purified glycosylphosphatidylinositols from Trypanosoma cruzi are potent proinflammatory agents. Embo J. 19, 1476-1485 (2000).
83. Massari, P. *et al.* Cutting Edge: Immune Stimulation by Neisserial Porins Is Toll-Like Receptor 2 and MyD88 Dependent. *The Journal of Immunology* **168**, 1533–1537 (2002).
84. Opitz, B. *et al.* Toll-like Receptor-2 Mediates *Treponema* Glycolipid and Lipoteichoic Acid-induced NF-κB Translocation. *Journal of Biological Chemistry* **276**, 22041–22047 (2001).
85. Leveque, G. *et al.* Allelic Variation in TLR4 Is Linked to Susceptibility to Salmonella enterica Serovar Typhimurium Infection in Chickens. *Infection and Immunity* **71**, 1116–1124 (2003).
86. Kogut, M. *et al.* Expression and function of Toll-like receptors in chicken heterophils. *Developmental & Comparative Immunology* **29**, 791–807 (2005).
87. Zarember, K. A. & Godowski, P. J. Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products, and Cytokines. *The Journal of Immunology* **168**, 554–561 (2002).
88. Heil, F. *et al.* The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *European journal of immunology* **33**, 2987–2997 (2003).
89. Heil, F. *et al.* Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8. *Science* **303**, 1526 (2004).

90. Hornung, V. *et al.* Quantitative Expression of Toll-Like Receptor 1-10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides. *The Journal of Immunology* **168**, 4531–4537 (2002).

91. Ameiss, K.A. *et al.* Influence of orally administered CpG-ODNs on the humoral response to bovine serum albumin (BSA) in chickens. *The Journal of Veterinary Immunology and Immunopathology.* 110, 257-267 (2006).

92. Dalloul, R.A *et al.* In vivo effects of CpG oligodeoxynucleotide on Eimeria infection in chickens. *The Journal of Avian Diseases.* 48, 783-790. (2004).

93. He H. *et al.* Identification of CpG oligodeoxynucleotide motifs that stimulate nitric oxide and cytokine production in avian macrophage and peripheral blood mononuclear cells. *The Journal of Developmental and Comparative Immunology.* 27, 621-627 (2003).

94. Roach J.C. *et al.* The evolution of vertebrate Toll-like receptors. *PNAS.* 102, 9577-9582 (2005).

95. Higgs, R. *et al.* Induction of a novel chicken Toll-like receptor following Salmonella enterica serovar Typhimurium infection. *The Journal of Infection and Immunity.* 74, 1692-1698 (2006).

96. Keestra, A.M. *et al.* Chicken TLR 21is an innate CpG DNA receptor distinct from mammalian TLR9. *The Journal of Immunology.* 185, 460-467 (2006).

97. Brownlie, R. *et al.* Chicken TLR21 acts as a functional homolog to mammalian TLR9 in the recognition of CpG DNA. *The Journal of Molecular Immunology.* 46, 3167-3170 (2009).

98. Glick B. The Immune Response in the Chicken: Lymphoid Development of the Bursa of

Fabricius and Thymus and an Immune Response Role for the Gland of Harder, *Poultry Science*, Volume 57, Issue 5, 1 September 1978, Pages 1441–1444, https://doi-org.ezproxylr.med.und.edu/10.3382/ps.0571441

99. Parra, Z. E., Mitchell, K., Dalloul, R. A. & Miller, R. D. A Second TCR Locus in Galliformes Uses Antibody-like V Domains: Insight into the Evolution of TCR and TCR Genes in Tetrapods. *The Journal of Immunology* **188**, 3912–3919 (2012).

100. Gobel, T. W. F. & Dangy, J.-P. Evidence for a Stepwise Evolution of the CD3 Family. *The Journal of Immunology* **164**, 879–883 (2000).

101. Gouaillard, C. *et al.* Evolution of T cell receptor alpha beta heterodimer assembly with the CD3 complex. *European Journal of Immunology*. 31, 3798-3805 (2001).

102. HÁLA, K. *et al.* Chicken major histocompatibility complex congenic lines differ in the percentages of lymphocytes bearing CD4 and CD8 antigens. Animal Genetics, 22: 279-284. (2001).

103. Tjoelker, L. W. *et al.* Evolutionary conservation of antigen recognition: the chicken T-cell receptor beta chain. *Proceedings of the National Academy of Sciences* **87**, 7856–7860 (1990).

104. Koskinen, R. *et al.* Cloning and modeling of the first nonmammalian CD4. *The Journal of Immunology*. 162, 4115-4121 (1999).

105. O'Regan, M.N. *et al.* A chicken homologue of the co-stimulating molecule CD80 which binds to mammalian CTLA-4. *The Jounrnal of Immunogenetics*. 49, 68-71 (1999).

106. Flajnik, M. F., Tlapakova, T., Criscitiello, M. F., Krylov, V. & Ohta, Y. Evolution of the B7

family: co-evolution of B7H6 and NKp30, identification of a new B7 family member, B7H7, and of B7's historical relationship with the MHC. *Immunogenetics* **64**, 571–590 (2012).

107. Mills, D. M. & Cambier, J. C. B lymphocyte activation during cognate interactions with CD4+ T lymphocytes: molecular dynamics and immunologic consequences. *Seminars in Immunology* **15**, 325–329 (2003).
108. Vainio, O. *et al.* B-L antigens of the chicken major histocompatibility complex control T-B cell interactions. *The Journal of Immunnogenetics.* 19, 131-140 (1984).
109. MacLennan, I.C. Germinal centres. *Annual Review of Immunology.* 12, 117-139 (1994).
110. Burns RB, Maxwell MH. Probable occurrence of IgE in the adult domestic fowl (Gallus domesticus) after horse serum stimulation. Vet Res Commun 1981; 5(1):67-72.
111. Chen CL, Lehmeyer JE, Cooper MD. Evidence for an IgD homologue on chicken lymphocytes. J Immunol 1982; 129(6):2580-2585.
112. Shimizu M, Nagashima H, Sano K, Hashimoto K, Ozeki M, Tsuda K et al. Molecular stability of chicken and rabbit immunoglobulin G. Biosci Biotechnol Biochem 1992; 56(2):270-274.
113. Warr GW, Magor KE, Higgins DA. IgY: clues to the origins of modern antibodies. Immunol Today 1995; 16(8):392-398.
114. Reynaud CA, Anquez V, Dahan A, Weill JC. A single rearrangement event generates most of the chicken immunoglobulin light chain diversity. Cell

1985; 40(2):283-291.

115. Weill JC, Reynaud CA. The chicken B cell compartment. Science 1987; 238(4830):1094-1098.

116. Reynaud CA, Anquez V, Grimal H, Weill JC. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. Cell 1987; 48(3):379-388.

117. McCormack WT, Tjoelker LW, Barth CF, Carlson LM, Petryniak B, Humphries EH et al. Selection for B cells with productive IgL gene rearrangements occurs in the bursa of Fabricius during chicken embryonic development. Genes Dev 1989; 3(6):838-847.

118. Parvari R, Ziv E, Lantner F, Heller D, Schechter I. Somatic diversification of chicken immunoglobulin light chains by point mutations. Proc Natl Acad Sci U S A 1990; 87(8):3072-3076.

119. Masteller EL, Lee KP, Carlson LM, Thompson CB. Expression of sialyl Lewis(x) and Lewis(x) defines distinct stages of chicken B cell maturation. J Immunol 1995; 155(12):5550-5556.

120. McCormack WT, Tjoelker LW, Thompson CB. Immunoglobulin gene diversification by gene conversion. Prog Nucleic Acid Res Mol Biol 1993; 45:27-45.

121. Kubo RT, Zimmerman B, Grey HM. The antigens. Academic press, 19730.

122. Walsh WE, Jr., Anderson BE, Ivancic D, Zhang Z, Piccini JP, Rodgers TG et al. Distribution of, and immune response to, chicken anti-alpha Gal immunoglobulin Y antibodies in wild-type and alpha Gal knockout mice. Immunology 2000; 101(4):467-473.

123. Shimizu M, Fitzsimmons RC, Nakai S. Anti-E.coli immunoglobulin Y isolated from egg yolk of immunized chickens as a potential food ingredient. Journal of food science 1998; 53(5):1360-1366.

124. Loeken MR, Roth TF. Analysis of maternal IgG subpopulations which are transported into the chicken oocyte. Immunology 1983; 49(1):21-28.

125. Hadge D, Ambrosius H. Evolution of low molecular weight immunoglobulins--IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA. Mol Immunol 1984; 21(8):699-707.

126. Horton J, Holden C, Ward P, MacDonald D, Sanderson A. Exploitation of phylogenetic distance in cell surface immune labeling: studies with Beta2-microglobulin. The journal of investigative dermatology 1984;(85):96-99.

127. Olovsson M, Larsson A. Biotin labelling of chicken antibodies and their subsequent use in ELISA and immunohistochemistry. Comp Immunol Microbiol Infect Dis 1993; 16(2):145-152.

128. Di Lonardo, A.D. *et al.* Egg yolk antibodies against the E7 oncogenic protein of human papillomavirus type 16. Archives of Virology. 146(1): 117-125 (2001).

129. Losch, U. *et al.* The chicken egg, an antibody source. Zentralbl. Veterinarmed. B 33, 609-619 (1986).

130. Schade, R. *et al.* Avian egg yolk antibodies. The egg laying capacity of hens following immunisaion with antigens of different kind and origin and the efficiency of egg yolk antibodies in comparison to mammalian antibodies. ALTEX, 11 75-84 (1994).

131. Larsson A, Wejaker PE, Forsberg PO, Lindahl T. Chicken antibodies: a tool to avoid interference by complement activation in ELISA. J Immunol Methods 1992; 156(1):79-83.

132. Larsson A, Karlsson-Parra A, Sjoquist J. Use of chicken antibodies in enzyme immunoassays to avoid interference by rheumatoid factors. Clin Chem 1991; 37(3):411-414.

133. Larsson A, Mellstedt H. Chicken antibodies: a tool to avoid interference by human anti-mouse antibodies in ELISA after in vivo treatment with murine monoclonal antibodies. Hybridoma 1992; 11(1):33-39.

134. Guss B, Eliasson M, Olsson A, Uhlen M, Frej AK, Jornvall H et al. Structure of the IgG-binding regions of streptococcal protein G. EMBO J 1986; 5(7):1567-1575.

135. Fischer M, Hlinak A. The lack of binding ability of staphylococcal protein A and streptococcal protein G to egg yolk immunoglobulins of different fowl species (short communication). Berl Munch Tierarztl Wochenschr

2000; 113(3):94-96.

136. Hoffman WL, Ruggles AO, Tabarya D. Chicken anti-protein A prevents Staphylococcus aureus protein A from binding to human and rabbit IgG in immunoassays and eliminates most false positive results. J Immunol Methods 1996; 198(1):67-77.

137. Lindahl TL, Festin R, Larsson A. Studies of fibrinogen binding to platelets by flow cytometry: an improved method for studies of platelet activation. Thromb Haemost 1992; 68(2):221-225.

138. Kammila, S.; Das, D.; Bhatnagar, P. K.; Sunwoo, H. H.; Zayas-Zamora, G.; King, M.; Suresh, M. R. A Rapid Point of Care Immunoswab Assay for SARS-CoV Detection. J. Virol. Methods 2008, 152(1–2), 77–84. DOI: 10.1016/j.jviromet.2008.05.023.

139. Lee, Y. C. *et l*. A Dominant Antigenic Epitope on SARS-CoV Spike Protein Identified by an Avian Single-Chain Variable Fragment (Scfv)-Expressing Phage Vet. Immunol. Immunopathol. 2007, 117(1–2), 75–85. DOI: 10.1016/j.vetimm.2007.02.001.

140. Bentes, G. A.; Lanzarini, N. M.; Lima, L. R.; Manso, P. P.; da Silva, A. O. S.; Mouta Junior, S. A. S.; Guimarães, J. R.; de Moraes, M. T.; Pelajo-Machado, M.; Pinto, M. A. Using Immunoglobulin Y as an Alternative Antibody for the Detection of Hepatitis A Virus in Frozen Liver Sections. Mem. Inst. Oswaldo Cruz 2015, 110(4), 577–579. DOI: 10.1590/0074-02760140457.

141. Shimizu, Y. K.; Shikata, T.; Beninger, P. R.; Sata, M.; Setoyama, H.; Abe, H.; Tanikawa,

Detection of Hepatitis A Antigen in Human Liver. Infect. Immun. 1982, 36, 1, 320–324.

142. Ferreira, C. T.; Vieira, S. M.; Kieling, C. O.; Silveira, T. R. Hepatitis A Acute Liver Failure: Follow-Up of Paediatric Patients in Southern Brazil. J. Viral Hepat. 2008, 15 (Suppl 2), 66–68. DOI: 10.1111/j.1365-2893.2008.01033.x.

143. Silva, A. O. S.; Vasconcelos, G. A.; Kappel, L. A.; Pinto, M. A.; Paula, V. S. An Immunoenzymatic Assay for the Diagnosis of Hepatitis A Utilising Immunoglobulin Y. Mem. Inst. Oswaldo Cruz 2012, 107, 7, 960–963.

144. Wilson, B.; Nakane, P. K. Recent Developments in the Periodate Method of Conjugating Horseradish Peroxidase (HRPO) to Antibodies; Elsevier/North-Holland: Vienna, Austria, 1978

145. Nafea, N. M.; Sabbah, M. A.; Al-Suhail, R.; Mahdavi, A. H.; Asgary, S. Development of Hen Antihepatitis B Antigen IgY-based Conjugate for ELISA Assay. Adv. Biomed. Res. 2015, 4, 100. DOI: 10.4103/2277-9175.156678.

146. He, J.; Wang, Y.; Sun, S.; Zhang, X. Evaluation of Chicken IgY Generated against Canine Parvovirus Viral-Like Particles and Development of Enzyme-Linked Immunosorbent Assay and Immunochromatographic Assay for Canine Parvovirus Detection. Viral Immunol. 2015, 28(9), 489–494. DOI: 10.1089/vim.2015.0030.

147. Zhang, L.; Li, D.; Liu, L.; Fang, J.; Xu, R.; Zhang, G. Development of a Colloidal Gold Immunochromatographic Strip for the Rapid Detection of Soft-Shelled Turtle Systemic Septicemia Spherical Virus. J. Virol. Methods 2015, 221, 39–45. DOI: 10.1016/j. jviromet.2015.04.016.

148. Zhang, L.; Li, D.; Liu, L.; Zhang, G. Rapid Immunochromatographic Test Strip to Detect Swimming Crab Portunus Trituberculatus Reovirus. Dis. Aquat. Organ. 2015, 117(1), 21–29. DOI: 10.3354/dao02921.

149. Dai, Y. C.; Wang, Y. Y.; Zhang, X. F.; Tan, M.; Xia, M.; Wu, X. B.; Jiang, X.; Nie, J. Evaluation of Anti-Norovirus IgY from Egg Yolk of Chickens Immunized with Norovirus P Particles. J. Virol. Methods 2012, 186(1–2), 126–131. DOI: 10.1016/j. jviromet.2012.07.002.

150. Dai, Y. C.; Zhang, X. F.; Tan, M.; Huang, P.; Lei, W.; Fang, H.; Zhong, W.; Jiang, X. A Dual Chicken IgY against Rotavirus and Norovirus. Antiviral Res. 2013, 97(3), 293–300. DOI: 10.1016/j.antiviral.2012.12.011.

151. Vega, C.; Bok, M.; Saif, L.; Fernandez, F.; Parreño, V. Egg Yolk IgY Antibodies: A Therapeutic Intervention against Group A Rotavirus in Calves. Res. Vet. Sci. 2015, 103, 1–10. DOI: 10.1016/j.rvsc.2015.09.005.

152. Vega, C.; Bok, M.; Chacana, P.; Saif, L.; Fernandez, F.; Parreño, V. Egg Yolk IgY: Protection against Rotavirus Induced Diarrhea and Modulatory Effect on the Systemic and Mucosal Antibody Responses in Newborn Calves. Vet. Immunol. Immunopathol. 2011, 142(3–4), 156–169. DOI: 10.1016/j.vetimm.2011.05.003.

153. Sarker, S. A.; Casswall, T. H.; Juneja, L. R.; Hoq, E.; Hossain, I.; Fuchs, G. J.; Hammarström, L. Randomized, Placebo-Controlled, Clinical Trial of Hyperimmunized Chicken Egg Yolk Immunoglobulin in Children with Rotavirus

Diarrhea. J. Pediatr. Gastroenterol. Nutr. 2001, 32, 1, 19–25.

154. Rahman, S. *et al.* Randomized Placebo-Controlled Clinical Trial of Immunoglobulin Y as Adjunct to Standard Supportive Therapy for Rotavirus-Associated Diarrhea among Pediatric Patients. Vaccine 2012, 30(31), 4661–4669. DOI: 10.1016/j.vaccine.2012.04.091.

155. Haese N, Brocato RL, Henderson T, Nilles ML, Kwilas SA, Josleyn MD, et al. Antiviral Biologic Produced in DNA Vaccine/Goose Platform Protects Hamsters Against Hantavirus Pulmonary Syndrome When Administered Post-exposure. PLoS Negl Trop Dis. 2015; 9(6): e0003803. https://doi.org/10.1371/journal.pntd.0003803 PMID: 26046641;

156. Fink AL, Williams KL, Harris E, Alvine TD, Henderson T, Schiltz J, et al. Dengue virus specific IgY provides protection following lethal dengue virus challenge and is neutralizing in the absence of inducing antibody dependent enhancement. PLoS neglected tropical diseases. 2017;11(7): e0005721.

157. Musso D, Nilles EJ, Cao-Lormeau VM. Rapid spread of emerging Zika virus in the Pacific area. Clin Microbiol Infect. 2014; 20(10):O595–6. doi: 10.1111/1469-0691.12707 PMID: 24909208

158. Moreira, J., T. M. Peixoto, A. Machado de Siqueira, and C. C. Lamas. 2017. Sexually acquired Zika virus: A systematic review. Clin. Microbiol. Infect. 23. doi: 10.1016/j.cmi.2016.12.027

159. Krauer, F., M. Riesen, L. Reveiz, O. T. Oladapo, R. Mart_ınez-Vega, T. V. Porgo, A.

Haefliger, N. J. Broutet, and N. Low, WHO Zika Causality Working Group 2017. Zika Virus infection as a cause of congenital brain abnormalities and Guillain-Barre´ Syndrome: Systematic Review. PLoS Med. 14: e1002203. doi: 10.1371/journal.pmed.1002203.

160. Dick, G.W.A., S. F. Kitchen, and A. J. Haddow. 1952. Zika virus (I). Isolations and serological specificity. Trans. R. Soc. Trop. Med. Hyg. 46: 509–520.

161. Diagne, C. T., D. Diallo, O. Faye, Y. Ba, O. Faye, A. Gaye, I. Dia, O. Faye, S.C. Weaver, A. A. Sall, et al. 2015. Potential of selected Senegalese Aedes spp. mosquitoes (Diptera: Culicidae) to transmit Zika virus. BMC Infect Dis. 15: 492. doi: 10.1186/s12879-015-1231-2.

162. Li, M. I., P. S. Wong, L. C. Ng, and C. H. Tan. 2012. Oral susceptibility of Singapore Aedes (Stegomyia) aegypti (Linnaeus) to Zika virus. PLoS Negl. Trop. Dis. 6: e1792. doi: 10.1371/journal.pntd.0001792.

163. Wong, P. S., M. Z. Li, C. S. Chong, L. C. Ng, and C. H. Tan. 2013. Aedes (Stegomyia) albopictus (Skuse): A potential vector of Zika virus in Singapore. PLoS Negl. Trop. Dis. 7: e2348. doi: 10.1371/journal.pntd.0002348.

164. Ledermann, J. P., L. Guillaumot, L. Yug, S. C. Saweyog, M. Tided, P. Machieng, M. Pretrick, M. Marfel, A. Griggs, M. Bel, et al. 2014. Aedes hensilli as a potential vector of Chikungunya and Zika viruses. PLoS Negl. Trop. Dis. 8: e3188. doi:10.1371/journal.pntd.0003188.

165. Guo, X. X., C. X. Li, Y. Q. Deng, D. Xing, Q. M. Liu, Q. Wu, A. J. Sun, Y. D. Dong, W. C. Cao, C. F. Qin, and T. Y. Zhao. 2016. Culex pipiens quinquefasciatus: A potential

vector to transmit Zika virus. Emerg. Microbes Infect. 5: e102. doi: 10.1038/emi.2016.102.

166. Aliota, M. T., S. A. Peinado, J. E. Osorio, and L. C. Bartholomay. 2016b. Culex pipiens and Aedes triseriatus mosquito susceptibility to Zika virus. Emerg. Infect. Dis. 22: 1857–1859.

167. Huang, Y. J., V. B. Ayers, A. C. Lyons, I. Unlu, B. W. Alto, L. W. Cohnstaedt, S. Higgs, and D. L. Vanlandingham. 2016. Culex species mosquitoes and Zika virus. Vector Borne Zoon. Dis. 16: 673–676.

168. Fernandes, R. S., S. S. Campos, A. Ferreira-de-Brito, R. M. Miranda, K. A. Barbosa da Silva, M. G. Castro, L. M. Raphael, P. Brasil, A. B. Failloux, M. C. Bonaldo, et al. 2016. Culex quinquefasciatus from Rio de Janeiro is not competent to transmit the local Zika virus. PLoS Negl. Trop. Dis. 10:e0004993. doi: 10.1371/journal.pntd.0004993.

169. Boccolini, D., L. Toma, M. Di Luca, F. Severini, R. Romi, M. E. Remoli, M. Sabbatucci, G. Venturi, G. Rezza, and C. Fortuna. 2016. Experimental investigation of the susceptibility of Italian Culex pipiens mosquitoes to Zika virus infection. Euro. Surveill. 21: doi: 10.2807/1560-7917.ES.2016.21.35.30328.

170. Hart, C. E., C. M. Roundy, S. R. Azar, J. H. Huang, R. Yun, E. Reynolds, G. Leal, M. R. Nava, J. Vela, P. M. Stark, et al. 2017. Zika Virus vector competency of mosquitoes, Gulf Coast, United States. Emerg. Infect. Dis. 23: doi:10.3201/eid2303.161636.

171. Ramirez, J. L., J. Souza-Neto, R. Torres Cosme, J. Rovira, A. Ortiz, J. M. Pascale, and G. Dimopoulos. 2012. Reciprocal tripartite interactions between the Aedes aegypti midgut microbiota, innate immune system and dengue virus influences vector competence. PLoS

Negl. Trop. Dis. 6: e1561. doi: 10.1371/journal.pntd.0001561.

172. Anderson, S. L., S. L. Richards, and C. T. Smartt. 2010. A simple method for determining arbovirus transmission in mosquitoes. J. Am. Mosq. Control Assoc. 26: 108–111.

173. Boorman, J. 1987. Induction of salivation in biting midges and mosquitoes, and demonstration of virus in the saliva of infected insects. Med. Vet. Entomol. 1: 211–214.

174. Weger-Lucarelli, J., C. Ru¨ ckert, N. Chotiwan, C. Nguyen, S. M. Garcia Luna, J. R. Fauver, B. D. Foy, R. Perera, W. C. Black, R. C. Kading, et al. 2016. Vector competence of American mosquitoes for three strains of Zika virus. PLoS Negl. Trop. Dis. 10: e0005101. doi: 10.1371/ journal.pntd.0005101.

175. Aliota, M. T., S. A. Peinado, J. E. Osorio, and L. C. Bartholomay. 2016b. Culex pipiens and Aedes triseriatus mosquito susceptibility to Zika virus. Emerg. Infect. Dis. 22: 1857–1859.

176. Chouin-Carneiro, T., A. Vega-Rua, M. Vazeille, A. Yebakima, R. Girod, D. Goindin, M. Dupont-Rouzeyrol, R. Lourenc¸o-de-Oliveira, and A. B. Failloux. 2016. Differential susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika virus. PLoS Negl. Trop. Dis. 10: e0004543. doi: 10.1371/journal.pntd.0004543.

177. Richard, V., T. Paoaafaite, and V. M. Cao-Lormeau. 2016. Vector competence of French

Polynesian Aedes aegypti and Aedes polynesiensis for Zika Virus. PLoS Negl. Trop. Dis. 10: e0005024. doi: 10.1371/journal.pntd.0005024.

178. Costa-da-Silva, A. L., R. S. Ioshino, H.R.C. de Araujo, B. B. Kojin, P.M.A. Zanotto, D.B.L. Oliveira, S. R. Melo, E. L. Durigon, and M. L. Capurro. 2017. Laboratory strains of Aedes aegypti are competent to Brazilian Zika virus. PLoS ONE 12: e0171951. doi 10.1371/journal.pone.0171951.

179. Heitmann, A., S. Jansen, R. Luhken, M. Leggewie, M. Badusche, B. Pluskota, N. Becker, O. Vapalahti, J. Schmidt-Chanasit, and E. Tannich. 2017. Experimental transmission of Zika virus by mosquitoes from central Europe. Euro. Surveill. 22: pii 30437; doi: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.2.30437.

180. Hall-Mendelin, S., A. T. Pyke, P. R. Moore, I. M. Mackay, J. L. McMahon, S. A. Ritchie, C. T. Taylor, F. A. Moore, and A. F. van den Hurk. 2016. Assessment of local mosquito species incriminates Aedes aegypti as the potential vector of Zika Virus in Australia. PLoS Negl. Trop. Dis. 10: e0004959. doi: 10.1371/journal.pntd.0004959.

181. Turell, M. J., D. J. Dohm, C. N. Mores, L. Terracina, D. L. Wallette, Jr., L. J. Hribar, J. E. Pecor, and J. A. Blow. 2008. Potential for North American mosquitoes to transmit Rift Valley fever virus. J. Am. Mosq. Control Assoc.24: 502–507.

182. Iranpour, M., M. J. Turell, and L. R. Lindsay. 2011. Potential for Canadian mosquitoes to transmit Rift Valley fever virus. J. Am. Mosq. Control Assoc. 27: 363–369.

183. Turell, M. J., S. C. Britch, R. L. Aldridge, L. L. Kine, C. Boohene, and K. J. Linthicum.

2013. Potential for mosquitoes (Diptera: Culicidae) from Florida to transmit Rift Valley fever virus. J. Med. Entomol. 50: 1111–1117.

184. Clarke, J. L. 1943. Studies of the flight range of mosquitoes. J. Econ. Entomol. 36: 121–122.

185. Szalanski, A. L., C. B. Owens, J. A. Lewter, and A. B. Broce. 2006. Genetic structure of Aedes vexans (Diptera: Culicidae) populations from central United States based on mitochondrial ND5 Sequences. Ann. Entomol. Soc. Am. 99: 157–163.

186. Francuski, L., V. Milankov, J. Ludo_ski, B. Krtini_c, J. O. Lundstro¨m, G. Kemenesi, and J. Ferenc. 2016. Genetic and phenotypic variation in central and northern European populations of Aedes (Aedimorphus) vexans (Meigen, 1830) (Diptera, Culicidae). J. Vector Ecol. 41: 160–171. doi: 10.1111/jvec.12208.

187. Vaughan, J. A., J. O. Mehus, C. M. Brewer, D. K. Kvasager, S. Bauer, J. L. Vaughan, H. K. Hassan, T. R. Unnasch, and J. A. Bell. 2012. Theoretical potential of passerine filariasis to enhance the enzootic transmission of West Nile virus. J. Med. Entomol. 49: 1430–1441.

188. Schofield, S., M. Tepper, and R. Gadawski. 2007. Field evaluation against mosquitoes of regular and polymer-based deet formulations in Manitoba, Canada, with comment on methodological issues. J. Med. Entomol. 44: 457–462.

189. Anderson, J. F., A. J. Main, P. M. Armstrong, T. G. Andreadis, and F. J. Ferrandino. 2015. Arboviruses in North Dakota, 2003-2006. Am. J. Trop. Med. Hyg. 92: 377–393.

190. Balenghien, T., F. Fouque, P. Sabatier, and D. J. Bicout. 2006. Horse-, bird-, and human-seeking behavior and seasonal abundance of mosquitoes in a West Nile virus focus of southern France. J. Med. Entomol. 43: 936–946.

191. Lu¨ hken, R., W. P. Pfitzner, J. Bo¨ rstler, R. Garms, K. Huber, N. Schork, S. Steinke, E. Kiel, N. Becker, E. Tannich, et al. 2014. A Field evaluation of four widely used mosquito traps in Central Europe. Parasit. Vectors 7: 268. doi: 10.1186/1756-3305-7-268.

192. Berec, L., I. Gelbic, and O. Sebesta. 2014. Worthy of their name: how floods drive outbreaks of two major floodwater mosquitoes (Diptera: Culicidae). J. Med. Entomol. 51: 76–88.

193. Bockov_a, E., A. Igl_odyov_a, and A. Ko_ci_sov_a. 2015. Potential mosquito (Diptera:Culicidae) vector of Dirofilaria repens and Dirofilaria immitis in urban areas of Eastern Slovakia. Parasitol. Res. 114: 4487–4492. doi: 10.1007/s00436-015-4692-8.

194. Kemenesi, G., K. Kurucz, A. Kepner, B. Dallos, M. Oldal, R. Herczeg, P. Vajdovics, K. Banyai, and F. Jakab. 2015. Circulation of Dirofilaria repens, Setaria tundra, and Onchocercidae species in Hungary during the period 2011- 2013. Vet. Parasitol. 214: 108–113. doi: 10.1016/j.vetpar.2015.09.010.

195. Merdi_c, E., G. Vignjevi_c, N. Turi_c, M. S. Bogojevi_c, J. Milas, I. Vru_cina, and I. Zahirovi_c. 2014. Mosquito survey during West Nile virus outbreak 2012 in northeast

Croatia. Coll. Antropol. 38: 423–428.

196. Fyodorova, M. V., H. M. Savage, J. V. Lopatina, T. A. Bulgakova, A. V. Ivanitsky, O. V. Platonova, and A. E. Platonova. 2006. Evaluation of potential West Nile virus vectors in Volgograd region, Russia, 2003 (Diptera: Culicidae): species composition, bloodmeal host utilization, and virus infection rates of mosquitoes. J. Med. Entomol. 43: 552–563.

197. Yaghoobi-Ershadi, M. R., F. Doosti, S. Schaffner, S. H. Moosa-Kazemi, K. Akbarzadeh, and N. Yaghoobi-Ershadi. 2016. Morphological studies on adult mosquitoes (Diptera: Culicidae) and first report of the potential Zika virus vector Aedes (Stegomyia) unilineatus (Theobald, 1906) in Iran. Bull. Soc. Pathol. Expt. 27: doi: 10.1007/s13149-016-0530-1.

198. Burkett, D. A., W. J. Ee, K. W. Lee, H. C. Kim, H. I. Lee, J. S. Lee, E. H. Shin, R. A. Wirtz, H. W. Cho, D. M. Claborn, et al. 2002. Late season commercial mosquito trap and host seeking activity evaluation against mosquitoes in a malarious area of the Republic of Korea. Korean J. Parasitol. 40: 45–54.

199. Wang, Z. M., D. Xing, Z. M. Wu, W. J. Yao, W. Gang, D. S. Xin, Y. F. Jiang, R. D. Xue, Y. D. Dong, C. X. Li, et al. 2012. Biting activity and host attractancy of mosquitoes (Diptera: Culicidae) in Manzhouli, China. J. Med. Entomol. 49: 1283–1288.

200. Tiawsirisup, S., J. R. Kinley, B. J. Tucker, R. B. Evans, W. A. Rowley, and K. B. Platt. 2008. Vector competence of Aedes vexans (Diptera: Culicidae) for West Nile virus and potential as an enzootic vector. J. Med. Entomol. 45: 452–457.

201. Vaidyanathan, R., J. D. Edman, L. A. Cooper, and T. W. Scott. 1997. Vector competence

of mosquitoes (Diptera: Culicidae) from Massachusetts for a sympatric isolate of eastern equine encephalomyelitis virus. J. Med. Entomol. 34: 346–352.

202. Hammon,W.M., andW. C. Reeves. 1943. Laboratory transmission of St. Louis encephalitis virus by three genera of mosquitoes. J. Exp.Med. 78: 241–253.

203. Ndiaye, el H., G. Fall, A. Gaye, N. S. Bob, C. Talla, C. T. Diagne, D. Diallo, B. A. Y. Dia, I. A. Kohl, A. A. Sall, et al. 2016. Vector competence of Aedes vexans (Meigen), Culex poicilipes (Theobald) and Cx. quinquefasciatus Say from Senegal for West and East African lineages of Rift Valley fever virus. Parasit. Vectors. 9: 94. doi: 10.1186/s13071-016-1383-y.

204. Ro¨ dl, P., V. B_ardos, and J. Ryba. 1979. Experimental transmission of Tahyna virus (California group) to wild rabbits (Oryctolagus cuniculus) by mosquitoes. Folia Parasitol. (Praha). 26: 61–64.

205. Takashima, I., N. Hashimoto, J. Arikawa, and K. Matsumoto. 1983. Getah virus in Aedes vexans nipponii and Culex tritaeniorhynchus: Vector susceptibility and ability to transmit. Arch. Virol. 76: 299–305.

206. Bell, J. A., N. J. Mickelson, and J. A. Vaughan. 2005. West Nile virus in hostseeking mosquitoes within a residential neighborhood in Grand Forks, North Dakota. Vector Borne Zoon. Dis. 5: 373–382.

207. Mehus, J. O., and J. A. Vaughan. 2013. Molecular identification of vertebrate and hemoparasite DNA within mosquito blood meals from eastern North Dakota. Vector Borne

Zoon. Dis. 13: 818–824.

208. Brasil P, Sequeira PC, Freitas AD, Zogbi HE, Calvet GA, de Souza RV, Siqueira AM, de Mendonca MCL, Nogueira RMR, de Filipis AMB, Solomon T. Guillain–Barre syndrome associated with Zika virus infection. Lancet 387, 1482 (2016).

209. Bautista, L. E. & Sethi, A. K. Association between Guillain–Barre syndrome and Zika virus infection. Lancet 387, 2599–2600 (2016).

210. de Oliveira WK, de França GVA, Carmo EH, Duncan BB, de Souza Kuchenbecker R, Schmidt MI. Infection-related microcephaly after the 2015 and 2016 Zika virus outbreaks in Brazil: a surveillance-based analysis. The Lancet. 2017;390(10097):861–70.

211. Cauchemez S, Besnard M, Bompard P, Dub T, Guillemette-Artur P, Eyrolle-Guignot D, et al. Association between Zika virus and microcephaly in French Polynesia, 2013–15: a retrospective study. The Lancet. 2016 May;387(10033):2125–32.

212. Martines, R. B, Bhatnagar J, Oliveira Ramos A.M, Davi H.P.F, Iglezias S.D, Kanamura CT, Keating MK, Hale G, Silva-Flannery L, Muehlenbachs A, Ritter J, Gray J, Rollin D, Goldsmith CS, Reagan-Steiner S, Ermias Y, Suzuki T, Luz KG, de Oliveria WK, Lanciotti R, Lambert A, Shieh WJ, Zaki SR. Pathology of congenital Zika syndrome in Brazil: a case series. Lancet 388, 898–904 (2016).

213. Lucey, D., Cummins, H. & Sholts, S. Congenital Zika syndrome in 2017. JAMA 317, 1368–1369 (2017).

214. Meneses J do A, Ishigami AC, de Mello LM, de Albuquerque LL, de Brito CAA, Cordeiro MT, et al. Lessons Learned at the Epicenter of Brazil's Congenital Zika Epidemic: Evidence From 87 Confirmed Cases. Clinical Infectious Diseases. 2017 May 15;64(10):1302–8.

215. Fajardo Á, Cristina J, Moreno P. Emergence and Spreading Potential of Zika Virus. Frontiers in Microbiology [Internet]. 2016 Oct 20

216. O'Donnell KL, Bixby MA, Morin KJ, Bradley DS, Vaughan JA. Potential of a Northern Population of Aedes vexans (Diptera: Culicidae) to Transmit Zika Virus. Journal of Medical Entomology. 2017

217. Katzelnick LC, Gresh L, Halloran EM, Mercado JC, Kuan G, Gordon A, Balmaseda A, Harris E. Antibody-dependent enhancement of severe dengue disease in humans. Science17 Nov 2017: 929-932

218. Munjal A, Khandia R, Dhama K, Sachan S, Karthik K, Tiwari R, Malik YS, Kumar D, Singh RK, Iqbal HMN, Joshi SK. Advances in Developing Therapies to Combat Zika Virus: Current Knowledge and Future Perspectives. Frontiers in Microbiology [Internet].

219. Stettler K, Beltramello M, Espinosa DA, Graham V, Cassotta A, Bianchi S, Vanzetta F, Minola A, Jaconi S, Mele F, Folierini M, Pedotti M, Simonelli L, Dowall S, Atkinson B, Percivalle E, Simmons CP, Varani L, Blum J, Baldanti F, Cameroni E, Hewson R, Harris E, Lanzavecchia A, Sallusto F, Corti D. Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. Science. 2016;353(6301):823–6.

220. Marinho CF, Azeredo EL, Torrentes-Carvalho A, Marins-Dos-Santos A, Kubelka CF, de Souza LJ, Cunha RV, de-Oliveria-Pinto L. Down-regulation of complement receptors on the surface of host monocyte even as in vitro complement pathway blocking interferes in dengue infection. PLoS One. 2014; 9(7):e102014. https://doi.org/10.1371/journal.pone. 0102014 PMID: 25061945;

221.Tirziu E, Seres M. Particularities of the avian immune system. 2010 TIMISOARA Immunology and Disease Prevention in Poultry [Internet].

222. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkkernagel RM, Aguet M. Function role of type I and type II interferons in antiviral defense. Science24 June 1994: 1918-1921.

223. WHO, Guidelines for plaque reduction neutralization testing of human antibodies to dengue viruses. IVB July, 2007

224. Diamond MS, Edgil D, Roberts TG, Lu B, Harris E. Infection of human cells by dengue virus is modulated by different cell types and viral strains. Journal of virology. 2000;74(17):7814–23.

225. Charles AS, Christofferson RC. Utility of a Dengue-Derived Monoclonal Antibody to Enhance Zika Infection In Vitro. PLoS Currents [Internet]. 2016

226. Chaichana P, Okabayashi T, Puiprom O, Sasayama M, Sasaki T, et al. (2014) Low Levels of Antibody-Dependent Enhancement in Vitro Using Viruses and Plasma from Dengue

Patients. PLOS ONE 9(3): e92173. https://doi.org/10.1371/journal.pone.0092173

227. Kostyuchenko VA, Lim EXY, Zhang S, Fibriansah G, Ng T-S, Ooi JSG, et al. Structure of the thermally stable Zika virus. Nature. 2016 May;533(7603):425–8.

228. Kollberg H, Carlander D, Olesen H, Wejåker P-E, Johannesson M, Larsson A. Oral administration of specific yolk antibodies (IgY) may prevent Pseudomonas aeruginosa infections in patients with cystic fibrosis: a phase I feasibility study. Pediatric pulmonology. 2003; 35(6):433±40. https://doi.org/10.1002/ppul.10290 PMID: 12746939

229. Hatta H, Tsuda K, Ozeki M, Kim M, Yamamoto T, Otake S. Hirasawa M, Katz J, Childers NK, Michalek SM. Passive Immunization against Dental Plaque Formation in Humans: Effect of a Mouth Rinse Containing Egg Yolk Antibodies (IgY) Specific to Streptococcus mutans Caries Research 31: 268-274

230. Torche AM, Le Dimna M, Le Corre P, Mesplede A, Le Gal S, Cariolet R, Le Potier MF. Immune responses after local administration of IgY loaded-PLGA microspheres in gut-associated lymphoid tissue in pigs. Veterinary Immunology and Immunopathology. 2006; 109(3±4):209±17. https://doi.org/10.1016/j.vetimm. 2005.08.016 PMID: 16219363

231. Vega CG, Bok M, Vlasova AN, Chattha KS, FernaÂndez FM, Wigdorovitz A, Parreno VG, Saif LJ. IgY antibodies protect against human Rotavirus induced diarrhea in the neonatal gnotobiotic piglet disease model. PloS one. 2012; 7(8):e42788. https://doi.org/10.1371/journal.pone.0042788 PMID: 22880110

232. Larocca RA, Abbink P, Peron JPS, de A. Zanotto PM, Iampietro MJ, Badamchi-Zadeh A,

Boyd M, Ng'ang'a D, Kirilova M, Nityanandam R, Mercado NB, Li Z, Moseley ET, Bricault CA, Borducchi EN, Giglio PB, Jetton D, Neubauer G, Nkolola JP, Maxfeild LF, De La Barrera R, Jarman RG, Eckels KH, Michael NL, Thomas SJ, Barouch DH. Vaccine protection against Zika virus from Brazil. Nature. 2016 Aug;536(7617):474–8.

233. Pardi N, Hogan MJ, Pelc RS, Muramatsu H, Andersen H, DeMaso CR, Dowd KA, Sutherland LL, Scearce RM, Parks R, Wagner W, Granados A, Greenhouse J, Walker M, Willis E, Yu JS, McGee CE, Sempowski GD, Mui BL, Tam YK, Huang YJ, Vanlandingham D, Holmes VM, Balachandran H, Sahu S, Lifton M, Higgs S, Hensley SE, Madden TD, Hope MJ, Kariko K, Santra S, Graham BS, Lewis MG, Pierson TC, Haynes BF, Weissman D. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. Nature. 2017 Feb 2;543(7644):248–51.
234. Dyer O. Trials of Zika vaccine are set to begin in North America. BMJ. 2016 Jun 28;i3588.
235. Magnani D, Rogers TF, Beutler N, Ricciardi MJ, Bailey VK, Gonzalez-Nieto L, Briney B, Sok D, Le K, Strubel A, Gutman MJ, Pedreño-Lopez N, Grubaugh ND, Silveira CGT, Maxwell HS, Domingues A, Martins MA, Lee DE, Okwuazi EE, Jean S, Strobert EA, Chahroudi A, Silvestri G, Vanderford TH, Kallas EG, Desrosiers RC, Bonaldo MC, Whitehead SS, Burton DR, Watkins DI. Neutralizing human monoclonal antibodies prevent Zika virus infection in macaques. Science Translation Medicine 2017 Oct 4.
236. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global

distribution and burden of dengue. Nature. 2013;496(7446):504–7. pmid:23563266

237. World Health Organization. Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition. WHO Guidelines Approved by the Guidelines Review Committee. Geneva2009.

238. Muller DA, Young PR. The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. Antiviral Res. 2013;98(2):192–208. pmid:23523765

239. Beatty PR, Puerta-Guardo H, Killingbeck SS, Glasner DR, Hopkins K, Harris E. Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. Sci Transl Med. 2015;7(304):304ra141. pmid:2635503

240. Avirutnan P, Zhang L, Punyadee N, Manuyakorn A, Puttikhunt C, et al. 2007. Secreted NS1 of dengue virus attaches to the surface of cells via interactions with heparan sulfate and chondroitin sulfate E. PLOSPathog. 3:e183

241. Puerta-Guardo H, Glasner DR, Harris E. 2016. Dengue virus NS1 disrupts the endothelial glycocalyx,leading to hyperpermeability. PLOS Pathog. 12:e1005738

242. Wan SW, Chen PW, Chen CY, Lai YC, Chu YT, et al. 2017. Therapeutic effects of monoclonal antibody against dengue virus NS1 in a STAT1 knockout mouse model of dengue infection. J. Immunol.199:2834–44

243. Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmeca S, et al. 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein

NS1 and complement.J. Infect. Dis. 193:1078–88

244. Youn S, Li T, McCune BT, Edeling MA, Fremont DH, et al. 2012. Evidence for a genetic and physical interaction between nonstructural proteins NS1 and NS4B that modulates replication of West Nile virus. J. Virol. 86:7360–71

245. Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CK, et al. 2009. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe 5:365–75

246. Mackenzie JM, Jones MK, Young PR. 1996. Immunolocalization of the dengue virus nonstructuralglycoprotein NS1 suggests a role in viral RNA replication. Virology 220:232–40

247. Scaturro P, Cortese M, Chatel-Chaix L, Fischl W, Bartenschlager R. 2015. Dengue virus non-structuralprotein 1 modulates infectious particle production via interaction with the structural proteins. PLOSPathog. 11:e1005277

248. Orozco, Susana et al. "Characterization of a model of lethal dengue virus 2 infection in C57BL/6 mice deficient in the alpha/beta interferon receptor"Journal of general virology vol. 93,Pt 10 (2012): 2152-7.

248. Chaichana P, Okabayashi T, Puiprom O, Sasayama M, Sasaki T, et al. (2014) Low Levels of Antibody-Dependent Enhancement in Vitro Using Viruses and Plasma from Dengue Patients. PLOS ONE 9(3): e92173. https://doi.org/10.1371/journal.pone.0092173

249. Balsitis, Scott J et al. "Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification" PLoS pathogens vol. 6,2 e1000790. 12 Feb. 2010,

doi:10.1371/journal.ppat.1000790

250. Chen Y, Pan Y, Guo Y, Qiu L, Ding X, Che X. 2010. Comprehensive mapping of immunodominant and conserved serotype- and group-specific B-cell epitopes of nonstructural protein 1 from dengue virus type 1. Virology 398:290–98

251. Jiang L, Zhou JM, Yin Y, Fang DY, Tang YX, Jiang LF. 2010. Selection and identification of B-cell epitope on NS1 protein of dengue virus type 2. Virus Res. 150:49–55

252. Falconar AK, Young PR, Miles MA. 1994. Precise location of sequential dengue virus subcomplex and complex B cell epitopes on the nonstructural-1 glycoprotein. Arch. Virol. 137:315–26

253. Lai YC, Chuang YC, Liu CC, Ho TS, Lin YS, et al. 2017. Antibodies against modifiedNS1 wing domain peptide protect against dengue virus infection. Sci. Rep. 7:6975

254. Hertz T, Beatty PR, MacMillen Z, Killingbeck SS, Wang C, Harris E. 2017. Antibody epitopes identified in critical regions of dengue virus nonstructural 1 protein in mouse vaccination and natural human infections. J. Immunol. 198:4025–35

255. Guy B, Noriega F, Ochiai RL, L'azou M, Delore V, Skipetrova A, et al. . A recombinant live attenuated tetravalent vaccine for the prevention of dengue. Expert Rev Vaccines (2017) 16:671–83. 10.1080/14760584.2017.1335201

256. World Health Organization. Updated Questions and Answers related to the dengue vaccine Dengvaxia® and its use. 2017. http://www.who.int/immunization/diseases/dengue/q_and_a_dengue_vaccine_dengvaxia_use/en/. Accessed: 16 April 2018.

257. Saez-Llorens X, Tricou V, Yu D, Rivera L, Tuboi S, et al. 2017. Safety and immunogenicity of one versus two doses of Takeda's tetravalent dengue vaccine in children in Asia and Latin America: interim results from a phase 2, randomised, placebo-controlled study. Lancet Infect. Dis. 17:615–25

258. Weiskopf D, Angelo MA, Bangs DJ, Sidney J, Paul S, et al. 2015. The human CD8+ T cell responses induced by a live attenuated tetravalent dengue vaccine are directed against highly conserved epitopes.J. Virol. 89:120–28

259. Angelo MA, Grifoni A, O'Rourke PH, Sidney J, Paul S, et al. 2017. Human CD4+ T cell responses to an attenuated tetravalent dengue vaccine parallel those induced by natural infection in magnitude, HLA restriction, and antigen specificity. J. Virol. 91:e02147-16

260. Laursen, N. S. *et al.* Universal protection against influenza infection by a multidomain antibody to influenza hemagglutinin. *Science* **362**, 598 (2018).