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Flavivirus-dependent packaging of Aedes aegypti saliva proteins into extracellular vesicles enhances infection

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Dissertation

FLAVIVIRUS-DEPENDENT PACKAGING OF *AEDES AEGYPTI* SALIVA PROTEINS INTO EXTRACELLULAR VESICLES ENHANCES INFECTION

by

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ABSTRACT

Flaviviruses are a genus of arboviruses, including dengue virus (DENV), Zika virus, yellow fever virus, and West Nile virus, that have the potential to cause severe disease in humans and represent a tremendous and growing threat to public health. Like all arboviruses, flavivirus infection is contracted upon the bite of an infected vector, a process during which the virus and saliva are injected into the host skin. A large body of work has already reported the infection-enhancing ability of proteins derived from vector saliva, suggesting the existence of selective pressures on the vector-to-host viral transmission process. Through the work described herein, we demonstrated that flavivirus infection of *Aedes aegypti* modulates the protein cargo of extracellular vesicles, a potential avenue for the delivery of pro-viral factors during transmission. In doing so, we identified one protein, AAEL002675 (ARGIL1- *Aedes aegypti* Arginase-like 1 Protein), a putative arginase, within specific fractions of *Aedes aegypti* saliva that displayed infection-enhancing activity. We also observed this ARGIL1 protein within extracellular vesicles derived from dengue-infected *Aedes aegypti* cells.

Importantly, treatment of cells with ARGIL1 resulted in an arginase-dependent enhanced level of DENV infection in vitro. In mammals, arginase is an important

regulator of excessive cellular inflammation. It catalyzes production of collagen and polyamines from L-arginine, therefore reducing the pool of L-arginine substrate available for inducible nitric oxide synthase (iNOS) to produce nitric oxide. Consistently, cells treated with ARGIL1 displayed decreased levels of iNOS upon DENV infection, suggesting that ARGIL1 treatment reshapes the cellular environment to be more permissive to viral replication upon mosquito bite. These findings provide further evidence that ARGIL1 is an arginase-like pro-viral factor enhancing vector-to-host DENV transmission. Altogether, a better understanding of the molecular processes driving DENV transmission, such as those described here, will be instrumental for the development of innovative preventative antiviral strategies against flaviviruses and arboviruses, including vaccines that target vector proteins.

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ADE	Antibody-Dependent Enhancement
ARG1	Human Arginase 1
ARGIL1	*Aedes aegypti* Arginase-like 1 Protein
B2M	Beta-2 Microglobulin
BSA	Bovine Serum Albumin
BEC	S-(2)-boronoethyl)-L-cysteine
C	Capsid Protein
cDNA	Complementary Deoxyribonucleic Acid
DENV	Dengue Virus
DENV-2	Dengue Virus Type-2 New Guinea C Strain
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
E	Envelope Protein
ER	Endoplasmic Reticulum
EV	Extracellular Vesicle
FBS	Fetal Bovine Serum
FFA	Focus Forming Assay
HPLC	High-Performance Liquid Chromatography
IFN	Interferon
IFNAR	Interferon-α/β Receptor

iNOS	Inducible Nitric Oxide Synthase
IRF	Interferon Regulatory Factor
ISG	Interferon-Stimulated Gene
ISGF-3	Interferon-Stimulated Gene Factor 3
ISRE	Interferon-Stimulated Response Elements
JAK1	Janus Kinase 1
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
M	Membrane Protein
MAVS	Mitochondrial Antiviral-Signaling Protein
MDA5	Melanoma-Associated Differentiation Antigen 5
MOI	Multiplicity of Infection
MyD88	Myeloid Differentiation Factor 88
NO	Nitric Oxide
NS	Non-Structural
NF-κB	Nuclear Factor Kappa B
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
prM	Precursor Membrane Protein
PRR	Pathogen Recognition Receptor
RIG-I	Retinoic Acid-Inducible Gene I
RT-PCR	Real-Time Quantitative Reverse Transcription PCR
RNA	Ribonucleic Acid

RNP	Ribonucleoprotein
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
sfRNA	Subgenomic Flaviviral RNA
STAT	Signal Transducer and Activator of Transcription
TBS	Tris-Buffered Saline
TBST	TBS Buffer with 0.05% Tween-20
TLR	Toll-Like Receptor
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon-β
TYK2	Tyrosine Kinase 2
YFV	Yellow Fever Virus
WNV	West Nile Virus
ZIKV	Zika Virus

INTRODUCTION

FLAVIVIRUS: EPIDEMIOLOGY & PUBLIC HEALTH IMPACT

Arthropod-borne disease represents a tremendous threat to public health, responsible for approximately 20% of the global tropical disease burden and accounting for about 1 million human deaths annually (Torto and Tchouassi 2021). Of these diseases, malaria, a disease caused by the mosquito-transmitted *Plasmodium* parasite, is the most burdensome killing more than 400,000 people every year (Talapko, Škrlec et al. 2019). In addition to malaria, there are several arthropod-borne viral diseases including dengue (DENV), Zika (ZIKV), and yellow fever (YFV), which constitute a substantial portion of this burden (Ushijima, Abe et al. 2021). The viruses responsible for this burden (arboviruses) are transmitted by a range of arthropod vectors such as mosquitoes, ticks, sandflies, and midges (Gubler 2006). Due to the geographic distribution of these vectors, particularly the *Aedes aegypti* and *Aedes albopictus* mosquitoes, which vector the most burdensome arboviruses, the burden of arboviral disease has historically been concentrated in tropical regions surrounding the equator in South America, Africa, and Asia (Wu, Yu et al. 2019). However, due to geographic expansion of arbovirus vectors, as well as population growth in already at-risk areas, there has been a rapid emergence and re-emergence of arthropod-borne diseases over the past 50 years, and the prevalence of these diseases is expected to grow in the near future (Wilder-Smith, Gubler et al. 2017, Kraemer, Reiner et al. 2019).

Flaviviruses, belonging to the family *Flaviviridae* and genus *Flavivirus*, are a group of arboviruses that cause extensive illness and death worldwide. The *Flavivirus*

genus is made up of about 73 arboviruses which infect a variety of vertebrates including pigs, birds, non-human primates, and humans (Daep, Muñoz-Jordán et al. 2014). This group of viruses includes several clinically relevant viruses transmitted by mosquitoes including DENV, ZIKV, and YFV, as well multiple tick-transmitted viruses including tick-borne encephalitis virus (Pierson and Diamond 2020). The presentation of flavivirus infection in humans ranges quite greatly from mild illness or asymptomatic infection, making up 50-80% of infections, to severe and life-threatening disease, including hemorrhagic fever, shock syndrome, encephalitis, paralysis, congenital defects, hepatitis, and hepatic failure (Pierson and Diamond 2020). While the first recorded epidemic of flavivirus-like disease was reported between 1779 and 1780, since then many of these pathogens have expanded to become endemic in tropical and subtropical areas (Gubler 2002, Daep, Muñoz-Jordán et al. 2014). Additionally, population movements, urbanization, deforestation, travel, and rising global temperatures continue to drive the spread of these viruses to previously non-endemic regions (Daep, Muñoz-Jordán et al. 2014).

One example of this is DENV, the flavivirus responsible for the greatest burden of human disease. DENV is estimated to cause about 400 million infections per year in over 125 countries, of which 100 million manifest in symptoms (Bhatt, Gething et al. 2013). As a result of recent increased epidemic activity of DENV and geographic expansion of the *Aedes aegypti* vector, dengue is now endemic in over 100 countries and about one half of the global population lives in areas environmentally suitable toward DENV transmission (Bhatt, Gething et al. 2013). And while the majority of cases in endemic

regions are asymptomatic or exhibit mild symptoms, the public health and economic burden of DENV in these regions cannot be ignored. Recent studies have estimated that in Vietnam alone DENV is responsible for 39,884 disability-adjusted life years annually, representing an economic burden of $94.7 million per year (Hung, Clapham et al. 2018). In addition to its current status as an emerging global health threat, due to climate change and population growth, models predict that 2.25 billion more people will be at risk for DENV in 2080 compared to 2015, bringing the total population at risk to over 6.1 billion (Messina, Brady et al. 2019). Studies have shown similar results for other flaviviruses including ZIKV and YFV, both of which are expected to expand in their geographic distribution in the near future (Kraemer, Reiner et al. 2019, Mordecai, Ryan et al. 2020).

Although there are safe and effective vaccines for flaviviruses, including YFV and Japanese encephalitis virus, the majority of flaviviruses remain without any approved vaccines, treatments, or prophylactics (WHO 2016, Douam and Ploss 2018, Pierson and Diamond 2020). Vaccines for other flaviviruses are in pre-clinical and clinical development, however challenges such as cross-reactivity of flavivirus-immune sera, response to vaccination by flavivirus-specific antibodies, and varying potency of immune response to vaccination have hindered development, emphasizing the need for effective treatment options (Low, Ooi et al. 2017, Pierson and Diamond 2020). Identifying molecules involved in the transmission of flaviviruses to the vertebrate host is a crucial first step in furthering our understanding of the transmission process and the development of new drugs and vaccines (Neelakanta and Sultana 2015, Low, Ooi et al. 2017).

DENGUE VIRUS

DENV exists as four genetically related, but antigenically distinct, serotypes transmitted by species of *Aedes* mosquito (Yung, Lee et al. 2015). Following inoculation with the virus by mosquito bite, all of these serotypes of DENV can cause disease in humans ranging from self-limited fever, commonly referred to as 'dengue fever', to a life-threatening condition, termed 'severe dengue.' Currently, DENV causes an estimated 390 million total infections, 100 million clinically symptomatic cases, and 500,000 presentations of severe dengue annually worldwide, with at least 2.5 billion people at risk of infection (Bhatt, Gething et al. 2013). Over the past 50 years, the number of annual dengue infections globally has grown rapidly, making DENV the most prevalent arthropod-borne viral disease (Bhatt, Gething et al. 2013). Due to the geographic distribution of the *Aedes aegypti* vector, dengue occurs widely in regions surrounding the equator, including the Americas, Asia, and Africa, and severe dengue occurs in all of these regions. While certain regions of the United States, such as the Gulf Coast, have experienced periodic outbreaks of dengue, sustained transmission has not occurred recently (Añez, Rios et al. 2012, Bouri, Sell et al. 2012). Future projections of DENV epidemiology, modelled accounting for climate change and population growth, predict rapid expansion of the range of dengue over the next 50 years (Messina, Brady et al. 2019).

Severe dengue is characterized by rapid onset of capillary leakage accompanied by thrombocytopenia and mild to moderate liver damage (Halstead 2007). While severe disease is accompanied by hemorrhagic symptoms, the greatest risk of mortality is

hypotension due to fluid loss into tissue (Nhan, Phuong et al. 2001). Unique to dengue, the incidence of severe disease differs between primary infection and secondary infection- infection of a host previously infected with a different serotype of DENV- with severe disease being more common in the case of secondary infection in children and adults (Burke and Kliks 2006, Halstead, Mahalingam et al. 2010). This clinical difference is believed to be due to a phenomenon known as antibody-dependent enhancement (ADE), in which existing antibodies from a previous infection with DENV enhance infection of monocytes by a different serotype of DENV during a subsequent infection (Halstead 2003). Consistent with this, incidence of severe dengue in infants, which can cause significant morbidity and mortality, occurs most often at about seven months of age when maternal serum antibodies transferred to the infant wane in their neutralizing ability and enhance infection rather than neutralize infectious virions (Simmons, Chau et al. 2007). As a result, severe dengue is more prevalent in infants and has a higher mortality rate compared to other age groups (Hammond, Balmaseda et al. 2005).

ZIKA VIRUS

ZIKV, a mosquito-borne flavivirus transmitted by species of *Aedes* mosquito, was first isolated from a rhesus monkey in 1947 in the Zika forest of Uganda (Dick, Kitchen et al. 1952, Mlakar, Korva et al. 2016). Prior to an outbreak in Yap Island of Micronesia in 2007, cases of ZIKV were rare and caused mild febrile illness in a small number of humans in Africa and parts of Asia (Hayes 2009). Following several major epidemics in island nations of the South Pacific in 2013 and 2014, ZIKV was introduced into Brazil and other regions of the Americas (Metsky, Matranga et al. 2017). The introduction of

ZIKV to South America began an epidemic resulting in millions of infections in countries throughout the Americas, the most affected of which was Brazil with estimates of 440,000 to 1.3 million autochthonous cases through December 2015 (Mlakar, Korva et al. 2016). Unlike previous ZIKV-associated disease, infection during this epidemic was observed with unique clinical manifestations, including neurological disease, Guillain-Barré syndrome, and congenital malformations, contributing to the morbidity and mortality of this virus (Mlakar, Korva et al. 2016, Musso, Bossin et al. 2018). Studies examining changes in ecological factors that may have driven the emergence of ZIKV in the Americas have shown that several factors resulting in increased *Aedes* vector populations and transmission, including climate change and human movement into urban areas, could have contributed to this epidemic (Ali, Gugliemini et al. 2017). Relatedly, studies examining genetic changes of pre- and post-epidemic ZIKV isolates have uncovered sequence differences associated with enhanced infection of the *Aedes aegypti* vector (Liu, Liu et al. 2017).

Similarly, several studies have been conducted examining genetic changes between pre- and post-epidemic strains of ZIKV, and how these changes may have affected the clinical presentation of ZIKV infection, specifically with regard to neurological disease. These studies identified 11 amino acid changes between older strains of ZIKV and the epidemic American ZIKV strain throughout the viral genome (Faria, Azevedo et al. 2016). Of these changes, several have been shown to enhance infection in neuroprogenitor cells in vitro and cause more severe microcephaly in neonatal mice in vivo, supporting the neuropathological symptoms observed in fetal cases

of ZIKV during the 2015 epidemic (Klase, Khakhina et al. 2016, Yuan, Huang et al. 2017).

Unique to ZIKV, compared to other arboviruses, is the ability to be sexually and vertically transmitted, both of which are important contributors to its spread and associated morbidity (Mead, Hills et al. 2018, Ades, Soriano-Arandes et al. 2021). Vertical transmission of ZIKV, when the virus is transmitted from an infected mother to a fetus in utero, has been shown to be associated with a group of birth defects known as congenital Zika syndrome (Ades, Soriano-Arandes et al. 2021). Interestingly, both the rate of vertical transmission and congenital Zika syndrome have been shown to decrease as pregnancy progresses, with the highest rates observed in maternal infections during the first trimester (Ades, Soriano-Arandes et al. 2021). Congenital Zika syndrome is a significant source of mortality for children, observed by population studies following the 2015 Epidemic, which showed that children born with congenital ZIKV were 10 times more likely to die during the first three years of life (Paixao, Cardim et al. 2022).

YELLOW FEVER VIRUS

YFV, characterized and named after the jaundice associated with severe infections, is thought to have emerged in Africa within the last 1,500 years before spreading to Europe and the Americas about 300 to 400 years ago (Bryant, Holmes et al. 2007). From the 15th to the 19th centuries, YFV was one of the most devastating epidemic diseases with large scale epidemics of high mortality frequently occurring in port cities and influencing the developing economies of the New World (Bryant, Holmes et al. 2007). Although most YFV infections result in asymptomatic cases, severe symptomatic

cases are associated with acute febrile illness that may result in hepatitis, renal failure, hemorrhage, and cardiovascular shock, resulting in a mortality rate of 20-60% in severe cases (Tuboi, Costa et al. 2007, Johansson, Vasconcelos et al. 2014, Monath and Vasconcelos 2015). Ultimately, outbreaks of YFV were hindered by the development of an effective vaccine, YFV-17D, in the 1930s and its widespread deployment in the 1940s (Monath 2005).

Despite the existence of an effective vaccine, YFV remains endemic and emerging in regions of Africa and South America, with an estimated 90% of YFV cases occurring in Africa (Barrett and Higgs 2007). Recent studies have estimated that about 130,000 severe cases of YFV occur annually in Africa, resulting in over 75,000 deaths (Hamlet, Jean et al. 2018). Although it is difficult to measure the overall burden YFV due to heterogeneity of disease, only about 12% of YFV cases in Africa are believed to cause severe illness, bringing the overall number of cases up to over 1 million annually (Johansson, Vasconcelos et al. 2014). Although the incidence of YFV in South America is much lower than in Africa, extended human activity in endemic areas and low vaccination rates in non-endemic areas has synergistically resulted in recent outbreaks outside of endemic regions of the Amazon, many of which occurred near major urban centers, supporting the expanding range of this virus (Chippaux and Chippaux 2018, Rezende, Sacchetto et al. 2018). Despite the existence of an effective vaccine, the growing spread of YFV into highly-populated areas with low vaccine coverage, such as these, further emphasizes the need for expanding vaccine coverage and developing novel

vaccination approaches better suited for rapid response to outbreaks (De Thoisy, Silva et al. 2020).

WEST NILE VIRUS

WNV, which cycles between *Culex* mosquitoes and birds, was first isolated in 1937 (Smithburn, Hughes et al. 1940). While mammals are not the natural host of WNV, this virus can infect and cause disease in several species of mammal, including humans, despite absence of effective transmission due to low-level viremia, effectively making these mammals 'dead-end' hosts (Hubálek and Halouzka 1999). However, previous studies demonstrating non-viremia transmission of WNV between co-feeding mosquitoes suggest that mammals could act as additional reservoirs (Higgs, Schneider et al. 2005). Historically, WNV caused temporally dispersed outbreaks of mild febrile illness in regions of the Eastern Hemisphere, including Africa, the Middle East, Asia and Australia (Hubálek and Halouzka 1999). However, there was a change in the epidemiology of WNV infection at some point in the 1990s and cases in Eastern Europe began to be associated with neurological disease (Hubálek and Halouzka 1999).

In 1999, WNV entered North America via an outbreak in New York and has since spread becoming the predominant circulating arbovirus in the United States. Between 1999 and 2019, there were a confirmed 51,747 cases of WNV, about half of which were associated with severe neurological disease, including meningitis and/or encephalitis, associated with over 2,000 deaths (Pierson and Diamond 2020). As a result of the geographic range of both the *Culex* vector and avian reservoir, WNV has spread to and circulated throughout the Western Hemisphere in North and South Americas. While

recent years have seen sporadic outbreaks of WNV in the Americas and Europe, WNV continues to evolve, with severe neurological disease and fatalities caused by a different genetic lineage, termed lineage 2 WNV, observed recently in cases in Europe (Veo, Della Ventura et al. 2019).

MOLECULAR BIOLOGY OF FLAVIVIRUSES

Flaviviruses are spherical enveloped particles about 50 nM in diameter with icosahedral symmetry (Barrows, Campos et al. 2018, Pierson and Diamond 2020). Each flavivirus contains a single 10.7 kb positive-sense RNA genome with a 5' cap, but no poly(A) tail (Barrows, Campos et al. 2018, Pierson and Diamond 2020). This RNA encodes for a single polyprotein that is co- and post-translationally cleaved into 10 proteins, three of which are structural proteins and seven of which are nonstructural (Barrows, Campos et al. 2018, Pierson and Diamond 2020). These structural proteins include capsid (C), membrane (M), and envelope (E), and together make up the physical structure of the virion. The exterior layer of the virion is composed of E dimers in complex with M proteins, while the interior is comprised of the viral genome complexed with C, forming the ribonucleoprotein (RNP) (Pierson and Diamond 2020). The seven non-structural proteins include an RNA-dependent RNA polymerase (NS5), a helicase/protease (NS3), and five other proteins (NS1, NS2A, NS2B, NS4A, and NS4B) that together form the viral replication complex (Barrows, Campos et al. 2018). Additionally, the region of the flavivirus RNA genome that encodes for this polyprotein is surrounded by two highly structured untranslated regions of about 100 nucleotides at the 5' end of the genome and 400-700 nucleotides at the 3' end, both of which contain

highly structured elements and are essential for replication and translation of the genome (Barrows, Campos et al. 2018).

The first step of the flavivirus life cycle is viral attachment and entry into the cell. While several viruses enter by specific receptor-mediated mechanisms, no single cellular receptor specifically binding to E and responsible for flavivirus entry has been identified (Laureti, Narayanan et al. 2018). Although a multitude of cell surface factors, including heparin sulfates, DC-SIGN, and heat shock proteins, have been found to be important regulators of flavivirus entry (Perera, Khaliq et al. 2008, Pierson and Kielian 2013), the ubiquitous expression of these factors across multiple cell lineages and tissues does not recapitulate the specific cellular tropism of flaviviruses, suggesting that more specific cellular receptors are yet to be uncovered. However, one cannot exclude that flavivirus cellular tropism is defined at the post-entry stage, and that flavivirus attachment and internalization into target cells remains a non-specific process. Following attachment to the cell surface, the virion is internalized by clathrin-mediated endocytosis (Chu and Ng 2004). Upon entry, endosome acidification causes conformational changes of the E protein. Exposure of the internal fusion peptide loop of E promotes the fusion of the viral and endosomal membranes, releasing the RNP into the cytoplasm (Mukhopadhyay, Kuhn et al. 2005).

Before translation and viral replication can begin, the RNP must be uncoated, releasing C and exposing the viral RNA to the translation machinery. This process is not well understood, yet it has been shown that ubiquitination and the cellular ATPase valosin-containing protein (VCP)/p97 are necessary for uncoating (Byk, Iglesias et al.

2016, Ramanathan, Zhang et al. 2020). Once the viral genome is exposed, translation is initiated by the host translation machinery, upon which the viral polyprotein is translated in a single open-reading frame as it is co- and post-translationally cleaved by host and viral protease machinery (Barrows, Campos et al. 2018). Following translation and accumulation of viral proteins, the viral genome is transitioned from being translated to being replicated by the RNA-dependent RNA polymerase NS5 (Khromykh, Sedlak et al. 1999). In the final steps of the flavivirus life cycle, newly replicated viral genomes are assembled with structural proteins in the ER, budding in the ER lumen and taking ER membrane as the lipid envelope in the maturing virion (Barrows, Campos et al. 2018). As the virion egresses through the Golgi, making its way through the trans-Golgi network, acidification results in conformational changes of E and maturation of M protein via cleavage of its precursor prM into pr and M. Following subsequent pH neutralization, mature virions are released into the extracellular space (Barrows, Campos et al. 2018).

FLAVIVIRUS TRANSMISSION IS A MULTIFACETED PROCESS

Flaviviruses are maintained in a constant cycling between two classes of organism: 1) the host- a vertebrate including humans, non-human primates, mammals, and birds, and 2) the vector- an arthropod including mosquitoes and ticks. Depending on the flavivirus, a certain vector may be more frequently involved in transmission than others due to vector permissiveness to infection and/or the geographic range of the vector. For example, the *Aedes aegypti* mosquito serves as the primary vector for dengue, while the *Aedes albopictus* vector serves as the secondary vector (Kraemer, Sinka et al. 2015). Similarly, depending on the feeding preferences of the vector and the ability of the

virus to productively infect a host, certain hosts are more likely to function as sites of viral amplification for further transmission, also known as reservoirs (Pandit, Doyle et al. 2018).

The transmission of clinically relevant flaviviruses, including DENV, ZIKV, and YFV, is thought to be split into two cycles: 1) the sylvatic cycle and 2) the urban cycle. The sylvatic cycle occurs in rural areas in which flavivirus is transmitted between vectors and hosts associated with these areas, such as jungle species of mosquitoes and non-human primates (Pandit, Doyle et al. 2018). The urban cycle occurs in more populated areas in which the virus is transmitted between a vector found in those areas, such as the *Aedes aegypti* mosquito and humans (Vasilakis and Weaver 2017). Historically, flavivirus epidemics have been thought to originate from a zoonotic spillover event from the sylvatic cycle to the urban cycle, upon which a novel pathogen is introduced into an urban setting before amplification and subsequent transmission (Vasilakis and Weaver 2017). However, given the recent growing establishment of several flaviviruses as endemic in multiple areas, it's thought that there is a constant cycling of these viruses in the urban cycle to maintain endemicity (Daep, Muñoz-Jordán et al. 2014).

In addition to vector and host preferences, there are other elements of flavivirus transmission that make it an intricate process and impact the distribution of these viruses. One such factor is the transmission to a host that is permissive to infection but unable to pass on infection, resulting in infection of an accidental host, also known as a 'dead end host.' In the case of WNV, this occurs when the virus is transmitted from its avian reservoir to a mammal, which has been the case in recent epidemics (Marm Kilpatrick,

Daszak et al. 2006). Another element that adds complexity to flavivirus transmission is vertical transmission, or the process by which the flavivirus is transmitted from an infected mother to their offspring. So far, vertical transmission of an arbovirus in humans has only been observed in those infected with ZIKV, and has been exemplified by the ability of ZIKV infection to promote ZIKV-induced microcephaly in the fetus (Lai, Zhou et al. 2020). However, many flaviviruses, including DENV, YFV, and WNV have been observed to be vertically transmitted from infected female mosquitoes to their offspring during the oogenesis and egg laying process, highlighting the importance of vertical transmission on the prevention and control of arboviral disease (Lai, Zhou et al. 2020).

During the flavivirus transmission event, in which an infected vector bites a host, the vector injects a bolus of saliva and virus into the skin, the primary site of infection. Following initial exposure to the virus, there is early infection of skin-resident epithelial and hematopoietic cells, including Langerhans cells in the epidermis, as well as dermal dendritic cells, macrophages, keratinocytes, fibroblasts and mast cells in the dermis (Ivory, Birchall et al. 2015, Begum, Das et al. 2019). Additionally, inflammation and hematopoietic cell infiltration in the skin, both of which are promoted by mosquito saliva, contribute to early stage viral amplification through the increased infection of recruited and resident myeloid cells (Pingen, Steven et al. 2016). After amplification in the skin, viral particles are thought to spread through the lymphatic system mainly as cell-free virus, but also potentially via dendritic cell-mediated shuttling, resulting in infection of lymph nodes (Merad, Manz et al. 2002, Schmid and Harris 2014). Further amplification in draining lymph nodes, especially in dendritic cells and monocytes, results in the

dissemination of virus to non-lymphoid organs via the blood circulation (Martina, Koraka et al. 2009). Once systemic infection is established, the infected host can become a novel viral reservoir and, depending on viremia level, transmit infectious viruses via naïve vectors to subsequent hosts (Martina, Koraka et al. 2009). Systemic spread of the virus can lead to further complications including more severe symptoms of flavivirus infection, including vascular shock and neurotropic disease (Pierson and Diamond 2020).

Following the initial biting event, a key mediator of the course of infection is the immune response, which can be split into two arms, the innate immune response and the adaptive immune response:

INNATE IMMUNE RESPONSE

Following infection, cells recognize viral RNA through several pathogen recognition receptors (PRRs), resulting in a multitude of immune responses with antiviral functions, notably production of type I interferon (IFN) and reactive nitrogen species, including nitric oxide (NO) (Chaturvedi and Nagar 2009, Schoggins 2018, Schoggins 2019). Flavivirus RNA found in the cytosol is detected primarily by the PRRs retinoic-acid inducible gene-I (RIG-I) and melanoma-associated differentiation antigen 5 (MDA5) preferentially as single-stranded and double-stranded forms, respectively (Ngono and Shresta 2018). Following binding of viral RNA, these PRRs undergo conformational changes, multimerize, and interact with the mitochondrial antiviral signaling protein (MAVS) (Suthar, Aguirre et al. 2013). Similar to viral RNA sensing by RIG-I and MDA5, within the endosomal compartment viral RNA is sensed by the toll-like receptors TLR3 and TLR7 in single- and double-stranded forms, respectively (Suthar, Aguirre et

al. 2013). Detection of viral RNA by these TLRs results in downstream signaling by the proteins myeloid differentiation factor 88 (MyD88) and TIR domain-containing adapter-inducing IFN-β (TRIF) (Suthar, Aguirre et al. 2013). Following sensing of viral RNA by these PRRs, there is downstream activation of interferon regulatory factor 3 (IRF3) and interferon regulatory factor 7 (IRF7), resulting in their phosphorylation, dimerization, and subsequent nuclear translocation (Ngono and Shresta 2018). Translocation of these IRFs, as well as activated nuclear factor kappa B (NF-κB), induces the expression of several genes including type I IFNs, IFN-α and IFN-β (Suthar, Aguirre et al. 2013).

Secreted IFN binds to the interferon-α/β receptor (IFNAR), resulting in activation through heterodimerization, and the activation of signaling molecules Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) by phosphorylation (Ngono and Shresta 2018). Activation of JAK1 and TYK2 results in the phosphorylation and heterodimerization of two signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT2, which subsequently join with IRF9, forming a complex known as interferon-stimulated gene factor 3 (ISGF-3), which is subsequently translocated to the nucleus (Ngono and Shresta 2018). Once in the nucleus, ISGF-3 binds to interferon-stimulated response elements (ISREs) in the genome, inducing the expression of hundreds of interferon-stimulated genes (ISGs) (Schneider, Chevillotte et al. 2014, Ngono and Shresta 2018). The expression of these ISGs can affect several cellular processes involved in the flavivirus life cycle, including RNA processing and translation, exerting a potent antiviral effector response.

In addition to the role of host factors involved in this described immune response, several pro-viral functions of flavivirus elements, including RNAs and NS proteins, have been described to counteract this coordinated immune response at multiple points (Ngono and Shresta 2018). Examples of this inhibition include: inhibition of sensing of viral RNA by RIG-I by DENV NS2B3 and DENV subgenomic flaviviral RNAs (sfRNAs), and inhibition of STAT1/2 phosphorylation and heterodimerization by multiple DENV and ZIKV NS proteins (Ngono and Shresta 2018). Elucidating the interplay of viral elements with host immune signaling factors is critical in furthering our understanding of the overall innate immune response to flavivirus infection.

ADAPTIVE IMMUNE RESPONSE

The adaptive immune response is comprised of the B cell response, responsible for the generation of anti-flavivirus antibodies, and the T cell response, which is responsible for cell-mediated immunity. Antibodies specific to flaviviruses are known to be important in the antiviral response due to their ability to employ protective effects via effector functions mediated by the Fc region of the antibody, including complement fixation, antibody-mediated cellular toxicity, and antibody-mediated opsonization, all of which can promote clearance of virus (Vogt, Dowd et al. 2011, Bournazos, Dilillo et al. 2015, Slon Campos, Mongkolsapaya et al. 2018). The majority of antibodies protective against flaviviruses target epitopes on the E protein of the virion, but have also been shown to bind to regions of the cell surface and secreted NS1 (Muller and Young 2013, Pierson and Diamond 2020). Like the humoral response, the T cell response, comprised of CD4+ and CD8+ T cells, is important in the defense and lost-lasting immunity to

flavivirus infection (Netland and Bevan 2013). CD4+ T cells are responsible for the memory T cell response elicited by prior flavivirus infection, but can also protect against infection via sustaining CD8+ T cell responses, aiding antibody responses, and lysing some infected cells (Netland and Bevan 2013, Weiskopf, Bangs et al. 2015). Due to their ability to lyse target cells, flavivirus-specific cytotoxic CD8+ T cells proliferate, release proinflammatory cytokines, such as IFN-γ and tumor necrosis factor (TNF), and lyse infected cells through delivery of perforin or granzymes, Fas-Fas ligand, or TNF-related apoptosis-inducing ligand (TRAIL) interactions (Netland and Bevan 2013). Together, the response of B cells and the dual response of T cells provides multiple mechanisms of lasting and specific immunity against flavivirus infection.

INFECTION-ENHANCING COMPONENTS OF VECTOR SALIVA PRESENT CHALLENGES TO HALTING DISEASE TRANSMISSION

Vector saliva, which is injected into the skin along with virus during an infectious biting event, has been shown to augment arbovirus infection in mammals (Styer, Kent et al. 2007, Schneider and Higgs 2008, Styer, Lim et al. 2011). Aside from its contribution to transmission, mosquito saliva contains a mixture of proteins that collectively function to allow the mosquito to acquire a blood meal by overcoming host barriers such as vasoconstriction, platelet aggregation, coagulation, and inflammation (Ribeiro and Francischetti 2003, Conway, Watson et al. 2014, Vogt, Lahon et al. 2018). Many of these proteins are known to be immunogenic, often resulting in allergic reactions ranging in severity and indicating the capacity of proteins in saliva to influence viral infection (Depinay, Hacini et al. 2006, Cantillo, Fernández-Caldas Rodríguez et al. 2015). Previous

studies examining the contents of *Aedes aegypti* saliva have shown that it contains over one hundred unique proteins, many of which have been shown to enhance or inhibit flavivirus infection (Conway, Watson et al. 2014, Conway, Londono-Renteria et al. 2016, Sun, Nie et al. 2020). Two such proteins that have been shown to enhance flavivirus infection include the *Aedes aegypti* serine protease CLIPA3, shown to augment DENV infection by proteolyzing extracellular matrix proteins, and the *Aedes aegypti* venom allergen-1, shown to enhance DENV and ZIKV infection by activating autophagy in host immune cells (Conway, Watson et al. 2014, Sun, Nie et al. 2020). In order for salivary proteins such as these to impact flavivirus infection in the host, they must either be secreted directly into the saliva or packaged in extracellular vesicles originating from the salivary gland then secreted into the saliva and later delivered during the transmission event (Vora, Zhou et al. 2018).

EXTRACELLULAR VESCILES REPRESENT AN UNDERSTUDIED COMPONENT OF VECTOR SALIVA

Extracellular vesicles, including microvesicles and exosomes, are believed to be secreted by all eukaryotic cells (Van Niel, D'Angelo et al. 2018, Raposo and Stahl 2019, Urbanelli, Buratta et al. 2019). Made up of a highly heterogenous population of membrane-bound vesicles of differing origin, size, and cargo, EVs are considered a mechanism of intercellular communication and as a result are involved in a multitude of physiological and pathological functions (Raposo and Stahl 2019, Urbanelli, Buratta et al. 2019). Only lately was it demonstrated that arthropod vectors secrete EVs, using a cell line derived from a clinically relevant arthropod (Vora, Zhou et al. 2018, Zhou, Woodson

et al. 2018, Reyes-Ruiz, Osuna-Ramos et al. 2019). While the role of arthropod EVs in flavivirus infection remains unclear, several recent studies have shown that mosquito and tick EVs are capable of mediating flavivirus transmission, and that flavivirus infection affects EV cargo (Hackenberg and Kotsyfakis 2018, Vora, Zhou et al. 2018, Zhou, Woodson et al. 2018). Similar results have been observed showing DENV-infection dependent modulation of EV cargo in the host, in this case in EVs derived from mammalian dendritic cells (Martins, Kuczera et al. 2018). Given their presence in vector saliva, EVs have the potential to be a critical component of the flavivirus transmission process, whether that be via delivery of virus or infectious RNA, or the packaging and delivery of pro-viral factors to host cells upon injection into the skin (Zhou, Woodson et al. 2018).

EXTRACELLULAR VESICLES AS A MECHANISM FOR THE DELIVERY OF PRO-VIRAL FACTORS

Due to their presence in vector saliva, capacity to harbor a wide range of cargo, and ability to deliver said cargo to a wide range of cell types, EVs represent a potential avenue for the delivery of pro-viral cargo that has yet to be investigated. While previous studies have examined the impact of flavivirus infection on the cargo of EVs originating from infected mammalian cells, mosquito EVs remain an understudied component of arbovirus infection and transmission. Herein, I describe the results of my work determining the impact of flavivirus infection on the protein cargo of *Aedes aegypti* EVs, and the potential of these infection-dependent changes to modulate virus transmission. Through the unique nature of this work, the results of my studies have the potential to

elucidate a novel mechanism of pro-viral factor delivery, further expanding on the existing canonical model of arbovirus transmission and the contribution of vector saliva to this process.

MATERIALS AND METHODS

Disclaimer: Portions of the text in this chapter were originally published as: (Gold, Feitosa-Suntheimer et al. 2020)

METHODS FOR IDENTIFICATION OF PRO-VIRAL FACTORS IN *AEDES AEGYPTI* SALIVA AND EXTRACELLULAR VESICLES

MOSQUITO REARING AND SALIVA COLLECTION

Aedes aegypti Rockefeller strain were kept in insectary conditions at 28°C, 80% humidity and light:dark cycle (12 hour:12 hour). Saliva from 8- to 10-day old female mosquitoes was collected following the protocols described previously with minimal modifications (Colton, Biggerstaff et al. 2005, Anderson, Richards et al. 2010). Briefly, a plexiglass (25.5 cm X 17.5 cm) was used as a platform to place the mosquitos. Sterile 200 μL pipette tips were then filled with 50 μL of sterile 1X phosphate buffered saline (PBS) and secured on the plexiglass using a clear tape to collect saliva. Wings and legs were removed from cold anesthetized mosquitos, and the proboscis were inserted into the pipette tip. Mosquitos were allowed to salivate for 20 minutes, then the pipette tip was removed and the content was pooled (200 mosquitoes per pool) in a sterile 1.5 mL tube. Saliva pools were kept at -80°C until further use. The protein concentration of the saliva was verified using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

CELL CULTURE CONDITIONS

Primary Dermal Fibroblast; Normal, Human, Adult (HDFa) (ATCC® PCS-202-012™, Manassas, VA, USA), Vero E6 cells, and 293T cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) (Gibco™ 11995065, Thermo Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Gemini 100-106) and 1% penicillin/streptomycin (Gibco™ 15140163, Thermo Scientific, Waltham, MA, USA) at 37°C with 5% CO_2. *Aedes aegypti* (ATC-10) (ATCC® CCL-125™, Manassas, VA, USA) cells were cultured in DMEM (Gibco™ 11995065) containing 10% FBS (Gemini 100-106), 1% penicillin/streptomycin (Gibco™ 15140163), and 1% tryptose phosphate broth (Gibco™ 18050039) at 30°C with 5% CO_2. Schneider's *Drosophila* Line 2 (S2) cells [*D. Mel.* (2), SL2] (ATCC® CRL-1963™, Manassas, VA, USA) were cultured in Schneider's *Drosophila* Medium (Gibco™ 21720024) containing 10% FBS (Gemini 100–106) and 1% penicillin/streptomycin (Gibco™ 15140163) at 28°C.

DETERMINATION OF ENHANCING SALIVARY PROTEINS BY HPLC AND LC-MS/MS

Mosquito saliva was fractionated by high-performance liquid chromatography (HPLC) on a nonporous reverse-phase column with a trifluoroacetic acid (TFA) buffer system into 80 fractions of 100 μL. Saliva fractions (final protein concentration 1 μg/mL) were incubated with DENV type-2 New Guinea C Strain (DENV-2) at a multiplicity of infection (MOI) of 0.1 for 15 minutes at 37°C. After this time, the saliva fraction/virus mixture was added to the cells. The wild-type (WT) group for this experiment was

fibroblasts treated with a saliva/virus mixture prepared the same way as those used to treat the experimental groups except using whole, unfractionated saliva. Fibroblasts were lysed 24 hours post-infection, and cellular RNA was isolated using the RNeasy Plus Mini Kit (Qiagen™ 74136, Hilden, Germany) according to the manufacturer's instructions. To determine the infection-modifying effect of each saliva fraction, this RNA was used to measure the DENV-2 viral load by quantifying the amount of DENV-2 viral RNA normalized to human β2 microglobulin (B2M) RNA by RT-PCR using QuantiFast SYBR Green PCR Kit (Qiagen™ 204056, Hilden, Germany), according to the manufacturer's instructions. RT-PCR was performed in duplicate using an RNA volume of 2.5 μL (40 ng). The primers used for the RT-PCR reactions to quantify DENV-2 RNA were designed to target the region of DENV-2 genome that encode for the virus E protein based on the sequence of DENV-2 (NC_001474) (Kinney, Butrapet et al. 1997). The primers used for the RT-PCR reactions to quantify human B2M were designed using the known human B2M gene sequence (NC_000015.10) (Zody, Garber et al. 2006). RT-PCR was performed using a Bio-Rad C1000 thermal cycler (Hercules, CA) combined with a Bio-Rad CFX96 detection module (Hercules, CA). The primers used include: DENV-2E_F: CATTCCAAGTGAGAATCTCTTGTCA, DENV-2E_R:CAGATCTCTGATGAATAACCAACG; Human B2M_F: CTCCGTGGCCTTAGCTGTG, Human B2M_R: TTTGGAGTACGCTGGATAGCC. The fractions that enhanced DENV-2 infection were submitted for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. This work was performed by the Interdisciplinary Center for Biotechnology Research at the University of Florida in

Gainesville, FL. An *Aedes aegypti* database was used for searching using Mascot-generated files from the MS/MS spectra.

AEDES AEGYPTI EXTRACELLULAR VESICLE ISOLATION AND PROTEIN PROCESSING

Aedes aegypti cells were treated with DENV-2 at MOI of 1. Following 2 hours of treatment with DENV-2, media and virus were aspirated, cells were washed three times with sterile PBS (Gibco™ 10010049), and then cells were cultured in DMEM containing 10% exosome-depleted FBS (Gibco™ A2720803, Thermo Scientific, Waltham), 1% penicillin/streptomycin (Gibco™ 15140163), and 1% tryptose phosphate broth (Gibco™ 18050039, Thermo Scientific, Waltham, MA, USA) for 72 hours. The cell supernatants following infection, or not, were collected and used to isolate extracellular vesicles using Invitrogen™ Total Exosome Isolation Reagent (Invitrogen™ 4478359, Thermo Scientific, Waltham, MA, USA), a method previously described for the isolation of EVs from *Aedes* cells (Tang, Huang et al. 2017, Vora, Zhou et al. 2018), according to the manufacturer's instructions. The proteins contained in these EVs were processed using the Thermo Scientific Pierce In-Solution Tryptic Digestion and Guanidination Kit, according to the manufacturer's instructions (Thermo Scientific™ 89895, Waltham, MA, USA).

MASS SPECTROMETRY

Each sample of digested peptides was desalted with a ZipTip® with 0.6 μL resin bed volume (Millipore, Burlington, MA, USA) according to the manufacturer's instructions. The eluent was dried and resuspended in 0.1% formic acid, and was analyzed using a Q Exactive HFX mass spectrometer connected to an Easy nLC 1200 ultra high-pressure

chromatography system (Thermo Scientific, Waltham, MA, USA). The samples were loaded onto a reverse-phase nano-trap column (75 μm interior diameter X 2 cm, Acclaim PepMap100 C18 3 μm, 100 Å, Thermo Fisher Scientific, Waltham, MA, USA) with mobile phase A (0.1% formic acid and 2% acetonitrile), and separated over an EASY-Spray column, (ES803A, 75 μm i.d. X 50 cm C18 2 μm, 100 Å, Thermo Fisher Scientific, Waltham, MA, USA) using a gradient (2% to 32% over 60 min) of mobile phase B (0.1% formic acid, 80% acetonitrile) at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 275°C and a potential of 2100 V applied to the emitter. All the data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution (60,000) MS precursor ion scan (350–1500 m/z range) was performed to select the 10 most intense ions for the subsequent fragmentation and MS/MS analysis using HCD (NCE 29 at 15,000 resolution) each duty cycle.

PEPTIDE IDENTIFICATION

The resulting RAW files were individually converted and searched using the MaxQuant platform (version 1.6.0.16; http://maxquant.org/) under standard settings. Searches were performed twice, once against all Uniprot entries in both Swiss-Prot and TrEMBL for *Aedes aegypti* (Taxonomy ID: 7159, downloaded 12 December 2018) and again against the vectorbase database for *Aedes aegypti* (downloaded 31 January 2019). Searches allowed for two missed trypsin cleavage sites and variable modifications of N-terminal acetylation and methionine oxidation. The carbamidomethylation of cysteine residues and guanidination of lysine residues were set as a fixed modification.

CLONING OF THE RECOMBINANT MOSQUITO PROTEIN AAEL002675

RNA from *Aedes aegypti* was isolated using the RNeasy Plus Mini Kit (Qiagen™ 74136, Hilden, Germany) according to manufacturer's instructions. A total of 5 μg of this RNA was then used to synthesize cDNA using the SuperScript III First-Strand Synthesis Super Mix (Thermo Fisher Scientific 18080400, Waltham, MA, USA). The gene encoding for AAEL002675 was amplified using this cDNA using the Phusion® High-Fidelity polymerase chain reaction (PCR) Master Mix with HF Buffer (New England BioLabs® M0531S, Ipswich, MA, USA) with primers designed for cloning of the PCR amplicon into the BglII and ApaI cut sites of the pMT/BiP/V5-His *Drosophila* expression vector (Invitrogen™ V413020). The PCR product of this reaction was cleaned using the QIAQuick PCR and Gel Cleanup Kit (Qiagen™ 28506, Hilden, Germany) according to the manufacturer's instructions. The PCR amplicon encoding for the mosquito protein of interest and the pMT/BiP/V5-His expression vector were digested using FastDigest BglII (Thermo Scientific™ FD0083, Waltham, MA, USA) and FastDigest ApaI (Thermo Scientific™ FD1414, Waltham, MA, USA) according to the manufacturer's instructions. The digested DNA was run on a 1% agarose gel then purified using the QIAQuick PCR and Gel Cleanup Kit (Qiagen™ 28506, Hilden, Germany) according to the manufacturer's instructions. The digested PCR amplicon encoding for the mosquito protein of interest was ligated with the digested pMT/BiP/V5-His expression vector using T4 DNA Ligase (New England BioLabs® M0202S, Ipswich, MA, USA), according to the manufacturer's instructions. The product of this ligation reaction was used to transform One Shot™ MAX Efficiency™ DH5α-T1^R Competent Cells (Invitrogen™ 12297016,

Thermo Scientific,Waltham, MA, USA) according to the manufacturer's instructions. The cloned plasmid was isolated from clones generated from the transformation reaction using the ZymoPURE™ Plasmid Miniprep Kit (Zymo Research D4212, Irvine, CA, USA). The successful cloning of the gene encoding the mosquito protein of interest in the pMT/BiP/V5-His *Drosophila* expression vector was then confirmed by sequencing the cloned plasmid.

EXPRESSION AND PURIFICATION OF MOSQUITO PROTEINS

S2 cells were seeded in T75 flasks and grown to about 80% confluency at 28°C. Each flask of these cells was then transfected with 10 μg of the previously cloned plasmid containing the gene encoding for the mosquito protein of interest using Effectene Transfection Reagent (Qiagen™ 301425, Hilden, Germany), according to the manufacturer's instructions, with the addition of $CuSO_4$ to a final concentration of 500 μM to induce protein expression. The cell supernatant was collected 72 hours post-transfection, and the recombinant mosquito protein of interest was purified using a HisPur Cobalt Spin Column (Thermo Scientific™ 89969,Waltham, MA, USA). The proteins were resolved via SDS-PAGE using a 4-20% Mini-PROTEAN® TGX™ precast protein gel (Bio-Rad 4651094, Hercules, CA, USA). Once resolved, the proteins were transferred to a nitrocellulose membrane using the Trans-Blot® Turbo transfer system (Bio-Rad 17001918, Hercules, CA, USA). The nitrocellulose membrane was incubated in 5% skim milk in TBST (blocking buffer) overnight at 4°C, then washed three times with TBST (washing buffer), and incubated with V5 Tag Monoclonal Antibody (Thermo Fisher Scientific R960–25, Waltham, MA, USA) overnight at 4°C, according to the

manufacturer's instructions. The nitrocellulose membrane was then washed three times and incubated with IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody (Li-Cor® Biosciences 925-32210, Lincoln, NE, USA) for two hours at room temperature, according to the manufacturer's instructions. The protein signal was detected using the Odyssey® CLX300 Near-Infrared Fluorescence Imaging System (Lincoln, NE, USA) (Figure S1). Once the purity was confirmed, the protein concentration was measured by the Micro BCA Protein Assay Kit (Thermo Scientific™ 23235, Waltham, MA, USA) according to the manufacturer's instructions.

TREATMENT OF FIBROBLASTS WITH MOSQUITO PROTEINS PRECEDING DENGUE INFECTION

Primary Dermal Fibroblast; Normal, Human, Adult (HDFa) (ATCC® PCS-201-012™, Manassas, VA, USA) were treated with proteins at a final concentration of 1 ng/mL for one hour and then infected with DENV-2, at a MOI of 0.1 and kept in the same conditions until use. In this case, the control group consisted of cells treated with AAEL001928 (*Aedes aegypti* Actin-1), which was previously observed in *Aedes aegypti* salivary gland extract and showed no enhancement or inhibition of DENV infection (Conway, Londono-Renteria et al. 2016). The AAEL001928 protein was cloned, synthesized, and purified using the same methods used to produce the AAEL002675 protein. The bovine serum albumin (BSA) group consisted of cells treated with BSA instead of mosquito protein. 24 hours post-infection, fibroblasts were lysed and cellular RNA was isolated using the RNeasy Plus Mini Kit (Qiagen™ 74136, Hilden, Germany) according to the manufacturer's instructions. The total RNA was used to measure the

DENV-2 viral load by quantifying the amount of DENV-2 viral RNA normalized to human B2M RNA by RT-PCR using the QuantiFast SYBR Green PCR Kit (Qiagen™ 204056, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed in duplicate using an RNA volume of 2.5 μL (40 ng). The primers used for the RT-PCR reactions to quantify DENV-2 RNA were designed to target the region of DENV-2 genome that encoded for the virus E protein based on the sequence of DENV-2 (NC_001474) (Kinney, Butrapet et al. 1997). The primers used for the RT-PCR reactions to quantify human B2M were designed using the known human B2M gene sequence (NC_000015.10) (Zody, Garber et al. 2006). RT-PCR was performed using a Bio-Rad C1000™ thermal cycler (Hercules, CA, USA) combined with a Bio-Rad CFX96™ detection module (Hercules, CA, USA). The primers used include: DENV-2E_F: CATTCCAAGTGAGAATCTCTTTGTCA, DENV-2E_R: CAGATCTCTGATGAATAACCAACG; Human B2M_F: CTCCGTGGCCTTAGCTGTG, Human B2M_R: TTTGGAGTACGCTGGATAGCC.

MEASURING EXPRESSION CHANGE OF AAEL002675 GENE UPON DENGUE INFECTION

Aedes aegypti (ATC-10) (ATCC® CCL-125™, Manassas, VA, USA) cells were infected with DENV-2, at a MOI of 0.1 and kept in the same conditions until use. Cells were lysed every 24 hours from 24 to 96 hours post-infection and cellular RNA was isolated using the RNeasy Plus Mini Kit (Qiagen™ 74136, Hilden, Germany) according to the manufacturer's instructions. RNA was used to measure the AAEL002675 expression levels by RT-PCR using the QuantiFast SYBR Green PCR Kit (Qiagen™ 204056,

Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed using a Bio-Rad C1000™ thermal cycler (Hercules, CA, USA) combined with a Bio-Rad CFX96™ detection module (Hercules, CA, USA). RT-PCR was performed in duplicate using an RNA volume of 2.5 μL (40 ng). The primers used for the RT-PCR reactions to quantify AAEL002675 RNA were designed based on the mRNA sequence of AAEL002675 (XM_001662007) (Nene, Wortman et al. 2007). The primers used for the RT-PCR reactions to quantify the *Aedes aegypti* actin RNA were designed based on the mRNA sequence of *Aedes aegypti* actin (DQ440059) (Ribeiro, Arcà et al. 2007). The expression levels were normalized to *Aedes aegypti* actin as the housekeeping gene. Log_2 expression was calculated using the normalized expression values of AAEL002675 calculated from the measured C_q values of AAEL002675 and *Aedes aegypti* actin from the RNA of infected and uninfected cells. The primers used include.: AAEL002675_F: CGGTATCCACGCTTTTGGGA, AAEL002675_R: GGAGCCTCAAGGACATCCAG; *Aedes aegypti* actin_F: GAACACCCAGTCCTGACA, *Aedes aegypti* actin_R: TGCGTCATCTTCTCACGGTTAG.

METHODS FOR DEMONSTRATION OF ARGIL1 AS A PRO-VIRAL FACTOR FOR FLAVIVIRUS INFECTION OF HUMAN CELLS

DENGUE INFECTION OF TARGET HUMAN CELLS

Primary Dermal Fibroblast; Normal, Human, Adult (HDFa) (ATCC® PCS-201-012™, Manassas, VA, USA) were seeded in 48 well plates at a density of 30,000 cells per well. The following day, media was aspirated and cells were infected with DENV-2 at a MOI of 1, mixed with DMEM, for 2 hours. After this time, virus and DMEM was removed and cells were washed with PBS (Gibco™ 10010049), and DMEM combined with 2% FBS (Gemini 100–106), 1% penicillin/streptomycin (Gibco™ 15140163) was added to cells. Cells were kept in culture at 37°C and 5% CO_2 until further use.

QUANTIFICATION OF DENGUE VIRUS INFECITON

RT-PCR

DENV-2 RNA was isolated from cell supernatants using the QIAamp Viral RNA Mini Kit (Qiagen™ 52906, Hilden, Germany) according to the manufacturer's instructions. This RNA was used to measure viral load by RT-PCR using the QuantiFast SYBR Green PCR Kit (Qiagen™ 204056, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed using a Bio-Rad C1000™ thermal cycler (Hercules, CA, USA) combined with a Bio-Rad CFX96™ detection module (Hercules, CA, USA). RT-PCR was performed in duplicate using an RNA volume of 2 μL. A standard curve generated using DENV-2 RNA standard was used for the absolute quantification of

DENV-2 RNA copies. The primers used for the RT-PCR reactions to quantify DENV-2 RNA were designed to target the region of DENV-2 genome that encoded for the virus E protein based on the sequence of DENV-2 (NC_001474) (Kinney, Butrapet et al. 1997). The primers used include: DENV-2E_F: CATTCCAAGTGAGAATCTCTTGTCA, DENV-2E_R:CAGATCTCTGATGAATAACCAACG

FOCUS FORMING ASSAY

Following DENV-2 infection, cell supernatants were collected and used to measure infection by focus forming assay. Vero E6 cells were seeded in 96 well plates at a density of 10,000 cells per well and cultured in DMEM (Gibco™ 11995065) containing 10% FBS (Gemini 100–106) and 1% penicillin/streptomycin (Gibco™ 15140163) at 37 °C with 5% CO_2. 24 hours after seeding, Vero E6 cells were treated with 100 μL of supernatants from DENV-2-infected cells in serial 10-fold dilution from undiluted to 10^{-7} diluted in DMEM (Gibco™ 11995065) for 2 hours at 37°C with 5% CO_2. After this time, supernatant was removed, cells were washed with PBS, and 100 μL of overlay consisting of a 1:1 ratio of half DMEM with 2% FBS and 1% penicillin/streptomycin and half 5% carboxymethyl cellulose in PBS was added to cells. Infection was allowed to progress for 7 days, after which the overlay was aspirated, cells were washed with PBS, then fixed with 4% paraformaldehyde (Thermo Scientific™ J19943.K2, Waltham, MA, USA) supplemented with 0.1% Triton X-100 ((Thermo Scientific™ A16046.AP, Waltham, MA, USA) for 30 minutes at room temperature. Fixing solution was then aspirated, cells were washed with PBS again, then blocked with 1% BSA (Millipore Sigma B6917) in PBS at

room temperature for 1 hour. Following this time, blocking buffer was removed and cells were incubated with flavivirus group antigen antibody (D1-4G2-4-15 (4G2)) (Novus Biologicals, NBP-52709, Centennial, CO, USA) diluted 1:1000 in 1% BSA in PBS for 2 hours at room temperature. After this time, antibody was removed, cells were washed 3 times with PBS, and cells were incubated with IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody (LI-COR® Biosciences, Lincoln, NE, USA) diluted 1:1000 in 1% BSA in PBS for 2 hours at room temperature. After this time, antibody was removed, cells were washed 3 times with PBS, PBS was aspirated, and cells were allowed to dry. Focus forming assays were performed in duplicate. Foci were identified by the detection of DENV-2 E-specific signal in the 700 nm channel using the Odyssey CLX300 Near-Infrared Fluorescence Imaging System (LI-COR® Biosciences, Lincoln, NE, USA).

ARG1/ARGIL1 TREATMENT OF FIBROBLASTS PRIOR TO DENGUE INFECTION

Primary Dermal Fibroblast; Normal, Human, Adult (HDFa) (ATCC® PCS-201-012™, Manassas, VA, USA) were seeded at a desired density in a 48 well plate and left to grow in DMEM (Gibco™ 11995065) containing 10% FBS (Gemini 100–106) and 1% penicillin/streptomycin (Gibco™ 15140163) at 37°C with 5% CO_2. 24 hours after cell seeding, media was aspirated and cells were treated with arginase proteins, either human arginase (ARG1) (Sino Biological Inc., 11558-H08H) or *Aedes aegypti* arginase-like protein 1 (ARGIL1), or BSA, mixed with DMEM (Gibco™ 11995065), or untreated, for 1 hour at 37°C with 5% CO_2. DENV-2 was then added to cells at a MOI of 1, and cells were left to incubate for 2 hours at 37°C with 5% CO_2. Following this time, the DENV-

2/protein/DMEM mixture was aspirated, cells were washed with PBS, then DMEM (Gibco™ 11995065) containing 2% FBS (Gemini 100–106) and 1% penicillin/streptomycin (Gibco™ 15140163) was added and cells were left at 37°C with 5% CO_2 until further use.

QUANTIFICATION OF ARG1 ARGINASE ACTIVITY

Arginase activity of ARG1 (Sino Biological Inc., 11558-H08H) was measured using the Arginase Activity Assay Kit (Sigma-Aldrich®, MAK112) according to the manufacturer's instructions. Arginase activity assays were performed in duplicate using 140ng of protein per reaction.

INHIBITION OF ARG1/ARGIL1 ARGINASE ACTIVITY

Arginase activities of ARG1 and ARGIL1 were inhibited using the competitive arginase inhibitor S-(2)-boronoethyl)-L-cysteine (BEC) (ApexBio Technology, C3215). BEC was diluted in tissue-culture grade water to a stock concentration of 10 mM. Protein was mixed with inhibitor diluted in either PBS (activity assays) or DMEM (cell treatment) and incubated for 20 minutes at 37°C. After this time, protein/inhibitor mixtures were either used to measure arginase activity by arginase activity assay, or to treat cells before infection with DENV-2.

Before using BEC for any infection experiments, the cytotoxic effect of BEC on cells was measured. Primary Dermal Fibroblast; Normal, Human, Adult (HDFa) (ATCC® PCS-201-012™, Manassas, VA, USA) were treated with BEC for 3 hours at 37°C with 5% CO_2. After this time, cell viability was measured in duplicate using the CellTiter-Glo® 2.0 Cell Viability Assay (Promega G9241) (Figure S2).

EXPRESSION OF RECOMBINANT ARGINASE PROTEINS

The genes encoding for both ARG1 and ARGIL1 were cloned into the mammalian expression vector pCDNA3 by restriction and ligation. The ARG1 gene was amplified by PCR from the bacterial plasmid HAI (Addgene plasmid #71316), and the ARGIL1 gene was amplified from *Aedes aegypti* cDNA as done previously in the cloning of AAEL002675. Once amplified, these PCR amplicons were restricted, then ligated with the restricted plasmid pCDNA3-GFP (Addgene, plasmid #74165) using T4 DNA Ligase (New England BioLabs® M0202S, Ipswich, MA, USA). The products of these ligation reactions were used to transform One Shot™ MAX Efficiency™ DH5α-T1^R Competent Cells (Invitrogen™ 12297016, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The cloned plasmids were isolated from clones generated from the transformation reaction using the ZymoPURE™ Plasmid Miniprep Kit (Zymo Research D4212, Irvine, CA, USA). The successful cloning of the genes encoding for ARG1 and ARGIL1 into the pCDNA3 mammalian expression vector was then confirmed by the sequencing of the cloned plasmid.

293T cells were transfected with 1 μg of plasmid using 5 μL of Cellfectin™ II Reagent (Gibco™, 10362100), according to the manufacturer's instructions. Following incubation with transfection complexes, transfection media was removed, cells were washed with PBS, then DMEM 2% FBS media was added and cells were left in culture at 37°C with 5% CO_2 until further use. To confirm protein expression, cells were lysed at 24, 48, and 72 hours post-transfection using RIPA buffer (Thermo Scientific, 89900) and proteins were resolved via SDS-PAGE using a 4-20% Mini-PROTEAN® TGX™ precast

protein gel (Bio-Rad 4651094, Hercules, CA, USA). Once resolved, the proteins were transferred to a nitrocellulose membrane using the Trans-Blot® Turbo transfer system (Bio-Rad 17001918, Hercules, CA, USA). The nitrocellulose membrane was incubated in 5% skim milk in TBS with 0.05% Tween 20 (blocking buffer) overnight at 4°C, then washed three times with TBS containing 0.05% Tween 20 (washing buffer), and incubated with His Tag Antibody (R&D Systems, MAB050) diluted in 5% skim milk in TBS with 0.05% Tween 20 overnight at 4°C, according to the manufacturer's instructions. The nitrocellulose membrane was then washed three times with TBS with 0.05% Tween 20 (washing buffer), then incubated with IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody (Li-Cor® Biosciences 925-32210, Lincoln, NE, USA) diluted in 5% skim milk in TBS with 0.05% Tween 20 for two hours at room temperature, according to the manufacturer's instructions. The protein signal was detected using the Odyssey® CLX300 Near-Infrared Fluorescence Imaging System (LI-COR® Biosciences, Lincoln, NE, USA) (Figure S3). Before lysis, cells transfected with pCDNA3-EGFP were imaged for GFP expression using the EVOS Cell Imaging System (Thermo Fisher) (Figure S3).

QUANTIFICATION OF INNATE IMMUNE GENE EXPRESSION

Cellular RNA was isolated from cells following DENV infection using the RNeasy Plus Mini Kit (Qiagen™ 74136, Hilden, Germany) according to the manufacturer's instructions. This RNA was used to measure expression of immune genes including IFN-α, IFN-β, and ISG-15 by RT-PCR using the QuantiFast SYBR Green PCR Kit (Qiagen™ 204056, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was

performed using a Bio-Rad C1000™ thermal cycler (Hercules, CA, USA) combined with a Bio-Rad CFX96™ detection module (Hercules, CA, USA). RT-PCR was performed in duplicate using an RNA volume of 2.5 μL (40 ng). Gene expression was normalized to the housekeeping gene human β2 microglobulin (B2M). The primers used for the RT-PCR reactions to quantify human IFN-α were designed using the known human IFN-α gene sequence (NM_006900.4). The primers used for the RT-PCR reactions to quantify human IFN-β were designed using the known human IFN-β gene sequence (NM_002176.4). The primers used for the RT-PCR reactions to quantify human ISG15 were designed using the known human ISG15 gene sequence (NM_005101.4). The primers used for the RT-PCR reactions to quantify human B2M were designed using the known human B2M gene sequence (NC_000015.10) (Zody, Garber et al. 2006).The primers used include: Human B2M_F: CTCCGTGGCCTTAGCTGTG, Human B2M_R: TTTGGAGTACGCTGGATAGCC; IFN-α_F: GCCTCGCCCTTTGCTTTACT, IFN-α_R: CTGTGGGTCTCAGGGAGATCA; IFN-β_F: ATGACCAACAAGTGTCTCCTCC, IFN-β_R: GGAATCCAAGCAAGTTGTAGCTC; ISG-15_F: CGCAGATCACCCAGAAGATCG, ISG15_R: TTCGTCGCATTTGTCCACCA.

MEASUREMENT OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION

Following infection of Primary Dermal Fibroblast; Normal, Human, Adult (HDFa) (ATCC® PCS-201-012™, Manassas, VA, USA) with DENV-2 at MOI 1, cells were lysed and lysates were used to quantify intracellular concentration of inducible nitric oxide

synthase (iNOS) using the Human iNOS enzyme-linked immunosorbent assay (ELISA) Kit (Abcam ab253217) according to the manufacturer's instruction

CHAPTER ONE: DENGUE INECTION OF *AEDES AEGYPTI* MODULATES EXTRACELLULAR VESICLE PROTEIN CARGO

Disclaimer: Data and portions of the text in this chapter were originally published as: (Gold, Feitosa-Suntheimer et al. 2020)

AEDES AEGYPTI SALIVA CONTAINS INFECTION ENHANCING PROTEINS

Mosquito saliva as a whole has been shown to enhance infection in mammals for several arboviruses (Cox, Mota et al. 2012, Pingen, Steven et al. 2016, Wichit, Diop et al. 2017), yet there is a lack of information regarding the identification of individual saliva proteins with an impact on viral infection. Our group and others have identified several proteins in *Aedes aegypti* saliva that have either enhancing or inhibitory effects on DENV infection in mammalian cells (Conway, Watson et al. 2014, Conway, Londono-Renteria et al. 2016, Sun, Nie et al. 2020). To measure the effect of individual salivary proteins on DENV infection, we used a method developed and optimized in our lab to separate (fractionate) saliva proteins, evaluate the impact of each fraction on DENV infection in human cells, and then identify the proteins present in fractions with infection-enhancing properties. Unlike previous studies (Sim, Ramirez et al. 2012, Sri-In, Weng et al. 2019), which isolated proteins from homogenized salivary gland tissues (termed salivary gland extract or SGE), here we used isolated mosquito saliva to ensure that the identified proteins have relevance in mosquito-to-human transmission in nature. To do this, saliva was collected from blood-fed *Aedes aegypti* and fractionated by high-performance liquid

chromatography (HPLC), which separated proteins into 80 fractions on the basis of their hydrophobicity (Figure 1.1).

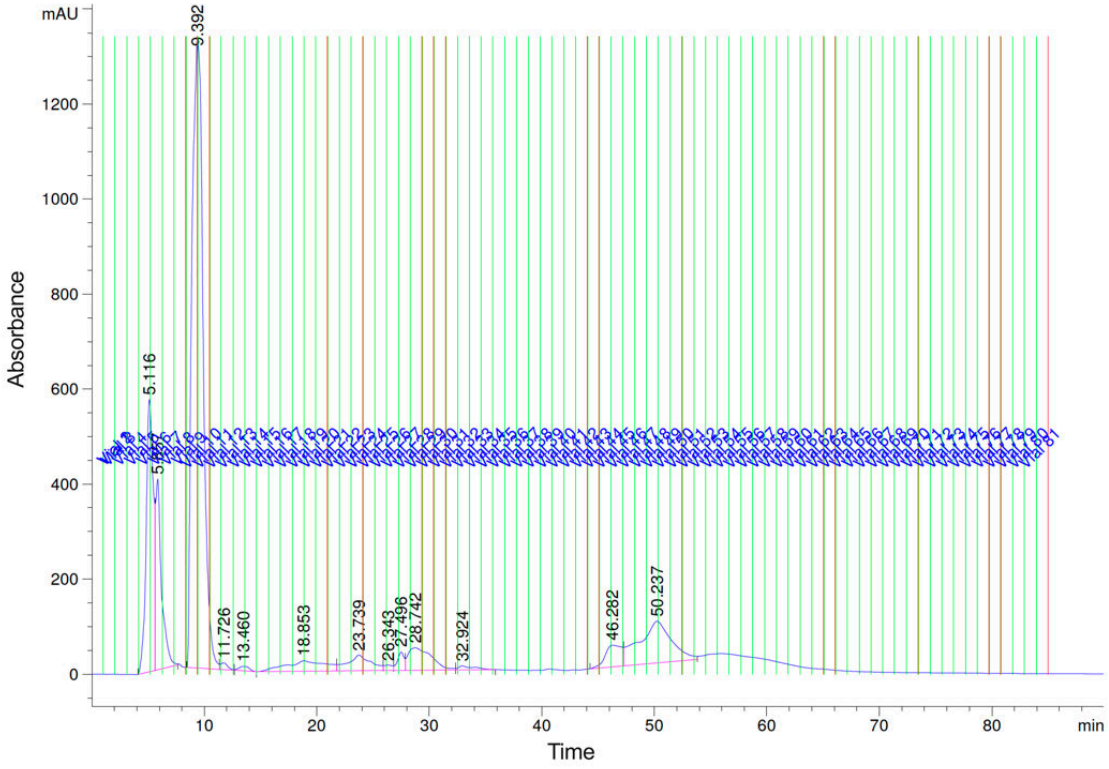

Figure 1.1 Fractionation of *Aedes aegypti* saliva by HPLC. Saliva was collected from female *Aedes aegypti* after blood feeding. Mosquito saliva was fractionated by HPLC on a nonporous reverse-phase column with a TFA buffer system into 80 fractions of 100 µL each.

Following fractionation, to assess the effect of salivary proteins on DENV infection, human fibroblasts were simultaneously treated with saliva fractions and infected with DENV. RNA was isolated from these fibroblasts 24 hours post-infection and used to quantify DENV infection by RT-PCR. When compared to control cells infected with DENV but treated with whole unfractionated saliva, many saliva fractions had an impact on infection, with several demonstrating substantial infection-enhancing activity (Figure 1.2). To identify which proteins were present and putatively responsible for greater levels of infection in the fibroblasts, these saliva fractions were subjected to

LC/MS-MS analysis, and the data generated from this analysis were used to compile a list of proteins found in each fraction (Table 1.1).

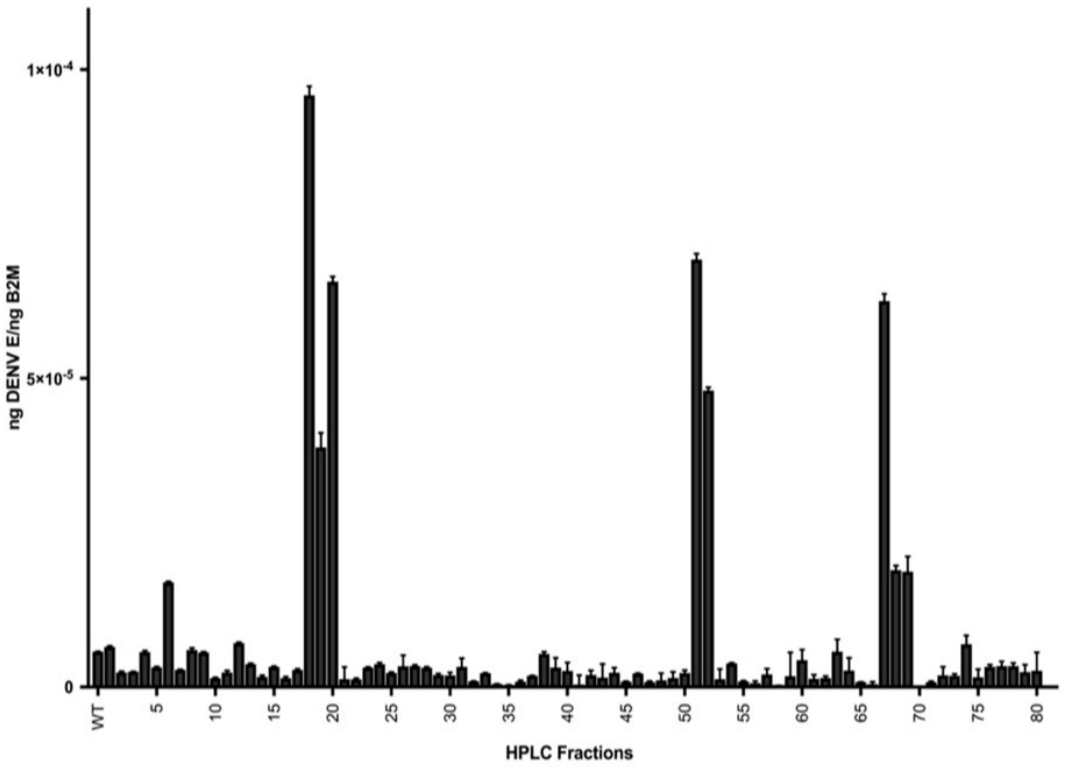

Figure 1.2 *Aedes aegypti* Saliva Fractions Enhance DENV Infection in Cells. Human primary dermal fibroblast cells were simultaneously treated with 80 *Aedes aegypti* saliva fractions and infected with DENV-2. WT cells were treated with whole saliva and DENV-2, instead of saliva fractions. 24 hours post-infection, cells were lysed and RNA was collected and used to quantify the DENV-2 viral load by RT-PCR. Data presented are ng RNA, calculated using previously determined standard curves for DENV-2 E mRNA and human B2M mRNA. Results are the mean (*n*=3) ± standard error of the mean of three biological replicates.

Protein SeqID	Fraction Observed	Putative Function-Vector Base[1]	Putative Function-Blastp[2]	Accession
AAEL000299-PA	A20	Unknown	Zinc finger protein 490 (*Aedes aegypti*)	XP_001654769.2
AAEL000794-PA	A18, A19,	Unknown	Clustered mitochondria	Q17N71.1

	A20, A51, A52		protein homolog	
AAEL000913-PA	A19, A20, A68	Cuticle Protein	Cuticle Protein	XP_001651656.2
AAEL002675-PA	A18, A19, A20, A52	Arginase	Arginase	XP_001662057.1
AAEL005493-PA	A18, A19, A20, A52, A69	Septin	Septin-1 isoform X2	XP_021704188.1
AAEL006525-PA	A20, A52	Kelch Repeat Protein	Kelch domain-containing protein 3	XP_001652008.2
AAEL006528-PA	A20	No Match	AAEL006528-PA (*Aedes aegypti*)	EAT41874.1
AAEL006844-PA	A19, A20, A25	GPCR Octopamine/Tyramine Family	Probably G-protein coupled receptor No18	XP_001652255.3
AAEL007354-PA	A67	Pseudouridylate synthase	tRNA pseudouridine synthase A, mitochondrial	XP_001658327.1
AAEL009062-PA	A19	Uncharacterized	Voltage-dependent calcium channel	XP_001679677.2
AAEL009533-PA	A68	F-box protein 25/32, Fox0 signaling pathway	F-box only protein 25 (*Aedes aegypti*)	XP_001660205.1
AAEL009824-PA	A20, A67	Ubiquitin specific protein 9/faf	Probably ubiquitin carboxyl-terminal hydrolase FAF (*Aedes*	XP_021705402.1

			aegypti)	
AAEL010440-PA	A67	Budd22/Serum response factor-binding protein 1	Nucleolin (*Aedes aegypti*)	XP_001660823.1
AAEL010962-PA	A18, A19	No match	Gustatory receptor 73 (*Aedes aegypti*)	NP_001345229.1
AAEL012615-PA	A67	No match	Uncharacterized protein LOC5576554 (*Aedes aegypti*)	XP_021705756.1

Table 1.1 Proteins Found in Enhancing Fractions of Mosquito Saliva

[1]Putative function according to VectorBase: Aedes aegypti protein database: https://vectorbase.org/

[2]Blastp-NCBI using non-redundant protein sequence (nr) and Aedes aegypti (taxid: 7159) databases at: https://blast.ncbi.nlm.nih.gov/Blast.cgi

DENGUE VIRUS INFECTION ALTERS PROTEIN CARGO OF *AEDES AEGYPTI* EXTRACELLULAR VESICLES

Although the ability of mosquito EVs to mediate flavivirus infection *in vitro* has been reported (Vora, Zhou et al. 2018), the protein cargo of these EVs, and any potential impact this cargo may have on flavivirus infection, remain to be determined. To assess how DENV infection affected extracellular vesicular protein packaging, EVs were isolated from DENV-infected and uninfected *Aedes aegypti* cells. Total proteins were isolated from these EVs, and samples were subjected to LC/MS-MS to determine which proteins were found in the EVs from DENV-infected cells, uninfected cells, or both EV sample types (infected and uninfected). The majority of the 88 proteins identified (58%)

were found in both uninfected and infected cells, which was expected as EVs contain a wide range of proteins that are necessary for their functions (Figure 1.3). However, several proteins were exclusively found in the EVs derived from DENV-infected cells (27%) (Figure 1.3 and Table 1.2), supporting an infection-dependent protein packaging strategy, similar to that previously observed in EVs derived from DENV-infected human dendritic cells (Martins, Kuczera et al. 2018).

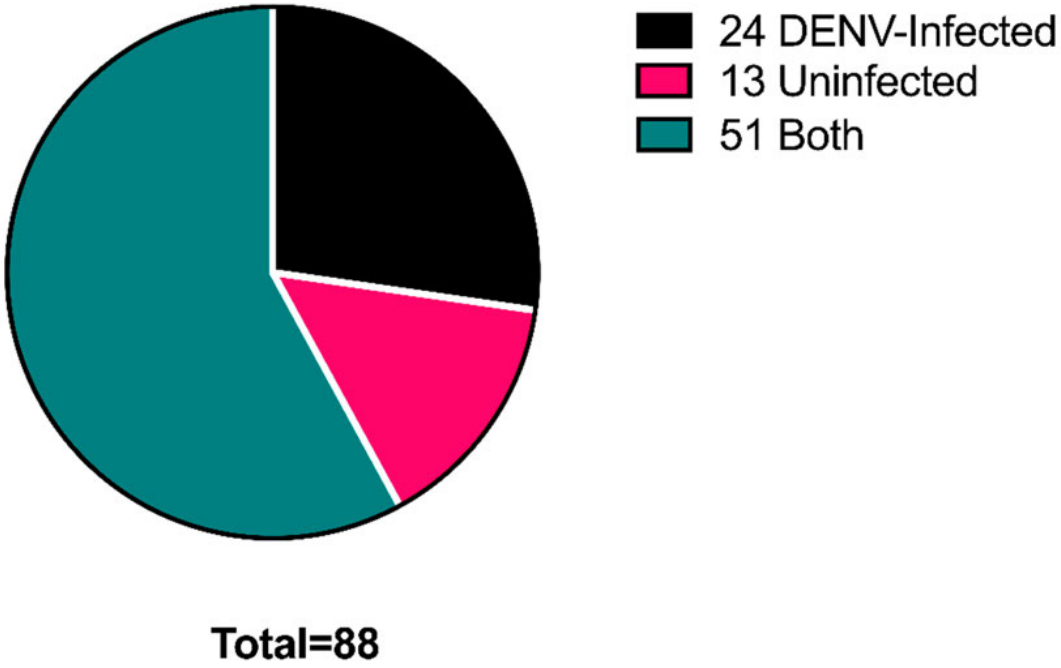

Figure 1.3 DENV Infection Modulates *Aedes aegypti* EV Protein Cargo. Extracellular vesicles were isolated from DENV-2 infected and uninfected *Aedes aegypti* cells. Proteins from both EV samples were processed and identified by mass spectrometry.

Gene ID VectorBase	Protein Vector Base	Putative Function-Vector Base[1]	Putative Function-Blastp[2]	Accession
AAEL000511	AAEL000511-PC	Acetylcholinesterase	Acetylcholinesterase isoform	XP_001656977.3

		(Fragment)	X1 (*Aedes aegypti*)	
AAEL001493	AAEL001493-PC	Laminin, N-terminal	Laminin subunit alpha-1 isoform X1 (*Aedes aegypti*)	XP_021700673.1
AAEL002675	AAEL002675-PA	Arginase	Arginase, hepatic (*Aedes aegypti)*	XP_001662057.1
AAEL003402	AAEL003402-PB	Sphingomyelin phosphodiesterase	AAEL003402-PB (*Aedes aegypti*)	EAT45277.1
AAEL003413	AAEL003413-PA	F-spondin	Spondin-1 (*Aedes aegypti)*	XP_001656777.2
AAEL003723	AAEL003723-PA	C-Type Lysozyme (Lys-A)	Lysozyme-like (*Aedes aegypti*)	XP_021699294.1
AAEL005951	AAEL005951-PC	Lipid storage droplets surface binding protein	Lipid storage droplets surface-binding protein 1 isoform X1 (*Aedes aegypti*)	XP_021693333
AAEL006240	AAEL006240-PA	Purple acid phosphatase, putative	Select seq ref|XP_001651840.1|	XP_001651840.1.1
AAEL006434	AEL006434-PA	Serine protease, putative	Serine protease 7 isoform X2 (*Aedes aegypti*)	XP_021703558.1
AAEL007992	AAEL007992-PB	Trypsin, putative	Serine protease 7 isoform X1 (*Aedes aegypti*)	XP_021693694.1
AAEL009038	AAEL009038-PB	Prolylcarboxypeptidase, putative	Putative serine protease F56F10.1 (*Aedes aegypti*)	XP_021697410.1
AAEL009345	AAEL009345-PA	Prohibitin	Protein 1(2)37Cc (*Aedes aegypti*)	XP_001653792.1
AAEL011271	AAEL011271-PA	PDCD6IP	AAEL011271-PA (*Aedes aegypti*)	EAT36654

AAEL012326	AAEL012326-PA	Calmodulin family	AAEL012326-PA (*Aedes aegypti*)	EAT35514.1
AAEL013620	AAEL013620-PA	Ras-related protein	AAEL013620-PA (*Aedes aegypti*)	EAT34116.1
AAEL013952	AAEL013952-PE	Prohibitin	AAEL013952-PA (*Aedes aegypti*)	EAT33777.1
AAEL014566	AAEL014566-PD	Wingless	AAEL014566-PA, partial (*Aedes aegypti*)	EAT32499.1
AAEL015038	AAEL015038-PA	Palmitoyl-protein thioesterase	Palmitoyl-protein thioesterase 1 (*Aedes aegypti*)	XP_001650360.2
AAEL015235	AAEL015235-PA	Flotillin subfamily	AAEL015235-PA, partial (*Aedes aegypti*)	EAT32605.1
AAEL017301	AAEL017301-PA	Elongation factor 1-alpha	AAEL017301-PA (*Aedes aegypti*)	EJY57625.1
AAEL017982	AAEL023321-PA	HSP70	Heat shock 70 Cb (*Aedes aegypti*)	ACJ64198.1
AAEL020330	AAEL020330-PA	Unknown	Heat shock protein 70 A1-like (*Aedes aegypti*)	XP_021693654.1
AAEL021904	AAEL021904-PA	Unknown	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 (*Aedes aegypti*)	XP_021702720.1
AAEL024406	AAEL024406-PB	Unknown	Uncharacterized protein LOC5572108 isoform X3	XP_021699023.1

			(*Aedes aegypti*)	

Table 1.2 Proteins Found in Extracellular Vesicles from DENV-Infected Mosquito Cells

[1]Putative function according to VectorBase: *Aedes aegypti* protein database: https://vectorbase.org/

[2]Blastp-NCBI using non-redundant protein sequence (nr) and *Aedes aegypti* (taxid: 7159) databases at: https://blast.ncbi.nlm.nih.gov/Blast.cgi

24 proteins were observed only in EVs isolated from DENV-infected *Aedes aegypti* cells (Table 1.2). Putative functions were identified for several of these proteins, including receptors, proteases, and cuticle proteins, among others. Of these proteins, AAEL002675 was the only protein found in EVs derived from only DENV-infected cells and in multiple fractions of mosquito saliva that displayed infection-enhancing activity in vitro (Figure 1.2). To demonstrate the intrinsic DENV infection-enhancing ability of AAEL002675, the recombinant protein was overexpressed in *Drosophila melanogaster* cells, purified, and used to pre-treat human fibroblast cells before infection with DENV. The addition of AAEL002675 to these cells resulted in the robust enhancement of the DENV intracellular viral RNA (Figure 1.4), greatly supporting the infection-enhancing ability of this protein.

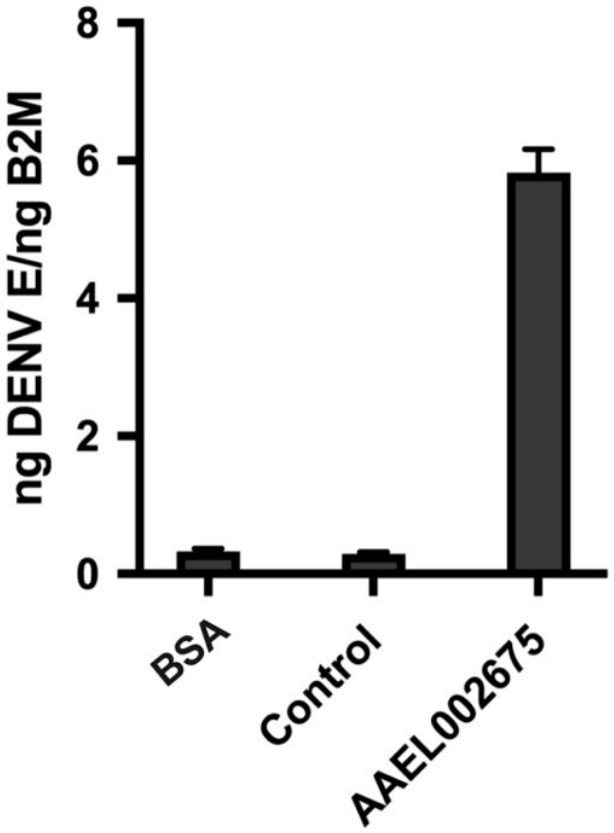

Figure 1.4 Treatment with AAEL002675 Enhances DENV Infection in Cells. Human primary dermal fibroblast cells were treated with either BSA, control (AAEL001928; Actin-1), or AAEL002675 for one hour before infection with DENV-2 at a MOI of 0.1. 24 hours post-infection, cells were lysed and RNA was collected and used to quantify the DENV-2 viral RNA by RT-PCR. Data presented here are ng RNA calculated using previously determined standard curves for DENV-2 E mRNA and Human B2M mRNA. Results are the mean (*n*=3) ± standard error of the mean of three biological replicates.

EXPRESSION OF AAEL002675 WAS UPREGULATED IN MOSQUITO CELLS UPON INFECTION

To better understand the DENV infection-dependent packaging of AAEL002675 in EVs, it was important to examine how DENV infection affected the expression of the AAEL002675 gene in *Aedes aegypti*. In doing so, *Aedes aegypti* cells were infected with

DENV and the level of AAEL002675 gene expression was measured from 24 to 96 hours post-infection (Figure 1.5). At all times following infection, an increase in the expression of the AAEL002675 gene was observed, with a pronounced peak of expression at 72 hours post-infection, suggesting that the infection-induced upregulation of the AAEL002675 gene may drive the increased packaging of this protein into EVs.

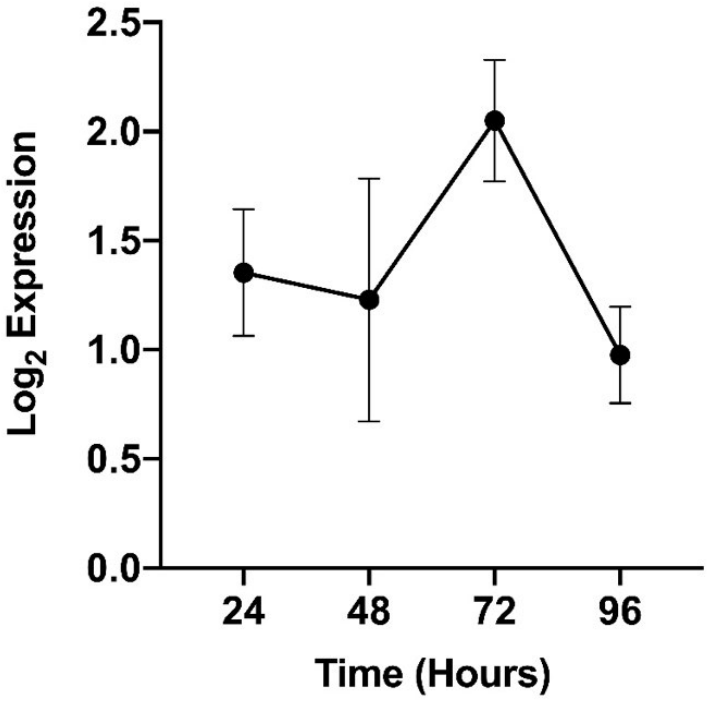

Figure 1.5 Expression of AAEL002675 Gene Increases upon DENV Infection. ***Aedes aegypti*** **cells were infected with DENV-2. At the given timepoints after infection, cells were lysed and RNA was collected and used to quantify AAEL002675 gene expression by RT-PCR. Results are the mean (*n=3*) ± standard error of the mean of three biological replicates.**

CHAPTER TWO: THE *AEDES AEGYPTI* PROTEIN ARGIL1 IS AN ARGINASE DEPENDENT PROVIRAL FACTOR

DESCRIPTION OF ARGIL1 AND ARG1 AS PRO-VIRAL FACTORS FOR FLAVIVIRUS INFECTION

While its enzymatic activity has not been directly demonstrated, sequence homology and metabolic studies heavily suggest that the *Aedes aegypti* protein AAEL002675, referred to as arginase-like protein 1 (ARGIL1), functions as an arginase (Von Dungern and Briegel 2001, Nene, Wortman et al. 2007, Isoe and Scaraffia 2013). Due to the role of ARG1 in metabolizing L-arginine, the precursor molecule of NO (a key effector mechanism in antiviral defense), and its transcriptional relationship with iNOS (the enzyme responsible for NO synthesis), this enzyme has been shown to play a pro-viral role in several viral infections, including Hepatitis C virus and Chikungunya virus (Cao, Sun et al. 2009, Burrack and Morrison 2014, Lombardi Pereira, Suzukawa et al. 2019). Although the function of ARG1 during DENV infection has not yet been elucidated, the function of NO during DENV infection is well characterized, and while the exact mechanism has yet to be determined, NO has been shown to inhibit DENV replication by impeding the polymerase activity of the viral protein NS5 (Takhampunya 2006, Chaturvedi and Nagar 2009). Given the ability of arginase to metabolize L-arginine and interfere with/decrease NO production, introduction of exogenous ARG1 or ARGIL1 in the extra- and intra-cellular compartment could then interfere with the induction of effective antiviral innate immune responses, ultimately creating an intracellular environment more permissive to viral replication.

ARIGIL1 ENHANCES DENGUE INFECTION IN HUMAN CELLS

Given that arginase has known roles as a pro-viral factor in the pathogenesis of several viruses (Cao, Sun et al. 2009, Burrack and Morrison 2014, Lombardi Pereira, Suzukawa et al. 2019), it was important to use a known arginase protein, in this case ARG1, as a control. Human adult primary dermal fibroblast cells were treated with increasing concentrations of ARG1, ARGIL1, BSA, or untreated, then infected with DENV. Groups treated with ARG1/ARGIL1 at a concentration of 500 nM displayed significantly greater number of viral particles in cell supernatants at 24 hours post-infection, as indicated by levels of viral RNA in supernatants, compared to those of cells treated with BSA (Figure 2.1).

To support the establishment of a greater level of productive infection following ARG1/ARGIL1 treatment, we conducted a longitudinal evaluation of the impact of ARGIL1 on DENV infection. Human fibroblasts were treated with ARG1, ARGIL1, and BSA at a concentration of 500 nM prior to infection with DENV. At 48 hours post-infection, cell supernatants were collected and used to collect viral RNA, and DENV viral RNA was quantified by RT-PCR, which showed that at both 24 (Figure 2.1) and 48 hours post-infection 500 nM ARG1/ARGIL1 treatment of cells resulted in significantly greater levels of DENV RNA copies in the cell culture supernatant, compared to those treated with BSA (Figure 2.2A). While viral RNA in the supernatant is a surrogate reflective of the number of viral particles in the supernatant, it is not an absolute quantification of the infectious virions present in a sample. In order to show that ARG1/ARGIL1 treatment resulted in the enhanced production of infectious virions, cell

supernatant samples used for previous RT-PCR (Figure 2.2A) were used to perform focus forming assays. The results of these assays showed that at 24 (Figure 2.2B) and 48 (Figure 2.2C) hours post-infection, consistent with our RT-PCR results (Figure 2.2A), ARG1/ARGIL1 treatment was associated with superior production of infection DENV particles in comparison to cells treated with BSA. Taken together, our results further support the role of ARGIL1 as a pro-viral factor and infection-enhancing protein during DENV transmission.

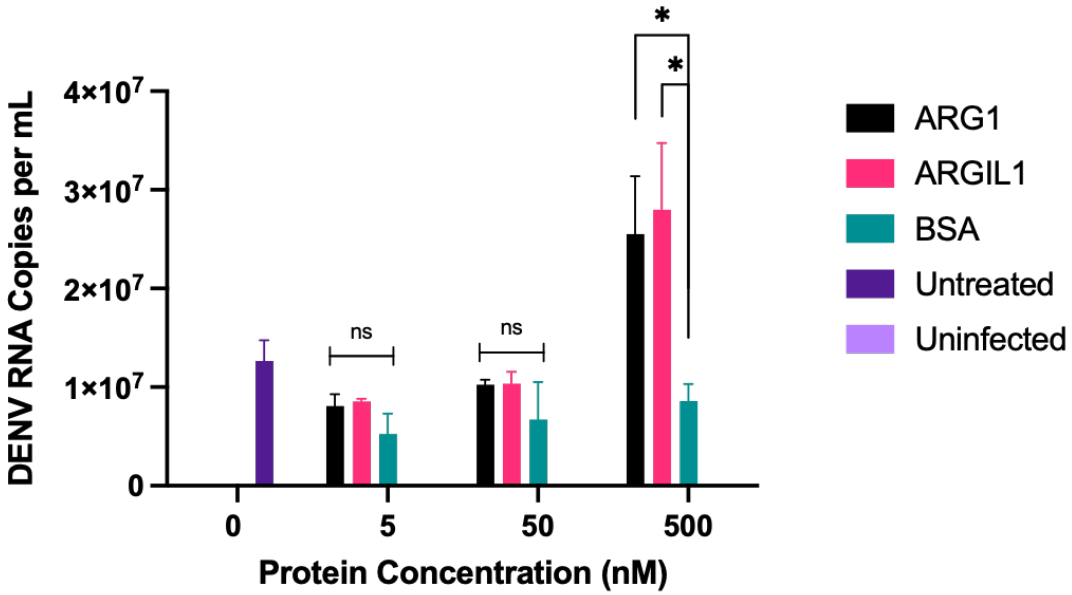

Figure 2.1 Treatment of Cells with Increasing Concentration of Arginase Enhances Dengue Infection. Adult human dermal fibroblasts were pre-treated with increasing concentration of proteins then infected with DENV-2 at MOI of 1. Supernatant viral RNA was collected 24 hours post-infection and used to quantify DENV-2 RNA by RT-PCR. Results are the mean (n=3) ± standard deviation of the mean of greater than three biological replicates. Welch's t-test. *$p \leq 0.05$.

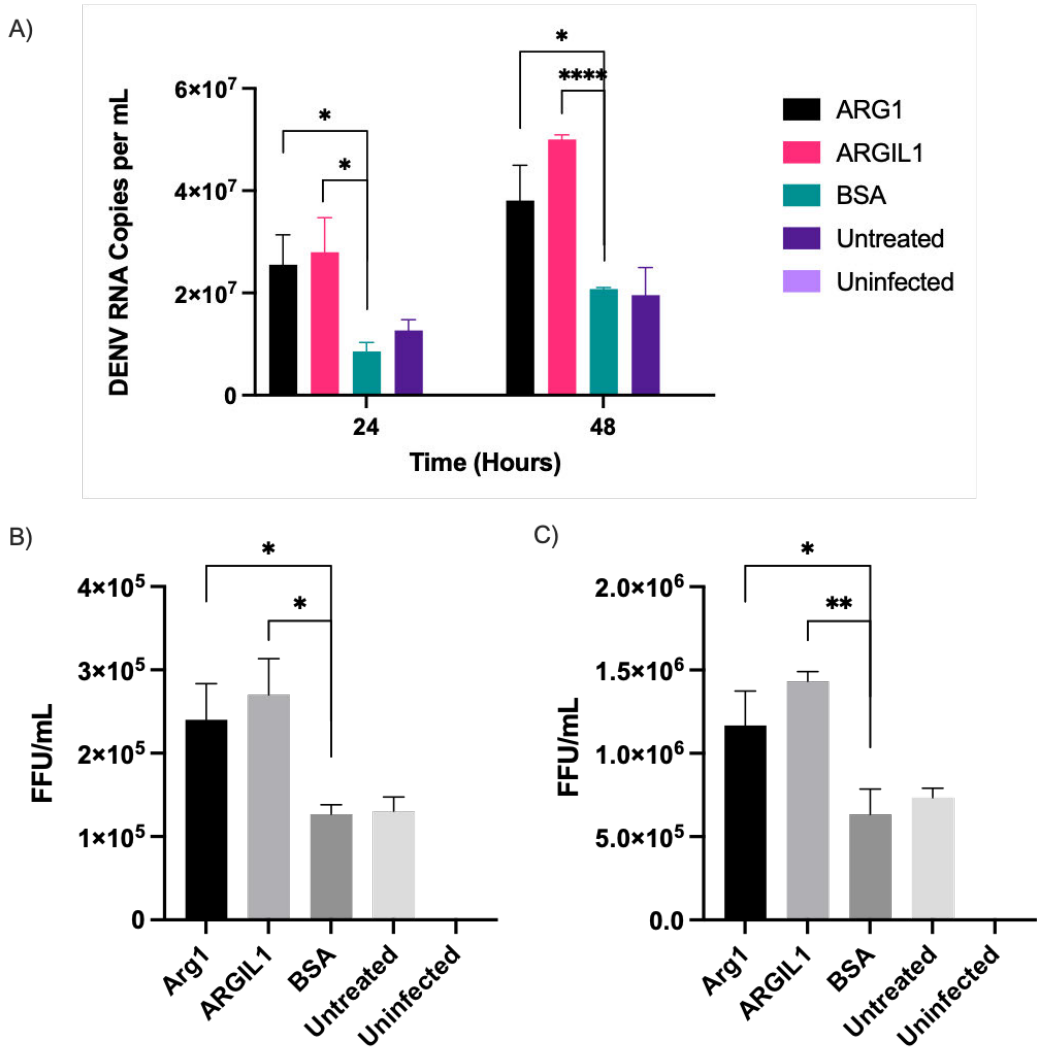

Figure 2.2 Enhanced Infection in Cells by Arginase Treatment is Maintained Over Time. Adult human dermal fibroblasts were pre-treated with protein at 500 nM then infected with DENV-2 at MOI of 1. Supernatant was collected at 24 and 48 hours post-infection. A) Viral RNA was collected from supernatants and used to quantify DENV-2 RNA by RT-PCR. Supernatants were also used to measure viral load at B) 24 and C) 48 hours post-infection by focus forming assay. Results are the mean (n=3) ± standard deviation of the mean of three biological replicates. Welch's t-test. *p≤0.05, **p≤0.01, ****p≤0.0001.

We then wanted to determine whether the extracellular origin of ARGIL1 is a key feature of its infection-enhancing function. To test this, we aimed to evaluate whether intracellular overexpression of ARGIL1 prior to infection can enhance DENV infection.

In doing so, 293T cells were transfected with mammalian expression vector encoding for ARG1, ARGIL1, or GFP, and then infected with DENV at increasing times post-transfection from 24 to 72 hours. Following infection, DENV RNA was measured by RT-PCR using RNA collected from cell supernatants at 24 and 48 hours post-infection. The results of these experiments showed that expression of ARG1/ARGIL1 did not result in greater levels of DENV RNA, compared to cells expressing GFP (Figure 2.3). While these results suggest that the phenotype observed following treatment with ARG1/ARGIL1 may be restricted to an extracellular-driven mechanism, future studies are needed to confirm that plasmid transfection does not promote differential post-translational modifications in comparison to our recombinant protein production protocols. However, this is unlikely as recombinant ARG1 was produced in 293T cells, but only resulted in infection enhancement upon extracellular treatment. Therefore, one can hypothesize that extracellular treatment with ARG1/ARGIL1 may result in a specific cellular compartmentalization of these proteins that facilitates L-arginine processing.

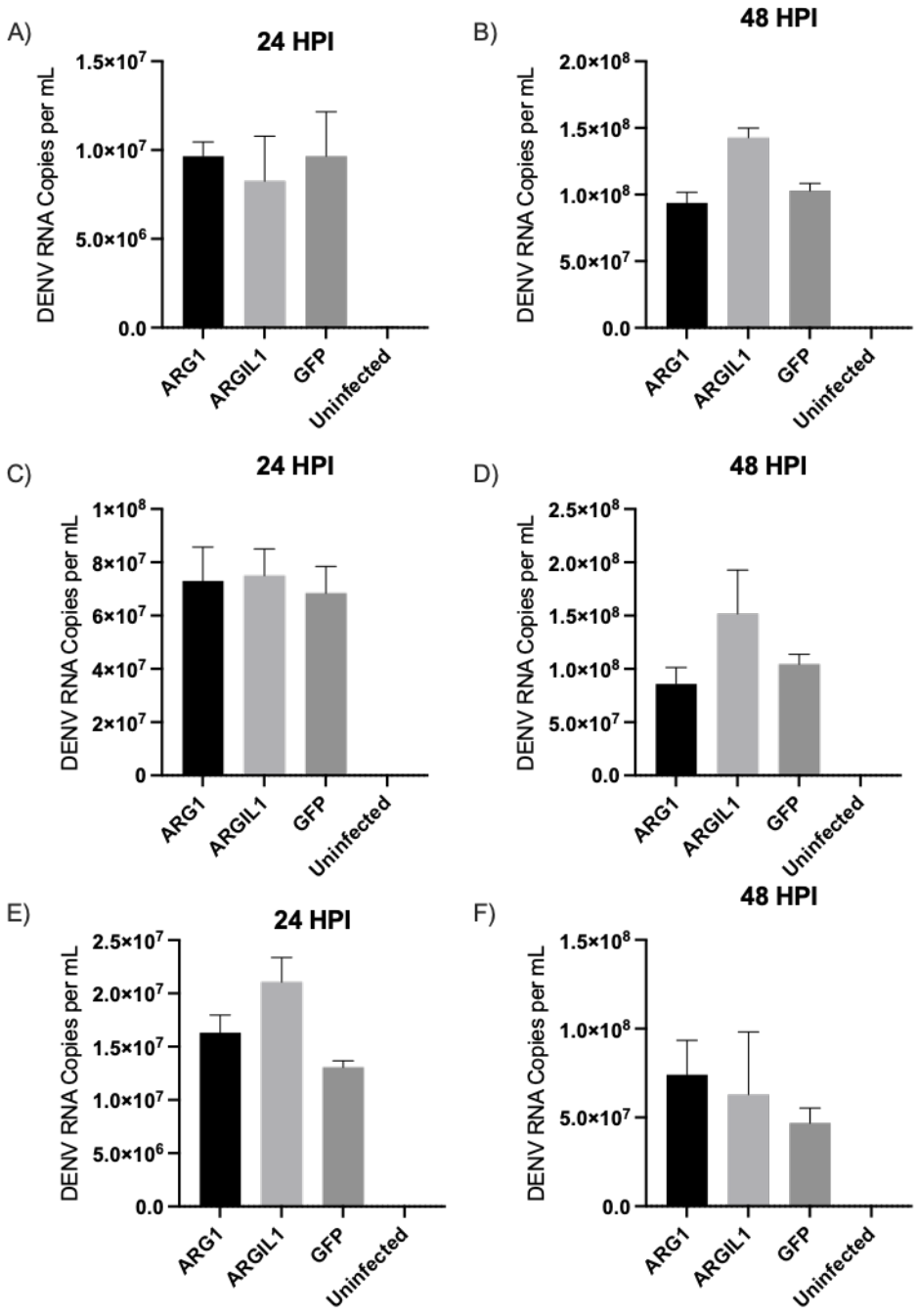

Figure 2.3 Expression of Arginase in Human Cells Does Not Affect Dengue Infection. 293T cells were transfected with plasmid encoding for ARG1, ARGIL1, or GFP. At 24 (A-B), 48 (B-C), and 72 (E-F) hours post-transfection, cells were infected with DENV-2 at a MOI of 1. Cell supernatants were collected at 24 (A, C, E) and 48 (B, D, F) hours post-infection, and viral RNA was isolated then used to measure DENV-2 viral RNA by RT-PCR. Results are the mean (n=3) ± standard deviation of the mean of three biological replicates.

THE ARGINASE ACTIVITY OF ARGIL1 IS RESPONSIBLE FOR ITS FLAVIVIRUS INFECTION-ENHANCING ACTIVITY

Although we hypothesize ARGIL1 to be an arginase, the arginase activity of ARGIL1 itself has not been demonstrated (Nene, Wortman et al. 2007). Following the establishment of the infection-enhancing phenotype of ARGIL1 (Figures 2.1 & 2.2), we therefore aimed to determine whether ARGIL1 was in fact an arginase and if this enzymatic activity is responsible for its infection-enhancing activity. To do so, we took advantage of a previously reported (Ckless, Lampert et al. 2008) competitive arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC). Before conducting infection studies, we first validated the ability of BEC to inhibit arginase activity using ARG1 as a prototypical arginase. ARG1 was treated with increasing concentrations of BEC, prior to quantification of its arginase activity. Expectedly, we found that treatment with 50 nM and 500 nM of BEC significantly decreased ARG1 arginase activity (Figure 2.4A). Following such validation, we then used BEC to pre-treat ARG1, ARGIL1, and BSA prior to incubation of treated proteins with fibroblasts and subsequent infection with DENV. Cell supernatants were collected 24 hours post-infection and DENV RNA in the cell culture supernatant was quantified by RT-PCR. Our results showed that cells exposed to ARG1/ARGIL1 pre-treated with 500 nM BEC did not exhibit significantly greater levels of DENV infection compared to those treated with BEC. In contrast, cells exposed to non-treated ARG1/ARGIL1 or to ARG1/ARGIL1 treated with an intermediate dose of BEC displayed higher levels of viral RNA in their supernatant in comparison to their respective BSA-treated and untreated controls (Figure 2.4B). The

abrogation of this infection-enhancing phenotype supports that ARGIL1 does exhibit arginase enzymatic activity, and that the infection-enhancing ability of both ARG1 and ARGIL1 is arginase-dependent.

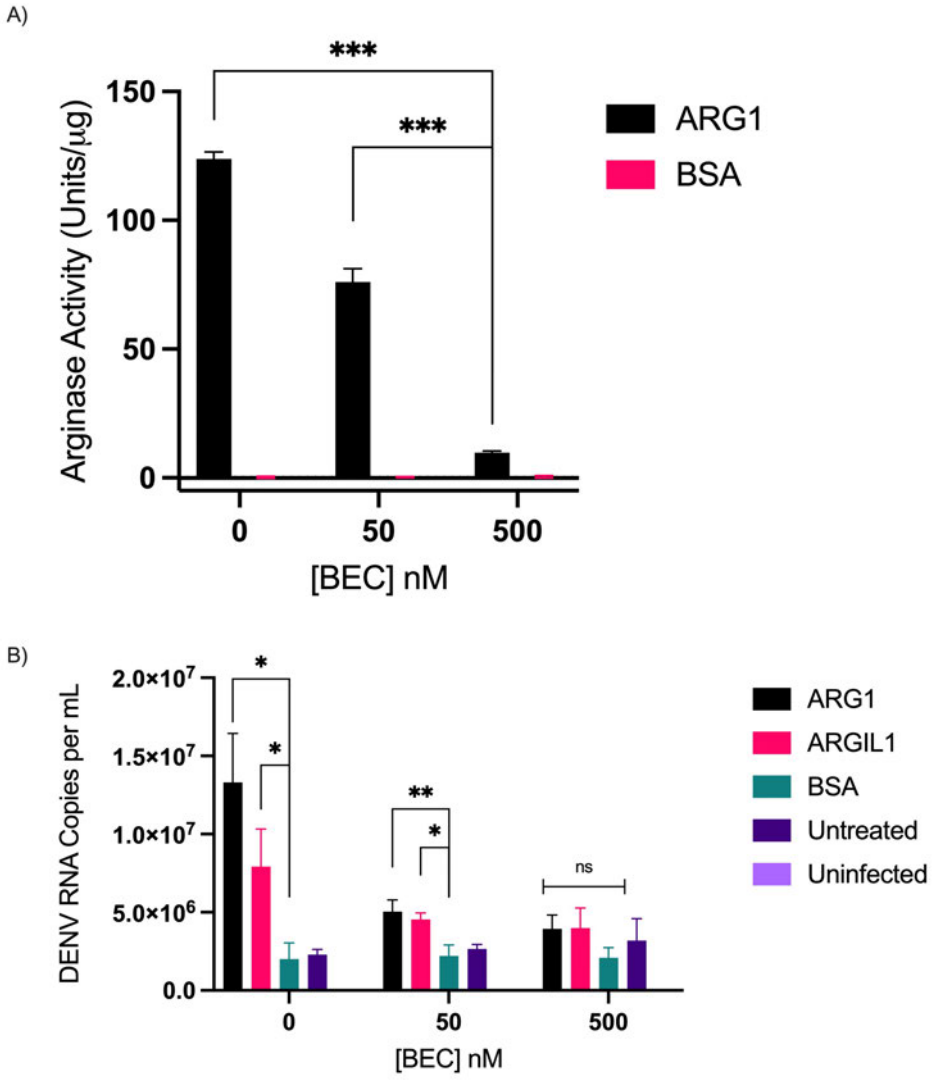

Figure 2.4 Enhancement of DENV Infection by ARGIL1 is Arginase-Dependent. A) ARG1 and BSA were pre-treated with increasing concentration of the competitive arginase inhibitor BEC before arginase activity was measured. B) Proteins were treated with increasing concentration of BEC, then used to pre-treat human adult dermal fibroblasts before infection with DENV-2. Supernatant RNA was collected 24 hours post-infection and used to quantify DENV-2 RNA by RT-PCR. Results are the mean (n=3) ± standard deviation of the mean of three biological replicates. Welch's t-test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

ARGIL1 MODULATES CELL INTRINSIC IMMUNITY TO INFECTION

To further investigate the mechanism behind the arginase-dependent enhancement of flavivirus infection by ARG1 and ARGIL1, it was important to determine whether ARGIL1-mediated infection enhancement associated with differential induction of innate immune responses. First, we focused on the type I IFN response, a key innate immune regulator in antiviral defense (Miorin, Maestre et al. 2017). In this case, the expression levels of type I IFN, including IFN-α (Figure 2.5 A, D), IFN-β (Figure 2.5 B, E), and the related ISG15 (Figure 2.5 C, F) were examined. As done in previous infection experiments, cells were treated with proteins then infected with DENV. At 24 and 48 hours post-infection, cells were lysed and cellular RNA was collected to quantify gene expression relative to the housekeeping gene B2M. While the results of these experiments were consistent with productive DENV infection, including robust induction of IFN-β and ISG15, there were no significant differences observed between groups treated with ARG1/ARGIL1 and those treated with BSA (Figure 2.5). These results indicate that the arginase-dependent enhancement of DENV infection by ARG1 and ARGIL1 does not function in an IFN-dependent manner.

In addition to interferon responses, formation of reactive nitrogen species is another antiviral mechanism. Given the connection between arginine metabolism and NO production, we aimed to examine how iNOS expression changed during ARG1/ARGIL1-dependent enhanced state of DENV infection. To look at iNOS expression, cells were similarly treated with ARG1, ARGIL1, or BSA, as done previously, then infected with DENV. Cells were lysed at 24 and 48 hours post-infection, and cell lysates were used to

measure intracellular concentrations of iNOS by ELISA (Figure 2.6). In contrast to experiments examining the interferon response, the results of these experiments showed a substantial decrease in iNOS production by cells treated with ARG1/ARGIL1, with statistical significance at 48 hours post-infection (Figure 2.6). The inhibition of iNOS expression following treatment with ARG1/ARGIL1 observed here is consistent with the described transcriptional relationship between arginase and iNOS, and indicative that this ARG1/ARGIL1-dependent enhanced DENV infection phenotype can be at least partially attributed to a decreased NO effector response of target cells. Additionally, reduced NO synthesis due to inhibition of iNOS expression would leave a greater amount of L-arginine available for metabolism by arginase. This metabolic shift towards arginase metabolism could result in greater intracellular levels of polyamines, a known pro-viral factor for several viruses (Firpo and Mounce 2020, Firpo, Mastrodomenico et al. 2021), which have the potential to promote several steps of the DENV replication cycle.

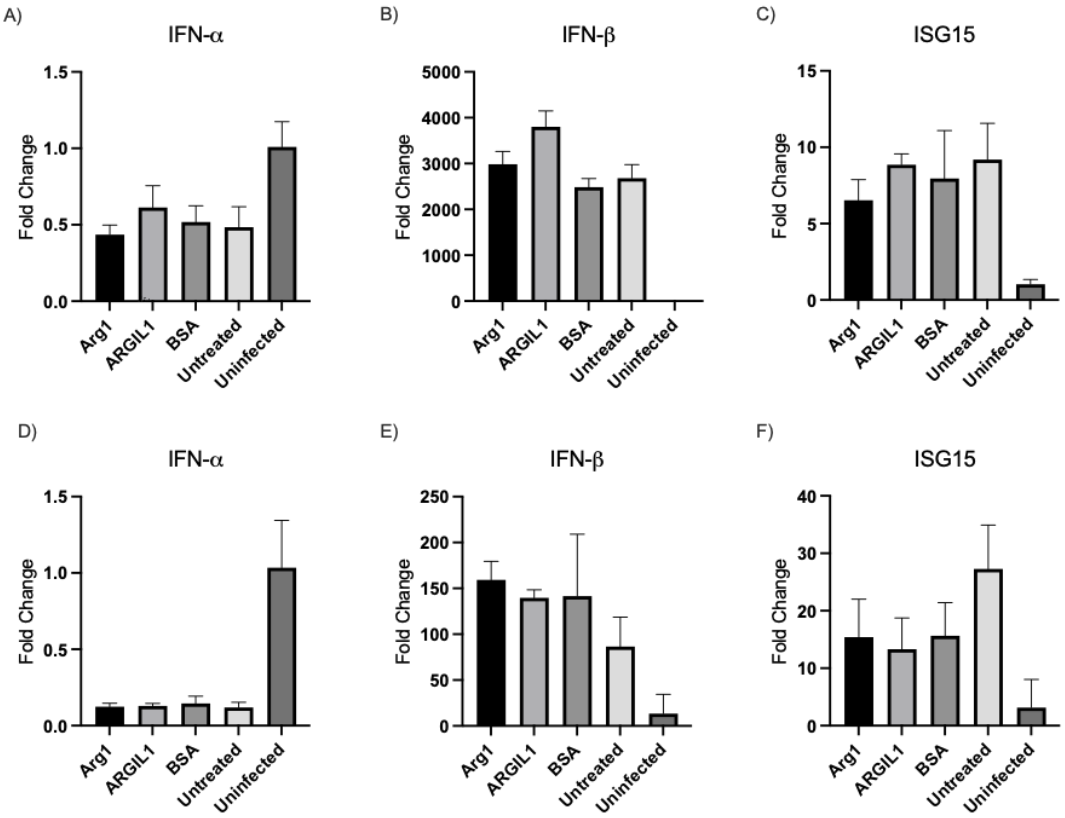

Figure 2.5 Treatment of Cells with Arginase Does Not Affect Type I IFN during DENV Infection. Adult human dermal fibroblasts were pre-treated with proteins at 500 nM then infected with DENV-2 at a MOI of 1. Cells were lysed at 24 (A-C) and 48 (D-E) hours post-infection, and lysates were used to measure expression of IFN-α, IFN-β, and ISG-15 by RT-PCR. Results are the mean (*n*=3) ± standard deviation of the mean of three biological replicates.

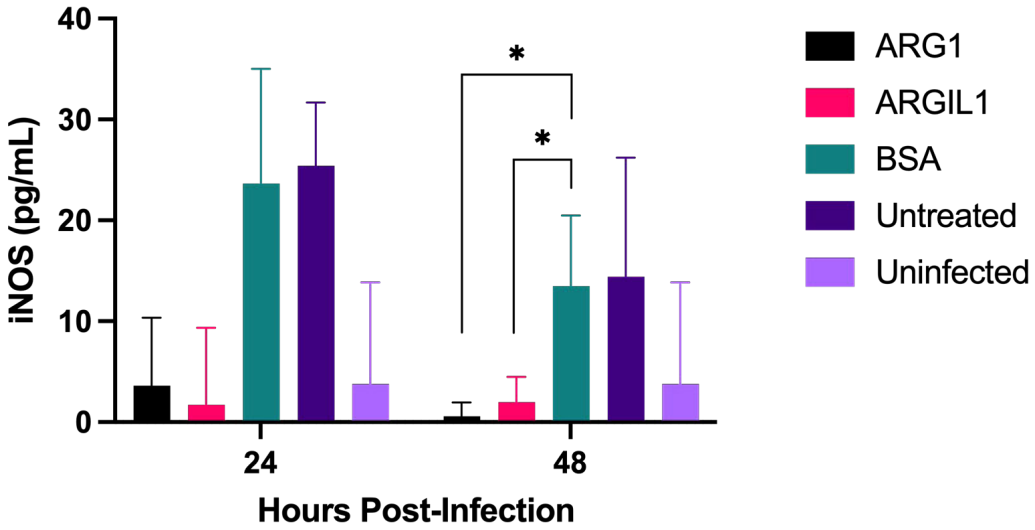

Figure 2.6 Arginase Treatment of Cells Results in Lower Levels of iNOS during DENV Infection. Adult human dermal fibroblasts were pre-treated with proteins at 500 nM then infected with DENV-2 at a MOI of 1. Cells were lysed 24 and 48 hours post-infection, and lysates were used to measure iNOS by ELISA. Results are the mean (n=3) ± standard deviation of the mean of three biological replicates. Welch's t-test. *$p \leq 0.05$.

DISCUSSION

Disclaimer: Portions of the text in this chapter were originally published as: (Gold, Feitosa-Suntheimer et al. 2020)

VECTOR SALIVA AND SALIVA PROTEINS ENHANCE FLAVIVIRUS INFECTION OF HUMAN CELLS

Proteins found in *Aedes aegypti* saliva have been shown to enhance flavivirus replication and spread (Conway, Watson et al. 2014, Surasombatpattana, Ekchariyawat et al. 2014, Sun, Nie et al. 2020). By using our unique method of saliva protein analysis (Figure 1.1 and Figure 1.2), we identified multiple proteins with suspected DENV infection-enhancing ability (Table 1.1). Additionally, while the ability of *Aedes* EVs, a recognized component of mosquito saliva, to facilitate DENV transmission has been demonstrated, the functions of the protein cargo of these EVs during infection remain to be determined (Vora, Zhou et al. 2018). Our data (Table 1.2 and Figure 1.3) show that DENV infection impacts the protein cargo packaging of *Aedes aegypti* EVs, a phenomenon previously characterized using EVs derived from DENV-infected mammalian dendritic cells (Martins, Kuczera et al. 2018). Several of the proteins found in EVs derived from infected cells, but not uninfected cells, have known infection-enhancing putative functions (Table 1.2). These results support the presence of a DENV infection-dependent protein packaging strategy, aimed at increasing viral transmission through the delivery of flavivirus infection-enhancing cargo by vesicular trafficking. Such a pro-viral strategy has been described for a multitude of other clinically relevant

viruses, including HIV, Epstein-Barr virus, Cytomegalovirus, and Hepatitis C virus, however this is the first report of this strategy as a mechanism of vector-borne flavivirus transmission (Urbanelli, Buratta et al. 2019).

ALTERATIONS OF EXTRACELLULAR VESICLE CARGO IMPACTS INFECTION OF HUMAN CELLS

Of the proteins found only in EVs from infected cells, two with well-characterized functions during DENV infection were AAEL017301, the *Aedes aegypti* elongation factor-1 alpha (EF1A), and AAEL017982, the *Aedes aegypti* heat shock protein 70 (HSP70) (Table 1.2). Human EF1A has been shown to function in the replication and pathogenesis of a diverse group of RNA viruses (Li, Wei et al. 2013). In the case of WNV, human EF1A has been shown to facilitate viral minus-strand RNA synthesis through interaction with the 3'-terminal stem-loop region of the viral genome (Blackwell 1997, Davis, Blackwell et al. 2007). Similarly, the EF1A of *Aedes albopictus* has been shown to bind to the 3' untranslated region of DENV in cells (De Nova-Ocampo, Villegas-Sepúlveda et al. 2002). Although neither of these studies comprehensively dissected the role of *Aedes aegypti* EF1A during flavivirus infection, the expression of AAEL017301 has been shown to increase following DENV infection, indicating a pro-viral role similar to that demonstrated in previous work (Raquin, Merkling et al. 2017).

Like EF1A, many studies have shown the involvement of HSP70 in flavivirus infection (Taguwa, Maringer et al. 2015, Kanlaya 2018). A HSP70 chaperone network has been shown to mediate the DENV viral cycle, in that cytosolic HSP70 isoforms are

required at distinct steps, including entry, RNA replication, and virus assembly (Taguwa, Maringer et al. 2015, Kanlaya 2018). A similar HSP70 chaperone network has been described for ZIKV, further indicating the role of HSP70 in the viral life cycle of all flaviviruses (Pujhari, Brustolin et al. 2019). Importantly, the inhibition of HSP70 blocks DENV replication as well as the replication of other mosquito-borne flaviviruses, supporting the role of *Aedes aegypti* HSP70 in the context of viral transmission and spread (Taguwa, Maringer et al. 2015). Similar to AAEL017301 and AAEL017982, another protein found in EVs derived from infected cells, AAEL002675, has a putative function known to affect viral infection (Nene, Wortman et al. 2007).

ARGINASE IS A PRO-VIRAL FACTOR OF FLAVIVIRUS INFECTION: THE POTENTIAL IMPACT ON VIRAL TRANSMISSION

AAEL002675, the *Aedes aegypti* putative arginase referred to as ARGIL1, was the only protein identified in both the DENV infection-enhancing saliva fractions (Table 1.1) and EVs derived from DENV-infected *Aedes aegypti* cells (Table 1.2) (Nene, Wortman et al. 2007). Although studies have elucidated the physiological role of ARGIL1, the function of this protein during flavivirus transmission and infection remains undefined (Von Dungern and Briegel 2001, Isoe and Scaraffia 2013). However, much more work has been performed characterizing the function of ARG1, the human homolog of ARGIL1. Physiologically, ARG1 metabolizes L-arginine to L-ornithine and urea, serving a fundamental role in the hepatic urea cycle (Munder 2009). Yet, several types of immune cells require L-arginine for their effector functions (Munder 2009). For example, activated macrophages consume L-arginine by converting it to L-citrulline, NO, and

reactive nitrogen species via iNOS as their primary mechanism of cytostatic or cytotoxic activity against viruses (MacMicking, Xie et al. 1997). Due to this role in the innate immune response, ARG1 has been shown to play a role in several viral infections (Burrack and Morrison 2014). For example, the expression of ARG1 has been shown to be associated with an increased viral load, disease severity, and persistence of Chikungunya virus, which like DENV is transmitted by the *Aedes aegypti* mosquito (Lombardi Pereira, Suzukawa et al. 2019). Unlike other proteins with potential pro-viral functions observed in EV samples, ARGIL1 is a putative enzyme that directly competes with its human ortholog for the same intracellular substrate, L-arginine, to regulate infection. By functioning as a "plug and play" enzyme which can directly regulate human intracellular L-arginine metabolism, ARGIL1 exposes a novel facet of the molecular arsenal carried by mosquito vectors to enhance human susceptibility to infection.

While the role of ARG1 during DENV infection is not well known, it has been shown to promote the persistence of Hepatitis C virus in human hepatoma cells (Cao, Sun et al. 2009). Additionally, the function of NO during DENV infection has been characterized, and although the exact mechanism has yet to be determined, it has been shown to inhibit DENV replication by impeding the polymerase activity of the viral protein NS5 (Takhampunya 2006, Chaturvedi and Nagar 2009). In this case, if an exogenous arginase, such as ARGIL1, was delivered to recently infected cells, cytosolic L-arginine would be converted to L-ornithine and urea, thereby reducing the cytosolic concentration of L-arginine that could be converted to NO and subverting the induction

of an optimal immune response (Munder 2009). Additionally, from the known reciprocal transcriptional relationship between arginase and iNOS, addition of arginase could result in a decrease in the expression of iNOS, further inhibiting the NO effector response (Munder 2009). In addition to its role in NO formation, secreted arginase has been observed to recruit T cells and inhibit their immune responsiveness (Munder 2006, Kropf, Baud et al. 2007, Czystowska-Kuzmicz, Sosnowska et al. 2019). While T cell responses are critical in regulating later stages of infection , they are thought to have limited impact during viral transmission given their antigen-specific nature and the predominance of the epithelial and myeloid responses characterizing dermis infection (Slon Campos, Mongkolsapaya et al. 2018, Pan, Cai et al. 2022). Nevertheless, one cannot exclude that arginase-dependent inhibition of T cells could potentially impact local priming of the adaptive immune response in the dermis upon viral transmission.

Aside from its ability to inhibit the antiviral innate immune response, arginase activity can increase the presence of a polyamines, a pro-viral factor that is a downstream metabolite of L-arginine. Following the conversion of L-arginine to L-ornithine and urea, L-ornithine is metabolized by ornithine decarboxylase into polyamines, including putrescine, spermine, and spermidine (Munder 2009). These small aliphatic metabolites have previously been shown to enhance multiple steps of the viral life cycle such as translation and replication, for a multitude of viruses, including ZIKV (Mounce, Poirier et al. 2016, Firpo and Mounce 2020, Firpo, Mastrodomenico et al. 2021). The ability of arginase activity to not only diminish the innate immune response by depleting NO, but also create a pro-viral cellular environment through the downstream synthesis of

polyamines, provides multiple mechanisms by which heightened arginase activity could enhance flavivirus infection. Consistent with the described function of arginase in innate immunity, our work showed that ARGIL1 treatment of human fibroblasts before infection with DENV resulted in greater levels of infectious virus production at 24 and 48 hours post-infection (Figure 2.2), strongly supporting the ability of this protein to enhance flavivirus infection. While we observed a two-fold increase in infection in ARG1/ARGIL1 treated groups, enhancement of infection could be greater at lower MOI, making infection studies with low MOI of high priority.

In addition to its described role in innate immunity of the mammalian host, arginase is thought to be an important contributor to insect immunity through its role in the synthesis of NO (Rivero 2006). Previous studies have demonstrated the function of NO as a key mediator in the immune response of invertebrates against several pathogens, including the *Plasmodium* parasite that causes malaria (Luckhart, Vodovotz et al. 1998, Rivero 2006). In the case of flaviviruses, while the role of NO in infection of *Aedes* mosquitoes has not been as well characterized, two previous studies conducted using DENV infection in vivo suggest that, similar to its function in mammals, NO functions as a mediator of antiviral defense in mosquitoes (Ramos-Castañeda, González et al. 2008, Terradas, Allen et al. 2017). Given the transcriptional relationship between arginase and iNOS, and the described immune function of NO in *Aedes*, increased expression of *ARGIL1* has the potential to make *Aedes* more permissive to flavivirus infection. This arginase-dependent enhancement of infection could potentially facilitate the rate of viral

dissemination and levels of infection in the salivary glands, both of which would have further implications on virus transmission.

While *ARGIL1* expression increased following infection of *Aedes aegypti* cells with DENV (Figure 1.5), supporting a DENV infection-dependent strategy for the delivery of this pro-viral factor during flavivirus transmission, further investigation are needed to determine whether *ARGIL1* expression regulates DENV infection in mosquito cells. This could be tested through extracellular treatment of *Aedes aegypti* cells with ARGIL1, and/or through ARGIL1-targeted knock-out, which could respectively result in an enhancement or decrease of viral replication.

ARGINASE-DEPENDENT FLAVIVIRUS INFECTION ENHANCEMENT AND REMODELING OF THE INNATE IMMUNE RESPONSE

Further studies investigating the infection-enhancing activity of ARGIL1 have more robustly demonstrated its ability, and the ability of ARG1, to enhanced flavivirus infection in a context of pre-treatment of human cells prior to infection (Figure 2.1 and Figure 2.2). While the concentration of ARG1 and ARGIL1 used to treat these cells (500 nM) was not representative of the amount of protein delivered to the skin by a mosquito bite, the ability of these proteins to enhance infection in vitro supports their ability to do so in vivo. In the context of flavivirus transmission, ARGIL1-dependent inhibition of the NO effector response in an early infected cell would result in an enhanced level of infection, characterized by more rapid and increased production of infectious virions. Following a mosquito bite, flavivirus infection spreads rapidly from early infected cells in the cutaneous compartment to secondary lymphoid tissues, especially lymph nodes. In

lymph nodes, flaviviruses undergo extensive viral amplification prior to diffusion into the peripheral circulation, resulting in viremia. Given the upstream nature of the mosquito bite in the course of infection, enhancement of infection in a small number of cells following virus delivery into the dermis could therefore have broad ramifications on later viral dissemination throughout the host and disease outcomes. Together, a better understanding of the role of vector saliva during flavivirus infection, such as the arginase-dependent mechanism of enhancement described here, is critical for the development of innovative antiviral strategies against arboviruses moving forward.

Interestingly, the enhanced-infection phenotype observed when cells were treated with ARG1/ARGIL1 prior to infection was not observed upon intracellular expression of these proteins (Figure 2.3). While these results are indicative that infection enhancement may be associated with an extracellular mechanism, further studies are required to validate this hypothesis and exclude any bias associated with ARGIL1-encoding plasmid transfection. Furthermore, whether ARGIL1 treatment of 293T cells enhance infection of these cells by DENV will need to be confirmed. Characterization of how this protein interacts with the cell extracellularly, the process by which it may be internalized, how and where it is translocated to mediate its function, and any interactions it may have with other host factors will be important to further our understanding of the role of ARGIL1 during flavivirus infection.

By using a competitive arginase inhibitor, further experiments demonstrated that the infection-enhancing abilities of ARG1 and ARGIL1 were arginase-dependent, strongly supporting the enzymatic activity of ARGIL1 as an arginase (Figure 2.4). While

our results support that ARGIL1 is an arginase, a more robust characterization of the enzymatic function of ARGIL1, including solving its crystal structure, identifying the active site and its key residues, and performing site directed mutagenesis screenings, is necessary before concluding that ARGIL1 is an arginase.

Given the known function of arginase in the antiviral innate immune response, it was finally critical to examine how innate immunity was affected during arginase-dependent enhanced state of flavivirus infection. Following probing for the expression of type I IFN and ISG15, the treatment of human cells with arginase preceding DENV infection did not result in any notable changes to the IFN response (Figure 2.5). However, when these same cells were probed for the expression of iNOS, the enzyme responsible for NO synthesis, cells treated with ARG1/ARGIL1 displayed significantly lower levels of iNOS (Figure 2.6), consistent with the described transcriptional relationship between arginase and iNOS (Munder 2009). Due to limitations posed by both our experimental design and the ELISA assay we used, the measured values for iNOS remained overall low across experimental conditions, which could put into question the significance of these data. While the difference in iNOS levels observed between ARG1/ARGIL1 treated samples and BSA controls was statistically significant and support the role of ARGIL1 in regulating iNOS, further investigation through the use of a highly selective inhibitor of iNOS or specific siRNAs targeting iNOS mRNA will be needed to strengthen the significance of our experimental model. While arginase treatment did not appear to directly inhibit the interferon response, modulation of the NO effector response by downregulation of the expression of NO supported the function of

arginase as a pro-viral factor. Although our results did not show an impact on the type I IFN response by ARG1/ARGIL1 treatment, a deeper exploration into the effect of these proteins on other aspects of the innate immune response, including pro- and anti-inflammatory cytokine and chemokine responses, will help create a clearer picture of how arginase remodels the cellular environment to be more favorable for viral replication.

LIMITATIONS & FUTURE DIRECTIONS

The results of these studies support a model of arginase-dependent enhancement of flavivirus infection by ARGIL1. However, a major limitation of this work is the lack of evidence that ARGIL1 enhances DENV infection upon transmission to a mammal host. Given that arbovirus transmission is a complex process that cannot be recapitulated in an in vitro setting, future in vivo studies are necessary to validate the function of ARGIL1 as a pro-viral factor. By using established mouse models of flavivirus infection, including wild-type mice or mice defective for type I IFN receptor expression (Graham, Swarts et al. 2017, Wong and Qiu 2018), not only can we further support that the presence of ARGIL1 at lower concentrations can enhance flavivirus infection and spread, but potentially also further characterize the mechanism by which it does so in ways not conducive to in vitro methods. For example, investigating the impact of ARGIL1 on innate immune responses of myeloid cells and their recruitment to the dermis upon infection would be informative in determining the mechanism of enhancement of ARGIL1. On one hand, depletion of NO and innate responses associated with hematopoietic recruitment by ARGIL1 could be beneficial for viral dissemination by

creating a cutaneous environment more permissive to infection. Alternatively, ARGIL1-dependent inhibition of hematopoietic-derived cell infiltration could deplete the myeloid reservoir for viral infection and be detrimental to infection and spread. Such in vivo investigations could be conducted through two major ways: 1) intradermal injection of mice with DENV combined with/without ARGIL1 and 2) siRNA-mediated knockdown of ARGIL1 in *Aedes aegypti* before infection with DENV and subsequent transmission studies using mice. Conducting in vivo studies like this will build on our existing understanding of the pro-viral function of ARGIL1, creating a more comprehensive and translational model of its contribution to flavivirus infection and transmission.

In this work, we showed that a 500 nM concentration of ARGIL1, but not lower concentration, associated with infection-enhancement. One significant question that emerges is whether such concentration is physiologically relevant, as it is unlikely to reflect the ARGIL1 concentration in mosquito saliva during transmission. One hypothesis could be to distinguish local vs. systemic ARGIL1 concentration. Indeed, upon viral transmission, ARGIL1 concentration may reach high levels at the site of virus delivery, affecting both extracellular and intracellular L-arginine concentrations. In vivo experiments, specifically inoculation of DENV virus locally in the skin mixed with a broad range of ARGIL1 concentrations, will allow investigation further into this important question and clarify whether in vivo and in the cutaneous compartment very low concentrations of ARGIL1 can significantly propel DENV infection. If this happens not to be the case, one cannot exclude that the enhancing role of ARGIL1 in the dermis may need to synergize its function with those mediated by other saliva proteins to

significantly enhance transmission at a level quantifiable phenotypically. In this case, the experimental design of our in vitro experiments, via the use of a high concentration of ARGIL1, would simply have unraveled, via function exacerbation, the enhancing function of a protein which, in vivo and excluded from a saliva context, would not have quantifiable impact on transmission. The results of the in vivo studies described above will assuredly strengthen evidence that ARGIL1 enhances flavivirus infection in the host, and also remove major concerns related to the potentially excessive protein concentration used for our in vitro studies.

Given the conserved function of arginase in the physiology of several arthropods and the involvement of arginase in innate immunity, it is likely that ARGIL1 may also have the potential to enhance transmission of other mosquito-borne flaviviruses, including WNV, YFV, and ZIKV, as well as potentially other arboviruses. However, it is worth noting that *ARGIL1* upregulation could also be DENV- and/or *Aedes aegypti*-specific, and not be recapitulated upon infections of *Aedes aegypti* or different mosquito or tick species by other arboviruses.

Future in vitro and in vivo studies, based on similar experimental approaches as those described above, will be required to determine if ARGIL1 is a pan-flavivirus/pan-arbovirus pro-viral factor. The identification of ARGIL1 as a pan-flavivirus/pan-arbovirus pro-viral factor will open avenues for the development of innovative antiviral approaches targeting a wide range of arboviral diseases, including transmission blocking vaccines.

Results of in vivo studies, such as those described here, will have greater translational implications, potentially validating ARGIL1 as a mediator of arbovirus transmission. Identifying and describing pro-viral factors like ARGIL1 is a key step in the development of antivirals and vaccines against flaviviruses. While there are no existing antivirals against flaviviruses, and given that all current vaccines against flaviviruses target viral antigens, vaccines and therapies preventing effective viral transmission, via the targeting of vector co-factors (proteins, miRNAs, etc.) regulating that process, like ARGIL1, could open avenues for the development of novel and complementary anti-flavivirus and anti-arbovirus countermeasures. For instance, targeting vector proteins through the use of a transmission blocking vaccine represents an alternative approach that would help mitigating flavivirus transmission and spread while circumventing several issues that have hindered traditional vaccine design progress, such as ADE and varying potency of the adaptive immune response.

APPENDIX

Disclaimer: Data and portions of the text in this chapter were originally published as: (Gold, Feitosa-Suntheimer et al. 2020)

EXPRESSION AND PURIFICATION OF MOSQUITO PROTEINS

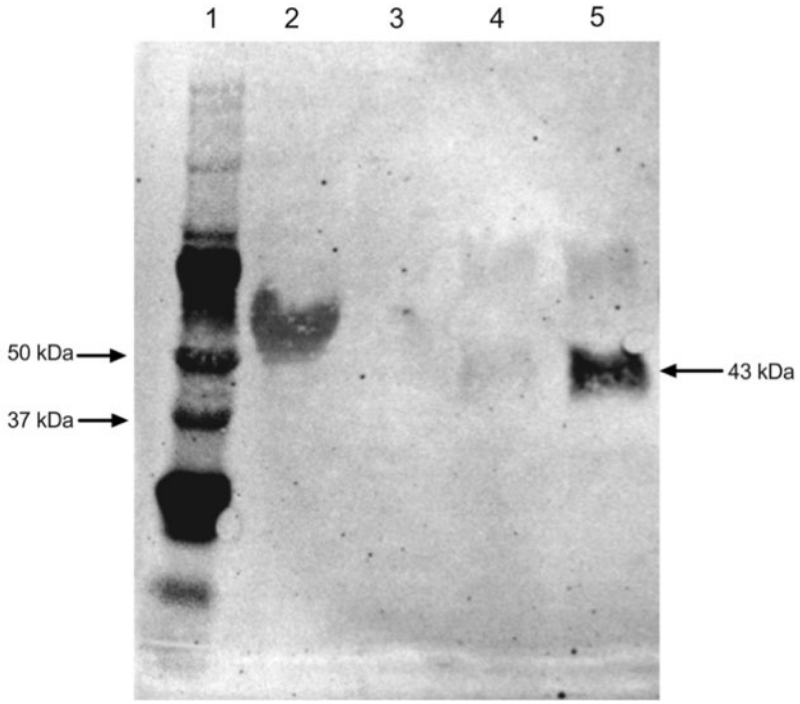

Figure S1. Detection of the Recombinant AAEL002675 Protein. Following purification of the recombinant AAEL002675 protein, proteins were resolved via SDS-PAGE and probed for the presence of a V5 tag on the protein of interest. Lane 1: Precision Plus Protein Dual Color Standards™ (Bio-Rad 1610374); lane 2: S2 supernatant HisPur column flow-through; lane 3: elution fraction 3; lane 4: elution fraction 2: lane 5: elution fraction 1.

INHIBITION OF ARG1/ARGIL1 ARGINASE ACTIVITY

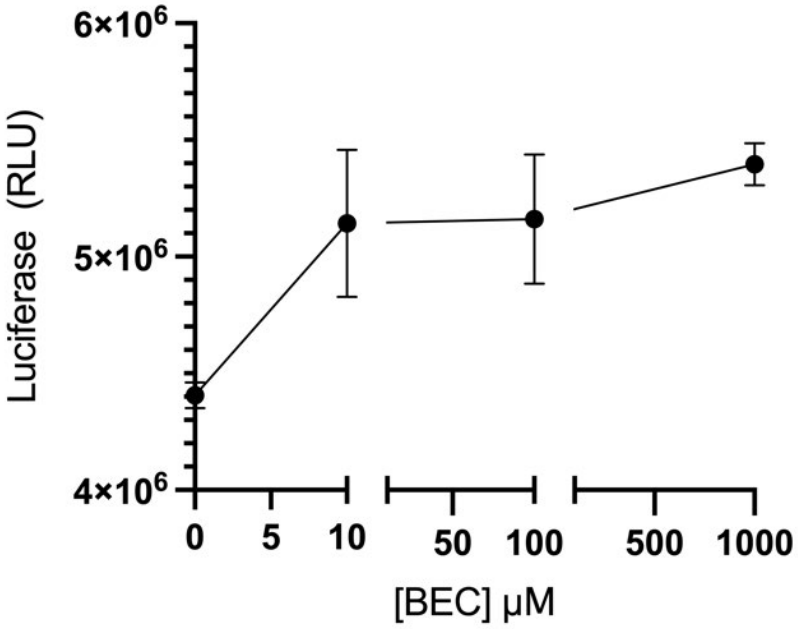

Figure S2. Inhibition of Arginase is Not Cytotoxic. Human adult dermal fibroblasts were treated with increasing concentration of BEC, then cell viability was measured by CellTiter-Glo® 2.0 Cell Viability Assay. Results are the mean (*n=3*) ± standard deviation of the mean of three biological replicates.

EXPRESSION OF RECOMBINANT ARGINASE PROTEINS

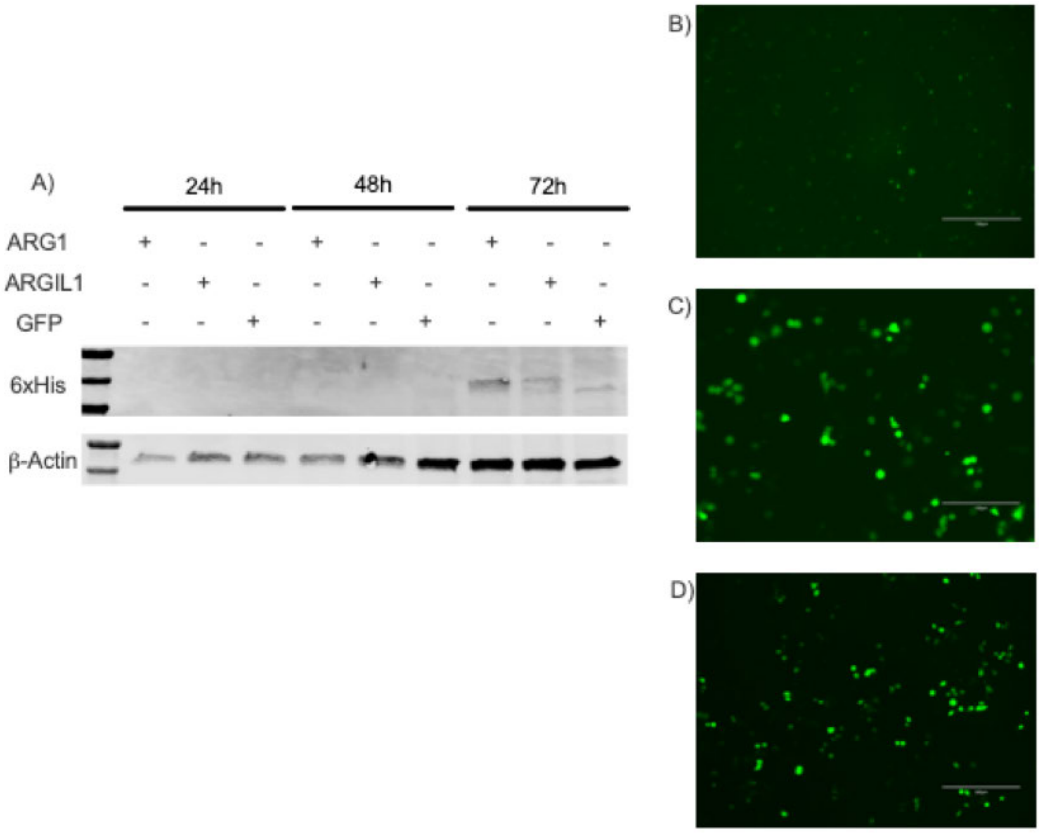

Figure S3. Expression of Recombinant Arginase Proteins. A) Following transfection of 293T cells with pCDNA3 encoding for arginase proteins and GFP, proteins were isolated and resolved via SDS-PAGE, and then probed for the presence of a 6xHis tag on the protein of interest by western blot. Additionally, cells transfected with pCDNA3-GFP were imaged before lysis at B) 24, C) 48, and D) 72 hours post-transfection.

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