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A Comparison of Chikungunya Virus Infection, Dissemination, and Cytokine Induction in Human and Murine Macrophages and Characterization of RAG2-/-γc-/- Mice as an Animal Model to Study Neurotropic Chikungunya Disease

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A dissertation submitted to the faculty of
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Doctor of Philosophy

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ABSTRACT

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Chikungunya virus (CHIKV) is classified as an alphavirus in the Togaviridae family. This virus is known to rely on *Aedes* arthropod vectors for its dissemination. Human infection is characterized by rash, high fever, and severe chronic polyarthritis that can last for years. Recently, efforts in developing animal models have been made in an attempt to better understand CHIKV pathogenesis.

CHIKV infection starts with a 7 to 10 day long febrile acute phase, in which most of the symptoms occur (rash, fever, and incapacitating pain in joints and muscle). Once the immune system clears most of the viral infection, a chronic phase starts in as many as 70% of the infected patients. Long term virus-related polyarthralgia is the hallmark of the CHIKV chronic phase. It is believed that CHIKV-infected macrophages infiltrate the joints during the acute phase, and CHIKV infects joint tissue and persists in it.

Research into the effects of CHIKV infection in human and murine macrophages revealed that CHIKV-infected human macrophages produce high amounts of virions as well as induce the production of pro-inflammatory cytokines and monocyte recruiting chemokines. This contrasts with murine macrophage infection where low quantities of the virus were detected as well as lower production of pro-inflammatory cytokines. This may contribute to the lack of polyarthritis in murine animal models. Current literature suggests that CHIKV's viral proteins bind and interact with human host cell machinery promoting viral replication more efficiently in humans than in mice.

CHIKV-related neuropathology is not the most common outcome of the disease. However, recent outbreaks suggest that this pathology is becoming more prevalent, affecting as many as 30% of confirmed patients. The role of adaptive and innate immunity in CHIKV disease amelioration has been extensively, yet separately, explored. A RAG2$^{-/-}\gamma c^{-/-}$ Balb/c mouse model was used to study the role of these immune pathways and their associated immune cells in CHIKV infection. The mice in this study developed local arthritis at the site of inoculation as well as showed signs of viral invasion in the brain. This study added to the hypothesis that both innate and adaptive immune responses are necessary to ameliorate the disease and that the lack of adequately matured lymphocytes and STAT6-activation deficient macrophages may result in more severe pathologies.

Keywords: Chikungunya virus, polyarthralgia, macrophage, cytokine, RAG2$^{-/-}\gamma c^{-/-}$ Balb/c mouse model, neuropathology

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"Master yourself, and become king of the world around you. Let no odds, chastisement, exile, doubt, fear, or ANY mental virii prevent you from accomplishing your dreams. Never be a victim of life; be its conqueror." ― Mike Norton

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ABBREVIATIONS

CHIKV = Chikungunya virus

CHIKF = Chikungunya fever

WNV = West Nile virus

ZIKV = Zika virus

DENV = Dengue virus

LACV = LaCrosse virus

SINV = Sindbis virus

VEEV = Venezuelan Equine Encephalitis virus

A. aegypti = *Aedes aegypti*

A. albopictus = *Aedes albopictus*

WHO = World Health Organization

BSL-3 = Bio-safety Level 3

CDC = Centers for Disease and Control

RA = Rheumatoid Arthritis

WEEV = Western Equine Encephalitis virus

VEEV = Venezuelan Equine Encephalitis virus

EEEV = Eastern Equine Encephalitis virus

MAYV = Mayaro virus

ONNV = O'Nyong Nyong virus

RRV = Ross River virus

BFV = Barmah Forest virus

PREFACE

To help the reader better understand the order and organization of this document, I will provide a brief explanation of such. Due to the large amount and varied content contained in chapter 1, it has been organized differently from chapters 2 through 4. This chapter has been dissected into three sections, each providing thorough background information, as well as a review of the current literature pertaining to the research that will be explained in chapters 2 through 4.

Chapters 2 through 4 have been organized like scientific articles, as that is how they were intended to be read. In these chapters, a summary is provided, followed by the introduction, methods, results, and discussion sections. Like chapter 1, the chapter number is presented first, and then the section number, followed by the subsection number.

Chapter 5 is the concluding chapter and contains three parts addressing future potential experiments and providing final discussions of the research outlined in chapters 2 and 3.

Chapter 1. Introduction and Review of the Literature

1.1 Introduction to Chikungunya fever and innate immunity

1.1.1 Arboviruses

Tropical viral diseases have recently caught the public interest and have become a health issue of the highest importance. The technology of the dawn of the 21st century did not only connect us even more through trading, internet, and air travel; but also exposed us to some of our greatest enemies, emerging infectious diseases. During August 1999, an unexpected outbreak of West Nile virus (WNV) in New York city infected five patients. It caused acute fever, severe myalgia, headache, conjunctivitis, and four out of the five patients ultimately developed flaccid paralysis and required ventilator support[1]. Until then, WNV was mostly found in Africa, the Middle East, Southwest Asia, with some isolated and sporadic cases in Australia and Europe. This outbreak showed that viral diseases that were thought contained in remote parts of the world could be easily carried to other regions of the planet and affect previously unexposed populations.

A distinguished group of emerging tropical diseases is viruses transmitted by arthropod vectors, also known as arboviruses. This group consists of the *Flaviviridae* family, which includes WNV, Zika virus (ZIKV), and Dengue virus (DENV). The *Bunyaviridae* family with La Crosse virus (LACV). Finally, the *Togaviridae* family with Sindbis virus (SINV), Venezuelan Equine Encephalitis virus (VEEV), and Chikungunya virus (CHIKV). All of these viruses produce febrile symptoms along with myalgia, and some do cause arthralgia, which makes traditional clinical diagnosis difficult. Flaviviruses and Togaviruses use *Aedes* mosquitoes as dissemination and infection vectors. *A. aegypti* tends to occupy urban areas, and *A. albopictus* is associated with thickets and arboreal vegetation environments. The aggressive nature of these

mosquitoes, inadequate prevention programs, and lack of effective vaccines have proven to play essential roles in the successful spread of many arboviruses.

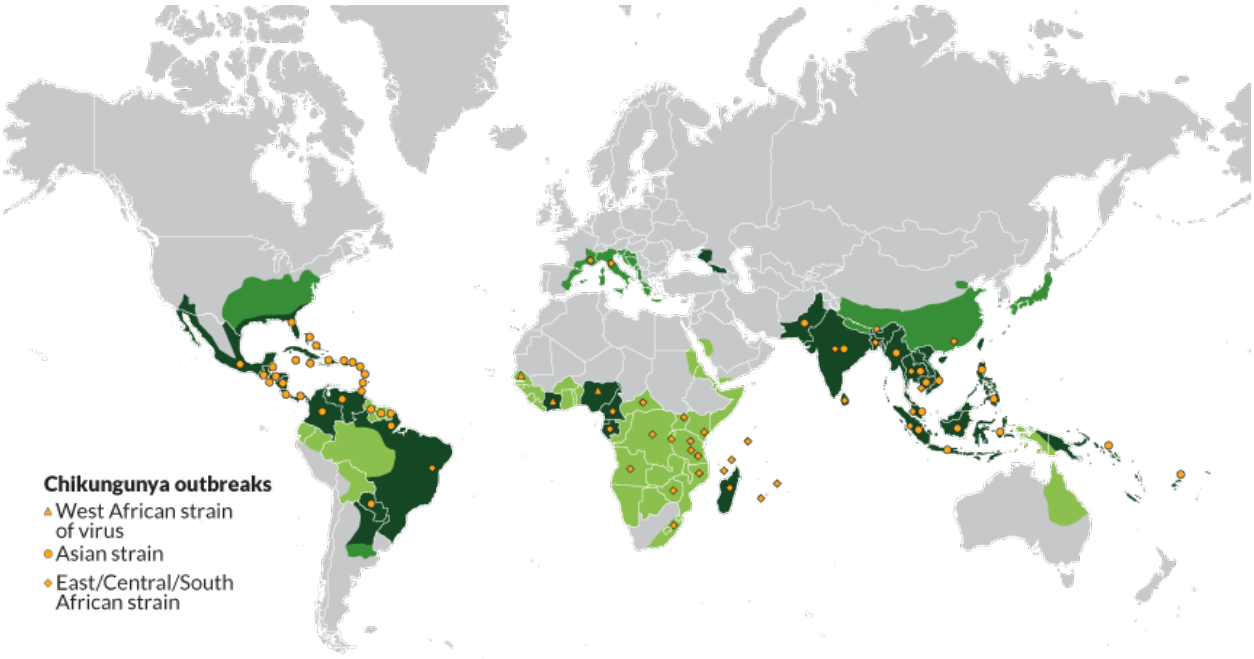

Figure 1-1. Global distribution of Chikungunya virus outbreaks. Chikungunya virus has broadly expanded its tropical range and made fleeting inroads into temperate zones. This map shows the phylogenic origin and location of significant epidemics since 1952. Mosquito ranges are approximations and hint at potentially vulnerable areas. World Health Organization (2018)

More recent outbreaks by emigrating Old World viruses have expanded rapidly in the Americas. In 2015, a ZIKV outbreak occurred in South America, and by the summer of 2016, it spread to other countries in Central America, North America, and the Caribbean[2–5]. Zika virus disease was quickly associated with a cluster of microcephaly and Guillain-Barre syndrome cases in Brazil. It has been estimated that 1.5 million people were infected in Brazil alone, with over 3,000 cases of the conditions described above[6–9]. Simultaneously, more than two million suspected cases of Chikungunya virus fever (CHIKF) were reported by the World Health Organization (WHO). These cases were spread from Brazil to Florida and effectively incapacitated many thousands of patients, causing massive economic losses to dozens of

countries[10,11]. A high percentage of the infected patients developed persistent arthralgia that lasted months, up to several years. These emerging diseases, along with more known and endemic viruses like DENV, and the ever present threat of yet unknown viruses, has forced a reassessment of research priorities and public health interventions[12].

Although CHIKF does not generally cause mortality, about one-third of CHIKV infected patients develop either arthralgia or chronic arthritis, with recent strains like La-Reunion causing up to 63.6% of the reported cases[13,14]. Economic analyses of the 2013-2015 CHIKV epidemic in the Americas reported an estimate of 40 million cases in the continent, which imposed an economic burden of 185 billion USD, in which chronic inflammatory rheumatism was the overwhelming attributable factor[12]. CHIKV's high rate of impairing chronic arthritis, along with an arthropod-based transmission. Which has been characteristically challenging for various health and environmental agencies around the globe, has led to its classification as a bio-safety level 3 (BSL-3) agent with potential bioweapon capabilities by the US Centers for Disease and Control (CDC) [11,15,16].

1.1.2 Alphaviruses and their replication cycle

Alphaviruses are a genus within the *Togaviridae* family of enveloped positive-sense RNA viruses. Clinically relevant alphaviruses are zoonotic diseases that use mosquito vectors for transmission into human hosts (Arboviruses). Historically, there have been three relevant alphaviruses in the United States: Western Equine Encephalitis (WEE), Venezuelan Equine Encephalitis (VEE), and Eastern Equine Encephalitis (EEE). These New World alphaviruses are nowadays very uncommon in the continental United States. On the other hand, Old World alphaviruses have become more relevant in the 21st century. Chikungunya Virus (CHIKV), Mayaro Virus (MAYV), O'Nyong Nyong Virus (ONNV), Ross River Virus (RRV), Barmah

Forest Virus (BFV), and Sindbis (SINV) have spread steadily through Eurasia, Africa and even invaded the American continent and the Caribbean[17–20].

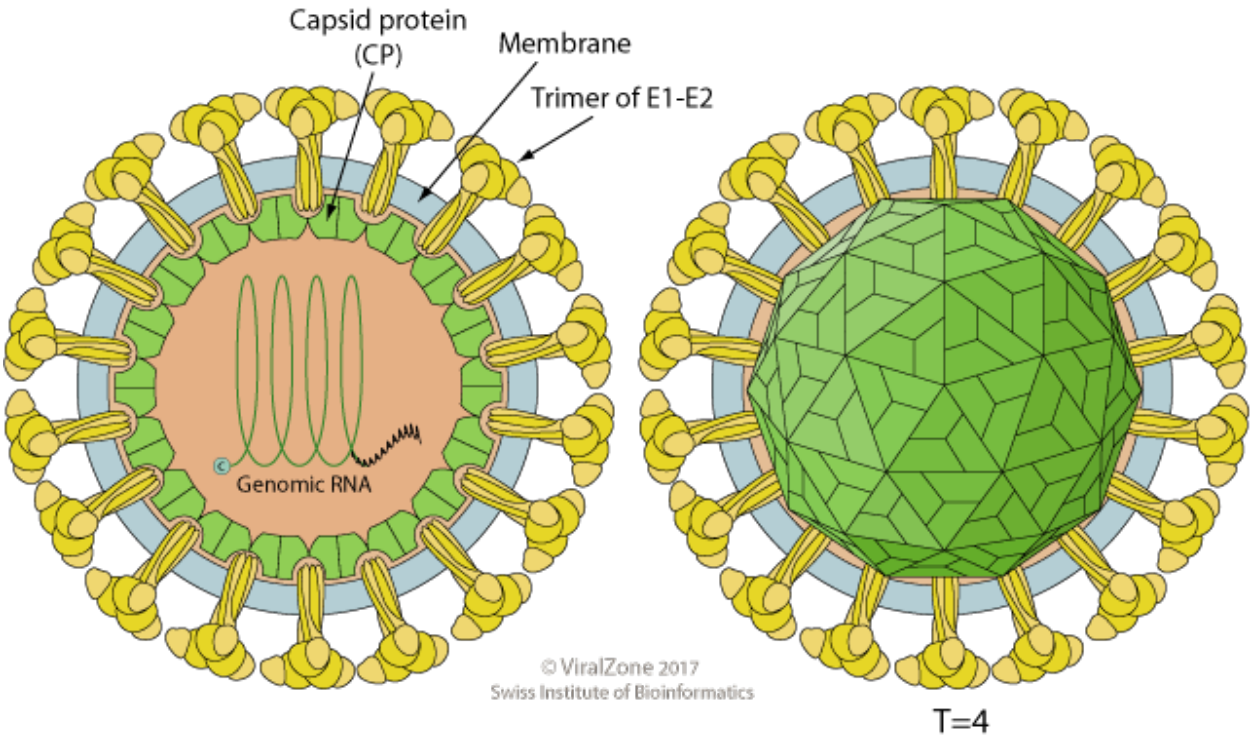

Figure 1-2. Alphavirus structure. Alphaviruses are spherical, enveloped, icosahedral, ~70nm in diameter. Form a capsid with a T=4 icosahedral symmetry. The envelope contains 80 spikes, consisting of a trimer of E1-E2 proteins. Swiss Institute of Bioinformatics. (2017)

These viruses are small enveloped spherical virions, 60 to 70 nm in diameter, which contains a positive-sense single strand of RNA, circa 11.8 kilobases long (Figure 1-2). The lipid envelope usually contains of two (rarely three) surface glycoproteins (E1, E2, and E3), which mediate cell invasion by attaching to host receptors. The viral replication cycle starts when the fusion of the viral envelop to the endosomal membrane is triggered by clathrin-mediated endocytosis (Figure 1-3). Releasing the spherical capsid to the cytosol[21]. Disassembly of the viral capsid by the host and viral proteases, release the virus single-stranded RNA genome which will eventually encode two polyproteins (one structural and one non-structural). The whole genome is translated into a non-structural polyprotein (nsP1234), which is processed by the protease

domain of nsP2[22,23]. An RNA-dependent-RNA-polymerase (nsP4) is found at the end (10%) of nsP1234. The nsP4 protein is expressed by suppression of termination, and also by cleavage at the nsP3/4 junction[24,25]. An nsP1/2 junction cleavage finishes the preparative steps to form the early replication complex (RC). Which initiates the replication of negative-sense viral RNA while a cleavage event completes the positive-sense producing machinery at the nsP2/3 junction. The resulting mature non-structural proteins, interact with host cell proteins forming the RC, producing positive-sense genomic (49S) and sub-genomic (26S) RNA molecules[26,27].

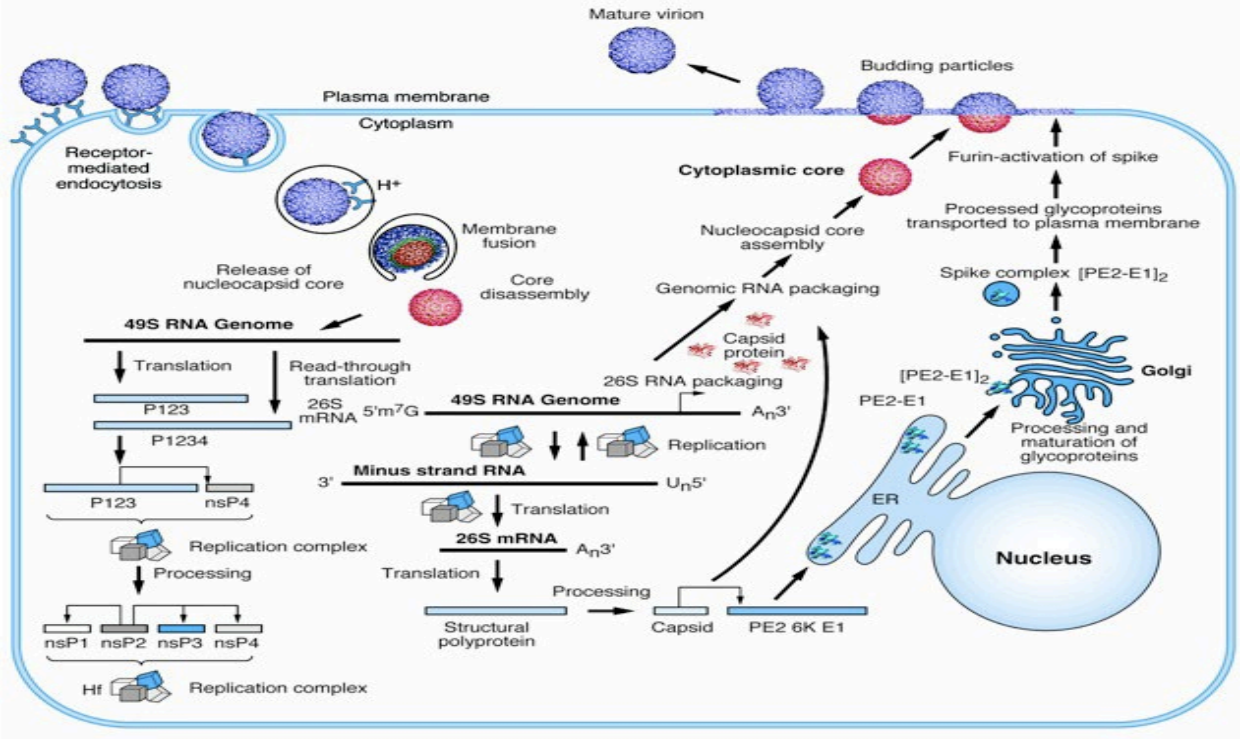

Figure 1-3. Chikungunya virus replication cycle. Chikungunya virus enters the cell by attaching to host receptor proteins using its viral E glycoproteins. Internalization to the cell is through clathrin-mediated endocytosis. Low pH in the endosome triggers viral fusion and the nucleocapsid is released into the cytoplasm. Viral (+)ssRNA is released and translated into a polyprotein, which is, in turn, cleaved into the non-structural proteins necessary for RNA replication and transcription. Non-structural proteins (nsP1-4), form replication complexes at the surface of endosomes. A dsRNA molecule is synthesized from the original (+)ssRNA, (-)ssRNA is then transcribed/replicated, thereby providing viral mRNA and new (+)ssRNA viral genomes. The expression of 26S sub-genomic RNA produces structural glycoproteins. These glycoproteins are processed through the Golgi and are transported to the plasma membrane. At the cytoplasm RNA binding to capsid proteins forms a nucleocapsid, which in turn is enveloped by budding at the plasma membrane exiting the host cell. Richard J. Kuhn (2018)

A single structural polyprotein is translated using the previously generated 26S sub-genomic positive-sense RNA, generating five structural proteins: The capsid (C), the envelope forming E1 and E2 proteins, and the two smaller E3 and 6K proteins, which are cleavage products[28,29]. The capsid protein contains two domains, an amino-terminal domain that regulates RNA packaging through cooperative functions of its three subdomains[30] and a C-termini globular protease domain, which executes two proteolytic cleavage events. First, it separates the C-termini from the C-E3-E2-6k-E1 polyprotein and the second, which leads to the release of the viral genome into the host's cytoplasm during initial steps of infection. The resulting envelope polyprotein E3-E2-6k-E1 is transported to the endoplasmic reticulum (ER) were host signalases cleave the polyprotein at the C- and N- termini of the 6k peptide, resulting in E3-E2, 6k, and E1 proteins[31]. A final cleavage event occurs at the Golgi apparatus during E3-E2 transport to the plasma membrane, where host furin or furin-like proteases separates E2 from E3[32–34]. Nucleocapsid formation occurs when 120 dimers of C protein capture and package a positive-sense viral RNA and form a spherical particle[35–37]. Finally, E1/E2 heterodimers form and accumulate at the plasma membrane, and C binds to E2 protein promoting cell exit or viral budding, which brings the alphavirus replication cycle completion[38,39].

1.1.3 Chikungunya fever

Some alphavirus cause diseases that may have been misdiagnosed for decades until their first descriptions and characterizations was achieved. It is believed that Chikungunya fever (CHIKF) was often confused with, and treated as Dengue fever, and it wasn't until 1995 that it was first described by Marion Robinson and W.H.R. Lumsden, following an outbreak on the Makonde plateau, close to the border between Mozambique and Tanzania[40]. The term "chikungunya" derives from the Makonde word *kungunyala*, meaning "to be contorted" or "that

which bends up." Its RNA genome has a high mutation rate, specifically in the structural proteins E1[41–44] and E2[45,46], and in the non-structural proteins nsP1, nsP3, and nsP4[44,47,48]. This high RNA mutation rate has produced interesting phenotypes such as E1-A226V mutation, which enhances CHIKV infectivity in *A. albopictus*[43]. As far as it is known, CHIKV is the sole alphavirus serotype that confers immunity to recovered patients from reinfection. However, there is enough variation to distinguish between 5 genotypes[49]: Central Africa (CA), West Africa strain (WA), East-South African strain (ESA), Indian Ocean strain (IO), and the Asian strain (Figure 1-4).

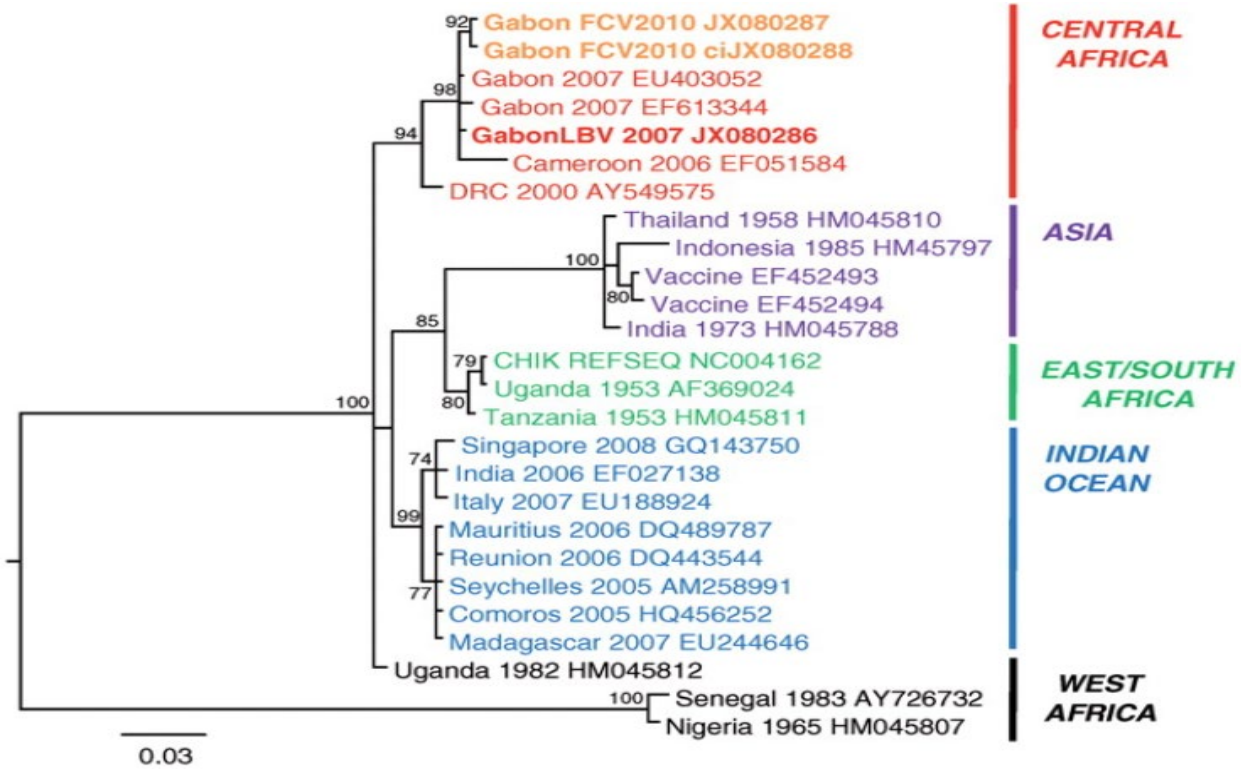

Figure 1-4. Chikungunya virus phylogenetic tree. Phylogenic tree of Chikungunya virus partial E gene sequence. All sequences isolated from clinical cases reported in Africa and representative sequences of CHIKV were included. West-African strains have evolved separately from the Central-Africa, Asia, East/South Africa and Indian Ocean strains. Caron et al, 2012.

Mosquitoes acquire the virus from a viremic host. After an incubation period that lasts for approximately ten days, the infection reaches a sufficient transmission titer and is capable of infecting susceptible hosts[50,51]. CHIKV is introduced through the mosquito bite directly into host's skin where it replicates inside dermis fibroblasts and is thought to reach blood vessels

where it disseminates to multiple tissues. In humans, CHIKV's incubation time ranges from two to ten days[6]. However, the percent of asymptomatic cases has historically varied between outbreaks, ranging from 3.8% to 27.7%[52]. CHIKF is characterized by an acute onset of fever, which typically lasts from 2 days to weeks, usually followed by severe polyarthritis, which can persist for months to several years [53]. Another not so typical symptom is maculopapular rash, which is only present in about 20% of confirmed patients. Typically, Old World alphaviruses are predominantly associated with polyarthritis and maculopapular rash. However, there are reports of recent outbreaks which show that CHIKV-infected patients can also develop symptoms more aligned with those of New World viruses which include meningoencephalitis in neonates and even some hemorrhagic disease[54,55].

Viral replication occurs in different tissues, which include muscle, joint, skin, liver, spleen, and meninges in neonates or immunocompromised patients. Fever onset usually correlates with viremia, in which the virus load can rapidly reach 10^9 RNA copies per milliliter of blood. This high level of viral replication triggers the innate immune response and the production of Type-I interferons. Fever dissipates within a week, which also coincides with low viremia titers. Antibody-based adaptive immunity clears the remaining virus with classic IgM anti-CHIKV antibodies. Typically, CHIKV does not cause any apparent damage in the healthy adult human brain. However, clinical evidence suggests neurotropic activity in neonates, young children, and elderly patients. Chronic CHIKV disease consists of persistent and relapsing joint pain that can last for several weeks, months, or even years. This virus-mediated arthralgia is coincident with anti-CHIKV IgM antibodies, which may be induced by constant exposure to specific CHIKV antigens.

The 2005-2006 epidemic on the French island of La Reunion was the first outbreak the virus was widely disseminated, and marked one of the highest CHIKV morbidity rates known. It was also the first time where severe adult cases, and deaths were attributed to CHIKF[14,56–64]. Severe cases were manifested in patients with underlying medical conditions like cardiovascular, neurological, and respiratory disorders. Acute incapacitating arthralgia was present in the affected joints of up to 50% of adult patients. This arthralgia lasted from 6 months to several years post infection[65]. Additionally, patients with post-CHIKV arthritic illnesses and progressive erosive arthritis were widely reported[56]. Contrary to classic rheumatoid arthritis (RA), levels of anti-cyclic citrullinated peptide and rheumatoid factor antibodies were not typically elevated. These observations suggest that post-CHIKV arthritis is a chronic inflammatory erosive arthritis[58,66].

Figure 1-5. Chikungunya virus outbreak in Acapulco, Mex. Chikungunya epidemic in Acapulco, Guerrero, accounted for over 400 CHIKV positive cases in May 2015. Emergency cases of Acapulco general hospital. (2015)

More recent outbreaks throughout the American continent and the Caribbean in which CHIKV strains were disseminated from Brazil to Florida, involved approximately one million people. These outbreaks produced interesting epidemiological data[67–69]. In 2015, Mexico suffered its first recorded CHIKV epidemic with 8,668 confirmed cases throughout 18 different states (Figure 1-5)[3,70,71]. The Mexican Diagnostic and Epidemiological Reference Institute (InDRE) isolated and sequenced two CHIKV strains: InDRE04 (Jalisco) which was isolated from a 33-year-old woman and identified as an imported case from the Caribbean, and InDRE51 (Chiapas) isolated from the first autochthonous case in Mexico, an eight-year-old girl[72].

Phylogenetic analysis indicated that both Mexican strains belonged to the Asian genotype, which is closely related to the 99659 strain, isolated in the British Virgin Islands. Interestingly, the E1 A226V mutation that enhances vector selection was not present in either genomes[72]. Severe clinical manifestations related to these strains were concentrated in the 20- to 24-year-old age groups. Acute fever, cephalea, and myalgia were present in over 90% of the cases; severe and light arthralgia was manifested in 70% of the cases; and a severe rash was present in 58% of the cases[53,73,74].

1.1.4 Innate immunity and inflammation

CHIKV infection in humans starts when an infected *Aedes* mosquito inoculates the virus into the skin through a bite. Once inside the body, it is thought that CHIKV replicates within susceptible cells, such as skin fibroblasts and monocytes[75–78]. It is also believed that mosquito saliva, which contains several proteins that prevent blood coagulation and downregulate host immune responses, enhances CHIKV infection[51]. SAAG-4 is an identified protein in *A. aegypti* saliva that promotes CD4 T cell induction of IL-4, thus promoting a Th2 response (Figure 1-6)[79].

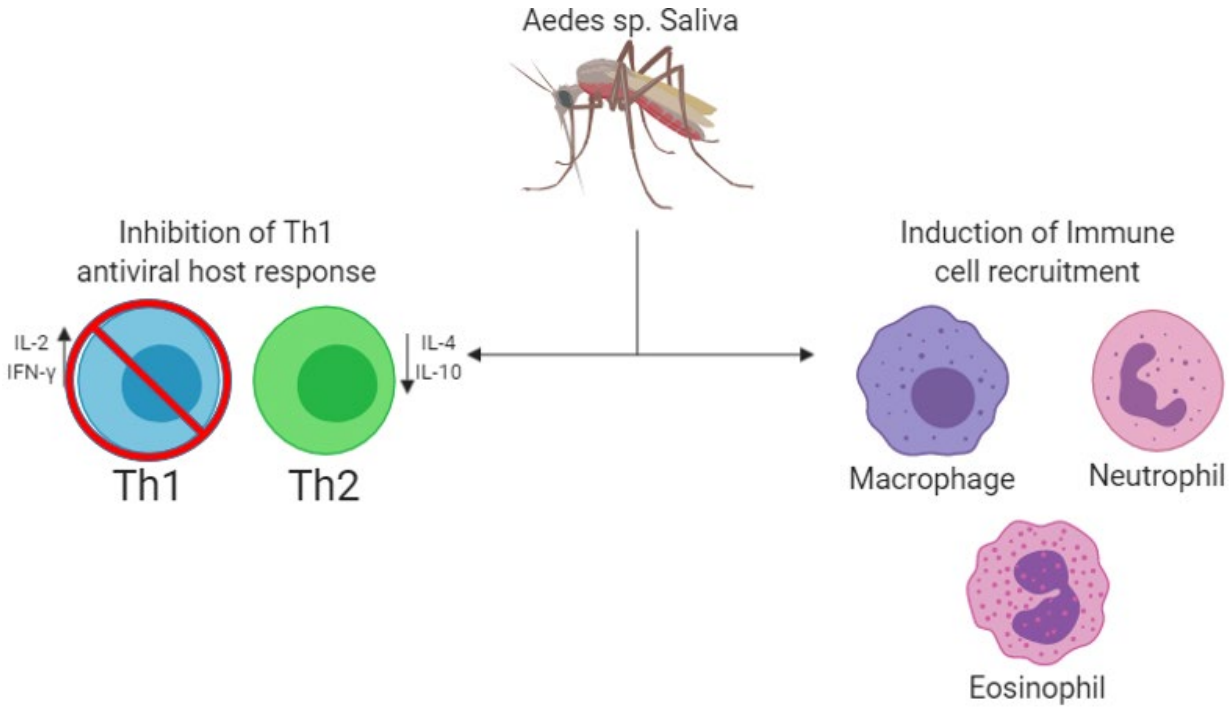

Figure 1-6. Effects of *Aedes* saliva on host immune response to Mosquito saliva. *Aedes* saliva is infectious as soon as two days after infection. Host Th1 cytokine immune response is significantly suppressed by mosquito saliva. Eosinophil, neutrophil, and macrophages are recruited to the site of inoculation in the presence of mosquito saliva.

Further studies have shown that mosquito saliva recruits eosinophils and neutrophils to the bite site, whereas these immune cells are absent at needle inoculation sites[80]. In mice, the resulting induction of a Th2 response decreases the classic anti-viral Th1 response, which produces a more susceptible host, thereby enhancing arboviral infections[81]. Although the early infection events have not been clearly defined, the acute blood phase is characterized by a brief but highly viremic period, where viral titers reach up to 10^9 viral copies per ml[82,83]. Previous studies have shown that mainly migrating monocytes, and to a lesser extent, B-cells and dendritic cells, are targeted during the acute blood phase[78,84].

In spite a robust innate immune response against CHIKV infection, the virus disseminates rapidly to the bloodstream. This viral dissemination could happen through the immune suppression of mosquito saliva discussed above. A Th2-dominated immune response is highly inefficient against viral infections. Another factor contributing to rapid viral dissemination

may be the migration of infected immune cells such as macrophages or dendritic cells to the lymph nodes. Once inside the lymph node, infected cells produce new viruses, which in turn infect more susceptible immune cells. During this phase, the infection may be contained or eliminated by the innate production of cytokines by different immune cells present in the lymph node. However, somehow the virus manages to escape and further disseminate to other tissues like joints, musculoskeletal tissue, and even brain by activating the endothelium and modifying the permeability of blood vessel barriers[85,86].

Once the virus reaches the bloodstream, it reaches the high average titers, which lasts between two and ten days in humans. The sudden decay of viral presence is thought to be due to a strong Type-I IFN response, to which CHIKV is highly sensitive[57,77].

Febrile and arthritic pathologies are likely immune-mediated. Infected patients typically exhibit a pro-inflammatory cytokine profile, which includes high levels of IL-1β, IL-6, and TNF-α[87–90]. It is thought that CHIKV induces an inflammatory loop in which a pro-inflammatory response causes arthralgia via infected fibroblasts, which expresses high levels of prostaglandins and contributes to the development of long-lasting chronic osteoarthritic joint pathology (Figure 1-7)[91–95].

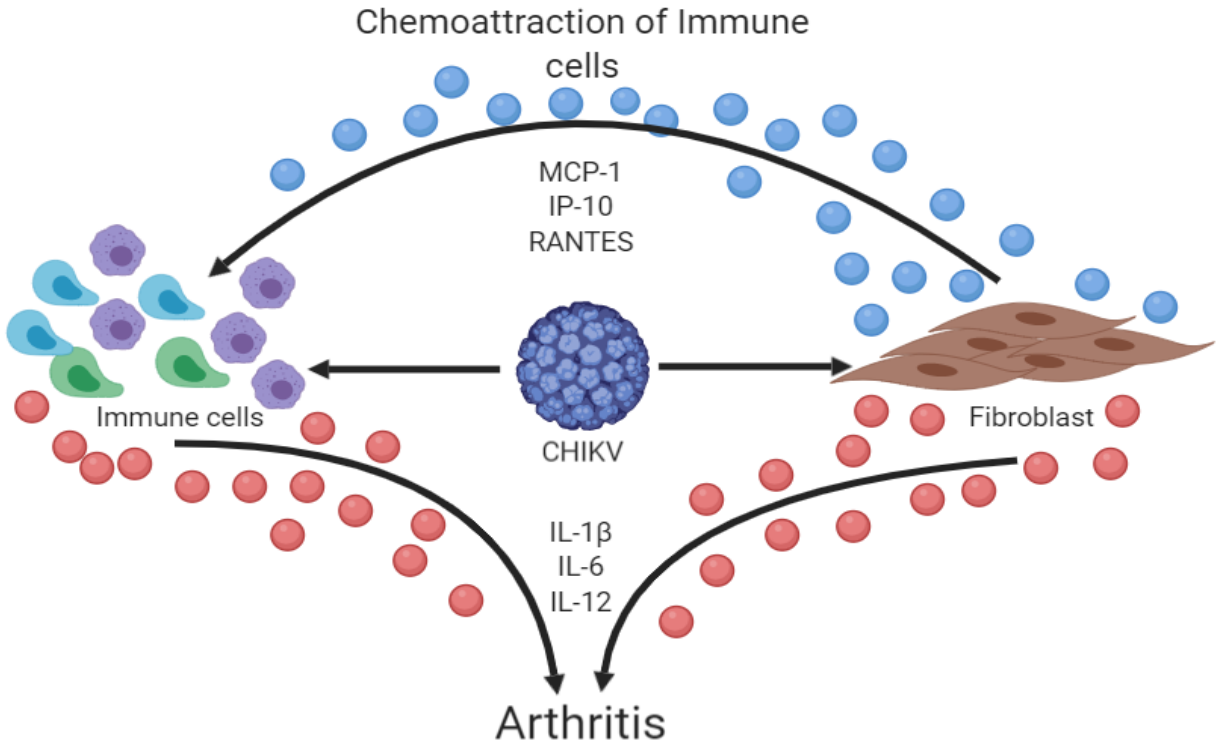

Figure 1-7. Chemoattraction of immune cells by infected fibroblast. Chikungunya virus infects fibroblasts and immune cells. Infected fibroblast secretes chemoattractant cytokines recruiting more immune cells to the site of infection. Infected cells secrete pro-inflammatory cytokines producing tissue damage leading to virus-induced arthritis.

The severity of the disease has been associated with high secretion levels of IL-1β, IL-6, IL-12, and a known T-cell chemokine RANTES, which has been useful for patient monitoring[88]. Patients with severe polyarthritis have shown higher levels of secreted MCP-1, IFN-α, IFN-γ, IL-6, and IP-10 than patients without polyarthritis. Indicating their pathologic role in the chronic phase of this disease[89,96]. Interestingly, these cytokines and chemokine profiles differ slightly from cohort to cohort, which has caused confusion as to which factors are more responsible for this CHIKV-mediated malady. It is possible that these differences may ultimately be attributed to ethnic and/or genetic differences between infected populations. However, understanding the role that pro- and anti-inflammatory factors play in Chikungunya virus disease (CHIKD) progression is still not completely understood. Further enlightenment of the molecular interplay between

viral factors and the host immune responses could elucidate potential targets to ameliorate polyarthritic pathology and stop disease progression.

1.2 Adaptive immunity and disease protection

It is widely accepted that after a primary infection, the immune system establishes an anti-CHIKV response, which may confer complete protection against reinfection. This is supported by epidemiologic studies where, contrary to other arboviral diseases, the re-emergence of CHIKV in previously infected populations does not occur[97]. This is supported by the fact that CHIKV re-emergence and epidemics occur every 7 to 8 years, with some instances where the virus was absent for up to 30 years[98–100].

T cells have an essential role in viral surveillance and elimination of infected cells, and it has been proven that they are also associated with CHIKV-induced pathology. In C57BL/6 mice, CD4+ and CD8+ T cells are found infiltrating inflamed joints of CHIKV-infected animals[101,102]. In two animal studies, CHIKV induced arthralgia appears to be mediated by the infiltration of CHIKV-specific CD4+ T cells (Figure 1-8). The same research also showed that CD4+ T cells do not mediate local inflammation via IFN-γ-mediated pathways[94]. Additionally, CD8+ T cells do not appear to have any antiviral activity or pathological role during CHIKV infection[94].

Interestingly, gene set enrichment studies with MHC-II and IFN-γ deficient mice showed an overlap in differentially expressed genes from RA and CHIKV-induced arthritis[103].

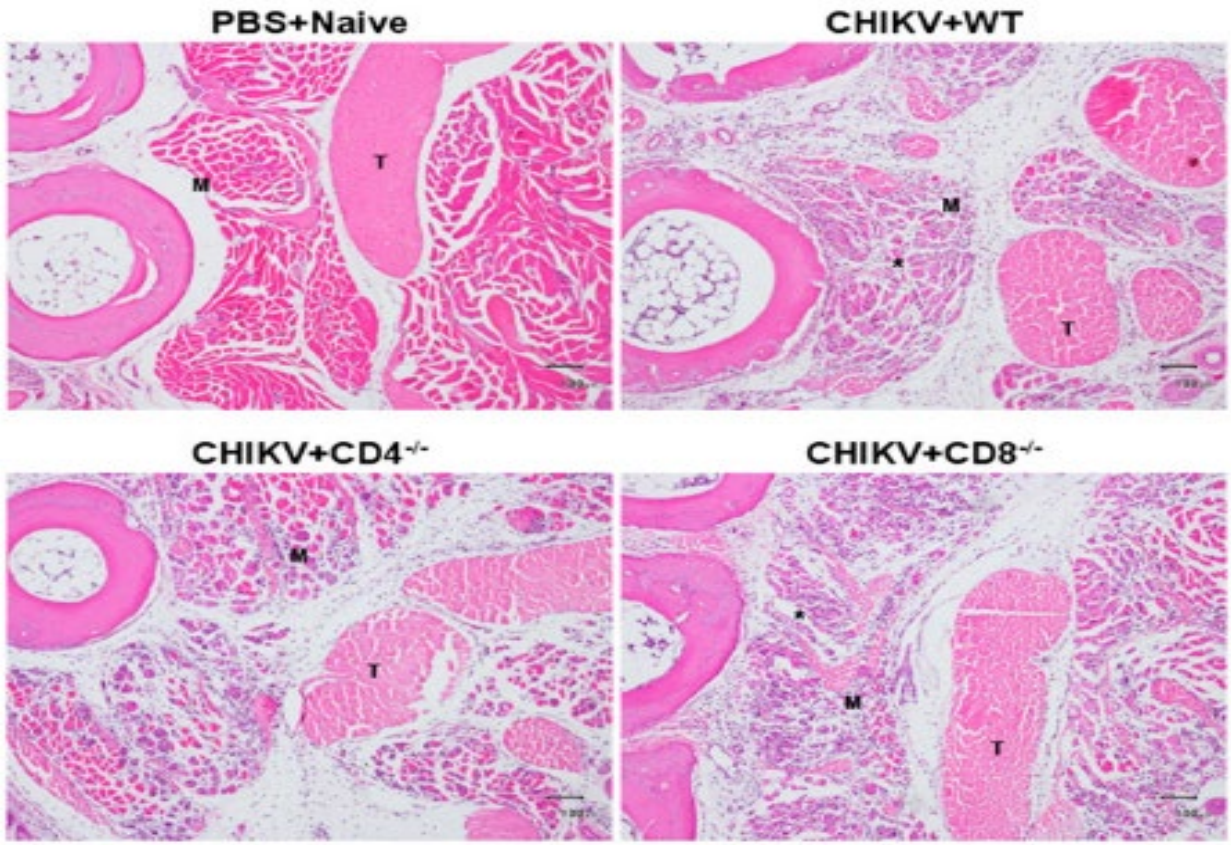

Figure 1-8. Depletion of CD4 T-cells ameliorates inflammation in mice. Reduction in joint pathology in $CD4^{-/-}$ mice. Representative histopathology photographs of swelling footpad in PBS+Naïve, CHIKV+WT, CHIKV+ $CD4^{-/-}$, and CHIKV+ $CD8^{-/-}$ mice on 6 dpi. H&E staining and transverse sectioning were done. The asterisks denote regions of severe infiltration and tissue damage. Scale bars, 100 µm. M, Muscle; T, tendon. Teo et at, 2013

B cells play an essential role in CHIKV clearance. This was demonstrated in µMT mice, where the absence of B cells allowed persistent CHIKV viremia for over a year[104]. CHIKF was more severe in these B cell knock-out mice, compared to wild type mice during the acute phase. Antibody protection against CHIKV has also been extensively addressed in conjunction with vaccine development, and structural glycoproteins have been shown to be successful surface targets for neutralizing antibodies against CHIKV (Figure 1-9)[105–109]. Despite the host's robust anti-viral response, CHIKV infection can persist in the host by evading neutralizing antibodies using a relatively unexplored cell-to-cell transmission mechanism (Figure 1-10). Co-culture of CHIKV infected and uninfected Hek293T cells, in the presence of a CHIKV Monoclonal

antibody, shows viral dissemination to previously uninfected cells. Genomic sequencing of these escape mutants reveals an E2.R82G mutation, which suggests the involvement of CHIKV E2 protein[107]. Notably, the E2 domain of other alphaviruses has been shown to interact with cell surface proteins, like heparan sulfate[110–113].

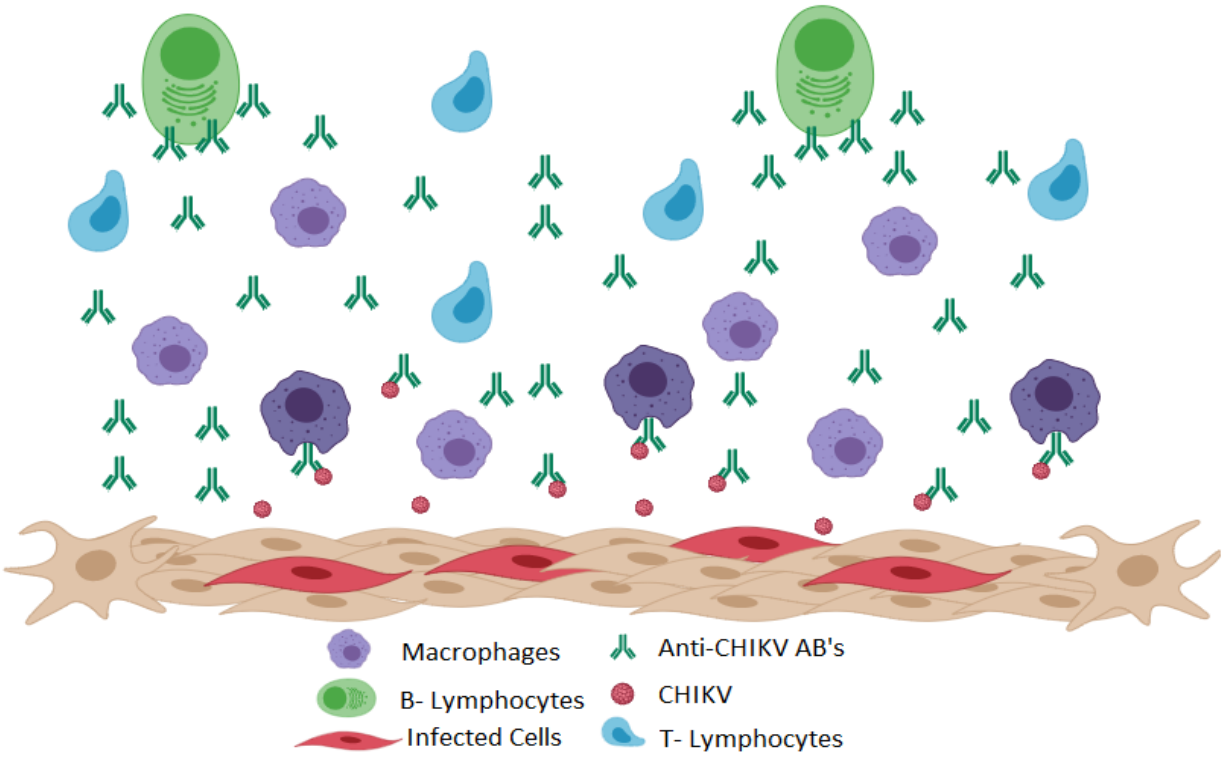

Figure 1-9. The immune system's robust response contains CHIKV infection. Macrophages and lymphocytes are recruited to the site of infection. T cell viral surveillance and elimination of infected cells mediate CHIKV-induced pathology. Activated macrophages infiltrate the affected tissue and promote a pro-inflammatory response. B cells and their antibody response are crucial for viral clearance.

Immunization with CHIKV virus-like particles introduces critical surface viral glycoproteins, which then can induce the production of neutralizing anti-CHIKV specific polyclonal Antibodies (pAbs). VLP vaccines have shown promising results by inducing CHIKV clearance in various mouse models, and non-human primates[114–121].

CHIKV-specific antibody therapy reduced viral infection and spread and neutralized reservoirs of the infectious virus; however, viral RNA persisted in the presence of Mab therapy

even when the infectious virus was not recovered from infected rhesus macaques[122]. It is still unclear why these cell populations are not eliminated by cytotoxic T cells or antibody-mediated effector mechanisms like phagocytosis or cellular cytotoxicity.

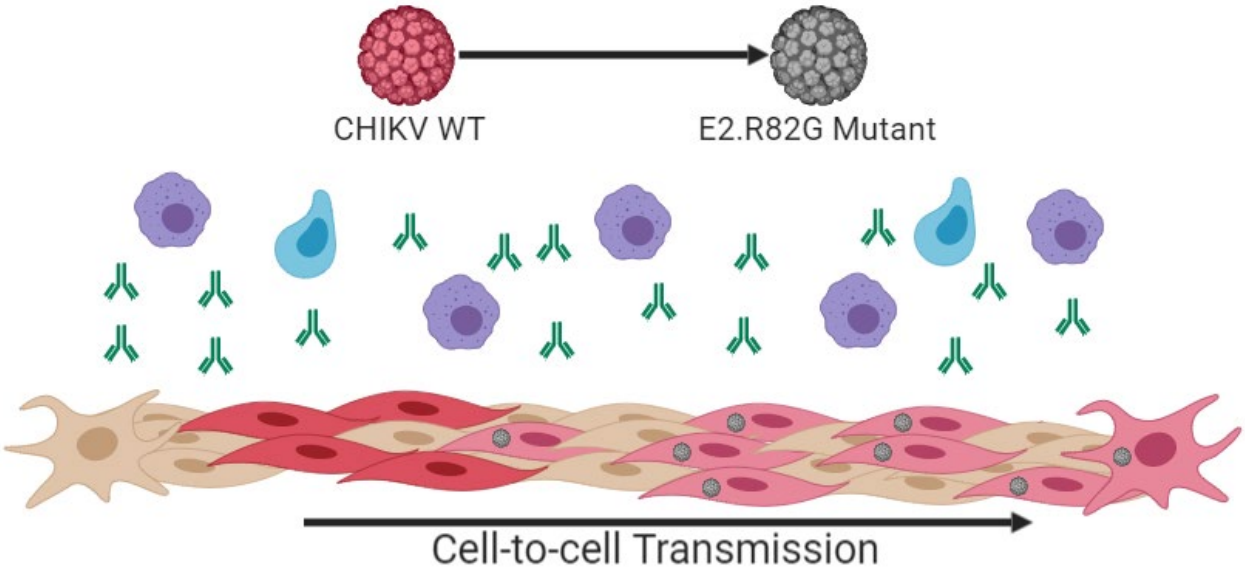

Figure 1-10. Mutation in CHIKV envelope protein 2 enhances cell-to-cell transmission. Chikungunya virus infection can evade neutralizing antibody response by undergoing genetic variation, improving long-term persistence. CHIKV's E2.R82G mutation is thought to induce a cell-to-cell transmission strategy, increasing evasion of the host immune response.

1.3 Animal models

1.3.1 Acute CHIKV disease models

CHIKV infection in humans is typically characterized by fever, arthritis, tenosynovitis, myositis, and myalgia. However, this pathophysiology of CHIKV infection in humans was mostly unknown before the La Reunion outbreak, due to the lack of an adequate animal model of infection.

An attempt to model acute CHIKV musculoskeletal disease was made using wild-type C57BL/6 (B6) mice, which, to date, is still the most used mouse strain to model the disease. Subcutaneous footpad inoculation of neonatal B6 mice produces disease signs with similarities to human pathologies. Which include joint swelling of the inoculated foot, tenosynovitis, myositis,

and periostitis[94,101,102,123]. Tissue damage induction by CHIKV has also been observed in affected footpads where the loss of trabecular and cortical bone correlates with that of human patients[56,124]. Osteoclastic bone resorption has also been identified as a component of CHIKV induced arthritis in 25-day-old and 8-week-old wild-type B6 mice[56,123]. Viremia in CHIKV-infected wild-type B6 mice is characterized by a high titer, which lasts for up to 10 days. Viral replication has been observed in various peripheral tissues, but joint-associated tissues contain the highest viral titer[94,101,102,125]. These observations have been confirmed in other strains such as ICR mice and CD-1 mice[95,126], wild-type 129 mice[127], and DBA/1J mice[128]. However, there were differences between these models (Figure 1-11). Wild-type 129 mice did not develop swelling of the inoculated foot, and immune cells infiltration was significantly milder than that reported for wild-type B6 mice. This suggests that the underlying genetics of the mouse strain can influence the development of acute musculoskeletal disease.

Additional studies indicate that the outcome of CHIKV infection is not only dependent on host genetics, but also on age. Wild-type B6 mice inoculated intradermally with CHIKV showed an age-dependent mortality. All 6-day-old mice succumbed to infection, 50% of 9-day-old mice succumbed to infection, and no mortality was observed in mice that were 12-days or older at the time of inoculation[77,101,129]. Other pathologies that were also age-dependent included foot swelling and tissue injury[130]. In correlation with clinical data where elderly humans harbor higher CHIKV viral titers[129], it was shown that older mice have prolonged viremia and elevated titers in tissues, when compared to 12-week-old mice[130].

In summary, wild-type C57BL/6 and other mouse strains have been extensively used by several research groups to investigate CHIKV's pathogenesis during the acute phase of the disease.

Musculoskeletal disease, innate and adaptive immunity, viremia and cell tropism, the influence of mosquito saliva on infection, and vaccine efficacy evaluations have all been investigated using CHIKV mouse models. However, none of these models have been able to produce polyarthritic mice, and in consequence, the mechanisms used by CHIKV to evade the immune response have remained unclear.

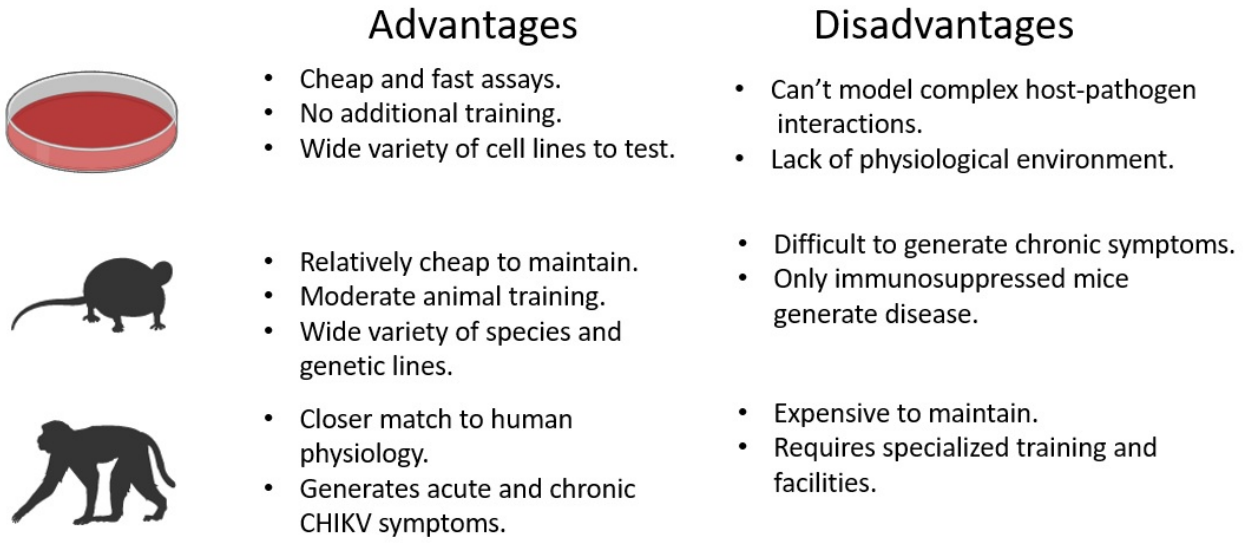

Figure 1-11. Advantages and disadvantages of different methods to model CHIKV disease. Progress of CHIKV research is limited to the current *in vitro* and *in vivo* models. *In vitro* models are cheap but lack the complexity needed to explore complex host-pathogen interactions. *In vivo,* mouse models have a wide variety of genetic backgrounds that can help explore the disease but cannot wholly mimic species-specific host-pathogen interactions. *In vivo,* non-human primate models develop disease closer to human pathology but maybe price restrictive.

1.3.2 Chronic phase and other severe outcomes of CHIKV infection

CHIKV persistence and disease relapse are one of the most debilitating aspects of disease caused by this virus, and it's been documented that it can last for months to years[14,131,132]. However, the mechanisms of chronic CHIKV disease pathogenesis are still not well understood. Experiments performed in cynomolgus macaques were the first to provide evidence of persistent CHIKV RNA in joint-associated tissues, muscle, and secondary lymphoid tissues 1-3 months after inoculation. In lymphoid tissues, viral antigen was localized in CD68+ macrophages,

suggesting that these cells can serve as a reservoir for persistent CHIKV infection and dissemination[133].

Following subcutaneous inoculation of the footpad in RAG1$^{-/-}$ mice, viral RNA and infectious virus were recovered in different tissues up to 112 days post-inoculation, but CHIKV was not detected in serum samples and muscle tissue of wild-type B6 mice after day seven. Collectively, these data suggest that T and B-cell mediated immunity controls CHIKV pathology in a tissue-specific manner[125].

Persistence of CHIKV in joint-associated tissue is associated with persistent synovitis and myositis, along with elevated levels of pro-inflammatory cytokines, which suggests that chronic CHIKV infection induces joint inflammation[95,125,130]. C57BL/6 mice, along with different genetic knockout models, have provided essential data on viral and host factors[95,125,130,134] that drive the persistent infection in joint-associated tissue, and culminate in the development of chronic arthritis.

Long-term CHIKV persistence is detectable not only in infected cynomolgus macaques[133] but also in rhesus macaques. Adult rhesus macaques that were inoculated intravenously with 10^7-10^{10}PFU, developed viremia lasting 3-4 days, lymphopenia, lymphadenopathy, fever, and maculopapular rash[114,135]. Histopathology analysis of various tissues from non-pregnant adults showed the absence of chronic joint inflammation and virus, which indicated a lack of chronic CHIKV pathologies in adult rhesus macaques[135]. In contrast, aged macaques showed viral RNA persistence in spleen tissue. However, this was strain-dependent, where the La Reunion strain displayed higher viral titers in spleen and serum[135].

CHIKV-associated encephalitis is usually found in neonates born to viremic mothers or exposed to the virus during birth, and rates of infection can reach 50%[136,137]. In mouse models,

severe morbidity developed in neonatal wild-type B6, ICR, and CD-1 mice. Mortality rates from these experiments were lower for ICR, and CD-1 neonate mice when compared to wild-type B6 (20% vs 100%, respectively)[138]. CHIKV can also disseminate to the central nervous system (CNS) of adult *Ifnar1*-/- B6 mice, which exhibit elevated viral titers in the brain, leading to severe morbidity and ultimately death[77,127,139,140]. Additional studies in *Ifnar*1-/-, as well as *Ifnar*7-/- and *Ifnar*3-/- B6 mice, showed that CHIKV infection is associated with hemorrhagic shock pathologies, which include vasculitis, hemorrhage, and thrombocytopenia[141].

Finally, these data suggest that CHIKV readily spreads and can cause severe pathologies only in neonatal mice, and adult mice with Type-I IFN pathway deficiencies. These models have provided systems to investigate CHIKV's mechanisms of acute and atypical outcomes as well as lethal challenges for vaccine evaluation and therapeutic trials.

Chapter 2. A Comparison of Chikungunya Virus Infection, Progression, and Cytokine Profiles in Human U937 and Murine RAW Monocyte-derived Macrophages

The following chapter is taken from an article published in PLOS One. All content and figures have been formatted for this dissertation, but it is otherwise unchanged.

2.1 Abstract

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that causes rash, fever and severe polyarthritis that can last for years in humans. Murine models display inflammation and macrophage infiltration only in the adjacent tissues at the site of inoculation, showing no signs of systemic polyarthritis. Monocyte-derived macrophages are one cell type suspected to contribute to a systemic CHIKV infection. The purpose of this study was to analyze differences in CHIKV infection in two different cell lines, human U937 and murine RAW264.7 monocyte derived macrophages. PMA-differentiated U937 and RAW264.7 macrophages were infected with CHIKV, and infectious virus production was measured by plaque assay and by reverse transcriptase quantitative PCR at various time points. Secreted cytokines in the supernatants were measured using cytometric bead arrays. Cytokine mRNA levels were also measured to supplement expression data. Here we show that CHIKV replicates more efficiently in human macrophages compared to murine macrophages. In addition, infected human macrophages produced around 10-fold higher levels of infectious virus when compared to murine macrophages. Cytokine induction by CHIKV infection differed between human and murine macrophages; IL-1, IL-6, IFN-γ, and TNF were significantly upregulated in human macrophages. This evidence suggests that CHIKV replicates more efficiently and induces a much greater pro-inflammatory cytokine profile in human macrophages, when compared to

murine macrophages. This may shed light on the critical role that macrophages play in the CHIKV inflammatory response.

2.2 Introduction

Chikungunya virus (CHIKV) is an alphavirus in the *Togaviridae* family. It consists of an outer membrane, an icosahedral capsid, and a positive sense RNA genome which encodes four structural proteins (C, E1, E2, and E3) and four non-structural proteins (nsP1, nsP2, nsP3, and nsP4) [142–144,]. CHIKV is a reemerging disease that has caused major outbreaks in Southeast Asia, Africa, and more recently, in southern Mexico and other South American countries [145–147]. This disease is transmitted by two widely disseminated mosquito vectors from the *Aedes* genus (*Aedes aegypti* and *Aedes albopictus*) [51,148–151]. Recent outbreaks like the one in La Reunion were associated with the atypical mosquito vector, *Aedes albopictus* [14,83,84]. The expansion of the CHIKV vector unequivocally boosted CHIKV dissemination, which included its rapid expansion in 2015 throughout South America, and as far north as southern Mexico [53,71,149,152,153]. The main clinical symptoms are sudden fever, myalgia, rash and debilitating polyarthralgia [14,56,89]. The incubation period for this virus is between 3 and 7 days, and asymptomatic CHIKV cases range from 3-28% [138,154].

CHIKV disease in humans is marked by two phases. The acute phase usually lasts for 7-12 days with a plasma viral load of 10^6-10^9 pfu/mL [145]. Higher levels of viremia are more likely to be detected in newborn and elderly CHIKV patients who usually require hospitalization. During the chronic phase of this disease, long term persistence of anti-CHIKV IgM antibodies has been reported for up to 24 months [14,56,107]. This could be an indication of persistent viral antigenic presence providing a continuous stimulation of the humoral response. This may very

well be the driving factor that leads to the development of chronic arthralgia, which can last for years [14,107].

The tropism of CHIKV in humans includes several human cell types such as primary epithelial and endothelial cells, monocyte-derived macrophages, and fibroblasts [155,156]. Similar to what happens with other alphaviruses, CHIKV-infected cells rapidly undergo apoptosis. Results from several biopsy studies have shown that CHIKV has a tendency to target muscle cells, skin fibroblasts, and joint tissue [77,156]. Additionally, there are also indications of endothelial tissue infections of the liver, spleen and brain [94,133,157,158]. Finally, the entry mechanism for CHIKV is still unclear, but there are indications that viral production is higher in human cells due to the interaction of viral proteins and certain human intracellular proteins. Interestingly, these interactions with mouse protein orthologs are lacking [75,159–161].

The lack of an effective vaccine or anti-viral treatment for CHIKV has resulted in substantial morbidity and considerable economic losses during outbreaks. In recent years, there have been some research efforts towards developing an animal model to build a better understanding of CHIKV pathogenesis; however, these rely on immune-deficient mice which develop swelling restricted to the inoculated foot, accompanied by higher levels of virus replication at the site of inoculation and little replication at distal sites [101,102,126,156,162,163]. This contrasts with the systemic infection seen in humans and the accompanying widespread arthritis. The reasons why mice are not the ideal model to study CHIKV pathogenesis are poorly understood.

In this study, we observed that CHIKV infection and replication efficiencies in human and murine monocytes are significantly different *in vitro*. Additionally, we observed significant differences in pro-inflammatory cytokine production induced by CHIKV infection in human and

murine macrophage cell lines. These results suggest that CHIKV replication in macrophage cell lines varies by host species. This study did not explore further which factors may be related to the higher rates of virus production in human macrophages, but previous research has shown that viral-host interactions are species-selective[160].

2.3 Materials and methods

2.3.1 Cell culture and virus propagation

U937 and RAW264.7 cell lines were propagated in RPMI 1640 (HyClone Cat. No. SH30027.01) media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Cat. No. SH3008703), 10,000 units of Penicillin/Streptomycin (HyClone Cat. No. SV30010), 2mM L-glutamine, and 10mM of HEPES Buffer (HyClone Cat. No. SH3023701). Baby Hamster Kidney (BHK) cells were propagated in DMEM (HyClone Cat. No. 11966025) media supplemented with 10% heat-inactivated FBS, and 10,000 units of Penicillin/Streptomycin. The cells were cultured in T-75 culture flasks (Greiner Bio-One Cellstar Cat. No. 658170) at 37°C in an incubator with 5% CO_2. U937 monocytes were transferred to 6-well tissue culture plates and induced to become adherent macrophage cells (5 X 10^5 cells/mL) by exposure to 5ng/ml of phorbol 12-mystrate 13-acetate (PMA) (Thermofisher Cat. No. P1585) and incubated in 3 mL of RPMI 1640 complete media at 37°C for 24 hrs.

CHIKV-LR strain was kindly provided by Dr. Jonathan Miner, Washington University, St. Louis, MO, and was propagated in Vero cells and stored for further use at -80°C. U937 cells were acquired from ATCC, while RAW264.7 cells stocks were donated by Dr. Kim O'Neill, Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT. Both U937 and RAW264.7 cell line stocks have been authenticated at University of Utah DNA sequencing core and University of Arizona Genetics core facilities, respectively.

2.3.2 Viral quantification by plaque assay

CHIKV-LR stocks and supernatant of infected cultures were titrated in BHK cells. Virus samples were diluted in serial 10-fold dilutions in DMEM + 2% FBS and inoculated in 6-well plates which contained ~90% confluent BHK cultures. Inoculated 6-well plates were incubated for 1 hour to allow virus infection and then a 1:1 mix of 2X MEM + 8% FBS and low-melt agarose was used to overlay. Cultures were incubated for 3 days, fixed with 10% formalin and stained with crystal violet for plaques. Titer was calculated as Log10 PFU/mL and determined by the following equation: PFU/mL = (plaque count/well) * dilution factor / (mL inoculum).

2.3.3 Infection assays

RAW264.7 and PMA-differentiated U937 macrophages were transferred to 12-well tissue culture plates at a cell density of 5 $X10^4$ cells/mL and cultured overnight in complete medium at 37 °C in 5% CO_2. Cultures were infected using CHIKV-LR virus at various multiplicities of infection and incubated in a 37°C incubator with 5% CO_2 for 2 hrs. Infected media was removed and cells were washed 3 times with PBS and fresh media was added and then incubated at the previously described conditions. Supernatant and intracellular RNA samples were taken at 2, 4, 6, 8, 12, 24, 36, and 48 hours' post-infection and stored for plaque assay, or in Trizol Reagent for RNA extraction.

2.3.4 RNA extraction

Intracellular RNA was extracted at previously mentioned time points using Trizol reagent (Thermofisher) and following the manufacturer's directions. Viral RNA in supernatant was extracted using QIAamp Viral RNA Extraction following the manufacturer's directions.

2.3.5 RT-qPCR quantification of viral RNA

Intracellular lysate and supernatant of CHIKV infected cells at MOI of 0.1 and 5 was quantified by RT-qPCR using Applied Biosystems Taqman Fast Virus 1-Step Master Mix (Cat. No. 4444432) using a specific probe and primers for the CHIKV E1 gene. Initial reverse transcription was set at 50°C for 5 minutes; reverse transcription inactivation and initial denaturing stage at 95°C for 20 s and 40 cycles of amplification at 95°C for 5 s and 60°C for 30 s. Final primer and probe concentrations were 400nM and 250nM, respectively. A positive control plasmid was assembled by reverse transcribing CHIKV RNA using Life Technologies SuperScript IV Reverse Transcriptase kit (Cat. No. 18090050) using random hexamers as primers following the manufacturer's directions. Amplification of the E1 gene was performed using primers containing a HindIII endonuclease restriction site in the reverse primer and an XbaI restriction site in the forward primer. Insertion of the PCR product into the pUC18 vector was performed by double restriction digest on the vector and insertion via HindIII-HF (NEB R3104S) and XbaI (NEB R0145S) restriction enzymes. The resulting plasmid, designated pUCE1, was transformed into *E. coli* chemically competent cells. Insertion of the E1 target sequence was confirmed by Sanger sequencing. A Ct standard curve for pUCE1 was done using nine 10-fold dilutions and obtaining the linear regression of the CT values; intercept of obtained experimental samples was analyzed and normalized to CHIKV genome copies per mL. Probe and primer sequences used in this method are shown in Table 1.

2.3.6 Flow cytometry

Infected PMA-differentiated U937 and RAW264.7 cultures were exposed to CHIKV virus at an MOI=1 and incubated for 2 hours at 37 °C, 5% CO_2 atmosphere at an MOI of 1. After 2 hours, the cultures were thoroughly washed with PBS three times and fresh media was added

and then incubated until 8 hpi. Cells were then Fc blocked for 30 min on ice with 10% human serum or mouse serum and 1% BSA in PBS. The cultures were then stained with either an anti-murine mCD11b-APC (ThermoFisher) or an anti-human hCD14-APC (ThermoFisher), and an anti-Chikungunya E1 protein antibody [CHK166; Antibody Research Corporation] previously conjugated with an Abcam Texas Red Conjugation kit (Cat. No. Ab195225) following the manufacturer's recommendations. Cells where fixed with 10% formalin for at least 1 hour before removing them from the BSL-3 suite. Quantification of infected cells was performed using an BD Accuri C6 cytometer and analyzed using FlowJo version 10.5.3.

2.3.7 RT-qPCR quantification of cytokine expression

Total RNA from PMA-differentiated U937 and RAW264.7 macrophages was reverse transcribed using Life Technologies SuperScript IV Reverse Transcriptase kit (Cat. No. 18090050) using random hexamers as RT primers following the manufacturer's directions. ThermoFisher Scientific's SYBR Select Master Mix was used for quantitative PCR assays. Specific primers for GAPDH, TNF, IL-1, IL-6, IL-10, IFN-α, IFN-γ and MCP-1 were designed to target the corresponding human and murine genes. GAPDH expression was used to normalize target mRNA expression, and fold expression changes were obtained by comparing CHIKV infected and uninfected cells using the ΔΔCT method. Probes and primers used in this method have been included in Table 1.

2.3.8 RT-qPCR quantification of Mxra8 expression

Total RNA from PMA-differentiated U937, undifferentiated U937 and RAW264.7 macrophages was reverse transcribed and PCR amplified following the same method previously described using Applied Biosystems Taqman Fast Virus 1-Step Master Mix (Cat. No. 4444432). Specific primers and probes were designed to target the human Mxra8 and GAPDH genes.

GAPDH expression was used to normalize target mRNA expression, and fold expression changes were obtained by comparing PMA-differentiated U937 vs undifferentiated U937 cells using the ΔΔCT method. Probe and primer sequences used in these experiments are listed in Table 1.

2.3.9 Cytometric bead array

Supernatant samples containing secreted cytokines from infected cultures were harvested at 24 hpi and stored at -80°C. Samples were fixed in 10% formalin for at least 1 hour before removing them from the BSL-3 suite. Cytokine standard serial dilutions were prepared on the same day and a linear regression was used to correlate the sample values. Quantification of secreted cytokines was done using BD Biosciences Cytometric Bead Arrays for human cytokines (Cat. No. 551811) detecting TNF, IL-1, IL-6, IL-8, IL-10, and IL-12; and for murine cytokines (Cat. No. 552364) detecting IFN-γ, IL-6, IL-10, IL-12, and TNF. Sample preparation was done following the manufacturer's directions and data was acquired in a BD Accuri C6 cytometer.

2.4 Safety protocols

All of the experimental work involving infectious CHIKV was performed in a Biosafety Level 3 environment and complying with all Brigham Young University Institutional Biosafety Committee requirements which were approved in protocol IBC-2018-0028.

2.5 Statistical analyses

Comparisons between groups were calculated in R (version 3.4.3) and analyzed with Welch's two-sample t-test which accounts for unequal variances between groups. We corrected for multiple comparisons using the Holm-Sidak method. *P* values of ≤ 0.05 were considered to be statistically significant. Graphics were generated using GraphPad Prism 8.0.1 for Windows, GraphPad Software, San Diego, California USA. Statistical results are included in Table 2.

2.6 Conflicts of interest

The authors declare no conflicts of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

2.7 Results

2.7.1 Chikungunya virus replicates to higher titers human macrophages than murine macrophages

CHIKV has a wide range of tropism in human cells including fibroblasts, muscle cells and macrophages[78,164]. However, to our knowledge, there has not been a direct comparison of CHIKV replication efficiency and innate immune responses in human versus murine macrophages, which may shed light on the differences in CHIKV pathogenesis between these two species. The human PMA-differentiated U937 and murine RAW264.7 macrophages were infected with CHIKV-LR (La Reunion strain) at low and high multiplicity of infection (MOI), and viral supernatants were then titered by plaque assay. For both sets of infections, we observed an approximately 10-fold higher production of infectious CHIKV in PMA-differentiated U937 cells at 8, 16, 24, 36 and 48 hours post infection (hpi) when compared to RAW264.7 cells (Figure 2-1). Viral RNA quantification of supernatant samples confirmed our plaque assay findings. Viral RNA levels increased at 8 hpi with a 10-fold difference between human and murine cultures, regardless of initial MOI (Figure 2-2A). Replication of viral RNA and infectious virus at a high MOI in PMA-differentiated U937 cultures increases over time until it reaches a plateau at 24 hpi. This stationary phase is observed until 36 hpi in RAW 264.7 cultures. Viral replication (viral RNA and infectious virus) at a low MOI shows a constant increase of viral RNA and infectious virus until 48 hpi (Figures 2-1 and 2-2B).

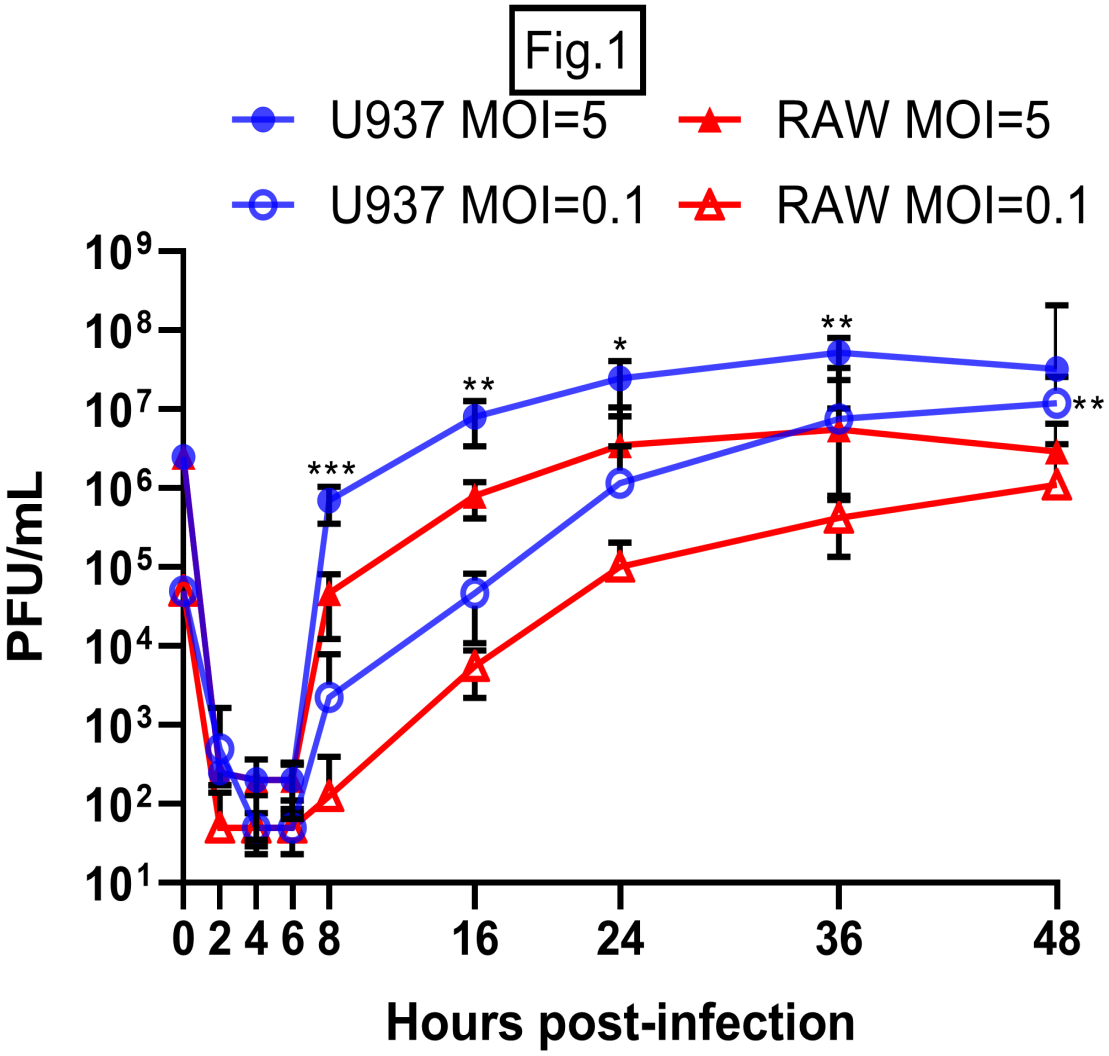

Figure 2-1. CHIKV replicates more efficiently in human macrophages than in murine macrophages. CHIKV infectious virus quantification was performed via plaque assay at the stated times (hpi). Data show mean values of three independent experiments with a total of n =9, MOI=0.1 and 5. Statistical significance was determined using multiple t-test corrected using Holm-Sidak method. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

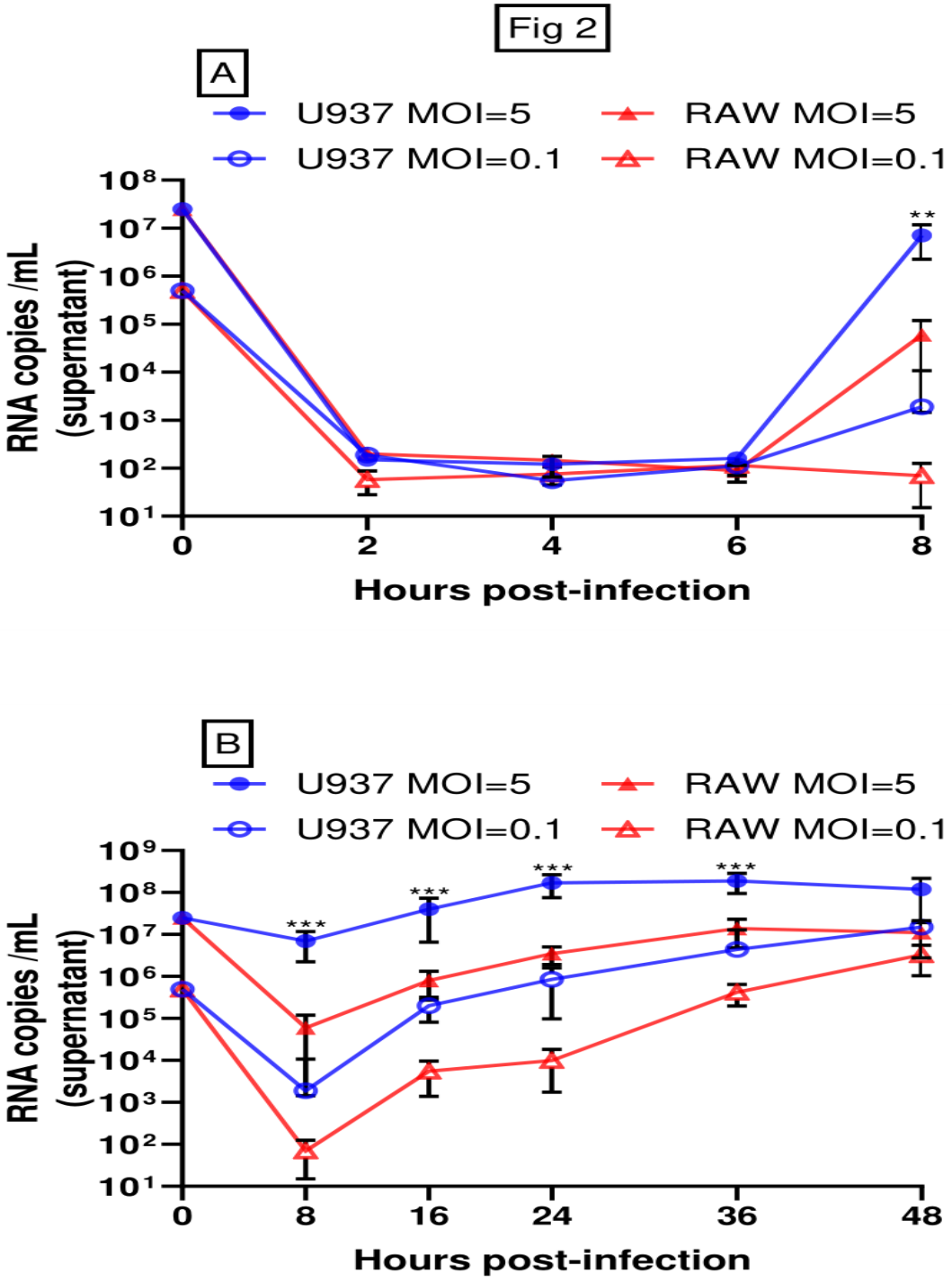

Figure 2-2. The CHIKV genome replicates more efficiently in human macrophages than in murine macrophages. A) RT-qPCR quantification of CHIKV RNA in supernatant samples collected from 2 to 8 hpi. B) RT-qPCR quantification of CHIKV RNA in supernatant samples collected from 8 to 48 hpi. Data show mean values of three independent experiments with a total of n=9, MOI=0.1 and 5. Statistical significance was determined using multiple t-test corrected using Holm-Sidak method. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

To determine if there is a difference in viral entry between murine and human macrophages, we measured both intracellular (Figure 2-3) and extracellular (Figure 2-2A) viral RNA during early time points of the first replication cycle. Our results showed that the majority of CHIKV RNA and infectious virus titer in supernatant decreases within 2 hpi in both cell lines, with no significant difference in intracellular viral RNA by cell type through 6 hpi (Figure 2-3). CHIKV infected PMA-differentiated U937 cells and RAW264.7 cells with similar efficiencies and it was not until 8 hpi that the amount of CHIKV RNA inside human macrophages increased about 2 logs greater than that in murine cells (Figure 2-3). These findings suggest a similar decrease in CHIKV titer in the supernatant but that it replicates better in human PMA-differentiated U937 macrophages.

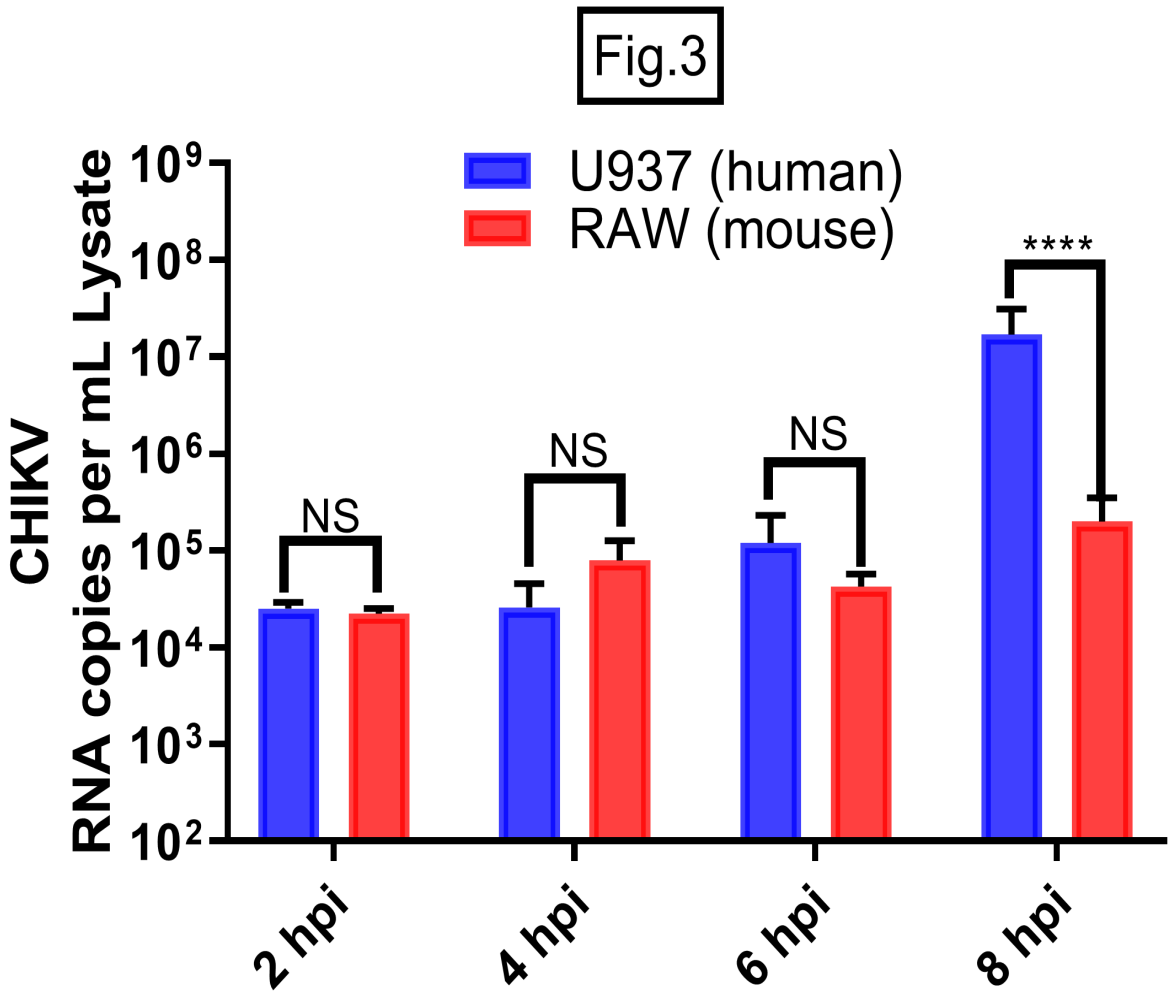

Figure 2-3. CHIKV genome levels in human and murine macrophages shortly after infection. Intracellular CHIKV RNA copies were quantified via RT-qPCR at stated times (hpi). Data show mean values of three independent experiments with a total of n=9, MOI =5. Statistically significant p values are denoted with an asterisk between compared groups. Statistical significance was determined using multiple t-test corrected using Holm-Sidak method. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

In addition, we explored the rate of productive CHIKV replication via flow cytometry. PMA-differentiated U937 and RAW264.7 cultures were infected at an MOI of 1 and quantified at 8 hpi using an anti-CHIKV antibody that targets the viral E1 glycoprotein. Results showed similar levels of E1 glycoprotein (an average of 60% positive cells) in both cell lines and no significant differences between human and murine macrophages (Figure 2-4).

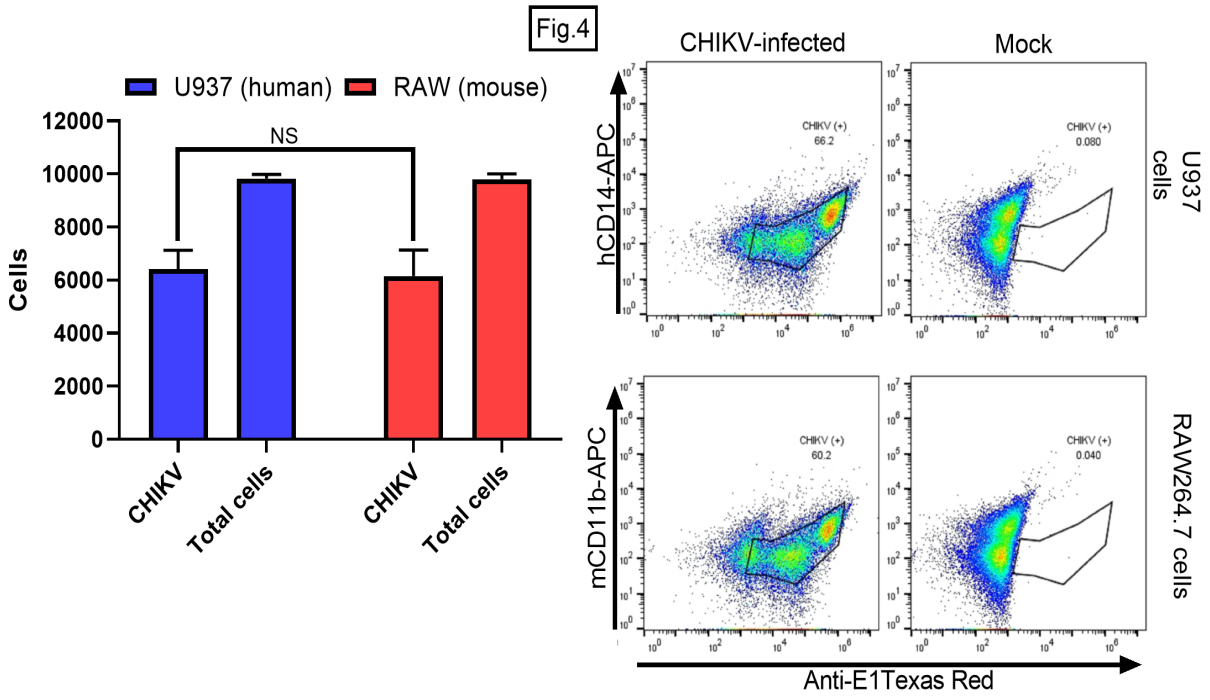

Figure 2-4. PMA-differentiated U937 and RAW264.7 cells display CHIKV envelope proteins at 8 hpi. PMA-differentiated U937 and RAW264.7 macrophages were exposed for 2 hours to CHIKV and then fixed and assayed at 8 hpi using flow cytometry and an anti-E1 protein fluorophore-conjugated monoclonal antibody. Data show mean values of three independent experiments with a total of n=9, MOI =1. Statistical significance was determined using multiple t-test corrected using Holm-Sidak method. *P<0.05; NS, not significant.

These accumulated data suggest that virus production is higher in PMA-differentiated U937 human macrophages versus murine RAW264.7 macrophages, and that CHIKV titers decrease in the supernatant, regardless of the cell line.

2.7.2 Production of pro-inflammatory cytokines following CHIKV infection shows species-specific differences.

Macrophages are one of the first lines of defense against infection and are responsible for the secretion of cytokine and chemokine signals to promote either anti- or pro-inflammatory pathways. Since systemic inflammation is a key difference in human versus murine infections, we examined a possible role in the mediation of this inflammation by cytokines secreted from infected human versus murine macrophages. CHIKV infection of PMA-differentiated U937

human macrophages showed a robust production of pro-inflammatory cytokines at 24 hpi when compared to PBS treatment as a mock-infection. Interleukins IL-1β, IL-6, IL-8, IL-10, IL-12p70, and Tumor Necrosis Factor (TNF) were significantly more abundant in CHIKV infected cell culture filtrates versus mock-infected ones (Figure 2-5).

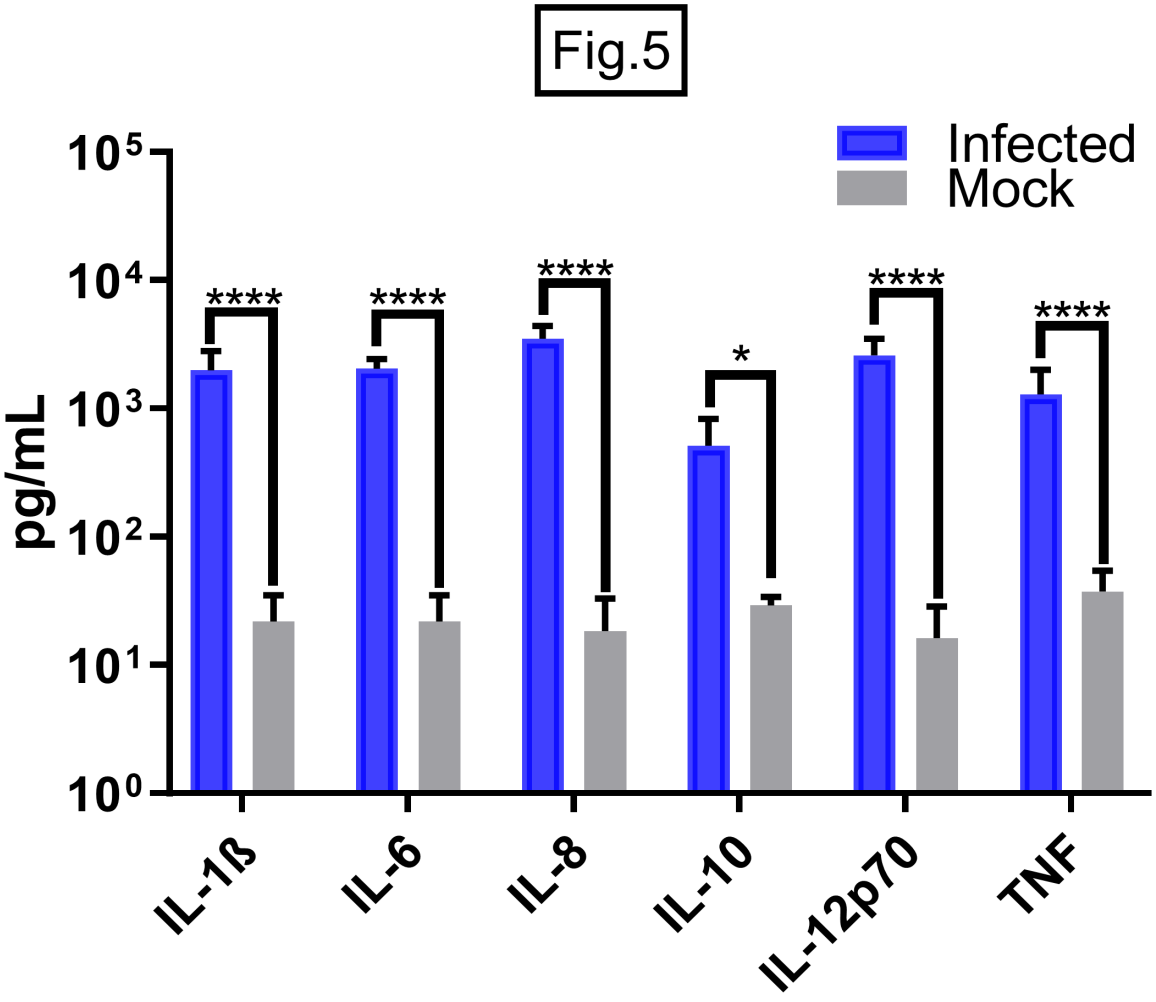

Figure 2-5. CHIKV infection induces pro-inflammatory cytokines in human macrophages. Secreted pro-inflammatory cytokine levels in CHIKV-infected PMA-differentiated U937 macrophages were quantified 24 hpi using cytometric bead arrays. Data show mean values of three independent experiments with a total of n=9, MOI =5. Statistical significance was determined using multiple t-test corrected using Holm-Sidak method. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

Conversely, we observed that infection of murine macrophages showed a significant increased secretion of only two pro-inflammatory cytokines (IL-12p70, and TNF) and one anti-

inflammatory cytokine (IL-10) in infected RAW264.7 cells, with similarly low levels of these cytokines in mock-infected cells (Figure 2-6). A direct comparison of secreted IL-6, IL-10, IL-12p70, and TNF concentrations in infected human and murine cultures indicate significant differences in all these cytokines but IL-10 (anti-inflammatory cytokine) (Figure 2-7). Cytokine responses in CHIKV-infected human patients have been extensively reported and can lead to a robust production of pro-inflammatory cytokines, compared to the relatively low levels observed in murine models[96,102,165–167].

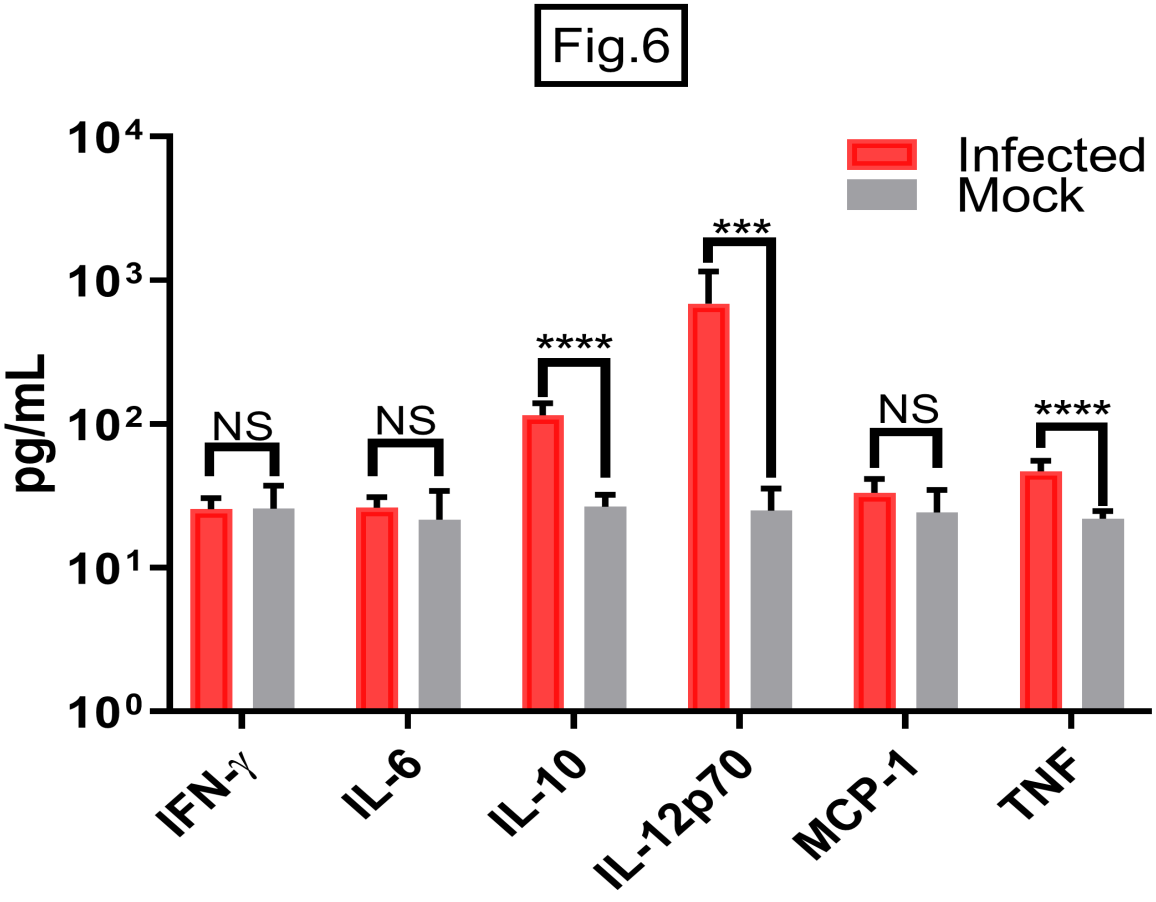

Figure 2-6. Cytokines induced in CHIKV-infected murine macrophages. Secreted cytokine levels in CHIKV-infected murine RAW264.7 macrophages were quantified 24 hpi using cytometric bead arrays. Data shows mean values of three independent experiments with a total of n=9, MOI =5. Statistical significance was determined using multiple t-test corrected using Holm-Sidak method. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

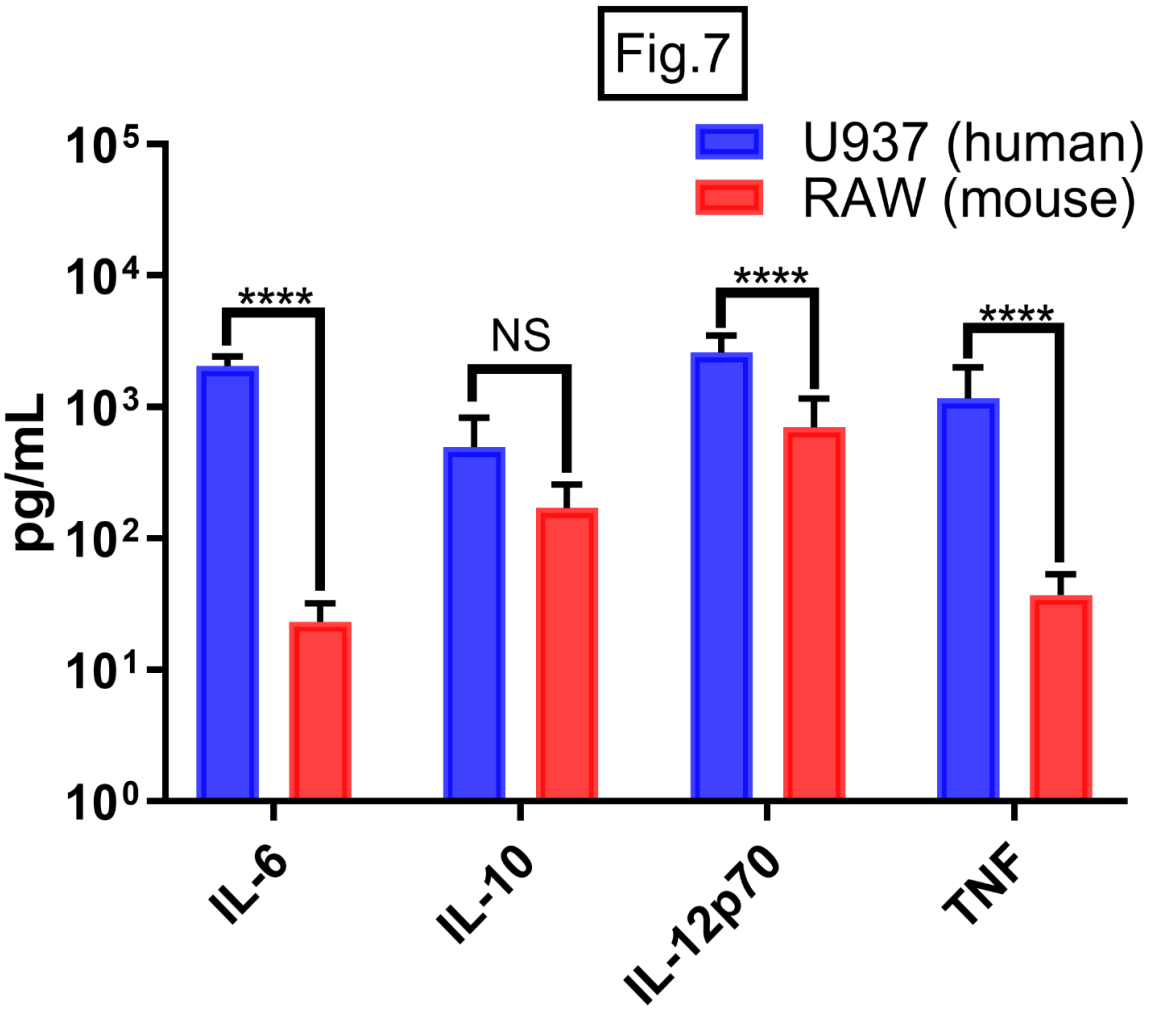

Figure 2-7. CHIKV-infected human macrophages show a more robust pro-inflammatory profile. Cytokine expression in CHIKV-infected human and murine macrophages was quantified by cytometric bead arrays at 24 hpi. Data show mean values of three independent experiments with a total of n =9, MOI=5. Statistical significance was determined using multiple t-test corrected using Holm-Sidak method. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

We also explored the differences in pro-inflammatory cytokine induction between CHIKV-infected human and murine macrophages by comparing the RT-qPCR (relative quantification) values for relevant pro-inflammatory cytokine mRNAs in human and murine cells, which confirmed differences in the expression of pro-inflammatory cytokines as measured by bead arrays. CHIKV-infected PMA-differentiated U937 and RAW264.7 macrophages displayed different gene expression profiles during CHIKV infection peak activity (24 hpi).

mRNA levels for IL-1, IL-6, IFN-α, IFN-γ, MCP-1, and TNF were significantly higher in human cells when compared to the expression levels of their murine counterparts (Figure 2-8). Again, IL-10 mRNA levels were similar in both human and murine cells, confirming our previous findings.

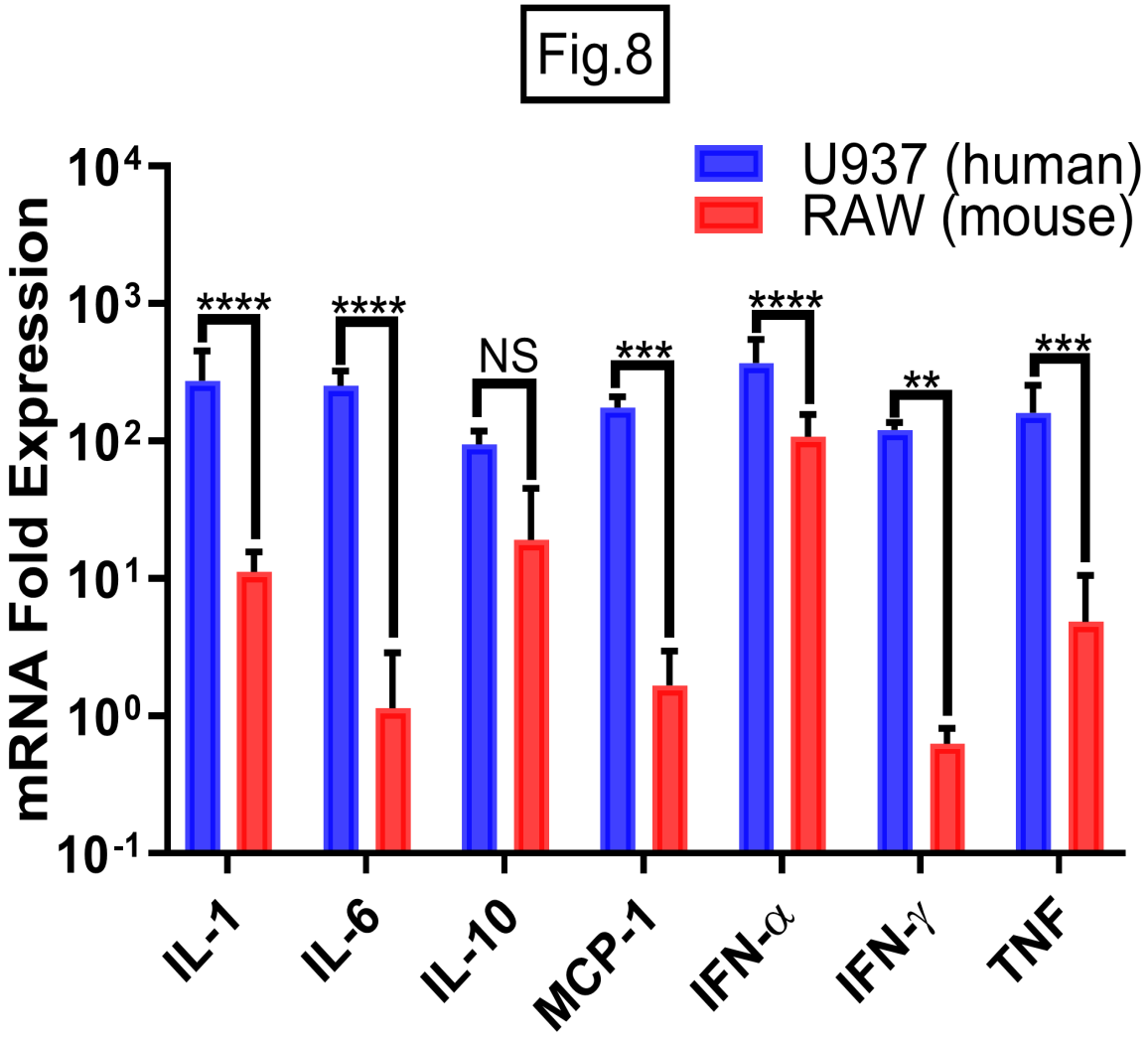

Figure 2-8. CHIKV infection upregulates pro-inflammatory cytokines mainly in human macrophages. Cytokine mRNA expression levels in CHIKV-infected human and murine macrophages was quantified by RT-qPCR at 24 hpi. Results were normalized relative to GAPDH expression levels. Data shows mean values of three independent experiments with a total of n=9, MOI=5. Statistical significance was determined using multiple t-test corrected using Holm-Sidak method. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

2.7.3 Mxra8 alphavirus entry mediator is upregulated in PMA-differentiated U937 macrophages

The matrix remodeling associated 8 (Mxra8) protein has been recently identified as an entry mediator for multiple arthritogenic alphaviruses, including CHIKV[168,169]. Therefore, we assayed the expression levels of Mxra8 in PMA-differentiated U937 macrophages and undifferentiated U937 monocytes using RT-qPCR (Figure 2-9). PMA-differentiated U937 cells showed a significant expression increase over undifferentiated U937 cells (P=0.0097).

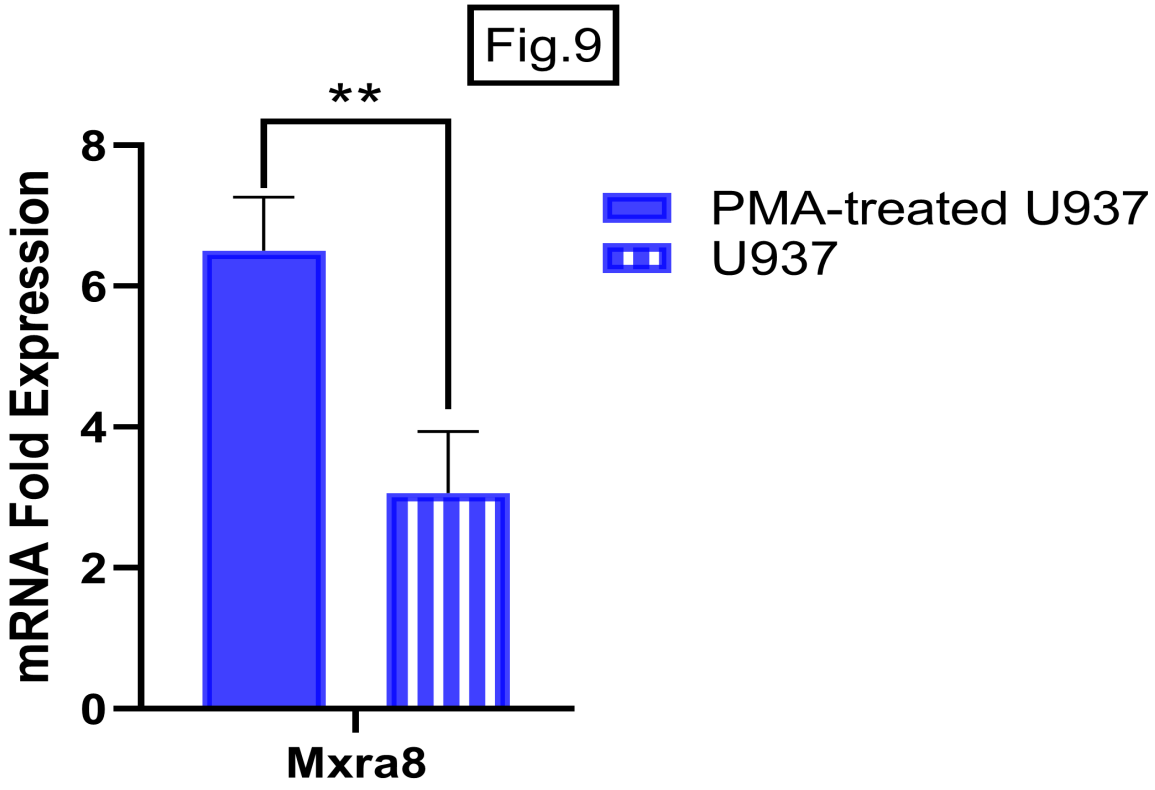

Figure 2-9. Mxra8 expression levels are higher in PMA-differentiated U937 macrophages. Mxra8 mRNA expression levels in PMA-differentiated macrophages and undifferentiated U937 monocytes was quantified by RT-qPCR. Results were normalized relative to GAPDH expression levels. Data shows mean values of three independent experiments with a total of n =9. Statistical significance was determined using a Welch two-sample t-test. **P<0.01.

2.8 Discussion

CHIKV has been shown to infect a wide variety of different cell types including immune, epithelial and endothelial cells. Murine *in vivo* and *in vitro* infection studies have shown that CHIKV infects brain tissue and glial cells [170], dendritic cells, macrophages [127], and epithelial cells[143]. In humans, CHIKV infects endothelial, epithelial, fibroblast, muscle satellite and macrophage cells [75,101,138,162,171,172]. Despite the similarities in cell types targeted across species, the stark differences in immune responses to infection between human and murine models are significant obstacles in using murine models to aid in understanding CHIKV pathogenesis [173].

CHIKV infection has been studied extensively in many murine models, however, these models have several inconsistencies when compared to symptoms present in human infections. Common manifestations seen in infected human patients like persistent polyarthritis, and chronic inflammation are not observed in current murine models [95,101,102].

The mechanisms involved in the dissemination of CHIKV within the host remain largely unknown. Macrophages seem to be involved in joint inflammation [165] in humans and non-human primate models, since significant infiltration of these cells has been detected in joints during the acute phase, and long after virus clearance from the blood [133]. To our knowledge, there has not been a study that directly compares CHIKV replication in human and murine monocytes or activated macrophages, and the differences in cytokine responses induced in these cells following CHIKV infection.

CHIKV infection of murine RAW264.7 has been previously explored[174]. This report compared viral infectivity and cytokine induction between RAW264.7 and a CTLL astrocyte cell line. CHIKV only infected 5% of the RAW264.7 populations whereas 100% of the CTLL cells were successfully infected at an MOI of 1. Additionally, viral kinetics in this mentioned study

showed that CHIKV RNA replication produced higher titers in CTLL cells compared to RAW264.7. Cytokine response showed upregulation of pro-inflammatory markers like TNF-α, IFN- α and ISG-56 at 24 hpi.

Using both RT-qPCR and plaque assays, we observed that about 10-fold higher levels of CHIKV was produced in PMA-differentiated U937 macrophages when compared to those produced in infected RAW264.7 macrophages (Figures 2-1, 2-2A, and 2-2B). Our CHIKV replication curves in both PMA-differentiated U937 and RAW264.7 macrophages correlated with the results reported by others, where CHIKV virions and viral RNA increased steadily, reaching a peak at 24 hpi, and then decreasing slightly until the end of the experiment at 48 hpi [78].

In comparison, our study showed poor innate immune response in cytokine gene expression and secretion, delayed virus production and lower titers, regardless of MOI. Our plaque assay, RT-qPCR, and flow cytometry results suggest that CHIKV infects and replicates in both human and murine macrophage cell lines within the first 8 hpi. However, CHIKV titer at 8 hpi is significantly lower in RAW264.7 versus PMA-differentiated U937 macrophages (Figure 2-1 and 2-2A). Additionally, delayed production of infectious virus titer and viral mRNA in supernatant was displayed in RAW264.7 macrophages from 8hpi until 48 hpi (Figures 2-1 and 2-2B). These results were consistent both at a MOI=5 and MOI=0.1. We decided to explore CHIKV RNA replication efficiencies within the first replication cycle to better understand these species-related differences. We quantified the RNA viral titer from our inoculum and tracked its presence in the cell supernatant. Within the first 6 hpi, we observed no significant differences in viral RNA levels between species, inside the infected macrophages (Figure 2-3). Flow cytometry quantification of CHIKV infected cells at 8 hpi showed similar levels of E1 glycoprotein on the

cell membranes of both human and murine cells (Figure 2-4), indicating that about 60% of both human and murine macrophage cell lines were infected. Our intention was to quantify the amount of CHIKV infected cells at a MOI=1 and assess a ratio of positive infected cells close to the first viral outpouring. As previously mentioned, CHIKV production seems to be tied to the species of the host cell.

Judith, et al explored CHIKV viral production in HeLa and MEF cells. Their results indicated that human NDP52, but not the murine orthologue, interacts with CHIKV nsP2, and that inhibiting synthesis of this protein reduces viral production [160]. An additional study performed in yeast indicated that nsP2 interacts with heterogeneous nuclear ribonucleoprotein K (hnRNP-K) and ubiquilin 4 (UBQLN4), resulting in CHIKV replication in vitro [143]. In total, this study identified 30 interactions between nsP2, nsP4, and E3 viral proteins and various human host factors. However, they also acknowledged that no cellular partners were found for the rest of the CHIKV proteins, which may reflect the technical limitations of their yeast two-hybrid system.

It is also noteworthy to mention the role of viral proteins with intracellular host factors, like NDP52, wherein murine cells CHIKV protein synthesis is inhibited, whereas, in humans, virus production is enhanced[143,160]. It becomes more evident that many factors between these cell lines are responsible for delayed or enhanced virus replication, which appear to be unique to the host species. It is possible that the presence of Mxra8 enhances CHIKV binding and fusion to the host cell. Once in the cytoplasm, species-specific interactions between viral proteins and host cell machinery further influence viral replication in the host cell.

We proceeded to explore the cytokine profiles of infected human and murine macrophages to understand inflammation differences between these species better. CHIKV

infection in human macrophages triggered secretion of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-12p70, and TNF (Figure 2-5). These data are consistent with pro-inflammatory cytokine profiles of infected patients and non-human primate models [27,52].

Interestingly, in Kumar et al. increased levels of TNF-α in CHIKV infected RAW264.7 macrophages decreased apoptosis susceptibility[176]. Additionally, they observed that CHIKV infected RAW264.7 macrophages did not produce significant levels of several interleukins, including IL-10. This lead to the conclusion that CHIKV infection in RAW264.7 macrophages leads to poor innate immune response, high TNF- α expression, and low apoptotic activity.

However, we observed a different cytokine response in murine macrophages, with only IL-10, IL-12p70 and TNF showing significant differences from uninfected controls (Fig 6). The absence of IFN-γ indicates a lack of monocyte/macrophage activation but the presence of high levels of IL-12p70 indicate that the exposed macrophages have recognized the presence of a pathogen. These results may indicate that RAW264.7 cells require interaction with IL-12-activated T_H1 cells, which were not present in our *in vitro* assays. In contrast, IFN-γ mRNA levels indicate upregulation in human PMA-differentiated U937 macrophages suggesting macrophage activation. Additionally, IL-12p70 levels in PMA-differentiated U937 macrophages indicate a possible autocrine induction of IFN-γ upregulation[177,178].

While we did not measure human MCP-1 by bead array, we did measure its mRNA levels by RT-qPCR. This cytokine plays an important role in macrophage recruitment and it was expressed at higher levels in CHIKV-infected PMA-differentiated U937 human macrophages, compared to the murine cell line (Fig 8). This could lead to fewer infections of circulating monocytes, effectively stalling the systemic spread of CHIKV in mice.

Macrophage infiltration of affected tissues has been extensively reported in CHIKV and other arthritis-causing alphaviruses [138,179–181]. Mice with macrophage recruitment deficiencies showed significant reductions of tissue infiltration and inflammation during CHIKV infection[182]. Other studies also confirmed that the inhibition of MCP-1 reduced inflammatory responses and infiltration of macrophages in CHIKV-infected mice[165,183]. The lack of expression of this important macrophage chemokine attractant by murine RAW264.7 cells could also contribute to the inability of murine animal models to mimic the polyarthritis which is a hallmark of many CHIKV human infections. An increase in TNF secretion by infected human macrophages indicates a robust systemic inflammatory response, whereas in contrast, murine macrophages display a mild induction of TNF production (Figures 2-6 and 2-7). The induction of pro-inflammatory cytokines in PMA-differentiated U937 human macrophages was significantly higher than that of murine RAW264.7 macrophages (Figure 2-7). Pro-inflammatory cytokine mRNA expression levels in CHIKV-infected human and murine macrophages showed similar species-specific differences. Upregulation of IFN-α in human and murine macrophages indicated that the cells recognized a viral infection and initiated antiviral signaling (Figure 2-8). However, murine macrophages did not significantly upregulate the expression of IL-6 or IFN-γ, which are critical factors for systemic inflammation. mRNA levels in murine RAW264.7 macrophages showed increases in the expression of MCP-1 (~2-fold), IL-1(~10-fold), IFN-α (over 100-fold), IL-10 (over 10-fold), and TNF (~5-fold), which indicated a discrete upregulation that correlated with our secreted cytokine results.

Interestingly, we observed significant gene expression upregulation and cytokine secretion of IL-10 in CHIKV infected RAW264.7 macrophages compared to mock infected ($P<0.00001$ and $P<0.001$, respectively) (Figures 2-6 and 2-8). However, gene expression and

secretion of IL-10 in CHIKV infected RAW264.7 macrophages showed no significant differences versus PMA-differentiated U937 macrophages (Figures 2-7 and 2-8).

In PMA-differentiated U937 macrophages displayed upregulation in all the screened cytokines including: IL-1 (~150 fold), IL-6 (~150 fold), MCP-1 (~100 fold), IFN-α (~150 fold), IFN-γ (~100 fold), and TNF (~100 fold), indicating a more robust activation of pro-inflammatory cytokine response during CHIKV infection (Figure 2-8). The induction of these Th1 pro-inflammatory cytokines in PMA-differentiated U937 macrophages, is similar to a previous report that observed Th1, Th2 and Th17 cytokine profile induction of undifferentiated U937 cells during CHIKV and Mayaro virus infection[184].

The role of Mxra8 as an arthritogenic alphavirus receptor was recently reported[168,169]. Deletion of this gene or blocking of the surface protein in human and murine cells resulted in reduced levels of viral infection. It was shown that Mxra8 binds directly to CHIKV E2 protein and enhances virus attachment and internalization into the cells. The increased presence of Mxra8 mRNA in PMA-differentiated U937 macrophages could explain why these cells are significantly more permissive to CHIKV infection (Figure 2-9) [185,186]. Other publications have shown that CD14+ peripheral blood mononuclear cells (PBMC) are susceptible to CHIKV infection, however, this report seems to encompass all CD14+ mononuclear cells[167], whereas the most relevant mononuclear subset to CHIKV infection is differentiated macrophages[90,133,182].

It has been suspected that CHIKV infection in humans induces a pro-inflammatory cytokine profile (Th1 and Th17) which in turn triggers persistent joint pain and polyarthritis pathology not only by activating host inflammatory cytokines, but also by the virus itself hijacking resident tissue macrophages, as has previously been described for other alphaviruses, such as Ross River Virus and Mayaro virus [24,59,61,62]. Here, we examined whether the outcomes

of CHIKV infection of macrophage lines from different species would differ, and if so, whether these differences could help explain the failure of the murine model to mimic polyarthritis and chronic inflammation seen in humans. We can conclude that CHIKV infects macrophages from both species, but replicates more efficiently in human macrophages.

Also, the cytokine profile of infected murine macrophages indicates the beginnings of an immune response towards infection by triggering the expression and secretion of IFN-α and IL-12p70. However, this stands in contrast to the robust pro-inflammatory cytokine response that infected human macrophages display. A graphical representation of these results has been summarized in Fig 10. Further research is needed to identify which intracellular interactions between host factors and viral components are most important for viral replication in human cells. Finally, our results suggest that the addition of human macrophages to a murine model, such as is available in humanized mouse models, could potentially bring the necessary components together to recapitulate the chronic polyarthritis seen in human infections.

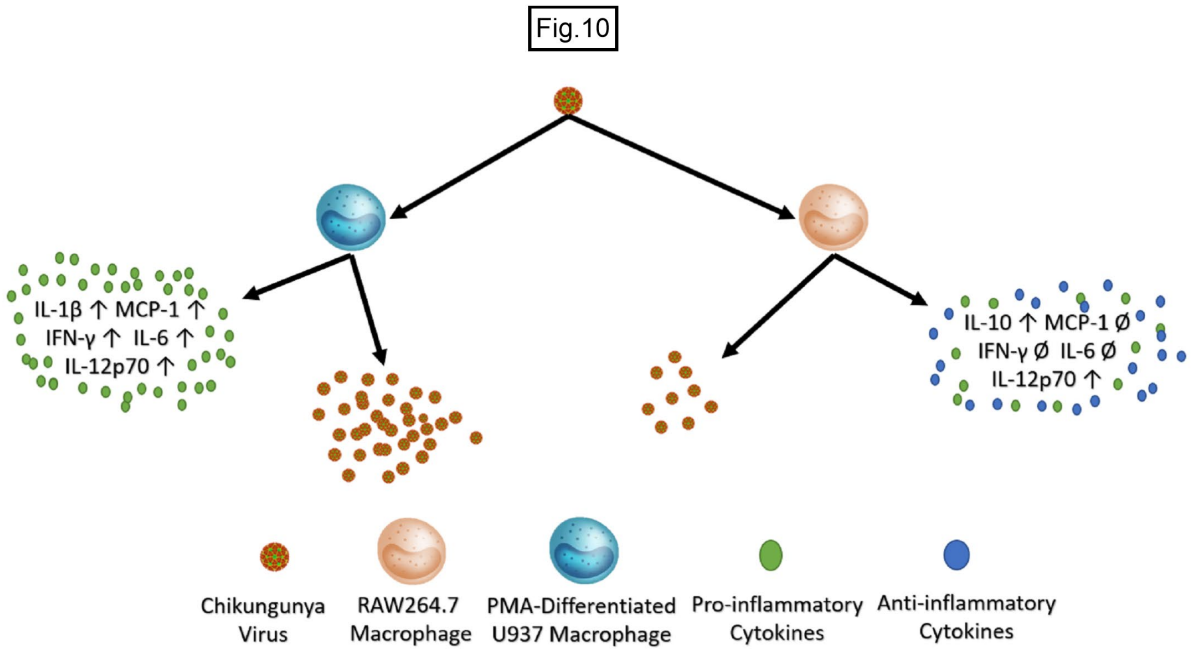

Figure 2-10. A graphic summary of CHIKV infection in human PMA-differentiated U937 macrophages and murine RAW264.7 macrophages. CHIKV infection in PMA- differentiated U937 macrophages produces higher amounts of virions and induces a more vigorous pro-inflammatory cytokine response. CHIKV infection in RAW264.7 cells results in lower quantities of virions and induction of more anti-inflammatory cytokines.

Chapter 3. A RAG2$^{-/-}\gamma c^{-/-}$ Balb/c Mouse Model to Study Chikungunya Virus Disease

3.1 Abstract

Chikungunya virus (CHIKV) is an emerging alphavirus that causes a febrile disease that is typically manifested by myalgia, maculopapular rash, and severe polyarthritis. Still, reports of patients with neurological disease have become increasingly common. In this study, we assessed the role of RAG2 and γc related immune functions and how their absence plays a role in CHIKV pathogenesis. We evaluated CHIKV infection of RAG2$^{-/-}\gamma c^{-/-}$ Balb/c and Balb/c wild-type mice to determine the role of host adaptive and innate immune systems in CHIKV pathogenesis. CHIKV-inoculated RAG2$^{-/-}\gamma c^{-/-}$ mice developed paw local inflammation and joint damage at 8 dpi. We also detected abundant viral RNA in serum, liver, spleen, and brain tissue of RAG2$^{-/-}\gamma c^{-/-}$ mice. Additionally, we detected a rise of IL-12p70, IL-6, and IFN-γ (pro-inflammatory cytokines) and MCP-1 (monocyte chemokine) in serum at 8 dpi in RAG2$^{-/-}\gamma c^{-/-}$ mice. This study provides a foundation for studying CHIKV-induced arthralgia and neuropathy in a RAG2$^{-/-}\gamma c^{-/-}$ model that closely resembles many aspects of CHIKV-associated human pathology.

3.2 Introduction

Chikungunya virus is an alphavirus that is transmitted by *Aedes* mosquitoes and causes Chikungunya fever in humans, which is mainly characterized by fever, myalgia, maculopapular rash, severe arthritis, and to a lesser extent neurologic pathologies[9,52,76,188]. In recent outbreaks, related neurologic symptoms have been manifested in about 20-33% of CHIKV infected patients, which include seizures, meningoencephalopathy, myelitis, and choroiditis[189,190]. These symptoms have been observed more commonly in neonates, elderly, and patients with co-morbidities[164,190–192]. Animal studies have shown that CHIKV infects the brain of neonate mice and to replicate in primary culture glial cells[156,170,193]. SCID and ICR mice also show a robust

replication of CHIKV in the brain[126], and a study using RAG1$^{-/-}$ C57BL6/J mice study reported the presence of infectious virus along with brain inflammation in 50% of their study mice at 28 days post-infection (dpi)[134].

Our RAG2$^{-/-}$γc$^{-/-}$ animal model consists of a double mutant mouse with an alymphoid phenotype, exhibiting defects in the genes encoding the recombinase activating gene (RAG2) and a common cytokine receptor gamma chain (γc). The RAG2 mutation prevents normal maturation of T and B lymphocytes, blocking their ability to generate antibodies or develop functional T-cell receptors [194]. The absence of γc (also known as interleukin-2 receptor subunit gamma) prevents cell activation by several interleukins and other cytokines, thereby inhibiting the expansion of lymphocytes, including Natural Killer cells[195,196]. Functional responses to IL-4 by monocytes and macrophages are affected by the absence of γc, which downregulates the expression of TNF[197] and reduces the activation capacity of STAT6[198], leading to the suppression of several innate and adaptive immune pathways[199–201].

In this study, we used wild-type and RAG2$^{-/-}$γc$^{-/-}$ Balb/c mice as a model system to explore the effects of these genes on the immune response during CHIKV infection. We observed that this particular strain and knockout do develop mild disease with a peak in viremia and inflammation at 8 dpi. We examined the cytokine immune response using a cytometric bead array, which measures IL-2, IL-6, IL12p70, TNF, and MCP-1 in serum. Additionally, we used immunohistochemistry and immunofluorescence to examine the effects of CHIKV and macrophage presence in the affected tissues. Finally, we examined the affected paws using micro X-ray Computed Tomography (micro-CT) to assess and quantify joint inflammation.

We hypothesize that the absence of 2 critical lymphocyte maturation factors (RAG2$^{-/-}$ and γc$^{-/-}$) leaves these Balb/c mice with few options to counter a CHIKV infection, which eventually

spreads through the host, reaches brain tissue and causes neurological damage[94,202,203]. The most successful animal model for Chikungunya disease is the Rhesus macaque, which has replicated human disease almost perfectly[135,204]. However, the development of a mouse model that mimics neurotropic symptoms of the disease not been described. We believe that these studies will provide a better understanding of the role of the immune cells in development of Chikungunya disease.

3.3 Methods

3.3.1 Mouse infection

Wild-type and RAG2$^{-/-}\gamma c^{-/-}$ knockout,6-8 week old male Balb/c mice were separated into two experimental and two control groups. Experimental groups were inoculated with 5,000 PFU of virus (CHIKV La Reunion strain) suspended in 30µl of sterile PBS, via footpad injection of the left hind paw. Control groups were instead inoculated with the same amount of sterile PBS. All animals were anesthetized during the procedure using the isoflurane open-drop method.

Table 3-1. Experimental and control groups used for CHIKV infection of RAG2$^{-/-}\gamma c^{-/-}$ Balb/c mice.

Experimental Groups	Control Groups
Balb/c RAG2$^{-/-}\gamma c^{-/-}$ (5,000 PFU)	Balb/c RAG2$^{-/-}\gamma c^{-/-}$ PBS-mock
Balb/c WT (5,000 PFU)	Balb/c WT PBS-mock

3.3.2 Foot inflammation measurement; blood and tissue harvest

The inflammation of paws was monitored daily using a digital caliper measuring the dorsal-ventral distance of both hind paws. At each blood draw day, about 70 µl of Blood was collected via tail vein bleed using a mouse restrainer and heparin treated capillary tubes. Whole blood was transferred to EDTA treated microcentrifuge tubes. Serum was separated by

centrifugation and then mixed with TRIzol reagent in a clean tube for storage at -20 °C. Liver, Brain, Spleen, and paw tissue samples were harvested at 7 dpi and 14 dpi. These samples where then incubated overnight at room temperature in 10% formalin to inactivate the virus or homogenized with a cell strainer in TRIzol reagent. Formalin treated tissues were embedded in paraffin using a Thermo Scientific Citadel 2000 Tissue Processor and sectioned into 5-7 µm slices.

3.3.3 Immunofluorescence and histochemistry

Mouse organs and tissues were treated with 10% formalin for 24 hours to inactivate the virus and fix the tissue. The samples were then transferred to70% EtOH for long term storage. The liver, spleen, and brain tissues were paraffin-embedded and sectioned into 5-7 µm slides and dried overnight in a heat block. Mouse paws were treated with a formic acid solution for two weeks to decalcify the bones. After decalcification, the paws were paraffin-embedded and sectioned into 5-7 µm slides, then dried overnight on a heat block. Tissue slides were processed for histological staining (Giemsa). For immunofluorescence, paraffin sections were stained with the following antibodies: monoclonal anti-CD11b APC conjugated (Thermofisher, 1:200) and monoclonal anti-E1 [CHK166] (Antibody Research Corporation, 1:200). Nucleus morphology was revealed by the addition of DAPI (Sigma, 100 ng/mg).

3.3.4 Micro-computed tomography imaging and analysis

Using the quantum GX micro-CT scanner (Perkin Elmer, Waltham, MA), left and right mouse paws were scanned under the following conditions: 90 kV, 88 µA, acquisition FOV 36 mm, reconstruction FOV 25 mm, Copper 0.1mm X-ray filter, high resolution, resulting in an acquisition time of 4 and 14 minutes. Images were analyzed using Caliper Analyzer 12.0 software (Analyze Direct, Inc., Overland Park, Ks). Histogram analysis was performed to

determine thresholds for bone and soft tissue. Using these thresholds, bone and soft tissue were extracted using a semi-automated segmentation process. Bone volumes and total paw volumes were calculated.

3.3.5 RT-qPCR quantification of viral RNA

Extracted RNA from serum and tissue samples were quantified by RT-qPCR using Applied Biosystems Taqman Fast Virus 1-Step Master Mix (Cat. No. 4444432) using a specific probe and primers for the CHIKV E1 gene. Initial reverse transcription was set at 50°C for 5 minutes; reverse transcription inactivation and initial denaturing stage at 95°C for 20 s and 40 cycles of amplification at 95°C for 5 s and 60°C for 30 s. Final primers and probe concentrations were 400nM and 250nM, respectively. A positive control plasmid was assembled by reverse transcribing CHIKV RNA using Life Technologies SuperScript IV Reverse Transcriptase kit (Cat. No. 18090050) using random hexamers as primers following the manufacturer's directions. Amplification of the E1 gene was performed using primers containing a HindIII endonuclease restriction site in the reverse primer and an XbaI restriction site in the forward primer. Insertion of the PCR product into the pUC18 vector was performed by double restriction digest of the vector (HindIII-HF (NEB R3104S) and XbaI (NEB R0145S) restriction enzymes), followed by hybridization and ligation. The resulting plasmid, designated pUCE1, was transformed into *E. coli* chemically competent cells. The insertion of the E1 target sequence was confirmed by Sanger sequencing. A Ct standard curve for pUCE1 was performed using nine 10-fold dilutions and obtaining the linear regression of the CT values; the intercept of collected experimental samples was analyzed and normalized to CHIKV genome copies per mL. The limit of detection for this assay was determined to be 70 RNA copies/mL.

3.3.6 Cytometric bead array

Serum samples containing secreted cytokines from infected and non-infected mice were harvested at 7 and 14 dpi. Samples were fixed in 10% formalin in a 1:1 ratio for at least 1 hour before removing them from the BSL-3 suite. Cytokine standard serial dilutions were prepared on the same day, and a linear regression was used to correlate the sample values. Quantification of secreted cytokines was done using BD Biosciences Cytometric Bead Arrays for murine cytokines (Cat. No. 552364) detecting IFN-γ, IL-6, IL-10, IL-12, and TNF. Samples were diluted 1:10 before proceeding with the protocol, following the manufacturer's directions. Data was acquired in a BD Accuri C6 cytometer and analyzed with FlowJo version 10.6.1 software.

3.4 Safety protocols

All of the experimental work involving infectious CHIKV was performed in a Biosafety Level 3 environment, complying with all Brigham Young University Institutional Biosafety Committee requirements, which were approved in protocol IBC-2018-0028.

3.5 Results

3.5.1 RAG2$^{-/-}$γc$^{-/-}$ mice develop Chikungunya disease showing elevated viral titers and local paw inflammation

To uncover the functional role of RAG2 and γc signaling pathways in CHIKV infection, adult RAG2$^{-/-}$γc$^{-/-}$ mice were inoculated in the left footpad with 5X10^4 PFU of CHIKV La Reunion Strain. We observed a higher viral RNA titer in infected RAG2$^{-/-}$γc$^{-/-}$ mice at 8 dpi, compared to the wild-type mice that showed a mild increase in CHIKV RNA at 10 dpi (Figures 3-1 and 3-2). Inflammation on the affected paws was also increased at 8 dpi for the RAG2$^{-/-}$γc$^{-/-}$ mice, compared with a milder increase at 10 dpi for the wild-type mice (Figures 3-1 and 3-2). These results show a direct correlation between viral titter and paw inflammation, and

demonstrate that RAG2 and γc genes are required for controlling and eliminating the virus and joint inflammation induced by CHIKV.

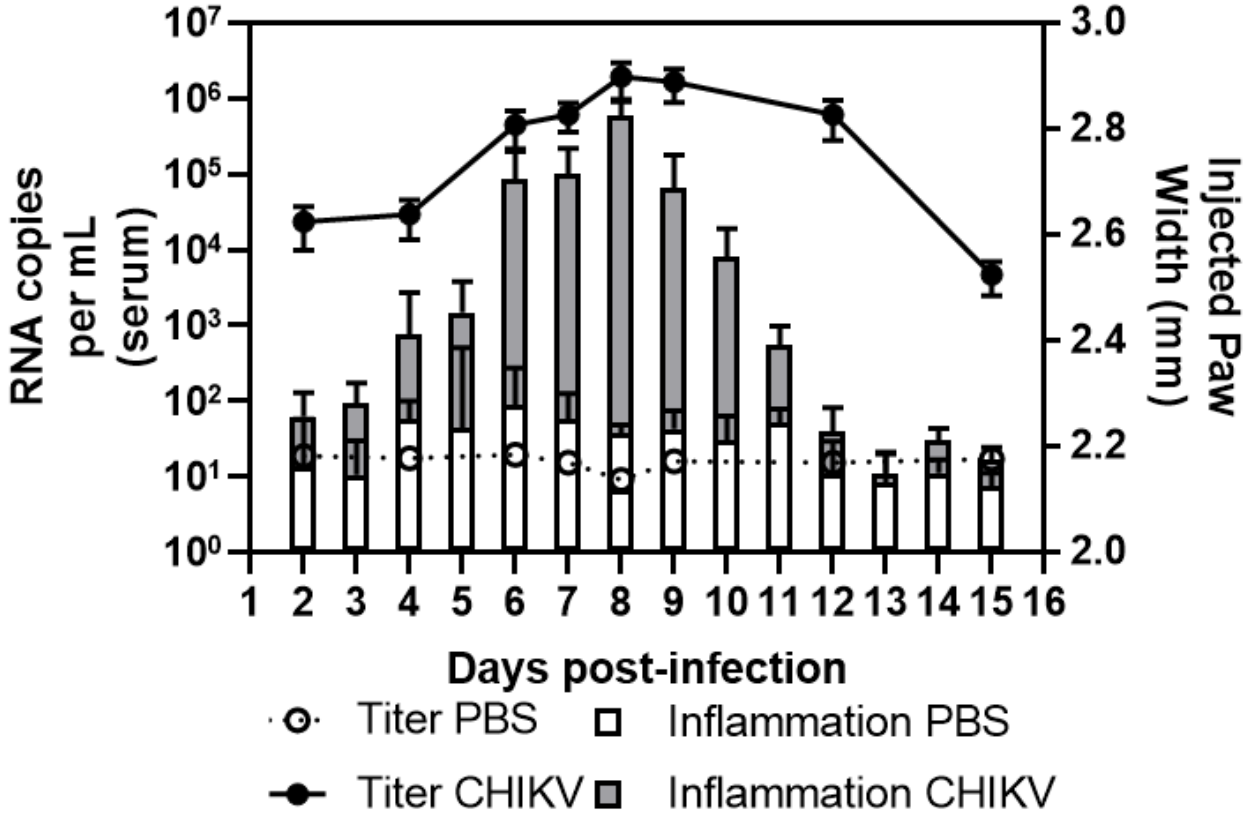

Figure 3-1. CHIKV RNA levels and paw inflammation at site of inoculation in RAG2$^{-/-}$γc$^{-/-}$ mice. Virus RNA was quantified via RT-qPCR using serum at the given time points (X-axis) and is displayed on the left-Y axis. Inflammation (measured by swelling) of inoculated paws is displayed on the right-Y axis.

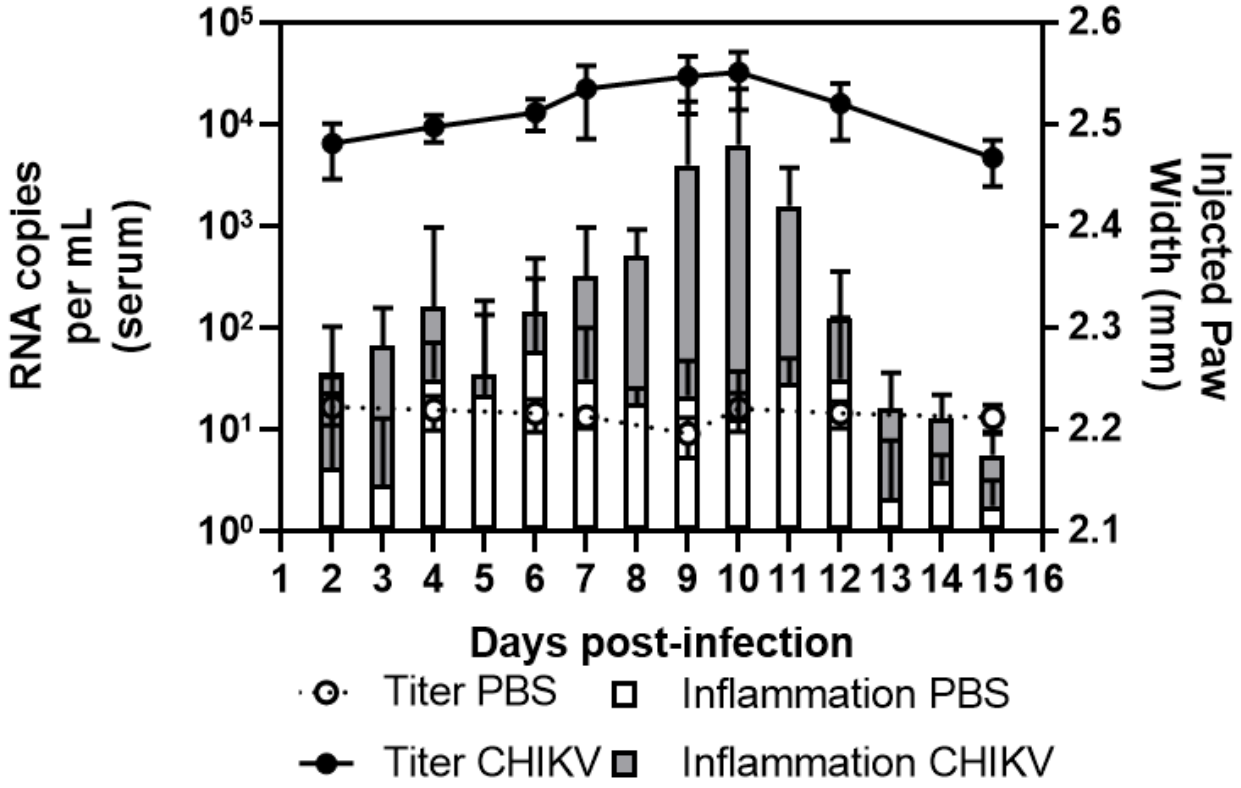

Figure 3-2. CHIKV RNA levels and paw inflammation at site of inoculation in wild-type mice. Virus RNA was quantified via RT-qPCR using serum at the given time points (X-axis) and is displayed on the left-Y axis. Inflammation (measured by swelling) of inoculated paws is displayed on the right-Y axis.

3.5.2 Chikungunya virus infects brain, liver, muscle and spleen tissue in RAG2$^{-/-}\gamma c^{-/-}$ mice

To assess tissue tropism, we harvested the organs of wild-type and RAG2$^{-/-}\gamma c^{-/-}$ mice inoculated with CHIKV or PBS. CHIKV was detected in both wild-type and RAG2$^{-/-}\gamma c^{-/-}$ mouse tissues. However, only half of the wild-type mice were positive for CHIKV RNA in the brain at 8 dpi, compared with 5 positive samples out of 6 RAG2$^{-/-}\gamma c^{-/-}$ mice (Table 2A). The number of CHIKV positive organs was reduced in the animals sacrificed at 15 dpi with spleen and muscle displaying presence of the virus in RAG2$^{-/-}\gamma c^{-/-}$ mice (Table 2B).

Table 3-2. Detection of viral RNA at 8dpi in organs of RAG2$^{-/-}\gamma c^{-/-}$ mice. Footpad inoculation with 5X10^4 pfu of Chikungunya virus La-Reunion strain.

	KO- PBS	WT- PBS	KO- CHIKV	WT- CHIKV
Brain	0/6	0/6	5/6	3/6
Liver	0/6	0/6	5/6	5/6
Spleen	0/6	0/6	6/6	4/6
Muscle	0/6	0/6	5/6	5/6

Table 3-3. Detection of viral RNA at 15 dpi in organs of wild-type and RAG2$^{-/-}\gamma c^{-/-}$ mice. Footpad inoculation with 5X10^4 pfu of Chikungunya Virus La-Reunion strain.

	KO- PBS	WT- PBS	KO- CHIKV	WT- CHIKV
Brain	0/3	0/3	1/3	0/3
Liver	0/3	0/3	1/3	1/3
Spleen	0/3	0/3	2/3	2/3
Muscle	0/3	0/3	2/3	0/3

At 8 dpi, the average levels of viral RNA in CHIKV positive RAG2$^{-/-}\gamma c^{-/-}$ mice were as follows: Brain 5.8X10^5 RNA copies/ mg, liver 2.9X10^3 RNA copies/mg, spleen 1.6X10^4 RNA copies/mg, and muscle 3.3X10^3 RNA copies/mg (Figure 3-3). Meanwhile, CHIKV RNA levels in wild-type mice did not exceed 3.4X10^2 RNA copies/mg except for muscle where the mean was 1.7X10^3 RNA copies/mg (Figure 3-3). Viral titers were significantly different between wild-type and RAG2$^{-/-}\gamma c^{-/-}$ mice in the brain, liver, and spleen tissues, with RAG2$^{-/-}\gamma c^{-/-}$ mice displaying the highest levels overall (Figure 3-3).

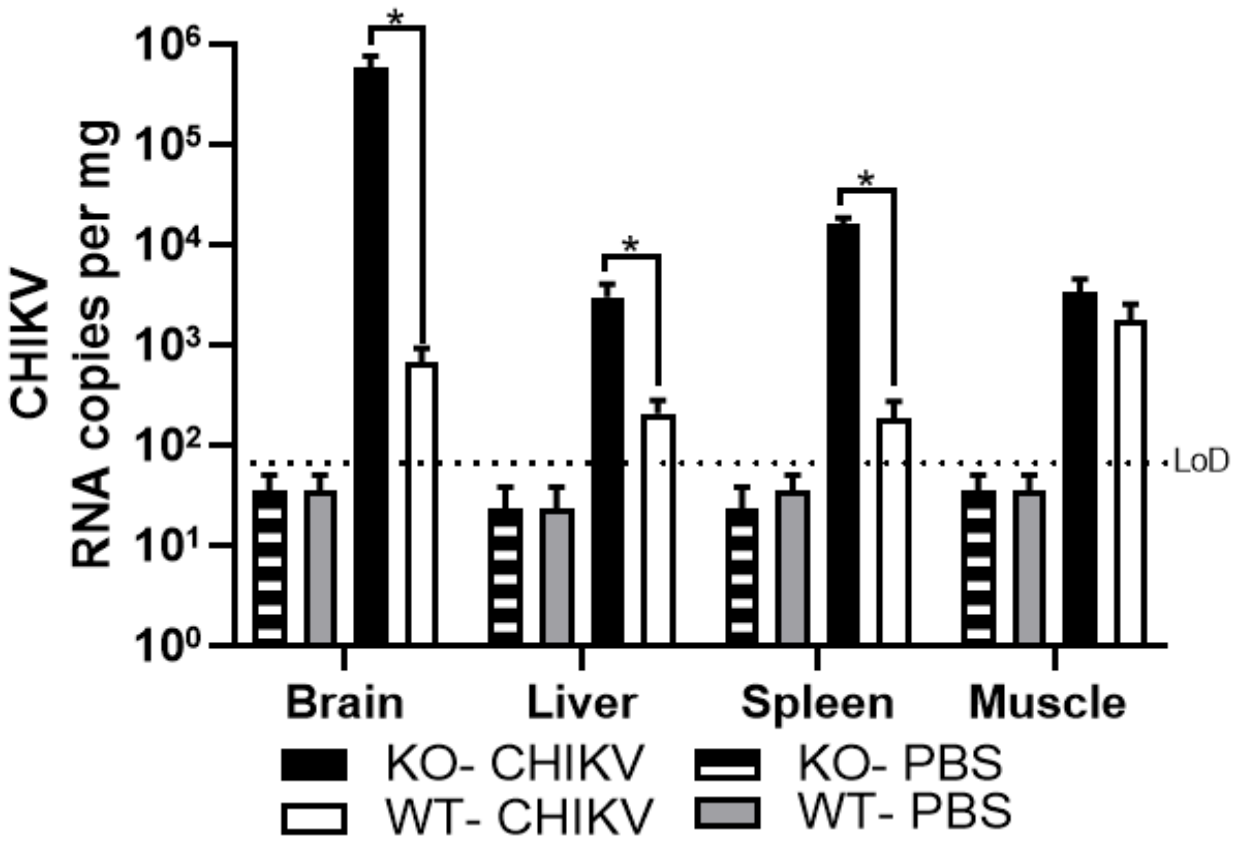

Figure 3-3. CHIKV RNA levels in different tissues from RAG2$^{-/-}\gamma c^{-/-}$ and wild-type mice. Virus RNA was quantified via RT-qPCR using tissue samples harvested at 8 dpi. Mice were inoculated with 5,000 pfu of CHIKV La-Reunion strain. Limit of detection (LoD) is shown as a horizontal dotted line.

3.5.3 Chikungunya virus infection of RAG2$^{-/-}\gamma c^{-/-}$ mice induces the secretion of pro-inflammatory cytokines in mouse sera

Cytokine secretion in mouse sera was quantified at 8 dpi using a cytometric bead array that binds to IL-12p70, TNF, IFN-γ, MCP-1, IL-10, and IL-6. Interleukins -12p70 and -6 were found at high levels in RAG2$^{-/-}\gamma c^{-/-}$ mice, and were significantly different than those of wild-type infected mice (Figure 3-4). The levels of IFN-γ, a crucial anti-viral cytokine, and the monocyte recruitment chemokine MCP-1 were also significantly higher in inoculated knockout mice, compared to their wild type counterparts (Figure 3-4). Interestingly, TNF and IL-10 showed a slight induction in inoculated wild-type, and RAG2$^{-/-}\gamma c^{-/-}$ mice, but no significant difference was detected between the CHIKV infected groups and the PBS control groups (Figure 3-4).

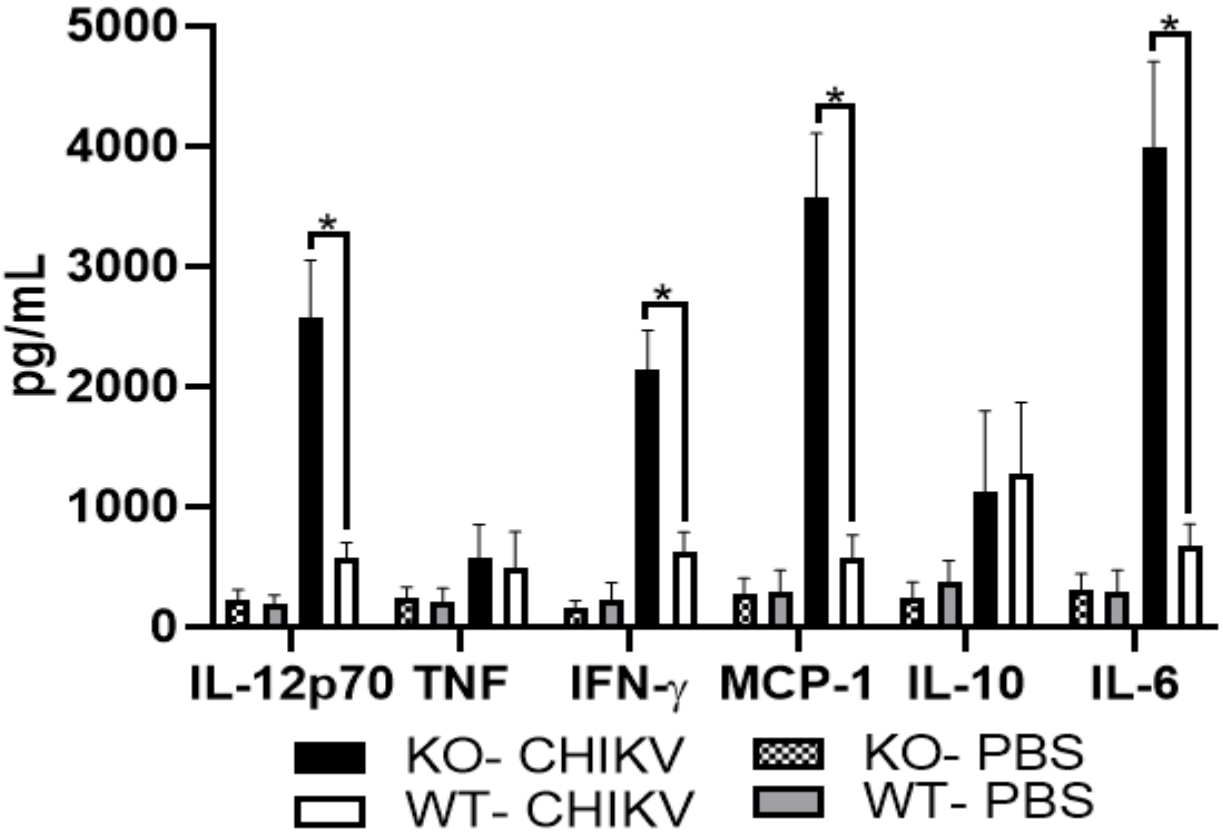

Figure 3-4. Cytokine levels in the serum of CHIKV and PBS inoculated RAG2$^{-/-}$γc$^{-/-}$ and wild-type mice. Secreted cytokines were quantified by cytometric bead array. Collected serum of RAG2$^{-/-}$γc$^{-/-}$ and wild-type mice was isolated from PBS and CHIKV infected groups at 8 dpi.

3.5.4 Histological analysis of brain, spleen, and liver indicates tissue inflammation and mononuclear cell infiltration

Formalin-fixed, paraffin-embedded tissues were sectioned, Giemsa stained, and analyzed. Brain tissue of RAG2$^{-/-}$γc$^{-/-}$ mice showed greater inflammation near ventricular sites and loss of general morphology compared to their wild-type counterparts. Macrophage infiltration was also higher in the knockout mice (Figure 3-5). Spleen sections from CHIKV-infected RAG2$^{-/-}$γc$^{-/-}$ mice also displayed inflammation and macrophage infiltration, as well as a mild increase in lymphocytic apoptosis (Figure 3-6). Giemsa stains of liver tissue sections did not show any signs of inflammation or increased levels of monocytes. However, some blood vessels appeared to have collapsed. There were also areas of spotty hepatocytic necrosis in the parenchyma, which

were identified at 8 dpi (Figure 3-7). PBS inoculated mice did not exhibit any sign of inflammation or an increase of monocytes in tissue.

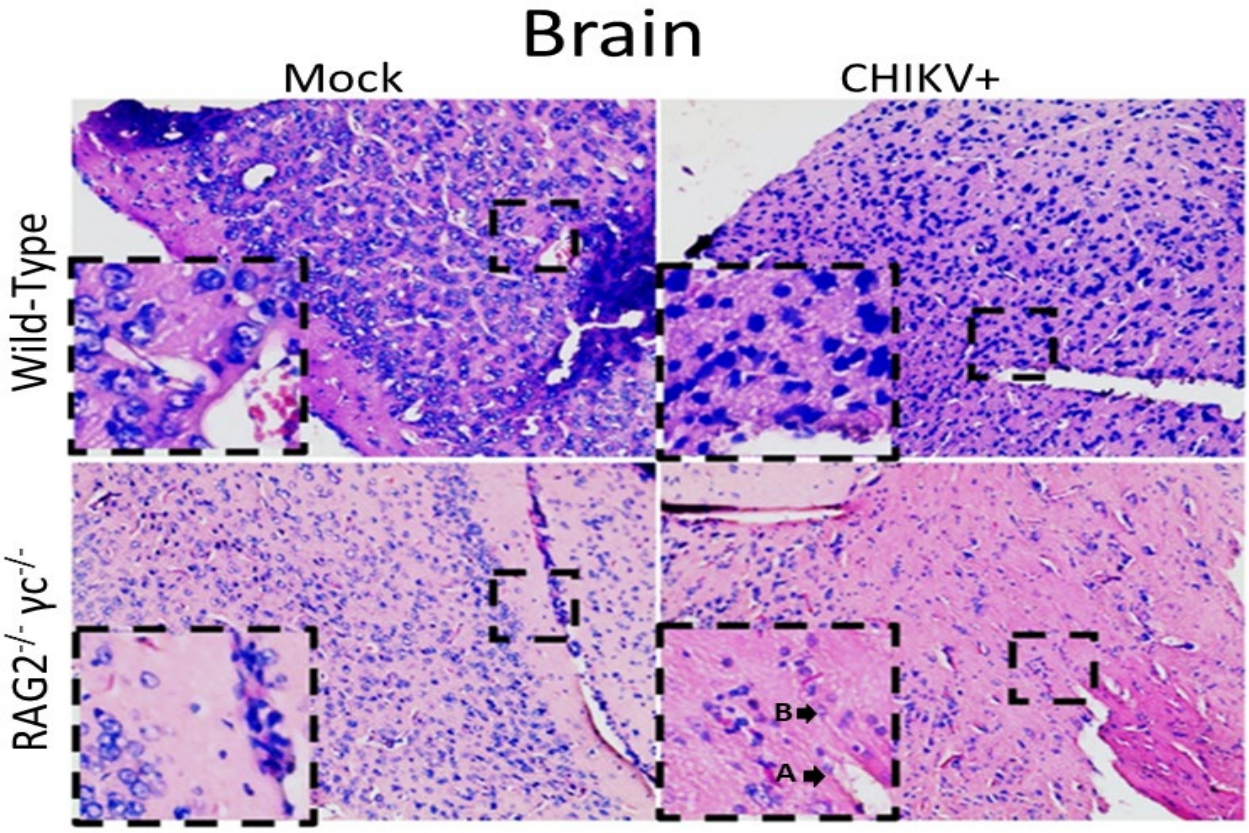

Figure 3-5. Brains sections from wild-type and RAG2$^{-/-}$γc$^{-/-}$ mice inoculated with CHIKV and PBS. Mice were inoculated by footpad injection with 5,000 pfu of CHIKV La-Reunion strain. Tissues were harvested at 8 dpi and paraffin embedded and Giemsa stained. A) Inflammation near the ventricular site. B) Macrophage infiltration of brain tissue.

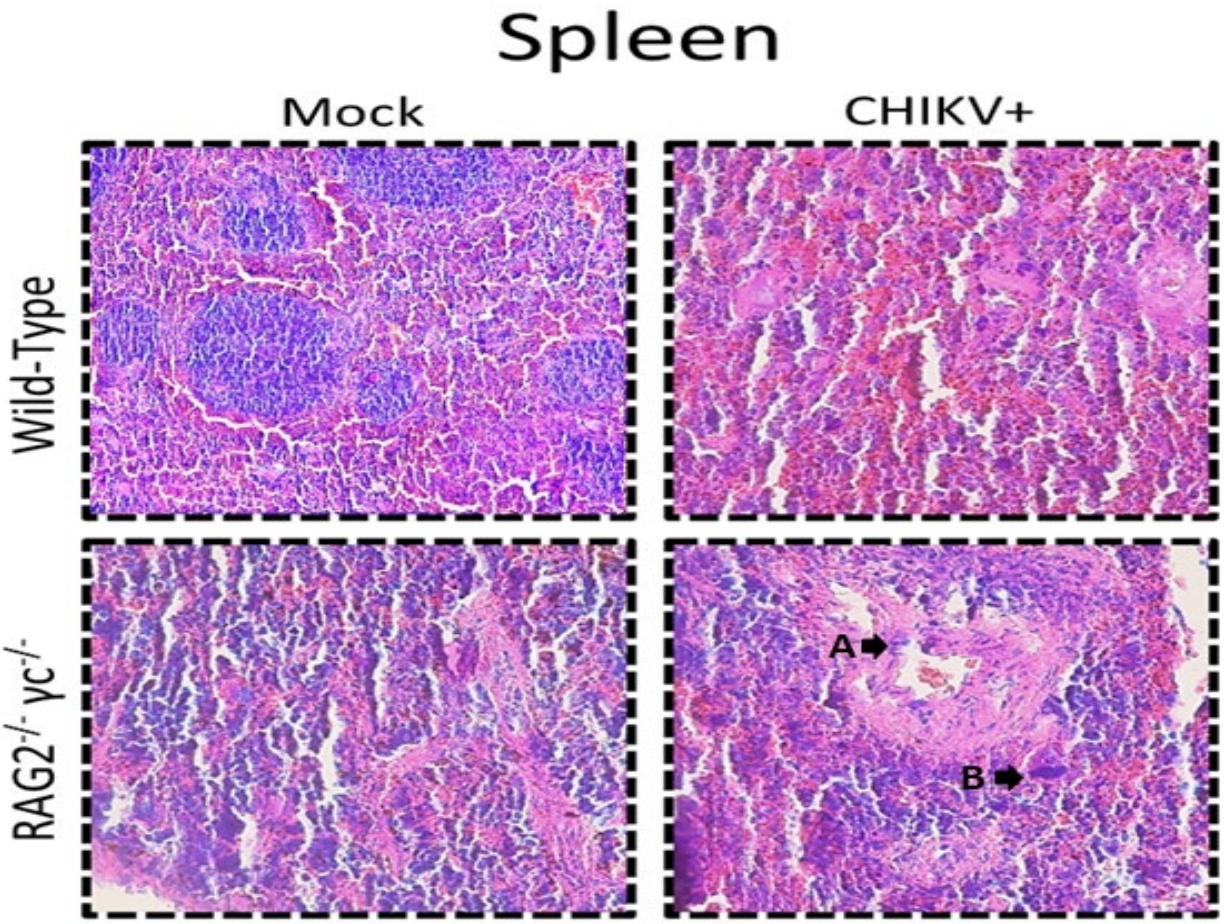

Figure 3-6. Spleen sections from wild-types and RAG2$^{-/-}$γc$^{-/-}$ mice inoculated with CHIKV and PBS. Mice were inoculated by footpad injection with 5,000 pfu of CHIKV La-Reunion strain. Tissues were harvested at 8 dpi and paraffin embedded and Giemsa stained. A) Macrophage infiltration. B) Mild increase of lymphocytic necrosis.

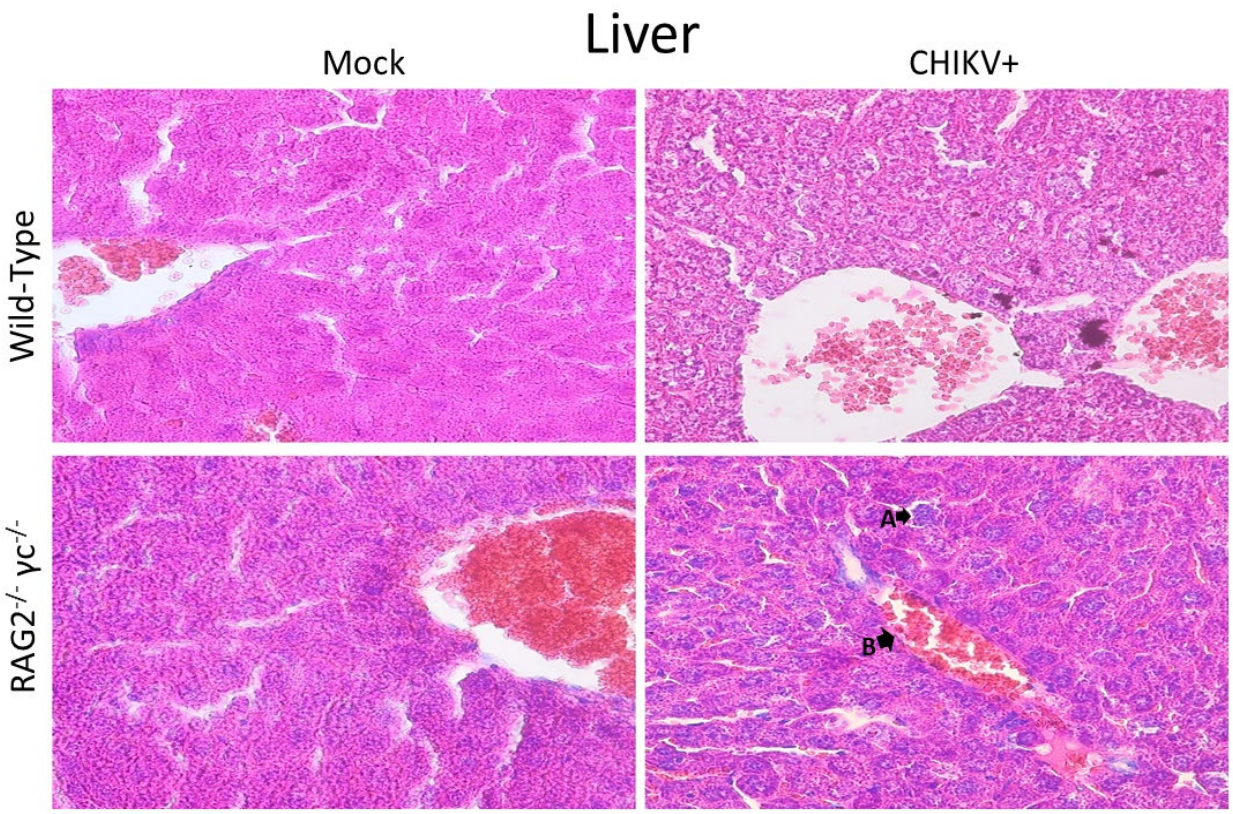

Figure 3-7. Liver sections from wild-type and RAG2$^{-/-}$γc$^{-/-}$ mice inoculated with CHIKV and PBS. Mice were inoculated by footpad injection with 5,000 pfu of CHIKV La-Reunion strain. Tissues were harvested at 8 dpi and paraffin embedded and Giemsa stained. A) Spotty hepatocytic necrosis evenly distributed in the parenchyma. B) Blood vessel appears to have lost structural integrity.

3.5.5 Chikungunya virus-induced monocyte infiltration in brain and spleen tissue is increased in RAG2$^{-/-}$γc$^{-/-}$ mice

Tissues from CHIKV-infected RAG2$^{-/-}$γc$^{-/-}$ and wild-type mice were analyzed by immunofluorescence staining for CD11b, as a murine monocyte marker, and DAPI for nuclei staining. CHIKV-infected RAG2$^{-/-}$γc$^{-/-}$ mice showed an increase of infiltrating monocytes in the brain and spleen compared to wild-type animals (Figure 3-8). The liver showed a slight increase in macrophages (Figure 3-8).

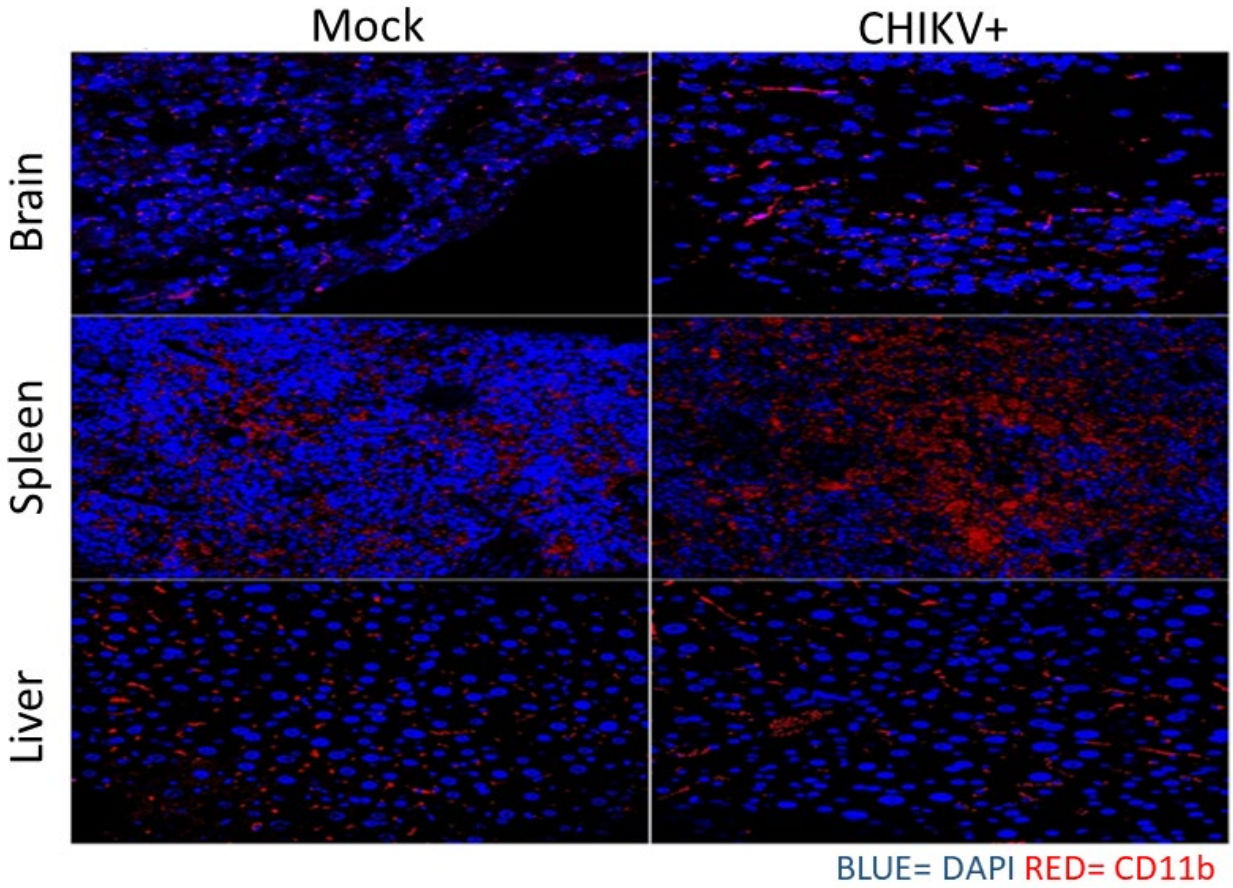

Figure 3-8. Macrophage infiltration of the brain, liver, and spleen in CHIKV and PBS inoculated RAG2$^{-/-}$γc$^{-/-}$ mice measured by immunofluorescence. Mice were inoculated by footpad injection with 5,000 pfu of CHIKV La-Reunion strain. Tissues were harvested at 8 dpi and paraffin embedded. Sections were stained with DAPI and an APC conjugated anti-mouse CD11b antibody.

3.5.5 Chikungunya virus replication is accompanied by monocyte infiltration in joint tissue in RAG2$^{-/-}$γc$^{-/-}$ mice

Paw sections from CHIKV-infected RAG2$^{-/-}$γc$^{-/-}$ and wild-type mice were analyzed by immunofluorescence staining for CHIKV E1 protein, CD11b as a murine monocyte marker, and DAPI for nuclei staining. RAG2$^{-/-}$γc$^{-/-}$ CHIKV infected mice showed increased levels of virus replication and macrophage infiltration (Figure 3-9).

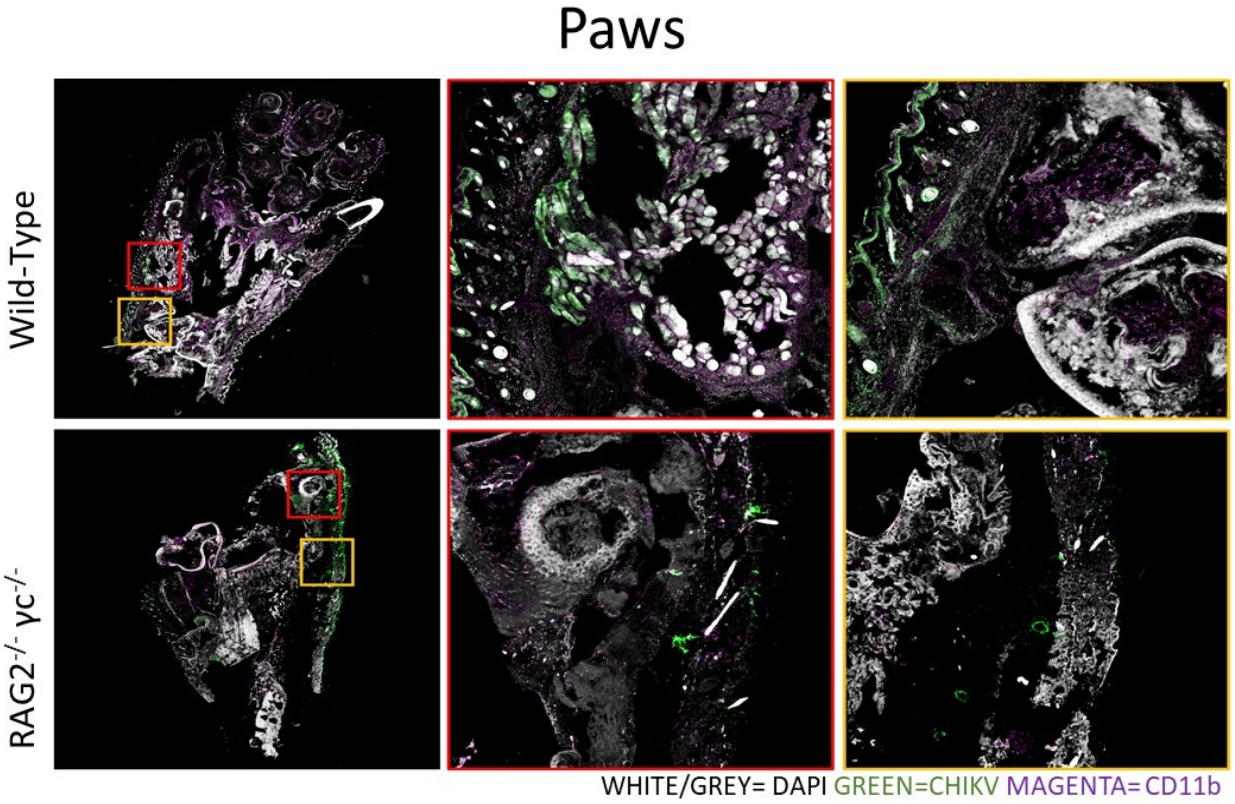

Figure 3-9. CHIKV replication and macrophage infiltration of mouse paws in CHIKV inoculated RAG2$^{-/-}$γc$^{-/-}$ and wild-type mice measured by immunofluorescence. Mice were inoculated by footpad injection with 5,000 pfu of CHIKV La-Reunion strain. Tissues were harvested at 8 dpi and paraffin embedded. Sections were stained with DAPI (white/grey), a Texas-Red conjugated anti-CHIKV antibody (green), and an APC conjugated anti-mouse CD11b antibody (magenta).

3.5.6 Micro-computed tomography analysis shows inflammation in joint tissues of CHIKV infected RAG2$^{-/-}$γc$^{-/-}$ mice

Micro-computed tomography (micro-CT) was used to evaluate tissue damage and inflammation in affected joints with increased resolution. Axial plane analysis of the dorsal side of inoculated paws shows a size reduction of the synovial cavity between the metacarpus and the first phalanx bones, indicating inflammation of the synovial cavity (Figure 3-10). A 3D image of the inoculated paws showed joint inflammation in CHIKV-infected paws. No bone structure damage was seen in either of the inoculated paws (Figure 3-11).

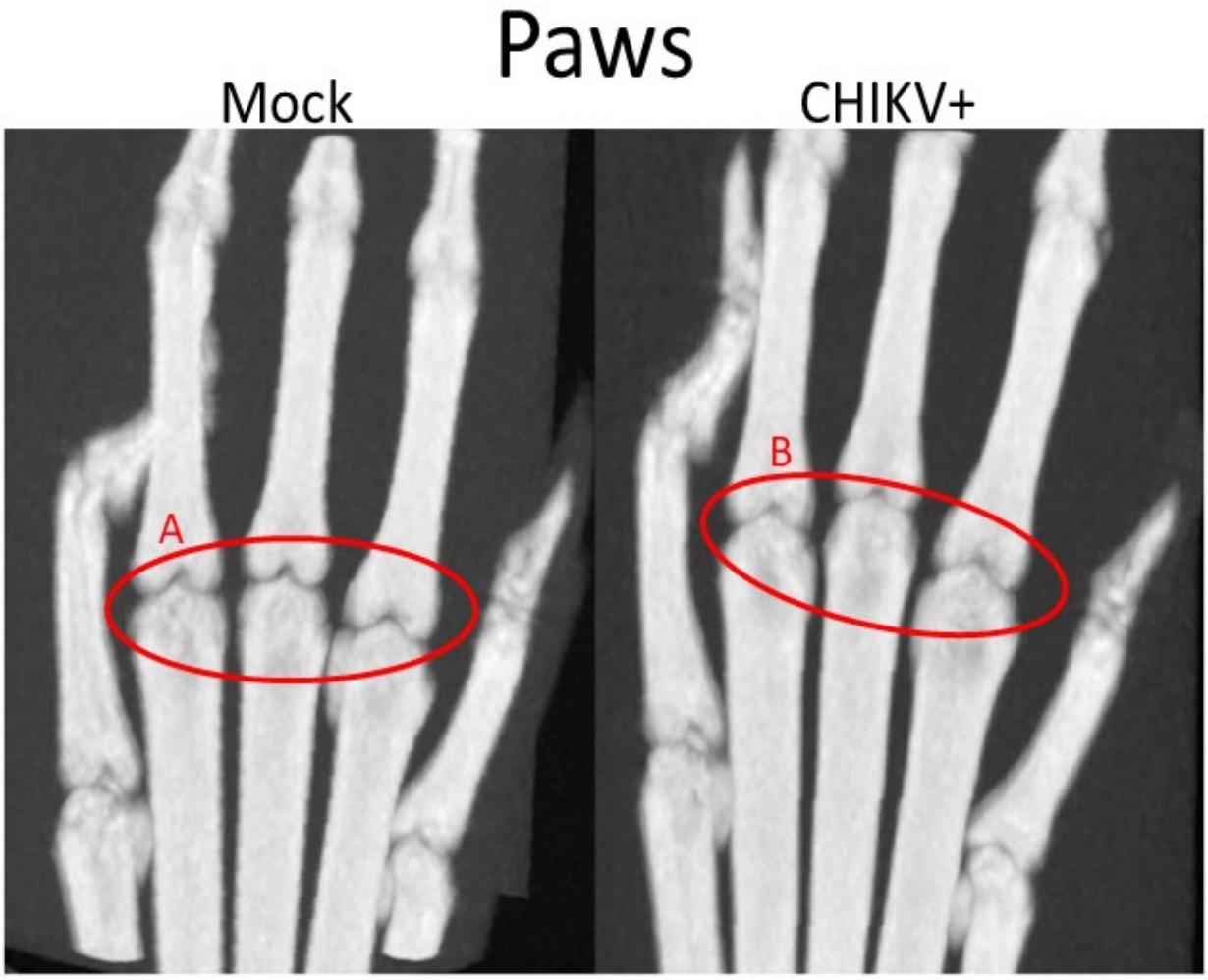

Figure 3-10. Micro-CT scan of CHIKV and PBS inoculated paws from RAG2$^{-/-}\gamma c^{-/-}$ mice. Mice were inoculated by footpad injection with 5,000 pfu of CHIKV La-Reunion strain. Tissues were harvested at 8 dpi, fixed in 10% formalin and stored in 70% ethanol. Inflamed joints are highlighted in red circles. A) Defined cartilage tissue shows no inflammation of the synovial cavity. B) Undefined cartilage tissue shows synovial cavity inflammation near the site of inoculation.

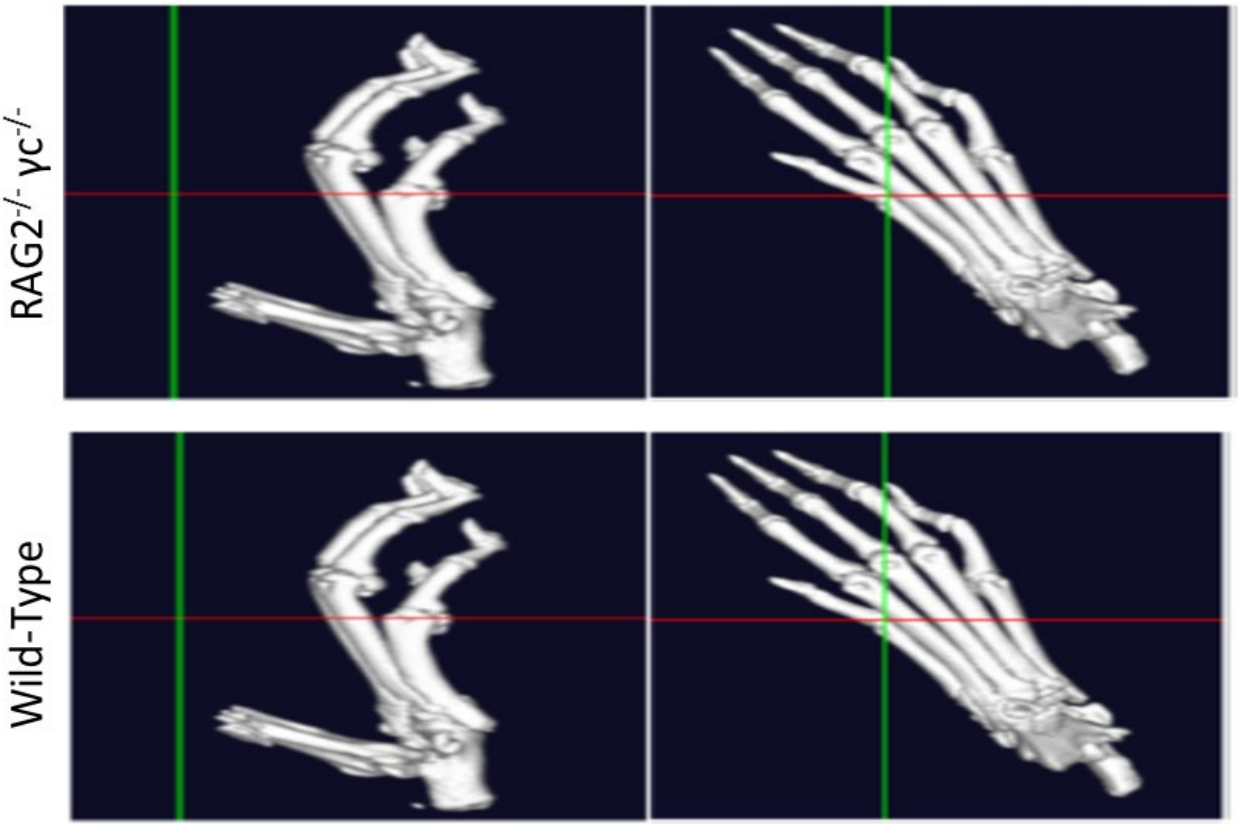

Figure 3-11. 3D rendering of micro-CT scan of mouse paws from CHIKV-inoculated RAG2$^{-/-}$γc$^{-/-}$ and wild-type mice. Mice were inoculated by footpad injection with 5,000 pfu of CHIKV La-Reunion strain. Tissues were harvested at 8 dpi, fixed in 10% formalin and stored in 70% ethanol.

3.6 Discussion

Understanding the consequences and pathogenesis of emerging arboviruses like Zika virus, West-Nile virus, and Chikungunya virus has become a more significant concern because of the potential threat these viruses can pose to the general public[9,11,136,145,164,205,206]. Vector adaptation has facilitated the widespread dissemination of these viruses to previously unaffected urban areas[41,43,51,150,164]. CHIKV is classified as an Old World alphavirus, typically known to be mostly arthritogenic [55,207,208]. However, case reports of human patients suffering from neurological complications following CHIKV infection have been increasing over the last ten years[132,209–213].

Since CHIKV does not efficiently infect wild-type mice, it is necessary to use the immune system knockout mice or to use a high infectious dose to model this disease[77,214,215]. Recent studies using RAG1 and RAG2 deficient C57BL/6 mice have shown the role of these immune factors in modulating virus-induced arthralgia, monocyte infiltration into affected tissues, and CHIKV brain invasion[94,95,134,216]. We used RAG2$^{-/-}\gamma c^{-/-}$ Balb/c mice to see if this model could mimic the symptoms of CHIKV infection observed in humans and non-human primates models[11,124,135,164,179,217].

Although we did not observe acute polyarthritis in either the wild-type or RAG2$^{-/-}\gamma c^{-/-}$ mice, CHIKV infection in the double knockout mice produced high viral titers in serum and several organs (Figures 3-1, 3-2, and 3-3), produced inflammation and joint tissue damage in the inoculated paws (Figures 3-1, 3-2, and 3-10), stimulated the production of several pro-inflammatory cytokines in serum (Figure 3-4), and induced monocyte infiltration of affected tissues, including the brain (Figures 3-5 and 3-8). These observations support the hypothesis that early antiviral cell-mediated immunity by lymphocytes and monocytes is necessary to control virus replication.

In our model, CHIKV-induced joint pathology reached its maximum inflammation at 8 dpi and persisted for 4 days correlating directly with peak viremia in serum (Figure 3-1 and 3-2). This contrasts with previous observations in RAG1$^{-/-}$ and RAG2$^{-/-}$ C57BL/6 mice where joint swelling occurred 2 days after peak viremia and immediately receded thereafter[94,134]. The different genetic backgrounds and additional γc knockout may explain the prolonged inflammation and viral persistence in our RAG2$^{-/-}\gamma c^{-/-}$ Balb/c mice. These other studies also used a higher concentrations of virus to inoculate their mice. As has been previously explained, the absence of a functional γc gene prevents the expansion of lymphocytes, thwarts functional

responses to IL-4 by monocytes and macrophages, downregulates the expression of TNF, and reduces the activation capacity of STAT6 leading to the suppression of several innate and adaptive immune pathways. Additionally, the genetic background of Balb/c mice predisposes them to generate a Th_2 immune response, which is not optimal for defense against viruses[218–220]. This makes our RAG2$^{-/-}\gamma c^{-/-}$ Balb/c mice extremely immunocompromised and more susceptible to viral infections, when compared to a C57BL/6 strain that is prone to induce a more effective anti-viral Th_1 immune response.

CHIKV tissue tropism in our RAG2$^{-/-}\gamma c^{-/-}$ Balb/c mice showed viral RNA replication in the brain, liver, spleen, and muscle (Figure 3-3). Interestingly, viral RNA in the muscle of both wild-type and RAG2$^{-/-}\gamma c^{-/-}$ mice was detected but without any significant difference between the two mouse strains. Additional histological analysis of muscle tissue was unable to be done. However, we acknowledge the importance of understanding CHIKV pathology in muscle tissue, especially when many alphaviruses tend to exhibit increased replication in fibroblast cells[89,161,221,222]. Interestingly, our model produced an increased number of CHIKV positive brain infections compared to a previous RAG1$^{-/-}$ C57BL/6 mouse model[134]. In a precious RAG1$^{-/-}$ C57BL/6 model, only 50% of the inoculated mice developed brain viremia. In contrast, our model produced detectable viral RNA in 83% of inoculated mice at 8 dpi (Tables 2A and 2B).

Cytokine analysis in CHIKV infected mice showed increased levels of IL-12p70, TNF, IFN-γ, MCP-1, IL-10, and IL-6 (Figure 3-4). However, TNF and IL-10 levels were not significantly different between wild-type and RAG2$^{-/-}\gamma c^{-/-}$ mice. Levels of IL-12p70, IFN-γ, MCP-1, and IL-6 were significantly higher in RAG2$^{-/-}\gamma c^{-/-}$ mice compared to infected wild-type animals. Previous research in RAG1$^{-/-}$ mice also reported increased levels of circulating TNF and IFN-γ, which are known anti-viral cytokines[223–225]. Despite our mouse strain's tendency towards

a Th_2 response, we observed stimulation of IL-12p70, a known Th_1-stimulating cytokine, during the inflammatory stage. Induction of IL-6 and type-I IFN during CHIKV infection are common markers used to determine disease severity in humans These hace also been correlated with increased mortality[88,226,227]. Additionally, we detected significantly higher levels of MCP-1 in our RAG2$^{-/-}\gamma c^{-/-}$ mice. MCP-1 is one of the key chemokines that regulate monocyte/macrophage infiltration and migration across the vascular endothelium into the tissue, as a response to inflammation[228]. Despite its crucial immunological activity, MCP-1 has been linked to increased arthritic pathologies during CHIKV infection[165,229]. The increased levels of MCP-1 correlate with peak joint inflammation and our immunofluorescence results, which show a recruitment of $CD11b^+$ monocytes into the brain and spleen (Figures 3-5 and 3-6).

Histology analysis of brain, spleen, and liver tissue in CHIKV-infected RAG2$^{-/-}\gamma c^{-/-}$ mice showed inflammatory signs in brain and spleen tissue (Figures 3-5, 3-6, and 3-7). Monocyte infiltration is also observable in CHIKV-infected brain and spleen tissue of RAG2$^{-/-}\gamma c^{-/-}$ mice (Figures 3-5, 3-6, and 3-8). Brain inflammation was observed near the ventricular sites (Figure 3-5), whereas spleen pathology does not appear to be confined to a specific region (Figure 3-6). To our knowledge, only one other study has reported neurological invasion using a mouse model[134].

Finally, the micro-CT examination of the affected joint tissue provided us with a detailed and high-resolution view of synovial cavity inflammation between the metacarpus and first phalanx bones (Figures 3-10 and 3-11). Typically, healthy joint tissue will show delimited gaps between bones, and inflamed joint tissue will display a reduction of this inter-bone gap. The synovial cavity of CHIKV-infected RAG2$^{-/-}\gamma c^{-/-}$ mice appeared to be significantly inflamed compared to both CHIKV-infected wild type, and PBS inoculated controls (Figure 3-10). This

correlating with other measures of joint inflammation (Figures 3-1 and 3-2). To our knowledge, this is the first time micro-CT has been used to examine CHIKV-infected joint tissue.

In summary, we have described a mouse model that effectively recapitulates CHIKV-induced local arthralgia and CHIKV-associated neuropathy. Our results suggest that cell-mediated innate and adaptive immune responses play a role in both inflammation induction and rapid viral clearance. We also observed increased levels of pro-inflammatory cytokines and monocyte recruitment chemokines in our RAG2$^{-/-}\gamma c^{-/-}$ Balb/c mice, which parallels reported human cytokine profiles during CHIKV infection. In addition, our particular mouse model is highly receptive to humanization via CD34^{+} engraftment[230–232], and may serve as a novel platform to further study the role of human immune cells during CHIKV infection.

Chapter 4. Alphavirus and Immune-Cells Host Interactions

The following chapter is written as a review article which will be sent to the journal Viruses for publication. All content and figures have been formatted for this dissertation, but it is otherwise unchanged.

4.1 Abstract

Human pathogens belonging to the *Alphavirus* genus, in the *Togaviridae* family, are predominantly arthropod-borne, being primarily transmitted by mosquitos. The signs and symptoms associated with these viruses can include fever and polyarthralgia, defined as joint pain and inflammation, as well as encephalitis. In the last decade avenues to increase understanding about the interaction between members of the alphavirus genus and the human host have intensified due to the re-appearance of the chikungunya virus in Asia and Europe, as well as its emergence in the Americas. Alphaviruses generally suppress host immunity, which makes comprehending alphavirus interactions with the components of the innate and the adaptive immune responses critical. In this review, we summarize the latest research in the field that focuses on alphavirus-host cell interactions, underlying mechanisms, and possible clinical applications.

4.2 Introduction

Alphaviruses are spherical enveloped viruses with a single-stranded positive-sense RNA genome that is between 11,000 and 12,000 nucleotides in length, contains a sub-genomic promoter, and has both a 5' cap and a poly-A tail[233]. The viral lipid envelope contains viral glycoproteins that protrude from the virions, endowing it with hemagglutination properties[234]. Viral protein production is achieved by two separate translation events that first produce the nonstructural polyprotein, which is encoded in the 5' end of the genome, followed by translation

of sub-genomic RNA of the structural proteins near the 3' end[20]. These two polyproteins are subsequently cleaved into four mature nonstructural proteins (nsp1-nsp4) and four mature structural proteins (C, E3, E2, and E1). This viral taxon has been shown to infect various vertebrates which include humans, fish, birds, rodents, as well as invertebrates which usually serve as transmission vector[235].

Alphaviruses are widely distributed around the globe and many are pathogenic in humans[17,18,97,236,237]. Arthritis, encephalitis, rash, and fever are some of the most commonly observed symptoms in alphavirus-related disease[5,44,66,74,124,217,238–240]. These viruses are naturally maintained in small rodents, birds, and mosquitoes. Larger mammals are generally dead-end hosts, with the exception of Venezuelan Equine Encephalitis virus (VEEV), which is maintained and amplified in horses[55,241,242].

Infection in humans begins when an individual is bitten by an infected mosquito, then as the virus enters the bloodstream it spreads rapidly by replicating mainly in fibroblast cells[39,76,156]. In rare cases, alphaviruses can also invade the Central Nervous System (CNS) where it will replicate in astrocytes and neurons, which can lead to fatal encephalitis[156,203]. In contrast, the subset of viruses that cause musculoskeletal and arthritis-like symptoms are not as well recognized or common (See Table 4-1). Articular manifestations in alphavirus-infected humans are principally caused by six viruses from the old-world alphaviruses group:

Chikungunya (CHIKV), O'Nyong Nyong(ONNV), Sindbis (SINV), Ross River (RRV), Mayaro (MAYV), and Barmah Forest (BFV). Old-world alphaviruses can cause quite remarkable acute diseases that may progress into prolonged chronic manifestations[57,93,241]. In most cases, arthritic manifestations are acute and transient. However, CHIKV infection causes

the most severe symptoms which include high fever, maculopapular rash, severe myalgia, persistent polyarthritis, and in rare cases hemorrhagic phenomena[88,164,191,217].

Viruses are generally recognized for stimulating the innate immune response. Viral RNA serves as a pathogen-associated molecular pattern (PAMPs) that is recognized by the host pattern recognition receptors (PRRs) such as Toll-like receptors 3,7 and 8 (TLR3, TLR7, and TLR8), and RIG-like receptors (RLRs)[243–245]. The interaction(s) between the viruses PAMPs and the PRRs in the host activate signaling pathways that lead to interferon production, which inhibits viral replication by inducing an anti-viral response[246,247]. Likewise, in the case of the genus Alphavirus the innate immune response, particularly type-I IFN[127,248–251], is the first line of host defenses.

An intermediate step in viral replication is the production of double-stranded RNA which induces the expression of IFN-α and IFNβ by the infected cell[164,243,249,252]. The type I Interferon pathway leads to Natural Killer cell activation through IFN-α and IFN-β[253,254]. Induction of the anti-viral response is triggered when IFN-α and IFN-β bind to the IFN α/β receptor[250,255]. Once bound, the JAK-STAT pathway is activated, which in turn induces the synthesis of several genes such as 2'-5'-oligoadenylate synthetase (2-5(A) synthetase), and activates ribonuclease L (RNase L) that will digest polyadenylated mRNA molecules[256]. Another type-I IFN-activated anti-viral gene includes dsRNA-dependent protein kinase (PKR), which blocks protein synthesis by phosphorylating the alpha subunit of the eukaryotic initiation factor (eIF2α). This phosphorylation leads to a depletion of protein synthesis by thwarting delivery of initiator tRNAs to the ribosome.

The purpose of this review is to scrutinize the pathogenesis of the currently relevant alphaviruses, and analyze the known or suspected role of immune cells in modulating and/or

enhancing the disease. We will also elucidate the overall pathogenic similarities between the old and new world groups which have become of great interest in recent years due to their potential use as biological weapons[257]. We will first provide a general overview of these viruses, then discuss their known pathogenesis, and finally summarize the host's immunological response. Our main goal is to understand where future research can improve human health by increasing the development and production of new treatments, prophylactics, and vaccines.

Table 4-1. Human diseases caused by Old- and New World Alphaviruses[54,258].

Alphavirus Species Name		Signs and Symptoms of Disease
Old World	Sindbis (SINV) Chikungunya (CHIKV) Ross River viruses (RRV) O'Nyong Nyong(ONNV) Mayaro (MAYV) Barmah Forest (BFV)	Fever, rash, arthritis, and arthralgia
New World	Eastern Equine Encephalitis (EEEV) Western Equine Encephalitis (WEEV) Venezuelan Equine Encephalitis virus (VEEV)	Fever, Encephalitis

4.3 Virus, epidemiology, and vectors

Arboviruses are vector-borne diseases which include the *alphaviridae*, *flaviviridae*, *bunyaviridae*, *reoviridae*, *rhabdoviridae*, and *orthomyxoviridae*. Alphaviruses are typically transmitted by arthropods of the *Aedes* family. Mosquito modulation of host haemotasis and immune defenses results in increased host susceptivity to infection. In mice, mosquito saliva can potentiate infection of many alphaviruses such as CHIKV, SFV, WEEV, and SINV[80,259,260]. It is thought that *Aedes* arthropods facilitate arbovirus transmission and infection by inhibiting type-I IFN responses, downregulating expression of Th1 cytokines and upregulating Th2 cytokines (Figure 4-1)[81,261–263].

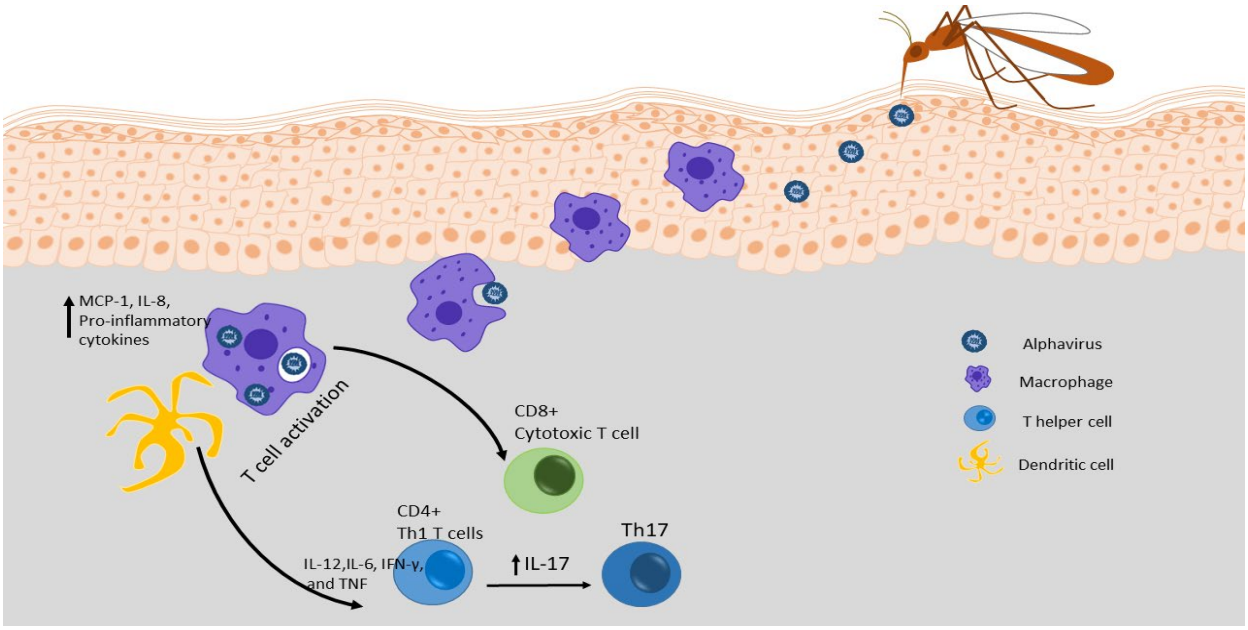

Figure 4-1. Stimulation of immune cells by alphaviruses at early time points of infection. Alphavirus are first encountered by resident macrophages. Infected macrophages secrete pro-inflammatory cytokines recruiting $CD4^+$ and $CD8^+$ T cells inducing a Th17 response.

4.3.1 Old world viruses

Old World alphaviruses (OWA), including Ross River (RRV), Barmah Forest (BFV), Mayaro (MAYV), O'Nyong Nyong (ONNV), Chikungunya (CHIKV) and Sindbis (SINV) viruses, are best known for causing arthritogenic fever in humans. These alphaviruses were

restricted by various factors to their own endemic regions. However, their adaptation to new and more effective arthropod vectors has increased their dissemination to new regions[18,41,264].

4.3.1.1 Ross river virus (RRV)

Ross river virus is an alphavirus endemic to the South Pacific region, including Australia, New Guinea and other islands[265–268]. It was initially described as "epidemic polyarthritis" after a series of outbreaks in Australia, but it took until 1959 to identify the virus in samples of *O. vigilax* mosquitoes near the river Ross in Queensland, Australia[124]. Further serological testing linked this new virus to previous patients that suffered from "epidemic polyarthritis"[269]. Signs and symptoms associated with RRV infection include fever, severe polyarthritis, extensive rash, lymph nodes enlargement. Recovery time varies wildly in adult patients, some recovering within 2 weeks and others taking up to 3 months[61,93].

4.1.1.2 Mayaro virus (MAYV)

Mayaro virus was first discovered in Trinidad in 1954 from the blood of 5 sick patients[270,271]. The virus rapidly expanded into new geographical areas as demonstrated by its isolation in the subsequent year from patients in the Amazon regions of Bolivia and Brazil[272,273]. It is thought that this virus is maintained in nature by a silent sylvatic transmission cycle[274,275]. Mounting evidence suggests that nonhuman primates and various arthropod mosquitoes act as natural reservoirs for MAYV[276,277]. Mayaro virus has also been isolated in *Haemagogus, Psorophora, Mansonia, Culex, and Sabethes* mosquitoes, with the *Haemagogus* genus being capable of sustaining the most strains of Mayaro virus (38 different strains)[237,277,278].

MAYV infection causes a febrile onset typically lasting 3 to 5 days, which is accompanied by headache, myalgia, diarrhea, maculopapular rash, and joint pain[272,279]. Although fatalities due Mayaro virus are rare, this disease does cause significant morbidity especially

among rural populations[272,276,279–281]. Each outbreak of this virus results in up to 60% of the infected people becoming immune[282].

4.1.1.3 Sindbis virus (SINV)

This alphavirus is considered the prototype alphavirus; it was originally isolated near Cairo in Sindbis, Egypt[283,284]. Sindbis virus (SINV) infections have been reported mainly in northern Europe and South Africa, and it is the causative agent of Ockelbo and Pogosta disease[235,285,286]. Its main amplifying host is birds and it is transmitted by mosquitoes within the *culex* genus[17,287,288]. SINV causes Sindbis fever in humans with signs and symptoms including arthralgia, rash and malaise[283–285,289].

4.1.1.4 Chikungunya virus (CHIKV)

This virus was originally discovered in 1955 in the area currently known as Tanzania[40], and is possibly the most virulent of the old world alphaviruses. It is transmitted by two different species of *Aedes* mosquitoes. *A. aegypti* was the traditional vector of transmission of this virus, usually spreading the virus in rural settlements[18,150,205,290]. The addition of *A. albopictus* as a viable vector introduced Chikungunya virus to more urban environments and is a significant factor for this virus's rapid expansion through Eurasia, Africa, and more recently the Caribbean islands and mainland America[49,164,291].

CHIKV causes a febrile disease that is accompanied by myalgia, maculopapular rash, neuropathy and severe polyarthritis that can persist for several months or years[3,52,84,136]. The most notable outbreak to date happened in the French island of La Reunion in 2006, where more than half of the population was infected with a highly arthritogenic strain[14,89,131,292].

4.1.1.5 Semliki Forest virus (SFV)

Semliki Forest virus was originally described by Smithburn and Haddow in 1942[293]. It was isolated from mosquitoes in the Semliki Forest in Uganda. SFV is widely distributed in Africa and human infection is relatively common[294]. This virus spreads mainly by mosquito bites and causes mild disease in humans[295]. Interestingly, its ability to cause lethal encephalitis in rodents has made it useful to model viral neuropathy[296].

4.3.2 New world viruses

New World (NW) alphaviruses are well known for their encephalitogenic phenotype[20]. The NW viruses include the Venezuelan equine encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV), and western equine encephalitis virus (WEEV), and have been classified as Category B priority biodefense agents due to their significant biological threat[297].

4.3.2.1 Eastern equine encephalitis virus (EEEV)

Eastern equine encephalitis virus is a highly pathogenic zoonotic pathogen, especially for the North American (NA) strains[298]. EEEV was first isolated from infected horses in 1933 in Virginia and New Jersey, followed by confirmed human cases in New England in 1938[299]. EEEV is found in North, Central, and South America and the Caribbean[300]. According to the Center for Disease Control and Prevention (CDC), the mortality rate for this pathogen is approximately 30%, with many survivors suffer from neurologic problems. EEEV is an important cause of disease in animals and humans. In the United States alone it causes approximately 7 reported human cases annually[301]. The most recent EEEV outbreak was in 2019 were 34 persons in the US were infected. Among the patients, 94% were diagnosed with encephalitis and 12 died as a result[301].

EEEV is a vector-borne disease that is transmitted to humans and animals by an infected mosquito *Culiseta melanura*, which is a principal vector among the avian population[302,303].

4.3.2.2 Western equine encephalitis virus (WEEV)

Western equine encephalitis virus is an alphavirus, classified as a group IV positive-sense single-stranded RNA virus. This virus is the closely related to EEEV, with its origin possibly due to a recombination event between EEEV and a virus similar to SINV[304]. WEEV was isolated back in 1930 from the brain of a horse in California, then later in 1938 from the brain of a child[305]. In 1987, 148 U.S cases of arboviral encephalitis cases were reported, of which 41 were WEEV[306]. It is suggested that WEEV encephalitis is milder that caused by EEEV. WEEV is primarily found in the western portion of the United States, western Canada and as far away as Argentina[307]. Phylogenetic analyses suggest that WEEV lineages in North and South American have evolved differently[308]. The main vector in United States is the *Culex tarsalis* mosquito, which prefers an avian host[309]. Epidemics in mules, horses, and birds generally lead to human disease[307].

4.3.2.3 Venezuelan equine encephalitis virus (VEEV)

VEEV was isolated and grown in a lab in 1938 after being discovered during outbreaks in Colombia, Venezuela, and Trinidad in 1935[297,310]. It identified in parallel in the United States during the 1930s; however it was during a major epidemic in south Texas in 1971 that resulted in human and animal (horse) fatalities, and where the infection rate of mosquitoes was reported to be 1:100[311].

VEEV is classified into six subtypes, designated I to VI, and consist of 9 strains[312]. VEEV strains have increased the number of viable mosquito vectors. The most widespread outbreaks involve a specific adaptation to *Ochlerotatus taeniorhynchus*, which is the most

common vector in coastal areas, it can also use the same *Culex* (*Melanoconion*) vector as EEEV and WEEV[313].

4.4 Pathogenesis

Although alphaviruses are transmitted via mosquitos, the infection caused by them is due to the various mechanisms of virus-host interaction and cytopathic effects [314,315]. From the Old World (OW) alphaviruses, CHIKV causes acute febrile illness and with decreasing severity for ONNV, MAYV, and RRV which are antigenically similar[316]. Overall, New World (NW) alphaviruses are more virulent with infection causing a very debilitating disease, with EEEV presenting the most severe form with disease severity decreasing for WEEV and VEEV[317] (Figure 4-2).

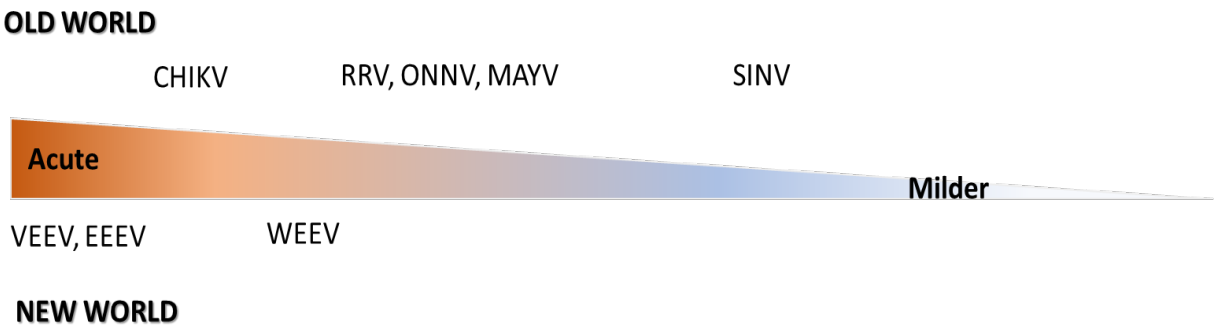

Figure 4-2. Distribution of disease severity for different alphaviruses. Old world alphaviruses are located on the top of the graph. New world alphaviruses are located on the bottom of the graph.

4.4.1 Old world viruses

4.4.1.1 Sindbis virus (SINV)

This virus has been extensively used as an acceptable model in mice for alphavirus replication and disease[318]. However, SINV-induced arthritis is relatively unexplored, unlike other alphaviruses that have been the focus of many recent studies[188]. After transmission via the bite of an infected mosquito, SINV spreads through the bloodstream to vital organs including the liver,

spleen, muscle, lymph nodes and joint associated tissues[285,319]. Viremia is usually followed by the activated innate immune response, which includes inflammation and infiltration of lymphocytes, Natural killer (NK) cells, and macrophages to the affected tissues[320–323]. Cell invasion occurs via receptor-mediated endocytosis and low pH-dependent membrane fusion[324–326]. Virions target the natural resistance-associated macrophage protein (NRAMP2) and use cell surface heparan sulfate and DC-SIGN and L-SIGN lectins as attachment factors[327–330]. Infection in many cells usually leads to aggressive cytopathic effects, but macrophages can be persistently infected leading to the induction of migration inhibitory factor (MIF), TNF, IL-1β, and IL-6[321,331,332]. Additionally, matrix metalloproteinases (MMP1 and- 2) are found in high quantities in the synovial fluid of affected patients, which may contribute to articular damage[321,333].

4.4.1.2 Ross river virus (RRV)

Ross River virus causes clinical disease in about 10% of infections. It manifests principally with myalgia, arthralgia, headache, fever, and maculopapular rash[267,268,334]. Symptoms commonly appear 7-10 days after infection and between 10-50% of patients continue to experience these symptoms for several years. Mononuclear cell infiltration as well as perivascular edema due to erythrocyte extravasation are usually present in rash lesions[155,182,334]. Similar to what is found in CHIKV, viral antigen is present in infiltrating monocytes and macrophages, but no infectious virus is recovered in the affected joints[335]. In addition to mononuclear cells, lymphocytes are the other predominant cell type with $CD8^+$ T lymphocytes being more prevalent than $CD4^+$ cells[155,202,336].

4.4.1.3 Chikungunya virus (CHIKV)

CHIKV infection in humans starts when an infected *Aedes* mosquito introduces the virus into the host during a blood meal. Once inside the host body, it is thought that CHIKV replicates

within susceptible cells such as skin fibroblasts and monocytes[75–78]. It is also thought that mosquito saliva enhances CHIKV infection through several proteins that prevent blood coagulation and downregulate the host immune system[51]. SAAG-4 is an identified protein in *A. aegypti* saliva that enhances CD4 T cell induction of IL-4 thus enhancing a Th2 response[79]. Further studies have shown that mosquito saliva recruits eosinophils and neutrophils to the bite site, whereas these immune cells are absent at needle inoculation sites[80]. In mice, the resulting induction of a Th2 response decreases the classic anti-viral Th1 response, which enhancers arboviral infection in susceptible hosts[81]. Although the early infection events have not been clearly defined, the acute blood phase is characterized by a brief but highly viremic phase, where viral titers reach up to 10^9 viral copies per ml[82,83]. Previous studies have shown that migrating monocytes, and to a lower extent B-cells and dendritic cells are targeted during the acute blood phase[78,84].

Despite a robust innate immune response against CHIKV infection, the virus disseminates rapidly to the bloodstream. This viral dissemination could be due to mosquito saliva that suppresses the host immune system and consequently induces a Th2 response. Such a response is highly inefficient against viral infections and affects the migration of infected immune cells such as macrophages or dendritic cells to the lymph nodes. Once inside the lymph node, infected cells produce new viruses which in turn infect more susceptible neighboring cells. During this phase, the infection may be contained or eliminated by the innate production of cytokines by the different immune cells present in the lymph node. However, the virus still manages to escape and further disseminate to other tissues like joints, musculoskeletal tissue, and even the brain by activating the endothelium and modifying the permeability of blood vessel barriers[85,86]. Once the virus reaches the bloodstream, it reaches the typical high titers which last

between 2 to 10 days in humans. The sudden decay of viral presence is thought to be due to the strong antiviral action of the Type-I IFN response, to which CHIKV is highly sensitive [57,77].

Febrile and arthritic pathologies are likely immune-mediated. Infected patients typically exhibit a pro-inflammatory cytokine profile which includes high levels of IL-1β, IL-6 and TNF-α[87–90]. It is thought that CHIKV induces an iterative cycle in which the pro-inflammatory response causes arthralgia, by infected fibroblasts that express high levels of prostaglandins which may contribute to the development of the chronic osteoarthritic joint pathology that persists for months or years after infection[91–95].

4.4.1.4 Semliki Forest virus (SFV)

The original L10 strain shows complete virulence in Balb/c mice. Intravenous infection is extremely lethal to mice, with a lethal dose, 50% (LD_{50}) of 1 pfu. Further studies attenuated the virus by chemical mutagenesis by affecting the efficiency of viral RNA synthesis[337]. This mutation decreased the lethality of SFV and allowed to identify CNS demyelination and teratogenesis as consequences of viral infection.

Intranasal infection exposes SFV to the olfactory bulb, allowing analysis of early events that follow CNS infection[338].

There is evidence that virulent strains of SFV spread rapidly in the CNS of mice, probably by axonal transport[339]. However, attenuated strains of SFV cross the BBB by infection of vascular endothelial cells, but do not spread rapidly in neurons[340,341].

4.4.2 New world viruses

4.4.2.1 Eastern equine encephalitis (EEEV)

EEEV is the most virulent of the NW alphaviruses[318]. It appears to directly infect neurons by a vascular route[298]. Infected individuals present severe symptoms that include high fever,

headache, vomiting, general or focal seizures, focal weakness, cranial nerve palsies, and coma; as well as long-term neurological sequelae which might cause both motor and cognitive impairments[342]. Experimental models used to understand the pathology of the EEEV are mice, hamsters, guinea pigs, rhesus monkeys, or marmosets, while histopathological studies are performed in equine and porcine cases[318].

Cellular targets of EEEV at the earliest time point (12 hours post-inoculation) showed local viral replication at the inoculation site. These results were quantified by EEEV RNA as well as antigen within fibroblasts, dendritic cells, lymph nodes and osteoblasts that appear to play a critical role in pathogenesis[298]. Other identified permissive cells are ovarian stromal cells, skin keratinocytes, and renal medullary interstitial cells. At later time points, viral nucleic acid and antigen were also found in the skeletal and cardiac myocytes, developing teeth, skin epithelium, ovaries, and renal papilla[298].

4.4.2.2 Western equine encephalitis (WEEV)

WEEV begins replication and viral RNA synthesis in local lymph nodes[307]. When the viral load is high, the virus can translocate to the central nervous system across the blood-brain barrier leading to inflammation (cerebral and meningeal inflammation, and necrosis)[307]. WEEV infection (mostly in the US) is asymptomatic or mild, and is relatively uncommon; however, the mortality rate is higher in infants and the elderly population[318]. Although this NW alphavirus is not as aggressive as EEEV, individuals still present with fever, headache, neck stiffness, photophobia, nausea and vomiting, weakness, tremors, and altered behavior[343]. It has been also reported that 15-30% of patients infected with WEEV develop secondary neurological damage[344].

4.4.2.3 Venezuelan equine encephalitis virus (VEEV)

The VEEV complex has 14 antigenic subtypes, of which subtype I variety AB and C are associated with major epizoonoses and human epidemics, while subtype IE can be neurovirulent in equines[345,346]. The virus replicates in non-lymphoid tissues such as heart, lung, kidney, and pancreas; as well as lymphoid tissues such as lymph nodes and the spleen, where it produces an inflammatory response characterized by cellular necrosis[347]. The virus appears in the brain 36-48 hours post-infection and the presence of inflammation is characterized by neurodegeneration, gliosis, and vacuolization of neutrophils[347]. VEEV infection in humans is asymptomatic during the incubation period of 1-5 days, followed by fever, headache, nausea, vomiting, arthralgia, myalgia, retro-ocular pain, chills, long periods of diarrhea, and lower back pain[348]. It has been reported that short febrile illness may progress into encephalitis, causing convulsions, hemiparesis, behavioral changes and alterations of consciousness[347]. VEEV can be transmitted in aerosol form and are highly infectious[318,347]. Aerosol exposure in humans affects the upper respiratory tract causing sore throat, pharyngeal erythema, neck pain, cervical lymphadenopathy, and even encephalitis[349].

Percutaneous acquired VEEV cases reported in humans have an incubation period of 1-4 days. Infected individuals present with fever, headache, myalgia, lethargy, chills, and somnolence/drowsiness; and hematological findings show an increase of lymphocytes, leukopenia and decreased number of neutrophils[350].

The virus replicates in the lymphoid tissue, and in dendritic cells after subcutaneous inoculation of VEEV in mouse models, leading to invasion of the CNS and causing encephalitis[317]. In another study subcutaneous inoculation was done in 5-week old mice (CD-1) and it was observed that VEEV disseminated to the brain through the olfactory system and the

trigeminal nerve which also enables spread to the periodontal membranes[351]. An aerosol and intranasal challenge in mouse models are characterized by lesson and viral load in the upper respiratory tract, nasal mucosa, and CNS[350].

4.5 Host immune response

4.5.1 Old world viruses

4.5.1.1 Innate immune response

Monocytes are amongst the largest of all white blood cells and are also the third most common circulating cell type (3-7%)[352]. They originate in the bone marrow from the pro-monocyte that is derived from the monoblast[353]. Monocytes are discharged by the bone marrow into the bloodstream, where they function as phagocytes for several days[354]. As they mature, their nucleus becomes oval-shaped, indented on one side, off-center, and often contorted with wrinkles[355]. The monocyte cytoplasm holds many hydrolytic enzyme-containing organelles called lysosomes that fuse to pathogen-containing phagosomes[356,357]. Once the monocytes leave the circulation they migrate to the tissue where they differentiate into tissue macrophages[358]. Macrophages are among the most versatile and important cells of the immune system since they function as patrol cells that can infiltrate almost any tissue and are involved in most of the immune reactions either directly or indirectly[359]. They are responsible for many types of specific and nonspecific phagocytic and cytopathic functions. Macrophages are also responsible for processing foreign molecules, presenting them to lymphocytes (T cells and B cells), and secreting cytokines that can promote inflammation, recruitment of other immune cells, and inhibition of immune reactions[353,359–361].

Alphaviruses can infect human macrophages, and they can even persist *in vitro* in macrophage cultures in the presence of neutralizing antibodies[362]. In addition, biopsies of

synovial fluid of affected joints in human patients show the persistence of RRV and CHIKV RNA, along with macrophage infiltration in the synovial cavity[216,363]. It is also known that RRV- and CHIKV infected macrophages secrete chemoattractants like MCP-1, IL-8 and IFN-α/β which trigger macrophage migration to the site of inflammation, where these immune cells can be susceptible to infection[155,165,182,235,364,365].

4.5.1.2 Adaptive immune response

Cell-mediated immune functions are critically important for host defense once alphavirus infection is established in skeletal muscle cells, fibroblasts or macrophages. IgM memory B cells represent the first line of defense against reinfection, and in the case of alphavirus, they appear 1-2 weeks after infection and usually persists for a few months[366]. $CD8^+$ T-cells and $CD4^+$ Th1 cells are the main components of the cell-mediated anti-viral immune response[367], with IL-12, IFN-γ, and TNF being secreted by activated Th1 cells[368,369]. IFN-γ induces an antiviral profile in cells, IL-2 acts indirectly by assisting in cytotoxic T-cell (CTL) recruitment and induction into an effector population[370–372]. Additionally, IL-2 and IFN-γ activate NK cells which play a major role in host defense during the first days of viral infection until a specific CTL response is developed[373]. Most pathogen-specific CTL response starts within 3-4 days after infection, peaks by 7-10 days, and then declines[374,375].

The role of T cells in alphavirus pathogenesis has not been clearly elucidated. Animal models have shown that the infiltration of $CD4^+$ and $CD8^+$ T cells occurs in the inflamed joint of CHIKV-infected animals[94,96,155,164,376]. These observations suggest active participation of T cells during the acute phase of CHIKV infection; however, the specific role that each T cell subtype plays remain largely undefined. Additional research with a $RAG2^{-/-}$, $CD4^{-/-}$, and $CD8^{-/-}$ mouse model demonstrated that CHIKV-related joint inflammation was partially mediated by

infiltration of CHIKV-specific $CD4^+$ T cells which do not appear to have an apparent antiviral role, and $CD8^+$ T cells do not appear to have any role in antiviral response or pathology enhancing activity during CHIKV infection[94]. Infection in IFN-γ $^{-/-}$ mouse models demonstrated that $CD4^+$ T cells do not mediate joint inflammation via the IFN-γ pathway[96]. There are conflicting reports on CHIKV-specific T cells involvement in joint pathology. Both reports were conducted in IFN-$\gamma^{-/-}$ mice, while one showed that IFN-γ is not a pro-inflammatory mediator of joint inflammation during CHIKV infection, another study concluded that IFN-γ producing T cells are present in the joint[77,94].

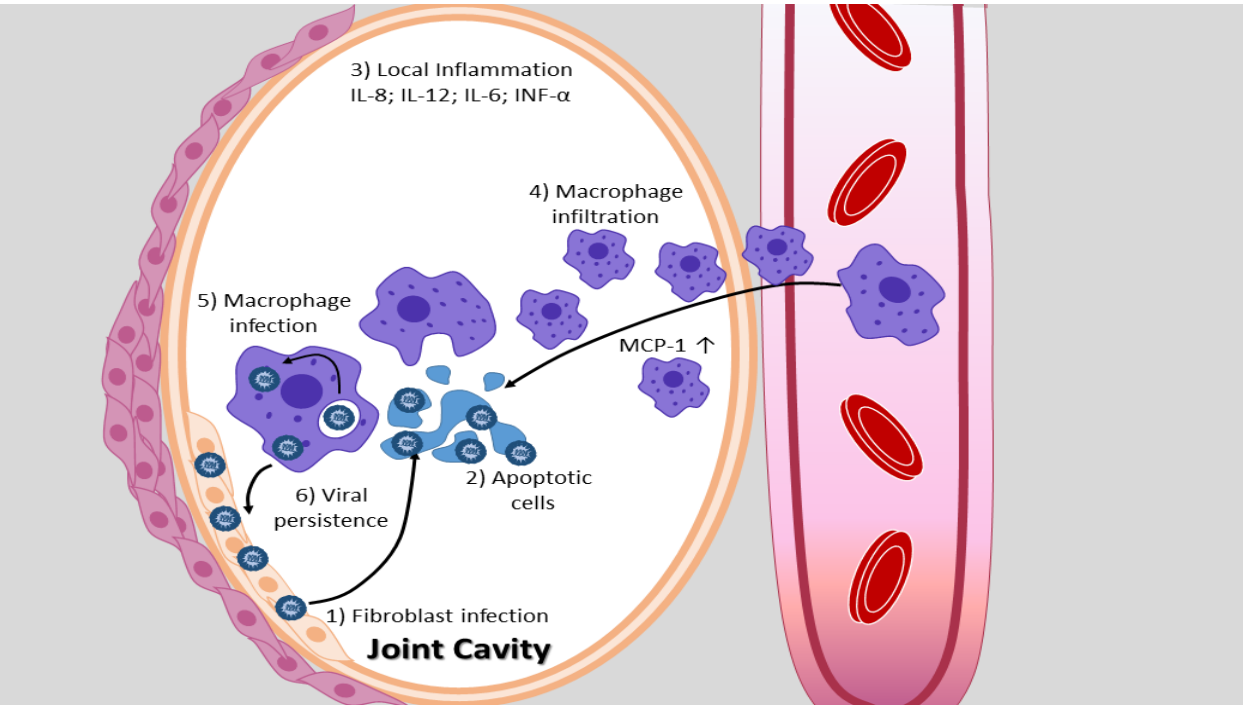

Figure 4-3. Macrophage infiltration and viral persistence to inflamed joint cavity. 1) Fibroblast are infected by alphavirus. 2) Infected fibroblast undergo apoptosis due to alphavirus cytopathic activity. 3)Local inflammation is induced by secretion of pro-inflammatory cytokines. 4) Macrophages are recruited by chemoattractant molecules and infiltrate the joint cavity. 5) Macrophage are infected by alphaviruses. 6) Resulting viral persistence renews the cycle producing virus-induced arthritis.

T cells are also associated with the generation of lesions of the myelin sheath in SFV infected mice[203]. Depletion of $CD4^+$ T cells reduced the extent of inflammation, whereas depletion of $CD8^+$ T cells reduced demyelination[203]. It is safe to assume that macrophages are

more closely associated with joint pathology, while $CD4^+$ T cells mediate inflammation via Th17- and Th1-related mechanisms as observed in rheumatoid arthritis[377]. T cells play a role as mediators of neuropathology and viral clearance in SINV infected mice[378]. Additionally, hippocampus damage has been reported by mononuclear cells which are recruited by $CD4^+$ T cells producing IFN-γ during SINV infection[208].

4.5.1.3 Cytokines

Just as the relationship between different immune cells is complex, so too is the cytokine network that governs the extracellular signaling system. Hundreds of small active molecules are constantly being secreted to regulate, stimulate, suppress, and otherwise control the many aspects of cellular development, inflammation, and immunity[379–382]. These cytokines are produced by several cell types, including monocytes, macrophages, lymphocytes, fibroblasts, and mast cells[383]. Their effects can be local or systemic and regulate pro- and anti-inflammatory responses, as well as recruit different cell types[384–386].

Elevation of cytokines associated with Th17, such as IL-1β, IL-6, and IL-17 have been reported during infection in humans and in CHIKV-infected murine models[377]. Patient cohorts have also shown an increase of Th1-associated cytokines including, IFN-γ, TNF-α, IL-2, IL-15, and IL-18, and chemokines like IL-10, Mig, MIP-1α/β[175,387]. In CHIKV-infected mouse models IFN-γ and TNF-α were elevated during the inflammatory phase[77,387,388]. Th1- stimulating cytokines like IL-12, IL-15, and IL-18 were elevated before the inflammatory phase, which suggests an expansion of Th1 $CD4^+$ T cells before the induction of inflammation[389,390].

4.5.2 New world viruses

4.5.2.1 Innate immune response

NW alphaviruses have evolved mechanisms to evade the innate immune response and to establish infection[391]. Both the innate and adaptive immune responses are activated during viral infection. Myeloid cells such as monocytes and macrophages respond to virus infection through the PRRs which are recognized by the PAMPs and promote the induction of systemic IFN-α/β[249,392]. Monocytes and macrophages change their cytokine/chemokine profile when infected, which helps viruses disseminate in the host[393]. Alphaviruses have developed mechanisms to evade myeloid cells and the antiviral immune response, ultimately, establishing infection in its host[394]. Studies done in mice infecting innate immune cells from the myeloid lineage with a IFN-resistant VEEV replicon that was packaged with structural proteins of SINV, EEEV and WEEV elucidated differences in the efficiency of infection of these cells, and in the capacity of the viral genomes to replicate[54].

EEEV replicates at a lower rate in the lymphoid tissues[395], while in murine animal models it has the tendency to infect fibroblasts and osteoblasts[351]. Invasion of the myeloid cells, including macrophages and dendritic cells, is limited[396]. This invasion is restricted by deficient binding to heparin sulfate (HS), an ideal receptor that is critical in increasing viral pathogenesis, neuron infection, and virus dissemination to the CNS[394,396,397]. Another reported mechanism that affects replication of EEEV in myeloid cells is a specific myeloid cell miRNA, miR-142-3 that binds to the 3' non-translated region of EEEV and suppresses viral replication in the myeloid cells[392]. The inability of the EEEV to invade innate cells facilitates the evasion of a strong IFN response[351].

Studies done in animal models suggest that the capacity of WEEV to cause disease and alter neurotropism are strain-specific, which suggests that genetic determinants affect their capacity to interact with the innate immune cells and produce an immune response[351].

VEEV induces cellular necrosis and an inflammatory response, while macrophage infiltration is seen as early as 24 hours post-infection[347]. Mouse models that were injected subcutaneously with VEEV showed that viral replication started on the draining lymph nodes within 4 hours post-infection[398]. This NW virus infects dendritic cells and macrophages in lymphoid tissues, as well as Langerhans cells, dendritic cells, and some skin macrophages[396]. In fact, the expression of macrophage chemotactic protein-1 (MCP-1) is upregulated in the brain of VEEV infected mice and plays an important role in the direct alteration of the blood-brain barrier (Figure 4-4)[399]. Other important innate immune receptors detected during the VEEV infection are toll-like receptors such as TLR3 and TLR9 which are expressed in dendritic cells and macrophages[400].

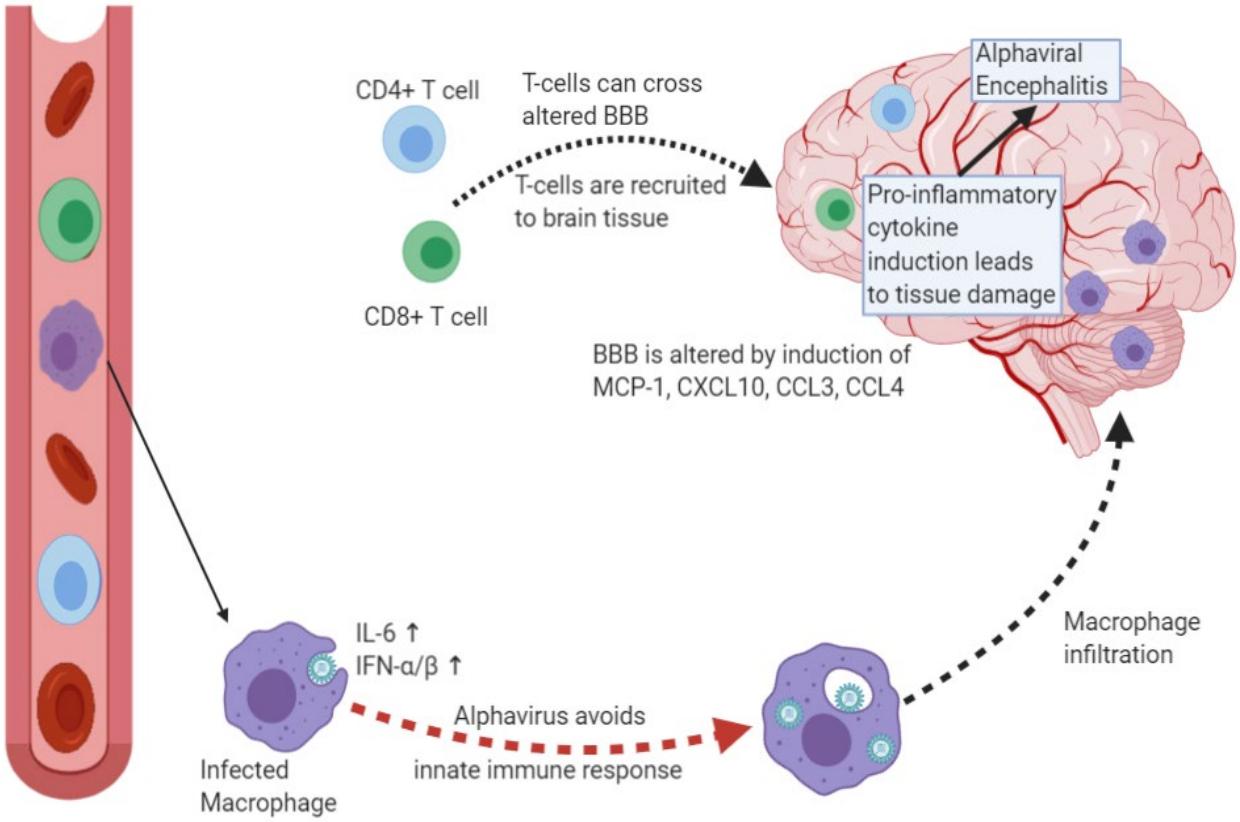

Figure 4-4. T-cell and macrophage infiltration of brain during infection of neurotropic alphaviruses. M1 macrophages are infected by alphavirus. Alphavirus avoids the innate immune response recruiting $CD4^+$ and $CD8^+$ lymphocytes to the site of infection. Infected macrophages infiltrate the brain. Chemoattractant molecules are upregulated in the brain alterating the blood-brain barrier recruiting additional immune cells to the brain. Pro-inflammatory cytokines trigger viral encephalitis.

Thus, the main difference between EEEV and VEEV/WEEV is their interaction with, and activation of, the host innate immune cells and further activation. The role of immunosenescence in the response to NW alphaviruses indicates that resident macrophages such as microglia in the brain can gradually lose their ability to carry out neuroprotective functions, changing to an inflammatory phenotype, contributing to the aggregation of amyloid β peptides, and damaging neurons, which increases the severity of encephalitis in the elderly population[401].

4.5.2.2 Adaptive immunity

Previous research done in 1989 in an animal model for EEEV suggested the need for multiple inoculations of the virus to produce a T helper immune response[402]. Years later, it was

clear that the inability of EEEV to replicate in the myeloid cells hurts the innate and the adaptive immune responses to this virus[394]. Production of systemic IFN-α/β by myeloid cells is important for T cell activation and differentiation, which leads to the development of effector and memory T cells that are important in the adaptive immune response[403].

VEEV is highly infectious via aerosol as mentioned above. Mice models exposed to aerosolized virus show that helper T cells ($CD4^+$) and not cytotoxic t cells ($CD8^+$) are important for protection against viral infection[404]. Another study done in nude and BALB/c mice between 28-35 days old using subcutaneous inoculation with an non-virulent strain of VEEV (TC-83) or a virulent strain (69Z1) observed that the lack of T cells in the nude mice contributed to higher viral loads in the CNS when compared to BALB/c mice[405]. Other studies have shown that re-stimulation with specific regions of the VEEV E2 surface protein can induce a memory $CD8^+$ $CD44^+$ T cell response, which elucidates a potential role for protective vaccines against this virus in the future[406]. VEEV subcutaneous infection in murine models have shown that the early influx of $CD3^+$ T cells confers protection, and that primed $CD4^+$ and $CD8^+$ cells have antiviral effects in the central nervous system upon adoptive transfer[407].

4.5.2.3 Cytokines

Type I IFNs are commonly the cytokines secreted during the immune host response to viral infection[408]. Secretion of IFN-β or IFN-α engages the type I IFN receptor complex, thereby activating the JAK/STAT signaling cascade, which conveys the signal into the nucleus and upregulates the antiviral interferon stimulation genes (ISGs) that inhibit alphavirus replication[394,409].

EEEV prevents IFN-α/β induction through the restriction of EEEV replication in myeloid cells by miR-142-3p. VEEV can be divided into two major categories: epizootic and epidemic[410].

VEEV induces both IFN I and II as well as interferon regulatory factors (IRF); however, both epizootic and epidemic strains inhibit STAT phosphorylation and translocation to the nucleous to prevent IFN α/β upregulation and interferon stimulated genes (ISG)[347,411]. Although there is currently an insufficient amount of information regarding the cytokine secretion process during WEEV infection, a study done in hamsters suggested that WEEV infection was reduced with the administration of a consensus-type interferon IFNα (IFN alfacon-1) when it was delivered before the viral challenge[412].

4.6 Current studies for alphavirus treatments

4.6.1 Old world

Over the last decade, efforts to counteract alphavirus disease have been focused on vaccine development; however, a growing interest in treatment alternatives has produced interesting results.

Antiviral treatments like ribavirin, glycyrrhizin, imatinib, and interferon-alpha have been widely studied as potential therapeutics against alphavirus infection. Ribavirin, glycyrrhizin, and interferon-alpha inhibit CHIKV and SFV replication and have been shown to reduce viral titers by as much as 5 $\log_{10}$ units[413–415]. Interestingly, these compounds appear to have synergistic activities. A combination of ribavirin and interferon-alpha was even more effective in *in vitro* studies, likewise high inhibitory effects of ribavirin used in combination with doxycycline were reported in a mouse model[414].

Imatinib mesylate is a compound that inhibits tyrosine kinases in the host cell and is an FDA-approved anti-cancer drug treatment which does have anti-viral activity. This small molecule reduces SINV replication in cultured cells, a result that could be easily explained as many metabolic enzymes require activation via phosphorylation from tyrosine kinases. Imatinib

treatment leads to a lower metabolic rate within the cell, effectively limiting the ability of the virus to hijack certain pathways leading to inefficient viral replication[416].

Antibody therapies are an interesting solution as they can provide rapid virus neutralization during the earlier stages of infection. Commercially available antibodies have been developed in recent years that can target different viral proteins effectively neutralizing cellular entry[106,417]. One example is a monoclonal antibody that targets the acid-sensitive region of the CHIKV E2 glycoprotein, which is of vital importance for viral entry into host cells[417]. This antibody has been proven to provide protection *in vitro* and in adult C57BL/6 mice[418]. A different approach using monoclonal antibodies prevents the release of CHIKV virions from infected cells[419]. This antibody targets the E1 glycoprotein and has only been tested *in vitro*.

The use of nucleic acids as viral replication inhibitors is a relatively new approach, which treats a viral infection with the same strategy as gene silencing. Small interfering RNAs (siRNAs), microRNAs (miRNAs) and short hairpin RNAs (shRNAs) have been evaluated for antiviral activity (against SFV and SINV) at the genome replication or protein translation level[420,421]. The use of anti-nsp3 and anti-nsP4 miRNAs displayed an attenuated spread into the central nervous system in adult Balb/c mice, greatly decreasing SFVs lethality[422]. Short-term protection (up-to 72 hpi) is also provided *in vitro* and *in vivo* models using siRNAs targeting the nsP3 and E1 viral proteins of CHIKV[423,424]. The siRNAs were transfected into Vero cells or injected into outbred mice and reduced CHIKV replication by over 99%[423,424]. Plaque reduction (in Vero cell cultures) of over 99.8% was achieved using miRNAs that targeted the CHIKV nsP1, nsP2, and capsid proteins[425]. Similar to the anti-E1 CHIKV monoclonal antibody study, treatment with an anti-E1 shRNA inhibited the viral replication of multiple strains of CHIKV and was proven effective *in vitro* and *in vivo*[426].

4.6.2 New world

The current diagnostic tests for EEEV rely on IgM antibodies found in the serum and/or cerebral spinal fluid (CSF), PCR, and virus isolation from the CSF[427]. Despite the severity of EEEV illness, treatment with intravenous immunoglobulin (IVIg) seems to be an alternative with positive clinical outcomes[427]. As with VEEV, EEEV and WEEV are of concern because of their potential use as a bioweapon that can be spread in aerosol form. Research for avenues that can protect against this threat has evaluated neutralizing antibodies against the EEEV E2 glycoprotein and shown a protective effect in mice after lethal subcutaneous or aerosol infection[428]. Another proposed vaccine to improve prophylactic measures is a trivalent vaccine composed of virus-like particles (VLPs) that generate neutralizing antibodies and protect mice and non-human primates against EEEV infection. Although this vaccine was not cross-protective in mice, it did show cross-protection in non-human primates, and also protected against aerosolized EEEV[429].

As with EEEV, WEEV, and VEEV, detection of infection can be performed by various assays including: IgM quantification using ELISA, neutralization assays, RT-PCR, indirect peroxidase for immunohistochemical detection, and virus isolation[264,318,430]. Treatments included vaccines with a formalin-inactivated virus for horses, which is also a treatment for the EEEV (with a combination of WEEV and EEEV)[318].

Promising vaccine strategies to protect against WEEV have been studied including: live attenuated strains of WEEV, envelope proteins, adenovirus vectors, DNA, and recombinant envelope proteins[431–435]. Murine studies have shown that treatment for WEEV infection using an adenovirus-mediated expression of interferon-α provided complete protection to mice; however, only partial protection was provided for post-exposure vaccination[436]. Phage display immune

libraries have been successfully used in the generation of murine, human and human-like antibodies against VEEV and more recently neutralizing human-like antibodies against WEEV[437–439].

We are not currently aware of animal models that confirm the efficacy of single-domain antibodies (sdAb) for alphavirus. Studies have used sdAb for binding E2/E3E2 sdAb suggesting that they may be a promising target as they could be neutralizing and protective, and they can be easily modified according to the desired functionality[440]. Other studies in murine models (BALB/c mice) using neutralizing and binding monoclonal antibodies reported protection against WEEV infection prior to aerosol exposure by triggering complement-mediated lysis or antibody-dependent cell-mediated lysis of infected cells[441].

VEEV vaccines are still under development; however, the most used are a live-attenuated virus, inactivated virus, recombinant subunit or chimeric virus, and virus-like particles[347]. Recently mRNA vaccines have been suggested as a potential candidate to treat infectious diseases such as VEEV, which seems to eliminate the need for live-attenuated vaccine, could be a safer alternative, and also provides protection for the aerosol VEEV challenge[442,443]. Other studies have proposed the use of a naturally occurring host antiviral peptide, LL-37, to inhibit VEEV replication in infected neuronal cells by inducing IFNβ1 expression has potential as a possible[444].

Alphaviruses in general possess a unique viral mRNA capping mechanism catalyzed by the viral nonstructural protein (nsP1), which is important for virus replication, and is involved in the formation of the viral mRNA cap-o structure for VEEV[445,446]. Reverse genetic studies demonstrated that mutation in the nsP1 affects virus replication and that drugs targeting this protein show promise as novel anti-VEEV drugs[445].

Src Family Kinases, which is a class of non-receptor of tyrosine kinases involved in cellular processes and important for virus survival, have been shown to interact with virus-encoded proteins [447]. Treatment with Src family inhibitors has been shown to block CHIKV infection. It has been suggested as an alternative treatment to block replication in other alphaviruses such as NNOV, MYV, RRV, and VEEV[448].

4.7 Conclusion

Alphaviruses can cause severe disease in humans that range from chronic arthritis to life-threatening encephalitis[180,190,449,450]. The aggressive nature and proximity of the current mosquito vectors, along with the potential aerosol transmission for some members of the alphavirus family, has lead government agencies to classify some of these pathogens as potential biological weapons[451].

These viruses have also evolved several mechanisms which makes them highly successful as pathogens. Some of these mechanisms include adaptation to urban-dwelling animal vectors[452], anti-interferon mechanisms[250,453], regulation of cytokine and chemokine responses[90,138,165], and a wide range of cell tropism[93,157,286,454].

Old-world alphaviruses are known to cause high fever and to induce arthritis in humans. The severity of these symptoms is dependent on the strain and host susceptibility. The most common anti-alphavirus strategies devised by the host immune system are rapid induction of the type-I interferon response[77,156], fast macrophage and T-cell recruitment to the site of infection[183,202,241], and creation of neutralizing anti-CHIKV antibodies[106,217,407,413]. However, alphaviruses like CHIKV, are able to evade the initial immune system response and in some cases persist in joint tissue for several months or even years[3,53,74,101,164,217,239].

Although studies have developed possible avenues to protect against alphavirus infection, still do not fully understand the mechanisms that these viruses use to evade the immune system and persist for long periods of time. Vaccine and therapeutic treatments have slowly been developed, with most of the progress being made in the CHIKV and EEEV fields. Further research efforts with humanized models could elucidate the specific role of immune cells and their molecular responses during alphavirus infection. A new generation of therapeutic treatments could target the viral replication cycle or regulate the immune response in favor of the host, providing protection or amelioration during infection. Novel vaccines are not currently available, but in the future could provide an effective way to immunize entire populations to help prevent the spread of these diseases.

Chapter 5. Conclusions and Future Directions

This work highlights several vital factors that potentially are of significant influence on Chikungunya virus pathogenesis in humans. There is accumulating evidence that immune cells, specifically macrophages, are constantly present in CHIKV-derived joint pathology. However, the typical polyarthritis that is found in humans is not present in any mouse model.

First, we explored the differences between human and murine macrophages during CHIKV infection, which indicated significant differences in viral replication and pro-inflammatory cytokine induction between human and murine macrophages. Second, we characterized a RAG2$^{-/-}\gamma c^{-/-}$ knockout mouse model, which recapitulates CHIKV-related arthralgia and neuropathology. Finally, our accumulated research provides evidence that macrophages are constantly involved in, a yet poorly understood role, CHIKV dissemination, replication, and may be involved in pathogen immune evasion.

5.1 Chikungunya virus replication is enhanced in human macrophages and is followed by the induction of pro-inflammatory cytokines

A viral infection is typically though in terms of specific infection of a virus to a very narrow selection of targets. Examples like these include Human Immunodeficiency Virus (HIV) infection of human immune cells, where the virus envelope glycoproteins target helper T- cells and macrophages using the host's cell receptors CD4+ and CCR5, respectively. However, alphaviruses, like CHIKV, are able to infect a wide variety of cell types. This broad tropism spectrum contributes to fast alphavirus replication in the host and extensive dissemination of the disease through zoonotic infection of arthropod reservoirs[75,161,185,204,454].

Despite its ability to infect various species of host, the infection efficiencies and disease progression differ from host to host. Non-human primates are, so far, the best model to mimic

CHIKV disease developing polyarthritis, acute viremia during the initial febrile stage, and viral RNA persistence during the chronic stage of the disease[135,204]. Mouse animal models have been restricted to use some sort of immune pathway knockout or infect adult mice with high virus titters[94,126,215,216]. Additionally, mouse models only appear to be able to partially mimic one of the most iconic symptoms of CHIKV disease, polyarthritis. This may be explained by research elaborated by Judith et al., where they proved that CHIKV's non-structural protein 2 (nsp2) interacts with the human autophagy receptor NDP52, but not with its mouse ortholog[160,455]. This interaction results in increased viral replication proving that viral protein interactions with host cell machinery are selective to host species and that it modulates the progression of the disease.

Our research provided evidence that CHIKV infection of human macrophages produces higher virion concentrations compared to murine macrophages. The cytokine profile produced by human macrophages during CHIKV infection indicated a more robust pro-inflammatory cytokine response, whereas cultures of CHIKV infected murine macrophages even produced anti-inflammatory cytokines like IL-10. Our *in vitro* results suggest that the increased viral titters of CHIKV infected human macrophages may help disseminate the virus more effectively through the human host and also suggest that these infected cells may contribute to joint tissue damage by the secretion of pro-inflammatory cytokines.

Finally, a noteworthy fact is that CHIKV infected human macrophages secrete MCP-1, which is a monocyte chemoattractant molecule that helps in the recruitment of blood circulating monocytes into the site of infection. The recruitment of additional macrophages into the site of infection may benefit CHIKV as it has been extensively that macrophages are susceptible to infection. Also, macrophages are some of the few cells that can easily infiltrate nearly any tissue, making them excellent Trojan horse type vehicles of pathogen dissemination.

Future research into these hypotheses and assumptions is certainly necessary to shed light on these issues. Humanized mouse models are interesting tools where genetically engineered mice which contain specific human factors. These platforms are potentially useful to CHIKV research; specifically, models were human immune cells are present. The potential infection of a $CD34^{+}$ engrafted humanized mouse, which harbors human T-cells, B-cells, Monocytes, NK-cells and has very low concentrations of its murine equivalents, could prove the macrophage "Trojan horse" hypothesis. However, a potential drawback is that CHIKV infects not only immune cells, but various organs and tissues, and these will not contain human factors that may be crucial to the progression of the disease. Additionally, interactions between immune and non-immune cells are very well documented, and some pathways require the intervention of non-immune related cells to induce or activate the immune system properly.

5.2 A neurotropic mouse model to study Chikungunya virus disease

In recent outbreaks, related neurologic symptoms have been manifested in about 20-33% of CHIKV infected patients, which include seizures, meningoencephalopathy, myelitis, and choroiditis[190]. These symptoms are more common in neonates, elderly, and patients with co-morbidities[164,190–192]. A recent $RAG1^{-/-}$ C57BL6/J study reported virions presence and brain inflammation of 1 out of 2 mice at 28 dpi[134].

Our study with CHIKV infected $RAG2^{-/-}\gamma c^{-/-}$, confirms that this particular strain and knockout do develop the mild disease with a peak in viremia and inflammation at 8 dpi. We also observed CHIKV RNA present in the brain, spleen, and muscle tissues in higher numbers than in liver samples, which correlate to macrophage infiltration. Histological analysis confirms brain inflammation in CHIKV RNA positive samples. Additionally, we observed an increased presence of macrophages in the spleen, brain, and liver of CHIKV infected mice.

Our results imply that the absence of two critical lymphocyte maturation factors ($RAG2^{-/-}$ and $\gamma c^{-/-}$) leaves the Balb/c mice with few options to counter a CHIKV viral infection, which then spreads through the host reaching brain tissue and causing neurotropic damage[94,202,203]. The most successful animal model for Chikungunya disease is the Rhesus macaque model, which has effectively replicated human disease almost perfectly[135,204]. However, the development of a mouse model that mimics neurotropic symptoms of the disease has been largely ignored.

As we have previously explained, the humanized mouse model may provide the necessary platform to explore further the interactions between CHIKV and human immune cells, which factors induce the progression of the disease and which others suppress it. Additionally, this model could be used as a therapeutic testing platform. Novel anti-inflammatory compounds and potential anti-viral molecules are being discovered or developed regularly, and some of them have shown promising results against CHIKV and other alphaviruses [201,414,416,456,457].

5.3 Final remarks

This work outlines several factors of CHIKV pathogenesis and how macrophages play a critical role in disease progression. It also provides additional evidence that CHIKV infection in mice cannot be fully modulated due, unexplored intracellular interactions between human host cell machinery and viral proteins. Our $RAG2^{-/-}\gamma c^{-/-}$ Balb/c model properly replicated CHIKV disease, recreating inflammation at the site of inoculation, induction of several pro-inflammatory cytokines and monocyte recruiting chemokines, macrophage infiltration in infected tissues, and viral infection of the brain. We predict that further research will lead not only to a better understanding of CHIKV's pathogenesis but ultimately to effective treatments and vaccines that ameliorate and prevent the extensive outbreaks like the ones suffered by the Latin-American communities in 2015.

REFERENCES

1. Nash D, Mostashari F, Fine A, et al. The Outbreak of West Nile Virus Infection in the New York City Area in 1999. *N Engl J Med*. 2001;344(24):1807-1814. doi:10.1056/NEJM200106143442401
2. Myrielle Dupont-Rouzeryol, Olivia O'Connor, Elodie Calvez, Maguy Daures, Michele John, Jean-Paul Grangeon A-CG. Co-infection with Zika and Dengue Viruses in 2 Patients, New Caledonia, 2014. *Emerg Infect Dis*. 2015;21(2):381-382.
3. Arredondo-Garcia J, Mendez-Herrera A, Medina-Cortina H. Arbovirus en Latinoamérica. *Acta Pediatr Mex*. 2016;37(2):111-131. doi:111-131
4. Tsetsarkin KA, Kenney H, Chen R, et al. A full-length infectious cDNA clone of Zika virus from the 2015 epidemic in Brazil as a genetic platform for studies of virus-host interactions and vaccine development. *MBio*. 2016;7(4):1-8. doi:10.1128/mBio.01114-16
5. Duncan J, Gordon-Johnson KA, Tulloch-Reid MK, et al. Chikungunya: important lessons from the Jamaican experience. *Rev Panam Salud Publica*. 2017;41(60):e60. http://www.ncbi.nlm.nih.gov/pubmed/28902273.
6. Lebrun G, Chadda K, Reboux A-H, Mertinet O, Gauzere B-A. Guillain-Barre Syndrome after Chikungunya Infection. *Emerg Infect Dis*. 2009;15:495.
7. Dos Santos T, Rodriguez A, Almiron M, et al. Zika Virus and the Guillain-Barre Syndrome - Case Series from Seven Countries. *N Engl J Med*. 2016;375(16):1598-1601. doi:10.1056/NEJMc1609015
8. Parra B, Lizarazo J, Jiménez-Arango JA, et al. Guillain–Barré Syndrome Associated with Zika Virus Infection in Colombia. *N Engl J Med*. 2016;375(16):1513-1523. doi:10.1056/NEJMoa1605564
9. Brasil P, Sequeira PC, Freitas ADA, et al. Guillain-Barré syndrome associated with Zika virus infection. *Lancet*. 2016;387(10026):1482. doi:10.1016/S0140-6736(16)30058-7
10. Kam YW, Ong EKS, Rénia L, Tong JC, Ng LFP. Immuno-biology of Chikungunya and implications for disease intervention. *Microbes Infect*. 2009;11(14-15):1186-1196. doi:10.1016/j.micinf.2009.09.003
11. Couderc T, Lecuit M. Chikungunya virus pathogenesis: From bedside to bench. *Antiviral Res*. 2015;121:120-131. doi:10.1016/j.antiviral.2015.07.002
12. Bloch D. The Cost and Burden of Chikungunya in the Americas. 2016.
13. Chan PSJ, Leung MH. A Case of Initially Undiagnosed Chikungunya Arthritis Developing into Chronic Phase in a Nonendemic Area. *Case Rep Med*. 2017;2017:4-7. doi:10.1155/2017/2592964
14. Borgherini G, Poubeau P, Jossaume A, et al. Persistent arthralgia associated with chikungunya virus: a study of 88 adult patients on reunion island. *Clin Infect Dis*. 2008;47(4):469-475. doi:10.1086/590003
15. Roberge LF. Chikungunya Virus Coming to America. *Biosafety*. 2014.
16. Morrison AC, Zielinski-Gutierrez E, Scott TW, Rosenberg R. Defining challenges and proposing solutions for control of the virus vector Aedes aegypti. *PLoS Med*. 2008;5(3):0362-0366. doi:10.1371/journal.pmed.0050068

17. Sammels LM, Lindsay MD, Poidinger M, Coelent RJ, Mackenzie JS. Geographic distribution and evolution of Sindbis virus in Australia. *J Gen Virol*. 1999;80(3):739-748. doi:10.1099/0022-1317-80-3-739

18. Powers AM, Brault AC, Tesh RB, Weaver SC. Re-emergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. *J Gen Virol*. 2000;81:471-479.

19. Halstead SB. Reappearance of Chikungunya , Formerly Called Dengue , in the Americas. 2015;21(4).

20. Strauss JH, Strauss EG. The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev*. 1994;58(3):491-562.

21. Martín CSS, Liu CY, Kielian M. Dealing with low pH: entry and exit of alphaviruses and flaviviruses. *Trends Microbiol*. 2009;17(11):514-521. doi:10.1016/j.tim.2009.08.002

22. Lachmi BE, Glanville N, Keranen S, Laariainen L. Tryptic peptide analysis on nonstructural and structural precursor proteins from Semliki Forest virus mutant-infected cells. *J Virol*. 1975;16(6):1615-1629. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1202249.

23. Clewley JP, Kennedy SIT. Purification and polypeptide composition of Semliki Forest virus RNA polymerase. *J Gen Virol*. 1976;32(3):395-411. doi:10.1099/0022-1317-32-3-395

24. Burge BW, Pfefferkorn ER. Isolation and characterization of conditional-lethal mutants of Sindbis virus. *Virology*. 1966;30(2):204-213. doi:10.1016/j.jiec.2015.11.018

25. Burge BW. Complementation Between Virus ' Mutants of Sindbis. *Virology*. 1966;223(2):214-223.

26. Kennedy SIT. Synthesis of Alphavirus RNA. In: *The Togaviruses*. ; 1980:351-368.

27. Bruton CJ, Kennedy SIT. Semliki forest virus intracellular RNA: properties of the multi stranded RNA species and kinetics of positive and negative strand synthesis. *J Gen Virol*. 1975;28(1):111-127. doi:10.1099/0022-1317-28-1-111

28. Garoff H, Simons K, Renkonen O. Isolation and characterization of the membrane proteins of Semliki Forest virus. *Virology*. 1974;61(2):493-504. doi:10.1101/gad.1986710

29. Simmons DT, Strauss JH. Replication of Sindbis virus. V. Polyribosomes and mRNA in infected cells. *J Virol*. 1974;14(3):552-559. doi:10.1111/j.1349-7006.2009.01161.x

30. Lulla V, Kim DY, Frolova EI, Frolov I. The Amino-Terminal Domain of Alphavirus Capsid Protein Is Dispensable for Viral Particle Assembly but Regulates RNA Encapsidation through Cooperative Functions of Its Subdomains. *J Virol*. 2013;87(22):12003-12019. doi:10.1128/JVI.01960-13

31. Wirth DF, Katz F, Small B, Lodish HF. How a single sindbis virus mRNA directs the synthesis of one soluble protein and two integral membrane glycoproteins. *Cell*. 1977;10(2):253-263. doi:10.1016/0092-8674(77)90219-7

32. Ozden S, Lucas-Hourani M, Ceccaldi P-E, et al. Inhibition of Chikungunya virus infection in cultured human muscle cells by furin inhibitors. *J Biol Chem*. 2008;283(32):21899-21908. doi:10.1074/jbc.M802444200

33. Voss JE, Vaney M-C, Duquerroy S, et al. Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. *Nature*. 2010;468(7324):709-712. doi:10.1038/nature09555

34. de Curtis I, Simons K. Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. *Proc Natl Acad Sci*. 1988;85(21):8052-8056. doi:10.1073/pnas.85.21.8052

35. Warrier R, Linger BR, Golden BL, Kuhn RJ. Role of Sindbis Virus Capsid Protein Region II in Nucleocapsid Core Assembly and Encapsidation of Genomic RNA. *J Virol*. 2008;82(9):4461-4470. doi:10.1128/JVI.01936-07

36. Perera R, Owen KE, Tellinghuisen TL, Gorbalenya AE, Kuhn RJ. Alphavirus Nucleocapsid Protein Contains a Putative Coiled Coil -Helix Important for Core Assembly. *J Virol*. 2001;75(1):1-10. doi:10.1128/JVI.75.1.1-10.2001

37. Tellinghuisen TL, Hamburger AE, Fisher BR, Ostendorp R, Kuhn RJ. In vitro assembly of alphavirus cores by using nucleocapsid protein expressed in Escherichia coli. *J Virol*. 1999;73(7):5309-5319. doi:10.1128/JVI.76.21.11128

38. Acheson NH, Tamm I. Replication of semliki forest virus: An electron microscopic study. *Virology*. 1967;32(1):128-143. doi:10.1016/0042-6822(67)90261-9

39. Brown DT, Waite MR, Pfefferkorn ER. Morphology and morphogenesis of Sindbis virus as seen with freeze-etching techniques. *J Virol*. 1972;10(3):524-536. doi:10.3233/jad-150254

40. Robinson MC, Lumsden WH. An epidemic of virus disease in Southern Province, Tanganyika territory, in 1952–1953. *Trans R Soc Trop Med Hyg*. 1955;49(1):28-32. doi:10.1016/0035-9203(57)90022-6

41. Arias-Goeta C, Mousson L, Rougeon F, Failloux AB. Dissemination and Transmission of the E1-226V Variant of Chikungunya Virus in Aedes albopictus Are Controlled at the Midgut Barrier Level. *PLoS One*. 2013;8(2). doi:10.1371/journal.pone.0057548

42. Kumar NP, Joseph R, Kamaraj T, Jambulingam P. A226V mutation in virus during the 2007 chikungunya outbreak in Kerala, India. *J Gen Virol*. 2008;89(8):1945-1948. doi:10.1099/vir.0.83628-0

43. Tsetsarkin K a., Vanlandingham DL, McGee CE, Higgs S. A single mutation in Chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathog*. 2007;3(12):1895-1906. doi:10.1371/journal.ppat.0030201

44. Schuffenecker I, Iteman I, Michault A, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med*. 2006;3(7):1058-1070. doi:10.1371/journal.pmed.0030263

45. Akahata W, Nabel GJ. A Specific Domain of the Chikungunya Virus E2 Protein Regulates Particle Formation in Human Cells: Implications for Alphavirus Vaccine Design. *J Virol*. 2012;86(16):8879-8883. doi:10.1128/JVI.00370-12

46. Tsetsarkin KA, McGee CE, Volk SM, Vanlandingham DL, Weaver SC, Higgs S. Epistatic roles of E2 glycoprotein mutations in adaption of Chikungunya virus to Aedes albopictus and Ae. Aegypti mosquitoes. *PLoS One*. 2009;4(8). doi:10.1371/journal.pone.0006835

47. Delang L, Guerrero NS, Tas A, et al. Mutations in the chikungunya virus non-structural

proteins cause resistance to favipiravir (T-705), a broad-spectrum antiviral. *J Antimicrob Chemother*. 2014;69(10):2770-2784. doi:10.1093/jac/dku209

48. Utt A, Das PK, Varjak M, Lulla V, Lulla A, Merits A. Mutations Conferring a Noncytotoxic Phenotype on Chikungunya Virus Replicons Compromise Enzymatic Properties of Nonstructural Protein 2. *J Virol*. 2015;89(6):3145-3162. doi:10.1128/JVI.03213-14
49. Caron M, Paupy C, Grard G, et al. Recent introduction and rapid dissemination of chikungunya virus and dengue virus serotype 2 associated with human and mosquito coinfections in gabon, central africa. *Clin Infect Dis*. 2012;55(6). doi:10.1093/cid/cis530
50. P.G. J, B.M. M, I. DS, P. D. Laboratory vector studies on six mosquito and one tick species with chikungunya virus. *Trans R Soc Trop Med Hyg*. 1981;75(1):15-19. http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed1a&NEWS=N&AN=6115488.
51. Dubrulle M, Mousson L, Moutailier S, Vazeille M, Failloux AB. Chikungunya virus and Aedes mosquitoes: Saliva is infectious as soon as two days after oral infection. *PLoS One*. 2009;4(6). doi:10.1371/journal.pone.0005895
52. Thiberville SD, Moyen N, Dupuis-Maguiraga L, et al. Chikungunya fever: Epidemiology, clinical syndrome, pathogenesis and therapy. *Antiviral Res*. 2013;99:345-370. doi:10.1016/j.antiviral.2013.06.009
53. Danis-Lozano R, Díaz-González EE, Trujillo-Murillo K del C, et al. Clinical characterization of acute and convalescent illness of confirmed chikungunya cases from Chiapas, S. Mexico: A cross sectional study. *PLoS One*. 2017;12(10):1-15. doi:10.1371/journal.pone.0186923
54. Ryman KD, Klimstra WB. Host Responses to alphavirus Infection. *Immunol Emerg Infect*. 2008;225(1):27-45. doi:10.1016/B978-0-7020-6285-8.00003-4
55. Garmashova N, Gorchakov R, Volkova E, Paessler S, Frolova E, Frolov I. The Old World and New World Alphaviruses Use Different Virus-Specific Proteins for Induction of Transcriptional Shutoff. *J Virol*. 2007;81(5):2472-2484. doi:10.1128/JVI.02073-06
56. Malvy D, Ezzedine K, Mamani-Matsuda M, et al. Destructive arthritis in a patient with chikungunya virus infection with persistent specific IgM antibodies. *BMC Infect Dis*. 2009;9:200. doi:10.1186/1471-2334-9-200
57. Simon F, Parola P, Grandadam M, et al. Chikungunya infection: An emerging rheumatism among travelers returned from Indian Ocean islands. Report of 47 cases. *Medicine (Baltimore)*. 2007;86(3):123-137. doi:10.1097/MD/0b013e31806010a5
58. Guruprasad SPMPVRUAPSSSSSKRABSNMIKCDRGPMPV. Clinical progression of chikungunya fever during acute and chronic arthritic stages and the changes in joint morphology as revealed by imaging. *Trop Med Hyg*. 2010;104(6):392-399.
59. Tesh RB. Arthritides Caused by Mosquito-Borne Viruses. *Annu Rev Med*. 1982;33(1):31-40.
60. Levine B, Hardwick JM, Griffin DE. Persistence of alphaviruses in vertebrate hosts. *Trends Microbiol*. 1994;2(1):25-28.
61. Suhrbier A, La Linn M. Clinical and pathologic aspects of arthritis due to Ross River virus

and othe alphaviruses. *Curr Opin Microbiol*. 2004;16(4):374-379.

62. Toivanen A. Alphaviruses: an emerging cause of arthritis. *Curr Opin Rheumatol*. 2008;20(4):486-490.
63. Fourie E, Morrison J. Rheumatoid arthritic syndrome after chikungunya fever. *South African Med J*. 1979;56(4):130-132.
64. Chopra A, Anuradha V, Lagoo-Joshi V, Kunjir V, Salvi S, Saluja M. Chikungunya virus aches and pains: An emerging challenge. *Arthritis Rheum*. 2008;58(9):2921-2922.
65. Santiago FW, Halsey ES, Siles C, et al. Long-Term Arthralgia after Mayaro Virus Infection Correlates with Sustained Pro-inflammatory Cytokine Response. *PLoS Negl Trop Dis*. 2015;9(10):e0004104. doi:10.1371/journal.pntd.0004104
66. Jaffar-Bandjee MC, Das T, Hoarau JJ, et al. Chikungunya virus takes centre stage in virally induced arthritis: possible cellular and molecular mechanisms to pathogenesis. *Microbes Infect*. 2009;11:1206-1218. doi:10.1016/j.micinf.2009.10.001
67. Henry M, Francis L, Asin V, Polson-Edwards K, Olowokure B. Chikungunya virus outbreak in Sint Maarten, 2013-2014. *Rev Panam salud publica*. 2017;41(5):e61. http://www.ncbi.nlm.nih.gov/pubmed/28902274.
68. Espinal M. Chikungunya: first emergent arbovirosis in the XXI century in the Americas. *Pan Am J Public Heal*. 2017;(41):e108.
69. Bloch D. The Cost and Burden of Chikungunya in the Americas. 2016. doi:10.1561/2200000016
70. Cigarroa-Toledo N, Blitvich BJ, Cetina-Trejo RC, et al. Chikungunya virus in febrile humans and aedes Aegypti mosquitoes, Yucatan, Mexico. *Emerg Infect Dis*. 2016;22(10):1804-1807. doi:10.3201/eid2210.152087
71. Rivera-ávila RC. Fiebre chikungunya en México : caso confirmado y apuntes para la respuesta epidemiológica. 2014;56(4):402-404.
72. Díaz-Quiñonez JA, Ortiz-Alcántara J, Fragoso-Fonseca DE, et al. Complete Genome Sequences of Chikungunya Virus Strains Isolated in Mexico: First Detection of Imported and Autochthonous Cases. *Genome Announc*. 2015;3(3). doi:10.1128/genomeA.00300-15
73. Garay-Moran C, Roman-Pedroza JF, Lopez-Martinez I, et al. Caracterizacion Clinica y Epidemiologica de Fiebre Chikungunya en mexico. *Pan Am J Public Heal*. 2017;41(58).
74. Murillo-Zamora E, Mendoza-Cano O, Trujillo-Hernández B, Alberto Sánchez-Piña R, Guzmán-Esquivel J. Persistent arthralgia and related risks factors in laboratory-confirmed cases of Chikungunya virus infection in Mexico TT - Artralgia persistente y factores de riesgos relacionados en casos confirmados por laboratorio de infección por el virus del Chikun. *Rev Panam Salud Publica*. 2017;41(72):e72-e72. http://www.scielosp.org/scielo.php?script=sci_arttext&pid=S1020-49892017000100230.
75. Ozden S, Huerre M, Riviere J-P, et al. Human muscle satellite cells as targets of Chikungunya virus infection. *PLoS One*. 2007;2(6):e527. doi:10.1371/journal.pone.0000527
76. Sourisseau M, Schilte C, Casartelli N, et al. Characterization of reemerging chikungunya virus. *PLoS Pathog*. 2007;3(6):0804-0817. doi:10.1371/journal.ppat.0030089
77. Couderc T, Chrétien F, Schilte C, et al. A mouse model for Chikungunya: young age and

inefficient type-I interferon signaling are risk factors for severe disease. *PLoS Pathog*. 2008;4(2):e29. doi:10.1371/journal.ppat.0040029

78. Her Z, Malleret B, Chan M, et al. Active infection of human blood monocytes by Chikungunya virus triggers an innate immune response. *J Immunol*. 2010;184(10):5903-5913. doi:10.4049/jimmunol.0904181

79. Schneider BS, Higgs ST. The enhancement of arbovirus transmission and disease by mosquito saliva is associated with modulation of the host immune response. *Trop Med Hyg*. 2008;102(5):400-408.

80. Thangamani S, Higgs ST, Ziegler SA, Vanlandingham DL, Tesh RB, Wikel S. Host immune response to mosquito-transmitted chikungunya virus differs from that elicited by needle inoculated virus. *PLoS One*. 2010;5(8):12137.

81. Schneider BS, Soong L, Zeidner NS, Higgs ST. Aedes aegypti salivary gland extracts modulate anti-viral and TH1/TH2 cytokine responses to sindbis virus infection. *Viral Immunol*. 2004;17(4):565-573.

82. Parola P, De Lamballerie X, Jourdan J, et al. Novel chikungunya virus variant in travelers returning from Indian Ocean islands. *Emerg Infect Dis*. 2006;12:1493-1499.

83. Vazeille M, Moutailler S, Coudrier D, et al. Two Chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, Aedes albopictus. *PLoS One*. 2007;2(11). doi:10.1371/journal.pone.0001168

84. Dupuis-Maguiraga L, Noret M, Brun S, Le Grand R, Gras G, Roques P. Chikungunya disease: Infection-associated markers from the acute to the chronic phase of arbovirus-induced arthralgia. *PLoS Negl Trop Dis*. 2012;6(3). doi:10.1371/journal.pntd.0001446

85. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med*. 2004;10:1366-1373.

86. McFarlane M, Arias-Goeta C, Martin E, et al. Characterization of Aedes aegypti Innate-Immune Pathways that Limit Chikungunya Virus Replication. *PLoS Negl Trop Dis*. 2014;8(7). doi:10.1371/journal.pntd.0002994

87. Dantzer R, Wollman E. Molecular mechanisms of fever: the missing links. *Eur Cytokine Netw*. 1998;9(1):27-31.

88. Ng LFP, Chow A, Sun YJ, et al. IL-1β, IL-6, and RANTES as biomarkers of Chikungunya severity. *PLoS One*. 2009;4(1):1-8. doi:10.1371/journal.pone.0004261

89. Hoarau JJ, Jaffar Bandjee MC, Krejbich Trotot P, et al. Persistent Chronic Inflammation and Infection by Chikungunya Arthritogenic Alphavirus in Spite of a Robust Host Immune Response. *J Immunol*. 2010;184(10):5914-5927. doi:10.4049/jimmunol.0900255

90. Chow A, Her Z, Ong EKS, et al. Persistent arthralgia induced by Chikungunya virus infection is associated with interleukin-6 and granulocyte macrophage colony-stimulating factor. *J Infect Dis*. 2011;203(2):149-157. doi:10.1093/infdis/jiq042

91. Fitzpatrick FA, Stringfellow DA. Virus and interferon effects on cellular prostaglandin biosynthesis. *J Immunol*. 1980;125:431-437.

92. Malfait AM, Schnitzer TJ. Towards a mechanism-based approach to pain management in osteoarthritis. *Nat Rev Rheumatol*. 2013;9:654-664.

93. Mejía CR, López-Vélez R. Tropical arthritogenic alphaviruses. *Reumatol Clin*. 2018;14(2):97-105. doi:10.1016/j.reuma.2017.01.006

94. Teo T-H, Lum F-M, Claser C, et al. A pathogenic role for CD4+ T cells during Chikungunya virus infection in mice. *J Immunol*. 2013;190:259-269. doi:10.4049/jimmunol.1202177

95. Poo YS, Rudd PA, Gardner J, et al. Multiple Immune Factors Are Involved in Controlling Acute and Chronic Chikungunya Virus Infection. *PLoS Negl Trop Dis*. 2014;8(12). doi:10.1371/journal.pntd.0003354

96. Wauquier N, Becquart P, Nkoghe D, Padilla C, Ndjoyi-Mbiguino A, Leroy EM. The acute phase of Chikungunya virus infection in humans is associated with strong innate immunity and T CD8 cell activation. *J Infect Dis*. 2011;204(1):115-123. doi:10.1093/infdis/jiq006

97. Laras K, Sukri NC, Larasati RP, et al. Tracking the re-emergence of epidemic chikungunya virus in Indonesia. *Trans R Soc Trop Med Hyg*. 2005;99(2):128-141. doi:10.1016/j.trstmh.2004.03.013

98. Lanciotti RS, Ludwig ML, Rwaguma EB, et al. Emergence of epidemic O'nyong-nyong fever in Uganda after a 35-year absence: genetic characterization of the virus. *Virology*. 1998;252:258-268.

99. Pavri KM. Editorial: epidemic of fever with severe joint pains: chikungunya virus. *Maharashtra Med J*. 1973;20:219-220.

100. Pavri KM. Disappearance of chikungunya virus from India and South East Asia. *Trans R Soc Trop Med Hyg*. 1986;80:491.

101. Morrison TE, Oko L, Montgomery SA, et al. A mouse model of chikungunya virus-induced musculoskeletal inflammatory disease: Evidence of arthritis, tenosynovitis, myositis, and persistence. *Am J Pathol*. 2011;178(1):32-40. doi:10.1016/j.ajpath.2010.11.018

102. Gardner J, Anraku I, Le TT, et al. Chikungunya Virus Arthritis in Adult Wild-Type Mice Chikungunya Virus Arthritis in Adult Wild-Type Mice. *J Virol*. 2010;84(16):8021-8032. doi:10.1128/JVI.02603-09

103. Nakaya HI, Gardner J, Poo YS, Major L, Pulendran B, Suhrbier A. Gene profiling of chikungunya virus arthritis in a mouse model reveals significant overlap with rheumatoid arthritis. *Arthritis Rheum*. 2012;64(11):3553-3563. doi:10.1002/art.34631

104. Lum F-M, Teo T-H, Lee WWL, Kam Y-W, Renia L, Ng LFP. An Essential Role of Antibodies in the Control of Chikungunya Virus Infection. *J Immunol*. 2013;190(12):6295-6302. doi:10.4049/jimmunol.1300304

105. Warter L, Lee CY, Thiagarajan R, et al. Chikungunya Virus Envelope-Specific Human Monoclonal Antibodies with Broad Neutralization Potency. *J Immunol*. 2011;186(5):3258-3264. doi:10.4049/jimmunol.1003139

106. Pal P, Dowd KA, Brien JD, et al. Development of a highly protective combination monoclonal antibody therapy against Chikungunya virus. *PLoS Pathog*. 2013;9(4):e1003312. doi:10.1371/journal.ppat.1003312

107. Lee CY, Kam YW, Fric J, et al. Chikungunya virus neutralization antigens and direct cell-to-cell transmission are revealed by human antibody-escape mutants. *PLoS Pathog*.

2011;7(12). doi:10.1371/journal.ppat.1002390

108. Kam YW, Simarmata D, Chow A, et al. Early appearance of neutralizing immunoglobulin G3 antibodies is associated with chikungunya virus clearance and long-term clinical protection. *J Infect Dis*. 2012;205(7):1147-1154. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L364428505%5Cnhttp://dx.doi.org/10.1093/infdis/jis033.

109. Kam Y-W, Lee WWL, Simarmata D, et al. Longitudinal Analysis of the Human Antibody Response to Chikungunya Virus Infection: Implications for Serodiagnosis and Vaccine Development. *J Virol*. 2012;86(23):13005-13015. doi:10.1128/JVI.01780-12

110. Dubuisson J, Rice CM. Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. *J Virol*. 1993;67(6):3363-3374. http://www.ncbi.nlm.nih.gov/pubmed/7684466%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC237680.

111. Bernard KA, Klimstra WB, Johnston RE. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology*. 2000;276(1):93-103. doi:10.1006/viro.2000.0546

112. Klimstra WB, Ryman KD, Johnston RE. Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *J Virol*. 1998;72(9):7357-7366. http://www.ncbi.nlm.nih.gov/pubmed/9696832%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC109960.

113. Ryman KD, Gardner CL, Burke CW, Meier KC, Thompson JM, Klimstra WB. Heparan Sulfate Binding Can Contribute to the Neurovirulence of Neuroadapted and Nonneuroadapted Sindbis Viruses. *J Virol*. 2007;81(7):3563-3573. doi:10.1128/jvi.02494-06

114. Akahata W, Yang Z-Y, Andersen H, et al. A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. *Nat Med*. 2010;16(3):334-338. doi:10.1038/nm.2105

115. Kam Y-W, Lum F-M, Teo T-H, et al. Early neutralizing IgG response to Chikungunya virus in infected patients targets a dominant linear epitope on the E2 glycoprotein. *EMBO Mol Med*. 2012;4(4):330-343. http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed10&NEWS=N&AN=2012200508.

116. Hallengard D, Kakoulidou M, Lulla A, et al. Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice. *J Virol*. 2014;88(5):2858-2866. doi:10.1128/JVI.03453-13

117. Garcia-Arriaza J, Cepeda V, Hallengard D, et al. A Novel Poxvirus-Based Vaccine, MVA-CHIKV, Is Highly Immunogenic and Protects Mice against Chikungunya Infection. *J Virol*. 2014;88(6):3527-3547. doi:10.1128/JVI.03418-13

118. Metz SW, Gardner J, Geertsema C, et al. Effective Chikungunya Virus-like Particle Vaccine Produced in Insect Cells. *PLoS Negl Trop Dis*. 2013;7(3). doi:10.1371/journal.pntd.0002124

119. S.W. M, B.E. M, P. van den D, et al. Chikungunya virus-like particles are more immunogenic in a lethal AG129 mouse model compared to glycoprotein E1 or E2

subunits. *Vaccine*. 2013;31(51):6092-6096. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L52866233%5Cnhttp://dx.doi.org/10.1016/j.vaccine.2013.09.045.

120. Roy CJ, Adams AP, Wang E, et al. Chikungunya vaccine candidate is highly attenuated and protects nonhuman primates against telemetrically monitored disease following a single dose. *J Infect Dis*. 2014;209(12):1891-1899. doi:10.1093/infdis/jiu014
121. van den Doel P, Volz A, Roose JM, et al. Recombinant Modified Vaccinia Virus Ankara Expressing Glycoprotein E2 of Chikungunya Virus Protects AG129 Mice against Lethal Challenge. *PLoS Negl Trop Dis*. 2014;8(9). doi:10.1021/ed500534k
122. Pal P, Fox JM, Hawman DW, et al. Chikungunya Viruses That Escape Monoclonal Antibody Therapy Are Clinically Attenuated, Stable, and Not Purified in Mosquitoes. *J Virol*. 2014;88(15):8213-8226. doi:10.1128/JVI.01032-14
123. Goupil BA, McNulty MA, Martin MJ, McCracken MK, Christofferson RC, Mores CN. Novel lesions of bones and joints associated with chikungunya virus infection in two mouse models of disease: New insights into disease pathogenesis. *PLoS One*. 2016;11(5):1-21. doi:10.1371/journal.pone.0155243
124. Chen W, Foo SS, Sims NA, Herrero LJ, Walsh NC, Mahalingam S. Arthritogenic alphaviruses: New insights into arthritis and bone pathology. *Trends Microbiol*. 2015;23(1):35-43. doi:10.1016/j.tim.2014.09.005
125. Hawman DW, Stoermer KA, Montgomery SA, et al. Chronic Joint Disease Caused by Persistent Chikungunya Virus Infection Is Controlled by the Adaptive Immune Response. *J Virol*. 2013;87(24):13878-13888. doi:10.1128/JVI.02666-13
126. Ziegler S a, Lu L, da Rosa AP a T, Xiao S-Y, Tesh RB. An animal model for studying the pathogenesis of chikungunya virus infection. *Am J Trop Med Hyg*. 2008;79(1):133-139. doi:79/1/133 [pii]
127. Gardner CL, Burke CW, Higgs ST, Klimstra WB, Ryman KD. Interferon-alpha/beta deficiency greatly exacerbates arthritogenic disease in mice infected with wild-type chikungunya virus but not with the cell culture-adapted live-attenuated 181/25 vaccine candidate. *Virology*. 2012;425(2):103-112. doi:10.1016/j.virol.2011.12.020
128. Dagley A, Ennis J, Turner JD, et al. Protection against Chikungunya virus induced arthralgia following prophylactic treatment with adenovirus vectored interferon (mDEF201). *Antiviral Res*. 2014;108(1):1-9. doi:10.1016/j.antiviral.2014.05.004
129. Werneke SW, Schilte C, Rohatgi A, et al. ISG15 is critical in the control of chikungunya virus infection independent of UbE1l mediated conjugation. *PLoS Pathog*. 2011;7(10). doi:10.1371/journal.ppat.1002322
130. Uhrlaub JL, Pulko V, DeFilippis VR, et al. Dysregulated TGF-β Production Underlies the Age-Related Vulnerability to Chikungunya Virus. *PLoS Pathog*. 2016;12(10):1-17. doi:10.1371/journal.ppat.1005891
131. Borgherini G, Poubeau P, Staikowsky F, et al. Outbreak of Chikungunya on Reunion Island: Early Clinical and Laboratory Features in 157 Adult Patients. *Clin Infect Dis*. 2007:1401-1407.
132. P. G, A. F, D. M, et al. Perceived morbidity and community burden after a Chikungunya outbreak: the TELECHIK survey, a population-based cohort study. *BMC Med*. 2011;9:5.

doi:10.1186/1741-7015-9-5

133. Labadie K, Larcher T, Joubert C, et al. Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages. *J Clin Invest*. 2010;120(3):894-906. doi:10.1172/JCI40104

134. Seymour RL, Adams AP, Leal G, Alcorn MDH, Weaver SC. A Rodent Model of Chikungunya Virus Infection in RAG1 -/- Mice, with Features of Persistence, for Vaccine Safety Evaluation. *PLoS Negl Trop Dis*. 2015;9(6):e0003800. doi:10.1371/journal.pntd.0003800

135. Messaoudi I, Vomaske J, Totonchy T, et al. Chikungunya Virus Infection Results in Higher and Persistent Viral Replication in Aged Rhesus Macaques Due to Defects in Anti-Viral Immunity. *PLoS Negl Trop Dis*. 2013;7(7). doi:10.1371/journal.pntd.0002343

136. Gérardin P, Couderc T, Bintner M, et al. Chikungunya virus–associated encephalitis. *Neurology*. 2016;86(1):94 LP - 102. doi:10.1212/WNL.0000000000002234

137. Barau G, Michault A, Bintner M, et al. Multidisciplinary Prospective Study of Mother-to-Child Chikungunya Virus Infections on the Island of La Reunion. *PLoS Med*. 2008;5(3):413-423. doi:10.1371/journal.pmed.0050060

138. Gasque P, Couderc T, Lecuit M, Roques P, Ng LFP. Chikungunya Virus Pathogenesis and Immunity. *Vector-Borne Zoonotic Dis*. 2015;15(4):241-249. doi:10.1089/vbz.2014.1710

139. Gerardin P, Couderc T, Bintner M, Michault A. Chikungunya virus–associated encephalitis. *Neurology*. 2016;86(1):94-102.

140. Gorchakov R, Wang E, Leal G, et al. Attenuation of Chikungunya Virus Vaccine Strain 181/Clone 25 Is Determined by Two Amino Acid Substitutions in the E2 Envelope Glycoprotein. *J Virol*. 2012;86(11):6084-6096. doi:10.1128/JVI.06449-11

141. Rudd PA, Wilson J, Gardner J, et al. Interferon Response Factors 3 and 7 Protect against Chikungunya Virus Hemorrhagic Fever and Shock. *J Virol*. 2012;86(18):9888-9898. doi:10.1128/JVI.00956-12

142. Santhosh SR, Dash PK, Parida MM, Khan M, Tiwari M, Lakshmana Rao P V. Comparative full genome analysis revealed E1: A226V shift in 2007 Indian Chikungunya virus isolates. *Virus Res*. 2008;135:36-41. doi:10.1016/j.virusres.2008.02.004

143. Bouraï M, Lucas-Hourani M, Gad HH, et al. Mapping of Chikungunya virus interactions with host proteins identified nsP2 as a highly connected viral component. *J Virol*. 2012;86(6):3121-3134. doi:10.1128/JVI.06390-11

144. Akhrymuk I, Kulemzin S V., Frolova EI. Evasion of the Innate Immune Response: the Old World Alphavirus nsP2 Protein Induces Rapid Degradation of Rpb1, a Catalytic Subunit of RNA Polymerase II. *J Virol*. 2012;86(13):7180-7191. doi:10.1128/JVI.00541-12

145. Staples JE, Breiman RF, Powers AM. Chikungunya fever: an epidemiological review of a re-emerging infectious disease. *Clin Infect Dis*. 2009;49(6):942-948. doi:10.1086/605496

146. Rashad AA, Mahalingam S, Keller PA. Chikungunya virus: Emerging targets and new opportunities for medicinal chemistry. *J Med Chem*. 2014;57(4):1147-1166. doi:10.1021/jm400460d

147. Chikungunya Fever - Emerging Epidemics - Bisen - Wiley Online Library.

148. Mangiafico J a. Chikungunya Virus Infection and Transmission in Five Species of Mosquito. *Am J Trop Med Hyg*. 1971;20(4):642-645.

149. Díaz-González EE, Kautz TF, Dorantes-Delgado A, et al. First report of Aedes aegypti transmission of chikungunya virus in the Americas. *Am J Trop Med Hyg*. 2015;93(6):1325-1329. doi:10.4269/ajtmh.15-0450

150. Nuckols JT, Ziegler SA, Huang Y-JS, et al. Infection of *Aedes albopictus* with Chikungunya Virus Rectally Administered by Enema. *Vector-Borne Zoonotic Dis*. 2013;13(2):103-110. doi:10.1089/vbz.2012.1013

151. McPherson RL, Abraham R, Sreekumar E, et al. ADP-ribosylhydrolase activity of Chikungunya virus macrodomain is critical for virus replication and virulence. *Proc Natl Acad Sci*. 2017;114(7):1666-1671. doi:10.1073/pnas.1621485114

152. Martínez-Sánchez A, Martínez-Ramos EB, Chávez-Angeles MG. Temas de actualidad Panorama situacional de México ante la pandemia del virus chikunguña Situational panorama of Mexico against the chikungunya virus pandemic. *Rev Med Inst Mex Seguro Soc*. 2015;53(2):200-205.

153. Díaz-quiñonez JA, Ortiz-alcántara J, Fragoso-fonseca DE, Garcés-ayala F, Escobar-escamilla N. Complete Genome Sequences of Chikungunya Virus Strains Isolated in Mexico : First Detection of Imported and Autochthonous Cases. *Genome Announc*. 2015;3(3):2014-2015. doi:10.1128/genomeA.00300-15.Copyright

154. Staples JE, Hills S, Powers AM. Infectious diseases related To travel. 2014 Yellow book. doi:10.1145/1529282.1529753

155. Morrison TE, Whitmore AC, Shabman RS, Lidbury B a, Mahalingam S, Heise MT. Characterization of Ross River virus tropism and virus-induced inflammation in a mouse model of viral arthritis and myositis. *J Virol*. 2006;80(2):737-749. doi:10.1128/JVI.80.2.737-749.2006

156. Couderc T, Lecuit M. Focus on Chikungunya pathophysiology in human and animal models. *Microbes Infect*. 2009;11(14-15):1197-1205. doi:10.1016/j.micinf.2009.09.002

157. Ashbrook AW, Burrack KS, Silva LA, et al. Residue 82 of the Chikungunya virus E2 attachment protein modulates viral dissemination and arthritis in mice. *J Virol*. 2014;88(21):12180-12192. doi:10.1128/JVI.01672-14

158. Polakowski N, Terol M, Hoang K, et al. HBZ Stimulates Brain-Derived Neurotrophic Factor/TrkB Autocrine/Paracrine Signaling To Promote Survival of Human T-Cell Leukemia Virus Type 1-Infected T Cells. *J Virol*. 2014;88(22):13482-13494. doi:10.1128/JVI.02285-14

159. Nazimuddin R Bin. COMPARISON OF CHIKUNGUNYA VIRUS CULTURE PROPAGATED IN TWO CELL LINES ; VERO AND C6 / 36 CELL LINES. *BSc Thesis, Univ Malaysia Sarawak*. 2015;(35197).

160. Judith D, Mostowy S, Bourai M, et al. Species-specific impact of the autophagy machinery on Chikungunya virus infection. *EMBO Rep*. 2013;14(6):534-544. doi:10.1038/embor.2013.51

161. Sourisseau M, Schilte C, Casartelli N, et al. Characterization of reemerging chikungunya virus. *PLoS Pathog*. 2007;3(6):0804-0817. doi:10.1371/journal.ppat.0030089

162. Ziegler SA. Characterization of the role of the innate immune system in the pathogenesis of chikungunya virus in mice. 2011. https://utmb-ir.tdl.org/handle/2152.3/785.

163. Ziegler SA, Nuckols J, McGee CE, et al. *In Vivo* Imaging of Chikungunya Virus in Mice and *Aedes* Mosquitoes Using a *Renilla* Luciferase Clone. *Vector-Borne Zoonotic Dis*. 2011;11(11):1471-1477. doi:10.1089/vbz.2011.0648

164. K.M. H, J.J.H. C. Insights into the interplay between chikungunya virus and its human host. *Future Virol*. 2011;6(10):1211-1223. doi:10.2217/fvl.11.101

165. Rulli NE, Rolph MS, Srikiatkhachorn A, Anantapreecha S, Guglielmotti A, Mahalingam S. Protection from arthritis and myositis in a mouse model of acute chikungunya virus disease by bindarit, an inhibitor of monocyte chemotactic protein-1 synthesis. *J Infect Dis*. 2011;204(7):1026-1030. doi:10.1093/infdis/jir470

166. Malvy D, Ezzedine K, Mamani-Matsuda M, et al. Destructive arthritis in a patient with chikungunya virus infection with persistent specific IgM antibodies. *BMC Infect Dis*. 2009;9. doi:10.1186/1471-2334-9-200

167. Her Z, Malleret B, Chan M, et al. Active Infection of Human Blood Monocytes by Chikungunya Virus Triggers an Innate Immune Response. *J Immunol*. 2010;184(10):5903-5913. doi:10.4049/jimmunol.0904181

168. Zhang R, Earnest JT, Kim AS, et al. Expression of the Mxra8 Receptor Promotes Alphavirus Infection and Pathogenesis in Mice and Drosophila. *Cell Rep*. 2019;28(10):2647-2658.e5. doi:10.1016/j.celrep.2019.07.105

169. Zhang R, Kim AS, Fox JM, et al. Mxra8 is a receptor for multiple arthritogenic alphaviruses. *Nature*. 2018;557(7706):570-574. doi:10.1038/s41586-018-0121-3

170. Das T, Hoarau JJ, Bandjee MCJ, Maquart M, Gasque P. Multifaceted innate immune responses engaged by astrocytes, microglia and resident dendritic cells against Chikungunya neuroinfection. *J Gen Virol*. 2014;96(Pt_2):294-310. doi:10.1099/vir.0.071175-0

171. Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. *Nat Rev Microbiol*. 2010;8:491-500. doi:10.1038/nrmicro2368

172. Tsetsarkin KA, Weaver SC. Sequential adaptive mutations enhance efficient vector switching by chikungunya virus and its epidemic emergence. *PLoS Pathog*. 2011;7(12). doi:10.1371/journal.ppat.1002412

173. Chan Y-H, Lum F-M, Ng L. Limitations of Current in Vivo Mouse Models for the Study of Chikungunya Virus Pathogenesis. *Med Sci*. 2015;3(3):64-77. doi:10.3390/medsci3030064

174. Kumar S, Jaffar-Bandjee MC, Giry C, et al. Mouse macrophage innate immune response to chikungunya virus infection. *Virol J*. 2012;9. doi:10.1186/1743-422X-9-313

175. Chirathaworn C, Poovorawan Y, Lertmaharit S, Wuttirattanakowit N. Cytokine levels in patients with chikungunya virus infection. *Asian Pac J Trop Med*. 2013;6(8):631-634. doi:10.1016/S1995-7645(13)60108-X

176. Kumar S, Jaffar-Bandjee MC, Giry C, et al. Mouse macrophage innate immune response to chikungunya virus infection. *Virol J*. 2012;9(313). doi:10.1186/1743-422X-9-313

177. Darwich L, Coma G, Peña R, et al. Secretion of interferon-γ by human macrophages

demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. *Immunology*. 2009;126(3):386-393. doi:10.1111/j.1365-2567.2008.02905.x

178. Trinchieri G. Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-γ). *Curr Opin Immunol*. 1997;9(1):17-23. doi:10.1016/S0952-7915(97)80154-9

179. Jaffar-Bandjee MC, Das T, Hoarau JJ, et al. Chikungunya virus takes centre stage in virally induced arthritis: possible cellular and molecular mechanisms to pathogenesis. *Microbes Infect*. 2009;11(14-15):1206-1218. doi:10.1016/j.micinf.2009.10.001

180. Franssila R, Hedman K. Viral causes of arthritis. *Best Pract Res Clin Rheumatol*. 2006;20(6):1139-1157. doi:10.1016/j.berh.2006.08.007

181. Okeoma CM. *Chikungunya Virus: Advances in Biology, Pathogenesis, and Treatment*.; 2016. doi:10.1007/978-3-319-42958-8

182. Herrero LJ, Sheng KC, Jian P, et al. Macrophage migration inhibitory factor receptor CD74 mediates alphavirus-induced arthritis and myositis in murine models of alphavirus infection. *Arthritis Rheum*. 2013;65(10):2724-2736. doi:10.1002/art.38090

183. Rulli NE, Melton J, Wilmes A, Ewart G, Mahalingam S. The molecular and cellular aspects of arthritis due to alphavirus infections: Lesson learned from Ross River virus. *Ann N Y Acad Sci*. 2007;1102:96-108. doi:10.1196/annals.1408.007

184. Danillo Lucas Alves E, Benedito Antonio Lopes da F. Characterization of the immune response following in vitro mayaro and chikungunya viruses (Alphavirus, Togaviridae) infection of mononuclear cells. *Virus Res*. 2018;256:166-173. doi:10.1016/j.virusres.2018.08.011

185. Wikan N, Sakoonwatanyoo P, Ubol S, Yoksan S, Smith DR. Chikungunya virus infection of cell lines: Analysis of the east, central and south African lineage. *PLoS One*. 2012;7(1). doi:10.1371/journal.pone.0031102

186. Zhang X, Huang Y, Wang M, et al. Differences in genome characters and cell tropisms between two chikungunya isolates of Asian lineage and Indian Ocean lineage. *Virol J*. 2018;15(1):101-111. doi:10.1186/s12985-018-1024-5

187. Herrero LJ, Nelson M, Srikiatkhachorn A, Gu R, Anantapreecha S. Critical role for macrophage migration inhibitory factor (MIF) in Ross River virus-induced arthritis and myositis. *PNAS*. 2011;108(29):12048-12053. doi:10.1073/pnas.1101089108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1101089108

188. Suhrbier A, Jaffar-Bandjee MC, Gasque P. Arthritogenic alphaviruses-an overview. *Nat Rev Rheumatol*. 2012;8(7):420-429. doi:10.1038/nrrheum.2012.64

189. Tournebize P, Charlin C, Lagrange M. Manifestations neurologiques du chikungunya: Propos de 23 cas colligés la Réunion. *Rev Neurol (Paris)*. 2009;165(1):48-51. doi:10.1016/j.neurol.2008.06.009

190. Rajapakse S, Rodrigo C, Rajapakse A. Atypical manifestations of chikungunya infection. *Trans R Soc Trop Med Hyg*. 2010;104(2):89-96. doi:10.1016/j.trstmh.2009.07.031

191. Sebastian MR, Lodha R, Kabra SK. Chikungunya infection in children. *Indian J Pediatr*. 2009;76(2):185-189. doi:10.1007/s12098-009-0049-6

192. Couderc T, Khandoudi N, Grandadam M, et al. Prophylaxis and therapy for Chikungunya

virus infection. *J Infect Dis*. 2009;200(4):516-523. doi:10.1086/600381

193. D. White J. Electron Microscopy of Chikungunya Virus Infection in the Nervous System of Suckling Mice. 1969:12.

194. Shinkai Y, Rathbun 0Gary, Lam KP, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 1992;68(5):855-867. doi:10.1016/0092-8674(92)90029-C

195. Caligiuri M a, Zmuidzinas A, Manley TJ, Levine H, Smith KA, Ritz J. Functional consequences of interleukin 2 receptor expression on resting human lymphocytes: Identification of a novel natural killer cell subset with high affinity receptors. *J Exp Med*. 1990;171(5):1509-1526. doi:10.1084/jem.171.5.1509

196. Carson BWE, Giri JG, Lindemann MJ, et al. Interleuking (IL) 15 Is a Nocel Cytokine That Activates Human Natural Killer Cells via Components of the IL-2 Receptor. *October*. 1994;180(October).

197. Bonder CS, Dickensheets HL, Finlay-Jones JJ, Donnelly RP, Hart PH. Involvement of the IL-2 receptor gamma-chain (gammac) in the control by IL-4 of human monocyte and macrophage proinflammatory mediator production. *J Immunol*. 1998;160(8):4048-4056. http://www.ncbi.nlm.nih.gov/pubmed/9558115.

198. Hart PH, Bonder CS, Balogh J, Dickensheets HL, Donnelly RP, Finlay-Jones JJ. Differential responses of human monocytes and macrophages to IL-4 and IL-13. *J Leukoc Biol*. 1999;66(4):575-578. doi:10.1002/jlb.66.4.575

199. Bancroft GJ, Schreiber RD, Unanue ER. Natural Immunity: A T-Cell-Independent Pathway of Macrophage Activation, Defined in the scid Mouse. *Immunol Rev*. 1991;124(1):5-24. doi:10.1111/j.1600-065X.1991.tb00613.x

200. Kirkham BW, Kavanaugh A, Reich K. Interleukin-17A: A unique pathway in immune-mediated diseases: Psoriasis, psoriatic arthritis and rheumatoid arthritis. *Immunology*. 2014;141(2):133-142. doi:10.1111/imm.12142

201. Gottlieb AB, Chamian F, Masud S, et al. TNF Inhibition Rapidly Down-Regulates Multiple Proinflammatory Pathways in Psoriasis Plaques. *J Immunol*. 2005;175(4):2721-2729. doi:10.4049/jimmunol.175.4.2721

202. Haist KC, Burrack KS, Davenport BJ, Morrison TE. Inflammatory monocytes mediate control of acute alphavirus infection in mice. *PLoS Pathog*. 2017;13(12):1-30. doi:10.1371/journal.ppat.1006748

203. Subak-Sharpe I, Dyson H, Fazakerley J. In vivo depletion of CD8+ T cells prevents lesions of demyelination in Semliki Forest virus infection. *J Virol*. 1993;67(12):7629-7633. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7901429.

204. Chen C-I, Clark DC, Pesavento P, et al. Comparative pathogenesis of epidemic and enzootic Chikungunya viruses in a pregnant Rhesus macaque model. *Am J Trop Med Hyg*. 2010;83(6):1249-1258. doi:10.4269/ajtmh.2010.10-0290

205. Diallo D, Dia I, Diagne CT, Gaye A, Diallo M. *Emergences of Chikungunya and Zika in Africa*. Elsevier Inc.; 2018. doi:10.1016/B978-0-12-811865-8.00004-0

206. Powers AM, Logue CH. Changing patterns of chikunya virus: Re-emergence of a zoonotic arbovirus. *J Gen Virol*. 2007;88:2363-2377. doi:10.1099/vir.0.82858-0

207. Markoff L. 153 - Alphaviruses. In: Bennett JE, Dolin R, Blaser MJ, eds. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*. Philadelphia: Content Repository Only!; 2015:1865-1874.e2. doi:https://doi.org/10.1016/B978-1-4557-4801-3.00153-3

208. Kimura T, Griffin DE. Extensive immune-mediated hippocampal damage in mice surviving infection with neuroadapted Sindbis virus. *Virology*. 2003;311(28-39).

209. Maity P, Roy P, Basu A, Das B, Ghosh US. A case of ADEM following chikungunya fever. *J Assoc Physicians India*. 2014;62(MAY):441-442.

210. Gauri LA, Ranwa BL, Nagar K, Vyas A, Fatima Q. Post chikungunya Brain stem encephalitis. *J Assoc Physicians India*. 2012;60(4):68-69.

211. Mehta R, Gerardin P, de Brito CAA, Soares CN, Ferreira MLB, Solomon T. The neurological complications of chikungunya virus: A systematic review. *Rev Med Virol*. 2018;28(3). doi:10.1002/rmv.1978

212. Ganesan K, Diwan A, Shankar SK, Desai SB, Sainani GS, Katrak SM. Chikungunya encephalomyeloradiculitis: Report of 2 cases with neuroimaging and 1 case with autopsy findings. *Am J Neuroradiol*. 2008;29(9):1636-1637. doi:10.3174/ajnr.A1133

213. Brizzi K. Neurologic Manifestation of Chikungunya Virus. *Curr Infect Dis Rep*. 2017;19(2). doi:10.1007/s11908-017-0561-1

214. Chu H, Das SC, Fuchs JF, et al. Deciphering the protective role of adaptive immunity to CHIKV/IRES a novel candidate vaccine against Chikungunya in the A129 mouse model. *Vaccine*. 2013;31(33):3353-3360. doi:10.1016/j.vaccine.2013.05.059

215. Gardner J, Anraku I, Le TT, et al. Chikungunya Virus Arthritis in Adult Wild-Type Mice. *J Virol*. 2010;84(16):8021-8032. doi:10.1128/jvi.02603-09

216. Hawman DW, Stoermer KA, Montgomery SA, et al. Chronic Joint Disease Caused by Persistent Chikungunya Virus Infection Is Controlled by the Adaptive Immune Response. *J Virol*. 2013;87(24):13878-13888. doi:10.1128/JVI.02666-13

217. Theilacker C, Held J, Allering L, et al. Prolonged polyarthralgia in a German traveller with Mayaro virus infection without inflammatory correlates. *BMC Infect Dis*. 2013;13(September 2011):369. doi:10.1186/1471-2334-13-369

218. Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. 2004;22(5):460-466.

219. Sellers RS, Clifford CB, Treuting PM, Brayton C. Immunological variation between inbred laboratory mouse strains: Points to consider in phenotyping genetically immunomodified mice. *Vet Pathol*. 2012;49(1):32-43. doi:10.1177/0300985811429314

220. Schulte S, Sukhova GK, Libby P. Genetically Programmed Biases in Th1 and Th2 Immune Responses Modulate Atherogenesis. *Am J Pathol*. 2008;172(6):1500-1508.

221. Tang BL. The cell biology of Chikungunya virus infection. *Cell Microbiol*. 2012;14(9):1354-1363. doi:10.1111/j.1462-5822.2012.01825.x

222. Phuklia W, Kasisith J, Modhiran N, et al. Osteoclastogenesis induced by CHIKV-infected fibroblast-like synoviocytes: A possible interplay between synoviocytes and

monocytes/macrophages in CHIKV-induced arthralgia/arthritis. *Virus Res*. 2013;177(2):179-188. doi:10.1016/j.virusres.2013.08.011

223. Hughes TK, Kaspar TA, Coppenhaver DH. Synergy of antiviral actions of TNF and IFN-gamma: evidence for a major role of TNF-induced IFN-beta. *Antiviral Res*. 1988;10(1-3):1-9.

224. Kuwano K, Kawashima T, Arai S. Antiviral effect of TNF-alpha and IFN-gamma secreted from a CD8+ influenza virus-specific CTL clone. *Viral Immunol*. 1993;6(1):1-11.

225. Hurgin V, Novick D, Werman A, Dinarello CA, Rubinstein M. Antiviral and immunoregulatory activities of IFN-γ depend on constitutively expressed IL-1α. *PNAS*. 2007;104(12):5044-5049.

226. Colavita F, Vita S, Lalle E, et al. Overproduction of IL-6 and type-I IFN in a lethal case of Chikungunya virus infection in an elderly man during the 2017 Italian outbreak. *Open Forum Infect Dis*. 2018;5(11). doi:10.1093/ofid/ofy276

227. Chang A, Tritsch S, Reid S, et al. The Cytokine Profile in Acute Chikungunya Infection is Predictive of Chronic Arthritis 20 Months Post Infection. *Diseases*. 2018;6(4):95. doi:10.3390/diseases6040095

228. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): An overview. *J Interf Cytokine Res*. 2009;29(6):313-325. doi:10.1089/jir.2008.0027

229. Ruiz Silva M, Van Der Ende-Metselaar H, Mulder HL, Smit JM, Rodenhuis-Zybert IA. Mechanism and role of MCP-1 upregulation upon chikungunya virus infection in human peripheral blood mononuclear cells. *Sci Rep*. 2016;6. doi:10.1038/srep32288

230. Berges BK, Wheat WH, Palmer BE, Connick E, Akkina R. HIV-1 infection and CD4 T cell depletion in the humanized Rag2-/-γc-/- (RAG-hu) mouse model. *Retrovirology*. 2006;3:1-14. doi:10.1186/1742-4690-3-76

231. Kuruvilla JG, Troyer RM, Devi S, Akkina R. Dengue virus infection and immune response in humanized RAG2-/-γc -/- (RAG-hu) mice. *Virology*. 2007;369(1):143-152. doi:10.1016/j.virol.2007.06.005

232. Strowig T, Rongvaux A, Rathinam C, et al. Transgenic expression of human signal regulatory protein alpha in Rag2 -/-γ c -/- mice improves engraftment of human hematopoietic cells in humanized mice. *Proc Natl Acad Sci U S A*. 2011;108(32):13218-13223. doi:10.1073/pnas.1109769108

233. Powers AM. Chikungunya. *Divostin Neolit Cent Serbia*. 1988;(801):419-446. doi:10.1207/s15327752jpa8502

234. Barrett AD, Weaver SC. Arboviruses: Alphaviruses, flaviviruses and bunyaviruses: Encephalitis; yellow fever; dengue; haemorrhagic fever; miscellaneous tropical fevers; undifferentiated fever. In: *Medical Microbiology*. Eighteenth. ; 2012:520-536. https://doi.org/10.1016/B978-0-7020-4089-4.00066-4.

235. Rulli NE, Melton J, Wilmes A, Ewart G, Mahalingam S. The molecular and cellular aspects of arthritis due to alphavirus infections: Lesson learned from Ross River virus. *Ann N Y Acad Sci*. 2007;1102:96-108. doi:10.1196/annals.1408.007

236. Chen KC, Kam Y-W, Lin RTP, Ng MM-L, Ng LF, Chu JJH. Comparative analysis of the

genome sequences and replication profiles of chikungunya virus isolates within the East, Central and South African (ECSA) lineage. *Virol J*. 2013;10(1):169. doi:10.1186/1743-422X-10-169

237. Hassing RJ, Leparc-Goffart I, Blank SN, et al. Imported Mayaro virus infection in the Netherlands. *J Infect*. 2010;61(4):343-345. doi:10.1016/j.jinf.2010.06.009
238. Marti-Carvajal A, Ramon-Pardo P, Javelle E, et al. Interventions for treating patients with chikungunya virus infection-related rheumatic and musculoskeletal disorders: A systematic review. *PLoS One*. 2017;12(6):e0179028. doi:10.1371/journal.pone.0179028
239. Adouchief S, Smura T, Sane J, Vapalahti O, Kurkela S. Sindbis virus as a human pathogen- epidemiology, clinical picture and pathogenesis. *Rev Med Virol*. 2016;26(March):221-241. doi:10.1002/rmv.1876
240. Bouquillard É, Combe B. A report of 21 cases of rheumatoid arthritis following Chikungunya fever. A mean follow-up of two years. *Jt Bone Spine*. 2009;76(6):654-657. doi:10.1016/j.jbspin.2009.08.005
241. Dhama K, Kapoor S, Pawarya RVS, Chakraborty S, Tiwari R, Verma AK. Ross river virus (RRV) infection in horses and humans: A review. *Pakistan J Biol Sci*. 2014;17(6):768-779. doi:10.3923/pjbs.2014.768.779
242. Forshey BM, Guevara C, Laguna-Torres VA, et al. Arboviral etiologies of acute febrile illnesses in western south America, 2000-2007. *PLoS Negl Trop Dis*. 2010;4(8):2000-2007. doi:10.1371/journal.pntd.0000787
243. Thompson JM, Iwasaki A. Toll-like receptors regulation of viral infection and disease ☆. 2008;60:786-794. doi:10.1016/j.addr.2007.11.003
244. Knudsen ML, Johansson DX, Kostic L, et al. The Adjuvant Activity of Alphavirus Replicons Is Enhanced by Incorporating the Microbial Molecule Flagellin into the Replicon. *PLoS One*. 2013;8(6):1-10. doi:10.1371/journal.pone.0065964
245. Matsumoto M, Seya T. TLR3 : Interferon induction by double-stranded RNA including poly (I : C) ☆. 2008;60:805-812. doi:10.1016/j.addr.2007.11.005
246. Shipley JG, Vandergaast R, Deng L, Mariuzza RA, Fredericksen BL. Identification of multiple RIG-I-specific pathogen associated molecular patterns within the West Nile virus genome and antigenome. *Virology*. 2012;432(1):232-238. doi:10.1016/j.virol.2012.06.009
247. Schlee M, Hartmann G. Discriminating self from non-self in nucleic acid sensing. *Nat Rev Immunol*. 2016;16:566. doi:10.1038/nri.2016.78
248. Seo YJ, Hahm B. *Type I Interferon Modulates the Battle of Host Immune System against Viruses*. Vol 73. 1st ed. Elsevier Inc.; 2010. doi:10.1016/S0065-2164(10)73004-5
249. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. *Nat Rev Immunol*. 2015;15(2):87-103. doi:10.1038/nri3787
250. Hengel H, Koszinowski UH, Conzelmann KK. Viruses know it all: New insights into IFN networks. *Trends Immunol*. 2005;26(7):396-401. doi:10.1016/j.it.2005.05.004
251. Hwang SY, Hertzog PJ, Holland KA, et al. A null mutation in the gene encoding a type I interferon receptor component eliminates antiproliferative and antiviral responses to interferons alpha and beta and alters macrophage responses. *Proc Natl Acad Sci U S A*. 1995;92(24):11284-11288. doi:10.1073/pnas.92.24.11284

252. Randall RE, Goodbourn S. Interferons and viruses: An interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol*. 2008;89(1):1-47. doi:10.1099/vir.0.83391-0

253. Welsh RM. Natural killer cells and interferon. *Crit Rev Immunol*. 1984;5(1):55—93. http://europepmc.org/abstract/MED/6085941.

254. French AR, Yokoyama WM. Natural killer cells and viral infections. *Curr Opin Microbiol*. 3AD;15(1):45-51.

255. Biron CA. Activation and function of natural killer cell responses during viral infections. *Curr Opin Immunol*. 1997;9(1):24-34.

256. Fros JJ, Pijlman GP. Alphavirus infection: Host cell shut-off and inhibition of antiviral responses. *Viruses*. 2016;8(6). doi:10.3390/v8060166

257. Zhang R, Hryc CF, Cong Y, et al. 4.4 Å cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. *EMBO J*. 2011;30(18):3854-3863. doi:10.1038/emboj.2011.261

258. Markoff L. 153 - Alphaviruses. In: Bennett JE, Dolin R, Blaser MJ, eds. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*. Philadelphia: Content Repository Only!; 2015:1865-1874.e2. doi:https://doi.org/10.1016/B978-1-4557-4801-3.00153-3

259. Pingen M, Bryden SR, Pondeville E, et al. Host Inflammatory Response to Mosquito Bites Enhances the Severity of Arbovirus Infection. *Immunity*. 2016;44(6):1455-1469. doi:10.1016/j.immuni.2016.06.002

260. Lim EXY, Lee WS, Madzokere ET, Herrero LJ. Mosquitoes as suitable vectors for alphaviruses. *Viruses*. 2018;10(2):1-17. doi:10.3390/v10020084

261. Cross ML, Cupp EW, Enriquez FJ. Differential modulation of murine cellular immune responses by salivary gland extract of Aedes aegypti. *Am J Trop Med Hyg*. 1994;51(5):690-696. doi:10.4269/ajtmh.1994.51.690

262. Zeidner NS, Higgs S, Happ CM, Beaty BJ, Miller BR. Mosquito feeding modulates Th1 and Th2 cytokines in flavivirus susceptible mice: An effect mimicked by injection of sialokinins, but not demonstrated in flavivirus resistant mice. *Parasite Immunol*. 1999;21(1):35-44. doi:10.1046/j.1365-3024.1999.00199.x

263. Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL. Viral Subversion of the Immune System. *Annu Rev Immunol*. 2000;18(1):861-926. doi:10.1146/annurev.immunol.18.1.861

264. Lambert AJ, Martin DA, Lanciotti RS. Detection of North American eastern and western equine encephalitis viruses by nucleic acid amplification assays. *J Clin Microbiol*. 2003;41(1):379-385. doi:10.1128/jcm.41.1.379-385.2003

265. Rosen L, Gubler DJ, Bennett PH. Epidemic polyarthritis (Ross River) virus infection in the Cook Islands. *Am J Trop Med Hyg*. 1981;30(6):1294-1302. doi:10.4269/ajtmh.1981.30.1294

266. Tesh RB, Mclean RG, Shroyer DA. Ross River infection in american samoa. *Trans R Soc Trop Med Hyg*. 1981;75(3):426-431.

267. Aaskov JG, Mataika JU, Lawrence GW. An epidemic of Ross River virus infection in Fiji.

Am J Trop Med Hyg. 981;30(5).

268. Scrimgeour EM, Aaskov JG, Matz LR. Ross River virus arthritis in Papua New Guinea. *Trans R Soc Trop Med Hyg*. 1987;81(5):833-834. doi:10.1016/0035-9203(87)90045-9
269. Mylonas AD, Brown AM, Carthew TL, et al. Natural history of Ross River virus-induced epidemic polyarthritis. *Med J Aust*. 2002;177(7):356-360.
270. CASALS J, WHITMAN L. Mayaro virus: a new human disease agent. I. Relationship to other arbor viruses. *Am J Trop Med Hyg*. 1957;6(6):1004-1011. doi:10.4269/ajtmh.1957.6.1004
271. Aitken THG, Downs WG, Anderson CR, Spence L, Casals J. Mayaro Virus Isolated from a Trinidadian Mosquito, Mansonia venezuelensis. *Science (80-)*. 1960;131(3405):986-986. doi:10.1126/science.131.3405.986
272. De Figueiredo MLG, Figueiredo LTM. Emerging alphaviruses in the americas: Chikungunya and mayaro. *Rev Soc Bras Med Trop*. 2014;47(6):677-683. doi:10.1590/0037-8682-0246-2014
273. Azevedo RSS, Silva EVP, Carvalho VL, et al. Mayaro fever virus, Brazilian amazon. *Emerg Infect Dis*. 2009;15(11):1830-1832. doi:10.3201/eid1511.090461
274. Tesh RB, Watts DM, Russell KL, et al. Mayaro Virus Disease: An Emerging Mosquito-Borne Zoonosis in Tropical South America. *Clin Infect Dis*. 1999;28(1):67-73. doi:10.1086/515070
275. Hotez PJ, Murray KO. Dengue, West Nile virus, chikungunya, Zika—and now Mayaro? *PLoS Negl Trop Dis*. 2017;11(8). doi:10.1371/journal.pntd.0005462
276. De Thoisy B, Gardon J, Alba Salas R, Morvan J, Kazanji M. Mayaro virus in wild mammals, French Guiana. *Emerg Infect Dis*. 2003;9(10):1326-1329. doi:10.3201/eid0910.030161
277. Monath TP, Pinheiro FP, LeDuc JW. Mayaro Virus Disease. *Arboviruses Epidemiol Ecol*. 2019;1(3):137-150. doi:10.1201/9780429280276-8
278. Torres JR, Russell KL, Vasquez C, et al. Family cluster of Mayaro fever, Venezuela. *Emerg Infect Dis*. 2004;10(7):1304-1306. doi:10.3201/eid1007.030860
279. LeDuc JW, Pinheiro FP, Travassos Da Rosa APA. An outbreak of Mayaro virus disease in Belterra, Brazil. II. Epidemiology. *Am J Trop Med Hyg*. 1981;30(3):682-688. doi:10.4269/ajtmh.1981.30.682
280. Powers AM, Aguilar P V., Chandler LJ, et al. Genetic relationships among Mayaro and Una viruses suggest distinct patterns of transmission. *Am J Trop Med Hyg*. 2006;75(3):461-469.
281. Esposito DLA, Fonseca BAL da. Will Mayaro virus be responsible for the next outbreak of an arthropod-borne virus in Brazil? *Brazilian J Infect Dis*. 2017;21(5):540-544. doi:10.1016/j.bjid.2017.06.002
282. Downs W, Anderson C. Distribution of immunity to Mayaro virus infection in the West Indies. *West Indian med j*. 1958;7(3):190-194.
283. Taylor RM, Hurlbut HS, Work TH, Kingston JR, Frothingham TE. Sindbis Virus: A Newly Recognized Arthropod-Transmitted Virus. *Am J Trop Med Hyg*. 1955;4(5):844-862.

284. H. Malherbe, M. Strickland-Cholmley ALJ. Sindbis Virus Infection in Man. *South African Med J*. 1963:547-552.

285. Laine M. Prolonged arthritis associated with Sindbis-related (Pogosta) virus infection. *Rheumatology*. 2002;41(7):829-830. doi:10.1093/rheumatology/41.7.829

286. Dubuisson J, Rice CM. Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. *J Virol*. 1993;67(6):3363-3374.

287. Jöst H, Bialonski A, Storch V, Günther S, Becker N, Schmidt-Chanasit J. Isolation and phylogenetic analysis of sindbis viruses from mosquitoes in Germany. *J Clin Microbiol*. 2010;48(5):1900-1903. doi:10.1128/JCM.00037-10

288. Karpf AR, Brown DT. Comparison of Sindbis Virus-Induced Pathology in Mosquito and Vertebrate Cell Cultures. *Virology*. 1998;240(2):193-201. doi:https://doi.org/10.1006/viro.1997.8914

289. Manni T, Kurkela S, Vaheri A, Vapalahti O. Diagnostics of pogosta disease: Antigenic properties and evaluation of sindbis virus IgM and IgG enzyme immunoassays. *Vector-Borne Zoonotic Dis*. 2008;8(3):303-311. doi:10.1089/vbz.2007.0623

290. Bautista-Reyes E, Núñez-Avellaneda D, Alonso-Palomares LA, Salazar MI. Chikungunya: Molecular aspects, clinical outcomes and pathogenesis. *Rev Investig Clin*. 2017;69(6):299-307. doi:10.24875/RIC.17002029

291. Yan-Jang S. Huang DLV and SH. *Viral Genetics of Chikungunya Virus and Zika Virus and Its Influence in Their Emergence and Application for Public Health Control Strategies*. Elsevier Inc.; 2018. doi:https://doi.org/10.1016/C2016-0-01089-6

292. Burt FJ, Chen W, Miner JJ, et al. Chikungunya virus: an update on the biology and pathogenesis of this emerging pathogen. *Lancet Infect Dis*. 2017;17(4):e107-e117. doi:10.1016/S1473-3099(16)30385-1

293. Smithburn KC, Haddow AJ. Semliki Forest Virus: I. Isolation and Pathogenic Properties. *J Immunol*. 1944;49(3):141-157. http://jimmunol.org/content/49/3/141.abstract.

294. Lundström JO. Mosquito-borne viruses in western Europe: a review. *J Vector Ecol*. 1999;24(1):1-39. http://www.ncbi.nlm.nih.gov/pubmed/10436876.

295. Mathiot CC, Grimaud G, Garry P, et al. An outbreak of human semliki forest virus infections in Central African Republic. *Am J Trop Med Hyg*. 1990;42(4):386-393. doi:10.4269/ajtmh.1990.42.386

296. Atkins GJ, Sheahan BJ, Dimmock NJ. Semliki Forest virus infection of mice: a model for genetic and molecular analysis of viral pathogenicity. *J Gen Virol*. 1985;66(3):395-408. doi:10.1099/0022-1317-66-3-395

297. Paessler S, Weaver SC. Vaccines for Venezuelan equine encephalitis. *Vaccine*. 2009;27 Suppl 4:D80-5. doi:10.1016/j.vaccine.2009.07.095

298. Vogel P, Kell WM, Fritz DL, Parker MD, Schoepp RJ. Early events in the pathogenesis of eastern equine encephalitis virus in mice. *Am J Pathol*. 2005;166(1):159-171. doi:10.1016/s0002-9440(10)62241-9

299. Broeck G Ten, Merrill MH. A Serological Difference Between Eastern and Western Equine Encephalomyelitis Virus. *Proc Soc Exp Biol Med*. 1933;31(2):217-220. doi:10.3181/00379727-31-7066C

300. Zacks MA, Paessler S. Encephalitic alphaviruses. *Vet Microbiol*. 2010;140(3-4):281-286. doi:10.1016/j.vetmic.2009.08.023

301. Prevention C for DC and. Eastern Equine Encephalitis - Statistics & Maps. https://www.cdc.gov/easternequineencephalitis/tech/epi.html.

302. Scott TW, Weaver SC. Eastern equine encephalomyelitis virus: epidemiology and evolution of mosquito transmission. *Adv Virus Res*. 1989;37:277-328.

303. Armstrong PM, Andreadis TG. Eastern equine encephalitis virus in mosquitoes and their role as bridge vectors. *Emerg Infect Dis*. 2010;16(12):1869-1874. doi:10.3201/eid1612.100640

304. Greenlee JE. Chapter 19 - The equine encephalitides. In: Tselis AC, Booss J, eds. *Handbook of Clinical Neurology*. Vol 123. Elsevier; 2014:417-432. doi:https://doi.org/10.1016/B978-0-444-53488-0.00019-5

305. Greenlee JE. Chapter 19 - The equine encephalitides. In: Tselis AC, Booss J, eds. *Handbook of Clinical Neurology*. Vol 123. Elsevier; 2014:417-432. doi:https://doi.org/10.1016/B978-0-444-53488-0.00019-5

306. Prevention C for DC and. Current Trends Arboviral Infections of the Central Nervous System -- United States, 1987. https://www.cdc.gov/mmwr/preview/mmwrhtml/00001082.htm.

307. Simon LV FMA. Western Equine Encephalitis. . doi:https://www.ncbi.nlm.nih.gov/books/NBK470228/ OP - Treasure Island (FL): StatPearls Publishing

308. Arrigo NC, Adams AP, Weaver SC. Evolutionary Patterns of Eastern Equine Encephalitis Virus in North versus South America Suggest Ecological Differences and Taxonomic Revision. *J Virol*. 2010;84(2):1014-1025. doi:10.1128/jvi.01586-09

309. Atkins GJ. The Pathogenesis of Alphaviruses. *ISRN Virol*. 2013;2013:22. doi:10.5402/2013/861912

310. Kubes V, Rios FA. The Causative Agent of Infectious Equine Encephalomyelitis in Venezuela. *Science (80-)*. 1939;90(2323):20-21. doi:10.1126/science.90.2323.20

311. SUDIA WD, NEWHOUSE VF, BEADLE LD, et al. Epidemic venezuelan equine encephalitis in north america in 1971: Vector studies1. *Am J Epidemiol*. 1975;101(1):17-35. doi:10.1093/oxfordjournals.aje.a112068

312. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, eds. Family - Togaviridae. In: *Virus Taxonomy*. San Diego: Elsevier; 2012:1103-1110. doi:https://doi.org/10.1016/B978-0-12-384684-6.00095-1

313. Weaver SC, Ferro C, Roberto Barrera 3**, Boshell J, Navarro J-C. VENEZUELAN EQUINE ENCEPHALITIS. *Annu Rev Entomol*. 2004;49(1):141-174. doi:10.1146/annurev.ento.49.061802.123422

314. Schmaljohn AL editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; MDOP-BS. Alphaviruses (Togaviridae) and Flaviviruses (Flaviviridae). 1996;Chapter 54. https://www.ncbi.nlm.nih.gov/books/NBK7633/.

315. Karpf AR, Brown DT. Comparison of Sindbis Virus-Induced Pathology in Mosquito and

Vertebrate Cell Cultures. *Virology*. 1998;240(2):193-201. doi:https://doi.org/10.1006/viro.1997.8914

316. Schmaljohn AL editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; MDOP-BS. Alphaviruses (Togaviridae) and Flaviviruses (Flaviviridae). 1996;Chapter 54.
317. Atkins GJ. The Pathogenesis of Alphaviruses. *ISRN Virol*. 2013;2013:22. doi:10.5402/2013/861912
318. Zacks MA, Paessler S. Encephalitic alphaviruses. *Vet Microbiol*. 2010;140(3-4):281-286. doi:10.1016/j.vetmic.2009.08.023
319. Ryman KD, Klimstra WB, Nguyen KB, Biron CA, Johnston RE. Alpha/Beta Interferon Protects Adult Mice from Fatal Sindbis Virus Infection and Is an Important Determinant of Cell and Tissue Tropism. *J Virol*. 2000;74(7):3366-3378. doi:10.1128/jvi.74.7.3366-3378.2000
320. Griffin DE, Johnson RT. Cellular immune response to viral infection: In vitro studies of lymphocytes from mice infected with Sindbis virus. *Cell Immunol*. 1973;9(3):426-434. doi:10.1016/0008-8749(73)90057-9
321. Assunção-Miranda I, Bozza MT, Da Poian AT. Pro-inflammatory response resulting from sindbis virus infection of human macrophages: Implications for the pathogenesis of viral arthritis. *J Med Virol*. 2010;82(1):164-174. doi:10.1002/jmv.21649
322. Macfarlan RI, Burns WH, White DO. Two Cytotoxic Cells in Peritoneal Cavity of Virus-Infected Mice : Antibody-Dependent Macrophages and Nonspecific Killer Cells Information about subscribing to The Journal of Immunology is online at : TWO CYTOTOXIC CELLS IN PERITONEAL CAVITY OF VIRUS- INFE. 2019.
323. Hirsch RL. Natural killer cells appear to play no role in the recovery of mice from Sindbis virus infection. *Immunology*. 1981;43(1):81-89.
324. Marsh M. The entry of enveloped viruses into cells by endocytosis. *Biochem J*. 1984;218(1):1-10. doi:10.1042/bj2180001
325. DeTulleo L, Kirchhausen T. The clathrin endocytic pathway in viral infection. *EMBO J*. 1998;17(16):4585-4593. doi:10.1093/emboj/17.16.4585
326. Smit JM, Bittman R, Wilschut J. Low-pH-dependent fusion of Sindbis virus with receptor-free cholesterol- and sphingolipid-containing liposomes. *J Virol*. 1999;73(10):8476-8484. http://www.ncbi.nlm.nih.gov/pubmed/10482600%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC112867.
327. Lozach PY, Burleigh L, Staropoli I, Amara A. The C type lectins DC-SIGN and L-SIGN: Receptors for viral glycoproteins. In: *Methods in Molecular Biology*. Vol 379. ; 2007:51-68. doi:10.1385/1-59745-393-5:51
328. Byrnes AP, Griffin DE. Binding of Sindbis virus to cell surface heparan sulfate. *J Virol*. 1998;72(9):7349-7356. http://www.ncbi.nlm.nih.gov/pubmed/9696831%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC109959.
329. Rose PP, Hanna SL, Spiridigliozzi A, et al. Natural resistance-associated macrophage

protein is a cellular receptor for Sindbis virus in both insect and mammalian hosts. *Cell Host Microbe*. 2011;10(2):97-104. doi:10.1016/j.chom.2011.06.009

330. Stiles KM, Kielian M. Alphavirus entry: NRAMP leads the way. *Cell Host Microbe*. 2011;10(2):92-93. doi:10.1016/j.chom.2011.07.008
331. Garmashova N, Gorchakov R, Frolova E, Frolov I. Sindbis Virus Nonstructural Protein nsP2 Is Cytotoxic and Inhibits Cellular Transcription. *J Virol*. 2006;80(12):5686-5696. doi:10.1128/jvi.02739-05
332. Assuncao-Miranda I, Cruz-Oliveira C, Da Poian AT. Molecular Mechanisms Involved in the Pathogenesis of Alphavirus-Induced Arthritis. *Biomed Res Int*. 2013;2013(973516):11. doi:10.1523/JNEUROSCI.3977-03.2004
333. Assuncao-Miranda I, Cruz-Oliveira C, Da Poian AT. Molecular mechanisms involved in the pathogenesis of alphavirus-induced arthritis. *Biomed Res Int*. 2013;2013. doi:10.1155/2013/973516
334. Fraser JRE. Epidemic polyarthritis and Ross River virus disease. *Clin Rheum Dis*. 1986;12(2):369-388.
335. Morrison TE, Oko L, Montgomery SA, et al. A Mouse Model of Chikungunya Virus–Induced Musculoskeletal Inflammatory Disease. *Am J Pathol*. 2011;178(1):32-40. doi:10.1016/j.ajpath.2010.11.018
336. Burrack KS, Montgomery SA, Homann D, Morrison TE. CD8 + T Cells Control Ross River Virus Infection in Musculoskeletal Tissues of Infected Mice . *J Immunol*. 2015;194(2):678-689. doi:10.4049/jimmunol.1401833
337. Atkins GJ, Sheahan BJ. Semliki forest virus neurovirulence mutants have altered cytopathogenesis for central nervous system cells. *Infect Immun*. 1982;36(1):333-341. doi:10.1128/iai.36.1.333-341.1982
338. Sheahan BJ, Ibrahim M, Atkins GJ. Demyelination of olfactory pathways in mice following intranasal infection with the avirulent A7 strain of Semliki Forest virus. *Eur J Vet Pathol*. 1996;2:117-125.
339. Kaluza G, Lell G, Reinacher M, Stitz L, Willems WR. Neurogenic spread of Semliki forest virus in mice. *Arch Virol*. 1987;93(1-2):97-110. doi:10.1007/BF01313896
340. Dropulić B, Masters CL. Entry of neurotropic arboviruses into the central nervous system: An in vitro study using mouse brain endothelium. *J Infect Dis*. 1990;161(4):685-691. doi:10.1093/infdis/161.4.685
341. Fazakerley JK, Pathak S, Scallan M, Amor S, Dyson H. Replication of the a7(74) strain of semliki forest virus is restricted in neurons. *Virology*. 1993;195(2):627-637. doi:10.1006/viro.1993.1414
342. Porter AI, Erwin-Cohen RA, Twenhafel N, et al. Characterization and pathogenesis of aerosolized eastern equine encephalitis in the common marmoset (Callithrix jacchus). *Virol J*. 2017;14(1 LB-Porter2017):25. doi:10.1186/s12985-017-0687-7
343. Beckham JD, Tyler KL. 91 - Encephalitis. In: Bennett JE, Dolin R, Blaser MJ, eds. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*. Philadelphia: Content Repository Only!; 2015:1144-1163.e3. doi:https://doi.org/10.1016/B978-1-4557-4801-3.00091-6

344. Liu JL, Shriver-Lake LC, Zabetakis D, Goldman ER, Anderson GP. Selection of Single-Domain Antibodies towards Western Equine Encephalitis Virus. *Antibodies*. 2018;7(4):44. https://www.mdpi.com/2073-4468/7/4/44.

345. Aguilar P V, Estrada-Franco JG, Navarro-Lopez R, Ferro C, Haddow AD, Weaver SC. Endemic Venezuelan equine encephalitis in the Americas: hidden under the dengue umbrella. *Future Virol*. 2011;6(6):721-740. doi:10.2217/FVL.11.5

346. Sharma A, Knollmann-Ritschel B. Current Understanding of the Molecular Basis of Venezuelan Equine Encephalitis Virus Pathogenesis and Vaccine Development. *Viruses*. 2019;11(2):164. doi:10.3390/v11020164

347. Sharma A, Knollmann-Ritschel B. Current Understanding of the Molecular Basis of Venezuelan Equine Encephalitis Virus Pathogenesis and Vaccine Development. *Viruses*. 2019;11(2):164. doi:10.3390/v11020164

348. Watts DM, Callahan J, Rossi C, et al. Venezuelan equine encephalitis febrile cases among humans in the Peruvian Amazon River region. *Am J Trop Med Hyg*. 1998;58(1):35-40. doi:10.4269/ajtmh.1998.58.35

349. Rusnak JM, Dupuy LC, Niemuth NA, Glenn AM, Ward LA. Comparison of Aerosol- and Percutaneous-acquired Venezuelan Equine Encephalitis in Humans and Nonhuman Primates for Suitability in Predicting Clinical Efficacy under the Animal Rule. *Comp Med*. 2018;68(5):380-395. doi:10.30802/AALAS-CM-18-000027

350. Rusnak JM, Dupuy LC, Niemuth NA, Glenn AM, Ward LA. Comparison of Aerosol- and Percutaneous-acquired Venezuelan Equine Encephalitis in Humans and Nonhuman Primates for Suitability in Predicting Clinical Efficacy under the Animal Rule. *Comp Med*. 2018;68(5):380-395. doi:10.30802/AALAS-CM-18-000027

351. Taylor A, Herrero LJ, Rudd PA, Mahalingam S. Mouse models of alphavirus-induced inflammatory disease. *J Gen Virol*. 2015;96(2):221-238. doi:10.1099/vir.0.071282-0

352. Chernecky CC, Berger BJ. Differential leukocyte count (diff) - peripheral blood. In: *Laboratory Test and Diagnostic Procedures*. ; 2013:440-446.

353. van Furth A, Beekhuizen H. Monocytes. In: *Encyclopedia of Immunology*. ; 1998:1750-1754.

354. Goldsby RA, Kindt TJ, Osborne BA. Mononuclear cells. In: *Kuby Immunology*. ; 1999:41-44.

355. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*. 2005;5(12):953-964. doi:10.1038/nri1733

356. Armstrong JA, Hart D. Response of cultured macrophages to Mycobacterium Tuberculosis, with observations on fusion of lysosomes with phagosomes. *J Exp Med*. 1971;134(3):713-740. doi:10.1084/jem.134.3.713

357. Christensen KA, Myers JT, Swanson JA. pH-dependent regulation of lysosomal calcium in macrophages. *J Cell Sci*. 2002;115(3):599-607.

358. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science (80-)*. 2010;327(5966):656-661. doi:10.1126/science.1178331

359. Epelman S, Lavine KJ, Randolph GJ. Origin and Functions of Tissue Macrophages.

Immunity. 2014;41(1):21-35. doi:10.1016/j.immuni.2014.06.013

360. Nikitina E, Larionova I, Choinzonov E, Kzhyshkowska J. Monocytes and Macrophages as Viral Targets and Reservoirs. *Int J Mol Sci*. 2018;19(9):2821. doi:10.3390/ijms19092821

361. Unanue ER. Antigen-Presenting Function of the Macrophage. *Annu Rev Immunol*. 1984;2(1):395-428. doi:10.1146/annurev.iy.02.040184.002143

362. Linn M La, Aaskov JG, Suhrbier A. Antibody-dependent enhancement and persistence in macrophages of an arbovirus associated with arthritis. *J Gen Virol*. 1996;77(3):407-411. doi:10.1099/0022-1317-77-3-407

363. Suhrbier A, La Linn M. Clinical and pathologic aspects of arthritis due to Ross River virus and other alphaviruses. *Curr Opin Microbiol*. 2004;16(4):374-379.

364. Soden M, Vasudevan H, Roberts B, et al. Detection of viral ribonucleic acid and histologic analysis of inflamed synovium in Ross River virus infection. *Arthritis Rheum*. 2000;43(2):365-369. doi:10.1002/1529-0131(200002)43:2<365::AID-ANR16>3.0.CO;2-E

365. Nair S, Poddar S, Shimak RM, Diamond MS. Interferon Regulatory Factor 1 Protects against Chikungunya Virus-Induced Immunopathology by Restricting Infection in Muscle Cells. *J Virol*. 2017;91(22):1-14. doi:10.1128/jvi.01419-17

366. Capolunghi F, Rosado MM, Sinibaldi M, Aranburu A, Carsetti R. Why do we need IgM memory B cells? *Immunol Lett*. 2013;152(2):114-120. doi:10.1016/j.imlet.2013.04.007

367. DeFranco AL, Locksley RM, Robertson M. Functions of TH1 Cells. In: *Cellular and Molecular Immunology*. Vol 7. ; 2007:140-142. doi:10.1084/jem.20090209

368. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today*. 1996;17(3):138-146. doi:10.1016/0167-5699(96)80606-2

369. Romagnani S. T-cell subsets (Th1 versus Th2). *Ann allergy, asthma Immunol*. 2000;85(1):9-18,21.

370. Durbin JE, Fernandez-Sesma A, Lee C-K, et al. Type I IFN Modulates Innate and Specific Antiviral Immunity. *J Immunol*. 2000;164(8):4220-4228. doi:10.4049/jimmunol.164.8.4220

371. Morrow AN, Schmeisser H, Tsuno T, Zoon KC. A Novel Role for IFN-Stimulated Gene Factor 3 II in IFN-γ Signaling and Induction of Antiviral Activity in Human Cells . *J Immunol*. 2011;186(3):1685-1693. doi:10.4049/jimmunol.1001359

372. Agaugué S, Marcenaro E, Ferranti B, Moretta L, Moretta A. Human natural killer cells exposed to IL-2, IL-12, IL-18, or IL-4 differently modulate priming of naive T cells by monocyte-derived dendritic cells. *Blood*. 2008;112(5):1776-1783. doi:10.1182/blood-2008-02-135871

373. Alerts E. Natural killer (NK) cells as a responder to interleukin 2 (IL 2). II . IL 2-induced interferon gamma production . This information is current as Why The JI ? Submit online . • Rapid Reviews ! 30 days * from submission to initial decision • No Triage ! 2019;2(Il 2).

374. Müllbacher A, Blanden R V. Murine cytotoxic T-cell response to alphavirus is associated mainly with H- 2Dk. *Immunogenetics*. 1978;7(1):551-561. doi:10.1007/BF01844044

375. Linn ML, Mateo L, Gardner J, Suhrbier A. Alphavirus-specific cytotoxic T lymphocytes

recognize a cross-reactive epitope from the capsid protein and can eliminate virus from persistently infected macrophages. *J Virol*. 1998;72(6):5146-5153. http://www.ncbi.nlm.nih.gov/pubmed/9573286%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC110085.

376. Teo T-H, Lum F-M, Lee WWL, Ng LFP. Mouse models for Chikungunya virus: deciphering immune mechanisms responsible for disease and pathology. *Immunol Res*. 2012;53(1-3):136-147. doi:10.1007/s12026-012-8266-x

377. Kulcsar KA, Baxter VK, Greene IP, Griffin DE. Interleukin 10 modulation of pathogenic Th17 cells during fatal alphavirus encephalomyelitis. *Proc Natl Acad Sci U S A*. 2014;111(45):16053-16058. doi:10.1073/pnas.1418966111

378. Burdeinick-Kerr R, Govindarajan D, Griffin DE. Noncytolytic Clearance of Sindbis Virus Infection from Neurons by Gamma Interferon Is Dependent on Jak/Stat Signaling. *J Virol*. 2009;83(8):3429-3435. doi:10.1128/jvi.02381-08

379. Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. *Front Biosci*. 1997;2:12-26.

380. Zhang JM, An J. Cytokines, inflammation, and pain. *Int Anesthesiol Clin*. 2007;45(2):27-37. doi:10.1097/AIA.0b013e318034194e

381. Ghirnikar RS, Lee YL, Eng LF. Inflammation in traumatic brain injury: Role of cytokines and chemokines. *Neurochem Res*. 1998;23(3):329-340. doi:10.1023/A:1022453332560

382. Watkins LR, Maier SF, Goehler LE. Immune activation: the role of pro-inflammatory cytokines in inflammation, illness responses and pathological pain states. *Pain*. 1995;63(3):289-302. doi:10.1016/0304-3959(95)00186-7

383. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J Clin Invest*. 2007;117(5):1175-1183. doi:10.1172/JCI31537

384. Naik E, Dixit VM. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *J Exp Med*. 2011;208(3):417-420. doi:10.1084/jem.20110367

385. Elenkov IJ, Chrousos GP. Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann N Y Acad Sci*. 2002;966:290-303. doi:10.1111/j.1749-6632.2002.tb04229.x

386. Opal SM, Depalo VA. Anti-inflammatory cytokines. *Chest*. 2000;117(4):1162-1172.

387. Venugopalan A, Ghorpade RP, Chopra A. Cytokines in acute chikungunya. *PLoS One*. 2014;9(10). doi:10.1371/journal.pone.0111305

388. Bao H, Ramanathan AA, Kawalakar O, et al. Nonstructural protein 2 (nsP2) of chikungunya virus (CHIKV) enhances protective immunity mediated by a CHIKV envelope protein expressing DNA vaccine. *Viral Immunol*. 2013;26(1):75-83. doi:10.1089/vim.2012.0061

389. Patil DR, Hundekar SL, Arankalle VA. Expression profile of immune response genes during acute myopathy induced by chikungunya virus in a mouse model. *Microbes Infect*. 2012;14(5):457-469. doi:10.1016/j.micinf.2011.12.008

390. Iijima N, Mattei LM, Iwasaki A. Recruited inflammatory monocytes stimulate antiviral Th1 immunity in infected tissue. *Proc Natl Acad Sci U S A*. 2011;108(1):284-289. doi:10.1073/pnas.1005201108

391. Trobaugh DW, Klimstra WB. Alphaviruses suppress host immunity by preventing myeloid cell replication and antagonizing innate immune responses. *Curr Opin Virol*. 2017;23:30-34. doi:10.1016/j.coviro.2017.02.004

392. Trobaugh D, Sun C, Klimstra W. Eastern equine encephalitis virus evades induction of the host immune response through miR-142-3p restriction of myeloid cell replication. *J Immunol*. 2018;200(1 Supplement):183.7-183.7.

393. Nikitina E, Larionova I, Choinzonov E, Kzhyshkowska J. Monocytes and Macrophages as Viral Targets and Reservoirs. *Int J Mol Sci*. 2018;19(9):2821. doi:10.3390/ijms19092821

394. Trobaugh DW, Klimstra WB. Alphaviruses suppress host immunity by preventing myeloid cell replication and antagonizing innate immune responses. *Curr Opin Virol*. 2017;23:30-34. doi:10.1016/j.coviro.2017.02.004

395. Gardner CL, Burke CW, Tesfay MZ, Glass PJ, Klimstra WB, Ryman KD. Eastern and Venezuelan equine encephalitis viruses differ in their ability to infect dendritic cells and macrophages: impact of altered cell tropism on pathogenesis. *J Virol*. 2008;82(21):10634-10646. doi:10.1128/JVI.01323-08

396. Gardner CL, Burke CW, Tesfay MZ, Glass PJ, Klimstra WB, Ryman KD. Eastern and Venezuelan equine encephalitis viruses differ in their ability to infect dendritic cells and macrophages: impact of altered cell tropism on pathogenesis. *J Virol*. 2008;82(21):10634-10646. doi:10.1128/JVI.01323-08

397. Zhu W, Li J, Liang G. How does Cellular Heparan Sulfate Function in Viral Pathogenicity? *Biomed Environ Sci*. 2011;24(1):81-87. doi:https://doi.org/10.3967/0895-3988.2011.01.011

398. Aronson JF, Grieder FB, Davis NL, et al. A single-site mutant and revertants arising in vivo define early steps in the pathogenesis of Venezuelan equine encephalitis virus. *Virology*. 2000;270(1):111-123. doi:10.1006/viro.2000.0241

399. Sharma A, Maheshwari RK. Oligonucleotide array analysis of Toll-like receptors and associated signalling genes in Venezuelan equine encephalitis virus-infected mouse brain. *J Gen Virol*. 2009;90(8):1836-1847. doi:doi:10.1099/vir.0.010280-0

400. Lester SN, Li K. Toll-like receptors in antiviral innate immunity. *J Mol Biol*. 2014;426(6):1246-1264. doi:10.1016/j.jmb.2013.11.024

401. Chan Y-H, Ng LFP. Age has a role in driving host immunopathological response to alphavirus infection. *Immunology*. 2017;152(4):545-555. doi:10.1111/imm.12799

402. Mathews JH, Roehrig JT. Specificity of the Murine T Helper Cell Immune Response to Various Alphaviruses. *J Gen Virol*. 1989;70(11):2877-2886. doi:doi:10.1099/0022-1317-70-11-2877

403. Huber JP, David Farrar J. Regulation of effector and memory T-cell functions by type I interferon. *Immunology*. 2011;132(4):466-474. doi:10.1111/j.1365-2567.2011.03412.x

404. Libbey JE, Fujinami RS. Chapter 10 - Adaptive immune response to viral infections in the central nervous system. In: Tselis AC, Booss J, eds. *Handbook of Clinical Neurology*. Vol 123. Elsevier; 2014:225-247. doi:https://doi.org/10.1016/B978-0-444-53488-0.00010-9

405. Taylor A, Herrero LJ, Rudd PA, Mahalingam S. Mouse models of alphavirus-induced inflammatory disease. *J Gen Virol*. 2015;96(Pt 2):221-238. doi:10.1099/vir.0.071282-0

406. Cohen C, Morazzani E, Kalinyak L, Glass P. T cell-mediated protection and epitope mapping with a trivalent alphavirus-like replicon particle vaccine (VIR2P.1167). *J Immunol*. 2015;194(1 Supplement):75.6-75.6.

407. Yun NE, Peng BH, Bertke AS, et al. CD4+T cells provide protection against acute lethal encephalitis caused by Venezuelan equine encephalitis virus. *Vaccine*. 2009;27(30):4064-4073. doi:10.1016/j.vaccine.2009.04.015

408. Welsh RM, Bahl K, Marshall HD, Urban SL. Type 1 interferons and antiviral CD8 T-cell responses. *PLoS Pathog*. 2012;8(1):e1002352-e1002352. doi:10.1371/journal.ppat.1002352

409. Welsh RM, Bahl K, Marshall HD, Urban SL. Type 1 interferons and antiviral CD8 T-cell responses. *PLoS Pathog*. 2012;8(1):e1002352-e1002352. doi:10.1371/journal.ppat.1002352

410. Wu JQH. Virulence determinants of New World alphaviruses and broad-acting therapeutic strategies. *Future Virol*. 2019;10:647+. http://link.galegroup.com/apps/doc/A415438763/AONE?u=byuprovo&sid=AONE&xid=fad12833.

411. Simmons JD, White LJ, Morrison TE, et al. Venezuelan Equine Encephalitis Virus Disrupts STAT1 Signaling by Distinct Mechanisms Independent of Host Shutoff. *J Virol*. 2009;83(20):10571-10581. doi:10.1128/jvi.01041-09

412. Julander JG, Siddharthan V, Blatt LM, Schafer K, Sidwell RW, Morrey JD. Effect of exogenous interferon and an interferon inducer on western equine encephalitis virus disease in a hamster model. *Virology*. 2007;360(2):454-460. doi:10.1016/j.virol.2006.10.031

413. Abdelnabi R, Neyts J, Delang L. Towards antivirals against chikungunya virus. *Antiviral Res*. 2015;121:59-68. doi:10.1016/j.antiviral.2015.06.017

414. Briolant S, Garin D, Scaramozzino N, Jouan A, Crance JM. In vitro inhibition of Chikungunya and Semliki Forest viruses replication by antiviral compounds: Synergistic effect of interferon-α and ribavirin combination. *Antiviral Res*. 2004;61(2):111-117. doi:10.1016/j.antiviral.2003.09.005

415. Guevara LE. In vitro Inhibition of Sindbis Virus Replication by Glycyrrhizin. *FASEB J*. 2010;24(1).

416. Focus F, Mesylate I, Costlow JL, Krow ES, Steel JJ. IMATINIB MESYLATE AS AN EFFECTIVE ANTI-VIRAL TREATMENT FOR ALPHAVIRUS INFECTIONS. 2017;3(March).

417. Smith SA, Silva LA, Fox JM, et al. Isolation and characterization of broad and ultrapotent human monoclonal antibodies with therapeutic activity against chikungunya virus. *Cell Host Microbe*. 2015;18(1):86-95. doi:10.1016/j.chom.2015.06.009

418. Clayton AM. Monoclonal antibodies as prophylactic and therapeutic agents against chikungunya virus. *J Infect Dis*. 2016;214:S506-S509. doi:10.1093/infdis/jiw324

419. Masrinoul P, Puiprom O, Tanaka A, et al. Monoclonal antibody targeting chikungunya virus envelope 1 protein inhibits virus release. *Virology*. 2014;464-465(1):111-117. doi:10.1016/j.virol.2014.05.038

420. Seyhan AA, Alizadeh BN, Lundstrom K, Johnston BH. RNA interference-mediated inhibition of Semliki Forest virus replication in mammalian cells. *Oligonucleotides*. 2007;17(4):473-484. doi:10.1089/oli.2007.0079

421. Seyhan AA, Vitiello D, Shields MT, Burke JM. Ribozyme inhibition of Alphavirus replication. *J Biol Chem*. 2002;277(29):25957-25962. doi:10.1074/jbc.M111360200

422. Ylosmaki E, Martikainen M, Hinkkanen A, Saksela K. Attenuation of Semliki Forest Virus Neurovirulence by MicroRNA-Mediated Detargeting. *J Virol*. 2013;87(1):335-344. doi:10.1128/jvi.01940-12

423. Dash PK, Tiwari M, Santhosh SR, Parida M, Lakshmana Rao P V. RNA interference mediated inhibition of Chikungunya virus replication in mammalian cells. *Biochem Biophys Res Commun*. 2008;376(4):718-722. doi:10.1016/j.bbrc.2008.09.040

424. Parashar D, Paingankar MS, Kumar S, et al. Administration of E2 and NS1 siRNAs Inhibit Chikungunya Virus Replication In Vitro and Protects Mice Infected with the Virus. *PLoS Negl Trop Dis*. 2013;7(9). doi:10.1371/journal.pntd.0002405

425. Saha A, Bhagyawant SS, Parida M, Dash PK. Vector-delivered artificial miRNA effectively inhibited replication of Chikungunya virus. *Antiviral Res*. 2016;134:42-49. doi:10.1016/j.antiviral.2016.08.019

426. Lam S, Chen KC, Ng MML, Chu JJH. Expression of Plasmid-Based shRNA against the E1 and nsP1 Genes Effectively Silenced Chikungunya Virus Replication. *PLoS One*. 2012;7(10). doi:10.1371/journal.pone.0046396

427. Julander JG, Siddharthan V, Blatt LM, Schafer K, Sidwell RW, Morrey JD. Effect of exogenous interferon and an interferon inducer on western equine encephalitis virus disease in a hamster model. *Virology*. 2007;360(2):454-460. doi:10.1016/j.virol.2006.10.031

428. Kim AS, Austin SK, Gardner CL, et al. Protective antibodies against Eastern equine encephalitis virus bind to epitopes in domains A and B of the E2 glycoprotein. *Nat Microbiol*. 2019;4(1):187-197. doi:10.1038/s41564-018-0286-4

429. Ko SY, Akahata W, Yang ES, et al. A virus-like particle vaccine prevents equine encephalitis virus infection in nonhuman primates. *Sci Transl Med*. 2019;11(492):12. doi:10.1126/scitranslmed.aav3113

430. Martin DA, Muth DA, Brown T, Johnson AJ, Karabatsos N, Roehrig JT. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. *J Clin Microbiol*. 2000;38(5):1823-1826.

431. Hulseweh B, Rulker T, Pelat T, et al. Human-like antibodies neutralizing Western equine encephalitis virus. *MAbs*. 2014;6(3):718-727. doi:10.4161/mabs.28170

432. Atasheva S, Wang E, Adams AP, et al. Chimeric alphavirus vaccine candidates protect mice from intranasal challenge with western equine encephalitis virus. *Vaccine*. 2009;27(32):4309-4319. doi:10.1016/j.vaccine.2009.05.011

433. Barabe ND, Rayner GA, Christopher ME, Nagata LP, Wu JQ. Single-dose, fast-acting vaccine candidate against western equine encephalitis virus completely protects mice from intranasal challenge with different strains of the virus. *Vaccine*. 2007;25(33):6271-6276. doi:10.1016/j.vaccine.2007.05.054

434. Nagata LP, Hu WG, Masri SA, et al. Efficacy of DNA vaccination against western equine encephalitis virus infection. *Vaccine*. 2005;23(17-18):2280-2283. doi:10.1016/j.vaccine.2005.01.032

435. Dupuy LC, Locher CP, Paidhungat M, et al. Directed molecular evolution improves the immunogenicity and protective efficacy of a Venezuelan equine encephalitis virus DNA vaccine. *Vaccine*. 2009;27(31):4152-4160. doi:10.1016/j.vaccine.2009.04.049

436. Wu JQH, Barabé ND, Huang Y-M, Rayner GA, Christopher ME, Schmaltz FL. Pre- and post-exposure protection against Western equine encephalitis virus after single inoculation with adenovirus vector expressing interferon alpha. *Virology*. 2007;369(1):206-213. doi:https://doi.org/10.1016/j.virol.2007.07.024

437. Duggan JM, Coates DM, Ulaeto DO. Isolation of single-chain antibody fragments against Venezuelan equine encephalomyelitis virus from two different immune sources. *Viral Immunol*. 2001;14(3):263-273. doi:10.1089/088282401753266774

438. Hunt AR, Frederickson S, Maruyama T, Roehrig JT, Blair CD. The first human epitope map of the alphaviral E1 and E2 proteins reveals a new E2 epitope with significant virus neutralizing activity. *PLoS Negl Trop Dis*. 2010;4(7):e739. doi:10.1371/journal.pntd.0000739

439. Rulker T, Voss L, Thullier P, et al. Isolation and characterisation of a human-like antibody fragment (scFv) that inactivates VEEV in vitro and in vivo. *PLoS One*. 2012;7(5):e37242. doi:10.1371/journal.pone.0037242

440. Liu JL, Shriver-Lake LC, Zabetakis D, Goldman ER, Anderson GP. Selection of Single-Domain Antibodies towards Western Equine Encephalitis Virus. *Antibodies*. 2018;7(4):44.

441. Burke CW, Froude JW, Miethe S, Hulseweh B, Hust M, Glass PJ. Human-Like Neutralizing Antibodies Protect Mice from Aerosol Exposure with Western Equine Encephalitis Virus. *Viruses-Basel*. 2018;10(4):7. doi:10.3390/v10040147

442. Maruggi G, Zhang C, Li J, Ulmer JB, Yu D. mRNA as a Transformative Technology for Vaccine Development to Control Infectious Diseases. *Mol Ther*. 2019;27(4):757-772. doi:https://doi.org/10.1016/j.ymthe.2019.01.020

443. Samsa MM, Dupuy LC, Beard CW, et al. Self-Amplifying RNA Vaccines for Venezuelan Equine Encephalitis Virus Induce Robust Protective Immunogenicity in Mice. *Mol Ther*. 2019;27(4):850-865. doi:https://doi.org/10.1016/j.ymthe.2018.12.013

444. Ahmed A, Siman-Tov G, Keck F, et al. Human cathelicidin peptide LL-37 as a therapeutic antiviral targeting Venezuelan equine encephalitis virus infections. *Antiviral Res*. 2019;164:61-69. doi:https://doi.org/10.1016/j.antiviral.2019.02.002

445. Ferreira-Ramos AS, Li CQ, Eydoux C, et al. Approved drugs screening against the nsP1 capping enzyme of Venezuelan equine encephalitis virus using an immuno-based assay. *Antiviral Res*. 2019;163:59-69. doi:10.1016/j.antiviral.2019.01.003

446. Li C, Guillén J, Rabah N, et al. mRNA Capping by Venezuelan Equine Encephalitis Virus nsP1: Functional Characterization and Implications for Antiviral Research. *J Virol*. 2015;89(16):8292-8303. doi:10.1128/jvi.00599-15

447. Pagano MA, Tibaldi E, Palù G, Brunati AM. Viral proteins and Src family kinases: Mechanisms of pathogenicity from a “liaison dangereuse.” *World J Virol*. 2013;2(2):71-

78. doi:10.5501/wjv.v2.i2.71

448. Broeckel R, Sarkar S, May NA, et al. Src Family Kinase Inhibitors Block Translation of Alphavirus Subgenomic mRNAs. *Antimicrob Agents Chemother*. 2019;63(4):20. doi:10.1128/aac.02325-18

449. Lokireddy S, Vemula S, Vadde R. Connective tissue metabolism in chikungunya patients. *Virol J*. 2008;5:1-5. doi:10.1186/1743-422X-5-31

450. Wu JQH. Virulence determinants of New World alphaviruses and broad-acting therapeutic strategies. *Future Virol*. 2019;10:647+.

451. Spurgers K, Glass P. *Vaccine Development for Biothreat Alpha Viruses*. Vol 01.; 2011. doi:10.4172/2157-2526.S1-001

452. Jacobs SC, Taylor A, Herrero LJ, Mahalingam S, Fazakerley JK. Mutation of a conserved nuclear export sequence in chikungunya virus capsid protein disrupts host cell nuclear import. *Viruses*. 2017;9(10). doi:10.3390/v9100306

453. Lester SN, Li K. Toll-like receptors in antiviral innate immunity. *J Mol Biol*. 2014;426(6):1246-1264. doi:10.1016/j.jmb.2013.11.024

454. Solignat M, Gay B, Higgs S, Briant L, Devaux C. Replication cycle of chikungunya: A re-emerging arbovirus. *Virology*. 2009;393(2):183-197. doi:10.1016/j.virol.2009.07.024

455. Gieffers C, Maul GG, Frey J. Molecular Characterization of NDP52, A Novel Protein of the Nuclear Domain 10, Which Is Redistributed upon Virus Infection and Interferon Treatment. 1995;130(1):1-13.

456. Bréhin AC, Casadémont I, Frenkiel MP, Julier C, Sakuntabhai A, Desprès P. The large form of human 2′,5′-Oligoadenylate Synthetase (OAS3) exerts antiviral effect against Chikungunya virus. *Virology*. 2009;384(1):216-222. doi:10.1016/j.virol.2008.10.021

457. Kaur P, Thiruchelvan M, Lee RCH, et al. Inhibition of Chikungunya virus replication by harringtonine, a novel antiviral that suppresses viral protein expression. *Antimicrob Agents Chemother*. 2013;57(1):155-167. doi:10.1128/AAC.01467-12