

Intersecting Threats: Exploring Obesity's Impact on Viral Pathogenesis and Transmission

Pallavi Rai

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James Weger-Lucarelli, Chair

Sally L. Paulson

Nisha K. Duggal

Tanya LeRoith

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ACADEMIC ABSTRACT

Malnutrition, including both undernutrition and obesity, affects millions of people globally and is persistently on the rise. Obesity affects ~13% of adults globally and was identified as a risk factor for worse disease outcomes after the H1N1 influenza pandemic of 2009 and has since been shown to aggravate disease outcomes of respiratory viruses like severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and mosquito-borne viruses like West Nile Virus (WNV), chikungunya virus (CHIKV) and Mayaro virus (MAYV) and reduce the vaccine efficacy for influenza and SARS-CoV-2. Obesity is associated with a chronic state of inflammation and dysregulated immune response which has been proposed to be one of the mechanisms driving the severity of coronavirus disease 2019 (COVID-19). These altered signatures or biomarkers might be associated with disease outcome and prognosis. Therefore, animal models reflecting the clinical outcomes and natural immune responses observed in humans are crucial to identifying reliable biomarkers. Using mouse hepatitis virus 1 (MHV-1) as a model for SARS-CoV-2, we established obesity as a risk factor and identified biomarkers and pathways associated with worse disease outcomes.

Obesity rates in low and middle-income countries (LMICs) are approaching levels found in high-income countries (HICs). Mosquito-borne viral diseases like dengue, chikungunya, and

Zika pose a significant threat to LMICs and cause huge health and economic losses. Obesity was shown to worsen alphavirus pathogenesis, but interestingly, it also reduced their transmission by mosquitoes. Given the global prevalence of obesity and mosquito-borne viruses, it is critical to understand how obesity drives reduced alphavirus transmission. Using a natural transmission cycle between lean and obese mice and mosquitoes, we confirmed that obesity reduced the transmission potential of alphaviruses like CHIKV and MAYV and activated the Toll pathway in mosquito midguts. Various genes and other pathways were also altered in response to obese bloodmeal at various time-points post-bloodmeal; however, one gene, AAEL009965, was downregulated in the mosquito midguts 1-day-post-bloodmeal and its knockdown led to reduced infection rates and titers in mosquitoes. Through this thesis, we aimed to utilize obesity as a tool to identify biomarkers to predict coronavirus disease outcomes and design effective alphavirus transmission control strategies.

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GENERAL AUDIENCE ABSTRACT

Obesity, a global epidemic associated with chronic systemic inflammation, has emerged as an independent risk factor following infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The impact of obesity on COVID-19 severity, largely based on epidemiological data or research with non-natural hosts of SARS-CoV-2, necessitates the use of natural models of obesity to study the host's response to coronaviruses. Here we used mouse models of diet-induced obesity and infected them with a mouse coronavirus to account for the host's natural immune response against the virus. We showed that obesity exacerbates disease outcomes and identified various genes associated with disease severity in obese mice that correspond to the gene expression patterns in severe COVID-19 patients. These findings underscore the critical role of obesity in disease outcomes and pave the way for further research in this area.

Obesity also worsens the disease outcome following infection with mosquito-borne alphaviruses like chikungunya (CHIKV) and Mayaro (MAYV), however, its impact on the vector's transmission potential is poorly understood. Using a natural transmission cycle between lean and obese mice and mosquitoes, we showed that obesity reduced alphavirus transmission by mosquitoes. We found altered expression of

specific genes and pathways, in mosquito midguts exposed to alphavirus-infected obese bloodmeal compared to the lean ones and their knockdown led to altered infection rates in mosquitoes suggestive of their role in viral infection of mosquitoes. We also established insulin as a potential antiviral factor in obese bloodmeal. Therefore, these studies provide the basic framework for understanding the impact of obesity on viral transmission and pathogenesis by using relevant animal models. Furthermore, these studies utilize obesity as a tool to identify predictors of severe disease outcomes and identify genes that could be used as potential transmission control strategies for various mosquito-borne viruses.

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“It is not the critic who counts: not the man who points out how the strong man stumbles or where the doer of deeds could have done better. The credit belongs to the man who is actually **in the arena**, whose face is marred by dust and sweat and blood, who strives valiantly, who errs and comes up short again and again, because there is no effort without error or shortcoming, but who knows the great enthusiasms, the great devotions, who spends himself in a worthy cause; who, at the best, knows, in the end, the triumph of high achievement, and who, at the worst, if he fails, at least he fails while daring greatly, so that his place shall never be with those cold and timid souls who knew neither victory nor defeat.”

—Theodore Roosevelt (Speech at the Sorbonne, Paris, April 23, 1910)

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Disclosure

Chapter 2 (published): Adenovirus transduction to express human ACE2 causes obesity-specific morbidity in mice, impeding studies on the effect of host nutritional status on SARS-CoV-2 pathogenesis. **Virology, 2021.**

Chapter 3 (published): Obesity fosters severe disease outcomes in a mouse model of coronavirus infection associated with transcriptomic abnormalities. **Journal of Medical Virology, 2024.**

Chapter 4 (published): Insulin reduces the transmission potential of chikungunya virus and activates the toll pathway in *Ae. aegypti* mosquitoes. **Insect Molecular Biology, 2023.**

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Chapter 1: Introduction and Literature Review

1.1 Rationale

(a) Relevance of obesity in viral pathogenesis and transmission

The 21st century has witnessed many pandemics, most of which were caused by RNA viruses¹, of which HIV/AIDS², H1N1/swine flu³, and SARS-CoV-2/COVID-19⁴ are some of the most significant ones. The HIV/AIDS pandemic which started in the 20th century, has claimed ~40 million lives due to increased global transmission and continues to be a major public health concern². The H1N1 “swine flu” outbreak of 2009 was declared a pandemic by the WHO, not due to disease severity, but because it spread to 74 countries and territories over a short span of 3 months⁵. The COVID-19 pandemic has emerged as the most significant global health crisis of the 21st century and continues to be a major public health concern due to several factors, particularly the emergence of new variants of concern (VOC) and their increased transmissibility⁶. All these viruses are significant public health threats due to their enhanced transmission potential despite the availability of vaccines and/or therapeutics. Contrary to these viruses, SARS-CoV-1, despite its comparatively high mortality rates, was less transmissible and effectively controlled⁷. **Therefore, in the context of viral emergence or re-emergence, both transmission and disease severity are crucial factors influencing whether a pandemic persists or is eradicated.** However, host biological factors impacting viral pathogenesis and transmission are poorly understood.

According to the World Health Organization (WHO), obesity affects approximately 800 million people globally and this figure is anticipated to surpass one billion by 2030⁸. Obesity impacts ~42% of adults in the US⁸ with a prediction that 50% of adults in the U.S. will be obese by 2030⁹. Obesity alters the host environment by altering the homeostasis of various metabolic factors like proteins, lipids, and carbohydrates¹⁰ and is also associated with adipocyte inflammation and apoptosis, altering their structure and function¹¹ which leads to reduced levels of negative regulators of inflammation¹²⁻¹⁴ and increased levels of pro-inflammatory cytokines, causing local and systemic inflammation¹⁵. In addition to dysregulated immune response, obese hosts also have reduced vaccine efficacy¹⁶⁻¹⁸ which might further aggravate viral pathogenesis and lead to poor disease outcomes. Obesity has been shown to increase the disease severity following respiratory viruses like influenza¹⁹ and SARS-CoV-2^{20,21} and mosquito-borne viruses like West Nile Virus (WNV)²², dengue virus (DENV)²³, chikungunya virus (CHIKV) and Mayaro virus (MAYV)²⁴. Furthermore, obese individuals infected with influenza or SARS-CoV-2 can have prolonged viral shedding^{25,26}, thereby increasing the risk of transmission. The global prevalence of obesity and its emergence as a significant risk factor for various viral infections establish it as an important biological factor for studying its impact on viral pathogenesis and transmission. **We hypothesized that obesity would impact viral pathogenesis and transmission which would be associated with altered gene expression that could be used to predict and prevent viral diseases.**

(b) Coronaviruses are medically relevant human pathogens

Coronaviruses are enveloped, positive-sense, single-stranded RNA viruses belonging to the *Coronaviridae* family and are subdivided into four genera: *Alpha*, *Beta*, *Gamma*, and *Delta* coronaviruses²⁷, of which betacoronaviruses are associated with major human outbreaks⁴. SARS-

CoV-2, the virus responsible for the COVID-19 pandemic, belongs to the genus *Betacoronavirus* and subgenus *Sarbecovirus*²⁸. The 30 kb long genomic RNA is capped at the 5' end and has a poly(A) tail at the 3' end²⁹. The first two-thirds of the viral genome encodes for open reading frames (ORFs) 1a and 1b which encode for 16 non-structural proteins (NSPs), while the last one-third of the genome encodes for the 4 structural proteins: spike (S), envelope (E), membrane (M) and nucleocapsid (N)- and the accessory proteins. The S protein has 2 subunits which are cleaved in the presence of furin into subunits S1 and S2³⁰. S1 binds to the angiotensin-converting enzyme 2 (ACE2) receptor while S2 has the fusion peptide which is activated by transmembrane protease, serine 2 (TMPRSS2), or cathepsin L to mediate membrane fusion on infecting new cells²⁷. The N protein is involved in viral packaging and modulating the host's innate immune response, the M protein is involved in virion assembly, and the E protein is a viroporin that retains the S protein at the sites of virion assembly and maturation³¹. The NSPs are critical in evading host immune responses³² with other major functions: nsp12 acts as the viral RNA-dependent RNA polymerase (RdRp), nsp13 as the helicase, nsp14 is the 3'-5' exonuclease responsible for proofreading, and nsp16 is involved in RNA capping³¹.

Common human coronaviruses (HCoVs), including 229E, NL63, OC43, and HKU1, usually cause mild common cold symptoms like runny nose, sore throat, headache, fever, and cough, but occasionally cause pneumonia or bronchitis in individuals with cardiopulmonary disease, weakened immunity, infants, and the elderly³³. The COVID-19 pandemic of 2019 has been preceded by two other HCoV outbreaks: the SARS-CoV-1 outbreak of 2003, infecting 8000 people in over 25 countries³⁴, and the Middle East Respiratory Syndrome (MERS) outbreak of 2012, infecting only 2000 people³⁵. While the SARS-CoV-1 outbreak was efficiently controlled due to low transmissibility of the virus and effective isolation and quarantine⁷, sporadic cases of

MERS continue to be reported³⁶ suggesting the probability of a MERS epidemic in the near future. The fatality rates for SARS-CoV-1 and MERS were 9.6%³⁷ and 40%³⁸ respectively. SARS-CoV-2, on the other hand, is highly transmissible with a fatality rate of <1%³⁹. As of 14th April 2024, SARS-CoV-2 was responsible for ~775 million confirmed cases and ~7 million deaths globally³⁹ and continues to emerge into new VOCs with increased transmissibility and pathogenesis⁴⁰. These VOCs have led to ongoing outbreaks globally^{41,42} posing a persistent threat to public health. Additionally, high mutation rates²⁷ and recombination between different VOCs lead to the emergence of novel variants⁴³ which reduce the efficacy of vaccines and other treatments^{44,45}.

(c) CHIKV and MAYV are medically relevant alphaviruses

Alphaviruses are enveloped, positive sense, single-stranded, RNA viruses ~11-12 kb long, with a 5' methyl guanylate cap and a 3' polyadenylated tail, with 5' and 3' untranslated regions (UTRs)⁴⁶. The genome encodes 2 open reading frames (ORFs)- the first ORF encodes a polyprotein that is cleaved into 4 non-structural proteins (NSPs): nsP1, nsP2, nsP3, and nsP4. There is a 26S subgenomic promoter between the non-structural and structural domains for transcription of subgenomic RNA⁴⁷. The second ORF is translated from the subgenomic RNA and encodes the structural polyprotein which is then cleaved into 5 structural proteins: capsid, E3, E2, 6K, and E1. During replication, the non-structural polyprotein, P1234 undergoes initial *cis* cleavage into P123 and nsP4, which form an unstable early replication complex facilitating the synthesis of negative strand RNA. Subsequently, P123 is *trans*-cleaved into nsP1, P23, and nsP4, which form a replication complex for genomic RNA synthesis⁴⁸. Further cleavage into all 4 non-structural monomers redirects replication activity toward genomic and subgenomic RNA synthesis⁴⁹, followed by assembly and budding. The E3 glycoprotein assists in the proper folding

of other structural proteins and prevents premature conformational changes of the E2-E1 heterodimer⁵⁰. The E2 protein has a multi-domain structure⁵¹ that enables it to mediate viral attachment and entry, while also serving as a primary target for the host immune response⁵². The 6K/TF membrane proteins have been predicted to be involved in glycoprotein processing and virion assembly and release^{53,54}. The E1 protein is a class II fusion protein involved in the fusion of viral membrane to the host lipid membrane^{55,56}.

Alphaviruses are classified as Old World and New World based on their geographical distribution⁵⁷ and most of them are maintained in a transmission cycle involving vertebrate hosts and mosquitoes⁵⁸. Old World alphaviruses, including Sindbis virus (SINV), O'nyong nyong virus (ONNV), Ross River virus (RRV), MAYV, and CHIKV, originated or are predominant in Africa, Europe, and Asia. They cause acute febrile illness, with rash and debilitating polyarthralgia⁵⁹. The New World alphaviruses, found mainly in the Americas, include Eastern, Western, and Venezuelan equine encephalitis viruses (EEEV, WEEV, VEEV), are equine pathogens and are mainly associated with encephalitis in humans⁵⁹.

Chikungunya was first identified in Tanzania in 1952 and is a Makonde word meaning “to walk bent over” which refers to the stooped posture of patients due to the debilitating joint pain⁶⁰ experienced by over 50% of individuals, months to years after initial infection⁶¹. CHIKV is classified into 3 genotypes, namely, West African, East/Central/South African (ECSA), and Asian, which caused sporadic outbreaks in Asia and Africa⁶² and would be sustained in a sylvatic cycle in non-human primates^{63,64}. However, since 2005, the ECSA strain of CHIKV has caused several outbreaks in the Indian Ocean⁶⁵, Europe⁶⁶, Asia^{67,68} and the US⁶⁹. The La Réunion outbreak in 2005-2006 was one of the biggest CHIKV outbreaks⁷⁰ attributed mainly to its

adaptation to the secondary vector, *Ae. albopictus*^{71,72}, and since then it has been reported in > 100 countries^{73,74}. During its emergence in the Americas from 2013-2017, it infected ~3 million people⁷⁵. As per the Pan American Health Organization (PAHO) for the Americas, CHIKV caused ~280,000 infections in 2022⁷⁶, ~410,000 in 2023⁷⁷, and has already infected ~136,000 people during the first quarter of 2024⁷⁷, suggesting the imminent threat posed by CHIKV in the region. Mayaro virus (MAYV) is a biosafety level 2 (BSL2) pathogen and is another emerging alphavirus with similar disease manifestations⁷⁸ and potentially the same vectors as CHIKV^{79,80}. MAYV has caused several outbreaks in South America and the Caribbean^{81,82} and has the potential to cause future outbreaks^{83,84}.

(d) Mosquitoes are crucial for alphavirus transmission

The increased emergence potential of CHIKV and MAYV is likely due to their adaptation to urban vectors^{85,86}, mainly *Ae. aegypti* and *Ae. albopictus* which are widely prevalent⁸⁷ and live in close proximity to humans. The ability of a vector to transmit a pathogen at a specific place and time is determined by its vectorial capacity⁸⁸, defined by Macdonald's equation, later modified by Black and Moore⁸⁹ and represented by the following equation:

$$V = \frac{m \times a^2 \times p^n \times b}{-\ln(p)}$$

$$-\ln(p)$$

where,

V = vectorial capacity

m = vector: host ratio

a = daily blood feeding rate

p = probability of daily survival

n = extrinsic incubation period (EIP)

b = transmission rates among exposed mosquitoes

$1/(-\ln p)$ = vector survival post EIP

An important factor in determining the vectorial capacity is b , the proportion of mosquitoes exposed to an infectious bloodmeal that can successfully transmit the virus, also known as vector competence⁹⁰. For effective transmission, alphaviruses must cross four major barriers inside the mosquito⁹¹: midgut infection barrier (MIB), midgut escape barrier (MEB), salivary gland infection barrier (SGIB) and salivary gland escape barrier (SGEB). These barriers are crucial for the vector competence of various virus-vector pairs and determine the EIP of the virus⁹². Midguts are the initial sites⁹³ of alphavirus infection and act as the first line of defense in mosquitoes⁹⁴ through various antiviral pathways like RNA interference (RNAi), Janus-kinase/signal-transducer and activator of transcription (JAK/STAT), Toll and immunodeficiency (IMD) pathways⁹⁵. Despite the key role of mosquito midgut in establishing infection, its response to alphavirus infection is understudied. The interaction between the virus and the innate immune response of the vector is the most critical element in determining the outcome of infection and viral transmission. **Therefore, a deeper understanding of vector-virus-host interactions is essential to develop effective transmission interventions.**

1.2 Research objectives: Establishing obesity's link to COVID-19 and alphaviruses

(a) Obesity and COVID-19

Obesity has been identified as an independent risk factor for hospitalizations⁹⁶⁻⁹⁸ and is directly correlated with mortality in COVID-19 patients⁹⁹. Recent studies have shown that severe COVID-19 outcomes have been associated with systemic inflammation¹⁰⁰⁻¹⁰² and are linked to dysregulated pro-inflammatory cytokines. High rates of hospitalizations in obese patients have been associated¹⁰³ and positively correlated¹⁰⁴ with low-grade inflammation. Moreover, the response towards vaccines and antivirals is diminished in obese individuals¹⁰⁵, which further exacerbates the disease outcome. Due to the ethical concerns of using humans, animal models are primarily used for studying various aspects of COVID-19¹⁰⁶. Therefore, selecting an animal model that accurately replicates the disease pathology observed in COVID-19 patients is crucial for these investigations. Several animal models including mice¹⁰⁷, ferrets¹⁰⁸, hamsters¹⁰⁹, minks¹¹⁰, and non-human primates (NHPs)¹¹¹, have been used particularly for studying the pathogenesis, transmission, and vaccine efficacy against SARS-CoV-2. Given that more severe forms of COVID-19 are linked to the host's immune response¹¹² and obesity increases the risk of severe outcomes⁹⁶, it is essential to consider a naturally obese coronavirus infection model that incorporates the host's natural immune response. The current animal models used for COVID-19 research suffer from these major drawbacks:

- a) The use of a modified host or virus might not mirror the natural immune response in humans.

- b) Most models exhibit only mild to moderate symptoms; therefore, we need models with a more severe form of the disease.
- c) Most models suffer from lethal encephalitis which is not a clinical finding in humans with severe COVID-19.

Severe COVID-19 manifests as a multisystem disease caused due to complex interactions among the immune system¹¹³, inflammatory responses¹¹², and coagulation cascades¹¹⁴. Therefore, the identification of reliable prognostic biomarkers would be beneficial in identifying and prioritizing individuals at risk of severe illness, facilitating prompt treatment, and reducing mortality risks. To address these limitations, this research was designed with the following objectives:

- a) Determine the impact of obesity on coronavirus disease severity using a relevant animal model.
- b) Identify biomarkers of severe disease outcome in easily accessible samples, like blood, at early time points of infection.

(b) Obesity and alphavirus transmission

An epidemiological study by Heath et al.¹¹⁵ showed obesity to be a risk factor for severe CHIK disease in humans, which was experimentally confirmed by our lab for various alphaviruses²⁴. Interestingly, we also found reduced transmission of MAYV by *Ae. aegypti* mosquitoes fed on MAYV-infected obese mice compared to those fed on their lean counterparts²⁴. Obesity is associated with dysregulation of various host factors, including glucose, lipids, insulin, and various cytokines¹¹⁶. Previous studies have shown that mammalian insulin reduces flavivirus replication in insect vectors by activating immune pathways like

JAK/STAT and Ras/ERK signaling^{117,118}. ERK pathway activation in the presence of insulin was also shown to inhibit infection of *Ae. aegypti* cells with the prototype alphavirus, Sindbis virus (SINV)¹¹⁹. However, the impact of insulin on CHIKV transmission has not been studied yet. These studies suggest that insulin's antiviral effect in mosquitoes might partly be attributed to the mosquito immune response. Therefore, we hypothesized that some component(s) in the alphavirus-infected obese bloodmeal impacts mosquito immune responses which might be driving the reduced transmission potential of *Ae. aegypti* mosquitoes. To test this hypothesis, we designed our study with the following objectives:

- a) Identify insulin's role in the transmission of chikungunya virus and mosquito immune response.
- b) Identify genes and/or pathways altered in mosquito midguts in response to alphavirus-infected obese bloodmeal.

1.3 Implications

The global prevalence and unending threat of obesity have increased awareness among researchers about its impact on various communicable diseases. However, most of the research done in the context of obesity focuses on how it aggravates a particular disease. Our research is novel in the sense that we are utilizing obesity to identify factors to predict and prevent disease outcomes. This research confirms the epidemiological findings linking obesity to severe COVID-19 outcomes and uses this model to identify biomarkers associated with worse disease outcomes. Further, we use obesity as a tool to identify potentially antiviral genes in mosquitoes that could be used to design effective transmission control strategies.

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Chapter 2: Adenovirus transduction to express human ACE2 causes obesity-specific morbidity in mice, impeding studies on the effect of host nutritional status on SARS-CoV-2 pathogenesis

Pallavi Rai¹, Christina Chuong¹, Tanya LeRoith¹, James W Smyth^{2,3,4}, Julia Panov^{5,6}, Moshe Levi⁷, Kylee Kehn-Hall¹, Nisha K. Duggal¹, and James-Weger Lucarelli^{1*}

¹Department of Biomedical Sciences and Pathobiology, Virginia Tech, VA-MD Regional College of Veterinary Medicine, Blacksburg, VA, United States of America.

²Center for Heart and Reporative Medicine, Fralin Biomedical Research Institute, Roanoke, VA, United States of America.

³Department of Biological Sciences, College of Science, Virginia Polytechnic State Institute and State University, Blacksburg, VA, United States of America.

⁴Department of Basic Science Education, Virginia Tech Carilion School of Medicine, Roanoke, VA, United States of America.

⁵Tauber Bioinformatics Research Center, Haifa 3498838, Israel.

⁶Sagol Department of Neurobiology, University of Haifa, Haifa 3498838, Israel.

⁷Department of Biochemistry and Molecular & Cellular Biology, Georgetown University,
Washington, DC, United States of America.

*Correspondence: weger@vt.edu

2.1 Abstract

The COVID-19 pandemic has paralyzed the global economy and resulted in millions of deaths globally. People with co-morbidities like obesity, diabetes and hypertension are at an increased risk for severe COVID-19 illness. This is of overwhelming concern because 42% of Americans are obese, 30% are pre-diabetic and 9.4% have clinical diabetes. Here, we investigated the effect of obesity on disease severity following SARS-CoV-2 infection using a well-established mouse model of diet-induced obesity. Diet-induced obese and lean control C57BL/6N mice, transduced for ACE2 expression using replication-defective adenovirus, were infected with SARS-CoV-2, and monitored for lung pathology, viral titers, and cytokine expression. No significant differences in tissue pathology, viral replication, or cytokine expression were observed between lean and obese groups. Notably, significant weight loss was observed in obese mice treated with the adenovirus vector, independent of SARS-CoV-2 infection, suggesting an obesity-dependent morbidity induced by the vector. These data indicate that the adenovirus-transduced mouse model of SARS-CoV-2 infection is inadequate for performing nutrition studies, and caution should be used when interpreting the resulting data.

2.2 Introduction

Coronavirus disease-2019 (COVID-19) is the third pandemic in the 21st century caused by a novel coronavirus, after severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 [1,2,3,4] and the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 [5,6,7]. COVID-19 is responsible for ~99 million confirmed cases, with over 2 million deaths globally, and ~25 million confirmed cases and ~400,000 deaths in the U.S. alone, as of January 26th, 2021 [8]. COVID-19 is characterized by fever and respiratory symptoms, which can progress to more severe and fatal disease [9]. Infection with SARS-CoV-2, the causative agent of COVID-19, is more likely to cause critical illness or deaths in the elderly, immunocompromised and individuals with co-morbidities such as obesity, diabetes, and hypertension [10,11,12,13].

Obesity and diabetes have been identified as risk factors for poor outcomes in other major pandemics involving respiratory tract infections like MERS [14] and influenza [15,16,17]. Like COVID-19, obesity too has become pandemic, affecting 12.5% of people globally and approximately 42% of Americans [18]. Obesity is the most frequently observed component of metabolic syndrome, a condition that clusters abdominal obesity, dyslipidemia (abnormally high blood lipid levels), hyperglycemia (increased blood sugar levels) and hypertension (increased blood pressure) [19]. Experiments using diet-induced obese mice to study the pathogenesis of influenza A virus have shown not only are obese mice more susceptible to infection [20] but that they also promote the emergence of more virulent strains of the virus [21]. To study the impact of obesity on SARS-CoV-2 pathogenesis and evolution, a similar mouse model is required; however, mice are not susceptible to infection with SARS-CoV-2 due to the lack of human

Angiotensin Converting Enzyme 2 (hACE2) receptor, required for entry of the virus [22,23]. Several strategies have been developed to circumvent this issue: transgenic mice expressing hACE2 (hACE2-Tg) [24,25,26,27], mouse-adapted SARS-CoV-2 strains [28,29], and transduction with adenovirus vectors expressing hACE2 (AdV-hACE2) [30,31]. The AdV-hACE2 approach has several advantages: it can be used with different strains of mice, is easy to manipulate, causes little pathology in wild-type mice, and allows for the use of wild-type (WT) SARS-CoV-2 [30,31].

In this study, we sought to investigate the effect of obesity on disease outcomes following SARS-CoV-2 infection using an adenovirus-transduced mouse model. Currently, no mouse models for studying the impact of nutritional status on COVID-19 have been described, limiting progress toward understanding the mechanisms leading to more severe disease in people with comorbidities. To this end, diet-induced obese and lean mice were transduced with AdV-hACE2 followed by SARS-CoV-2 infection and monitored for morbidity, mortality, virus replication, and cytokine expression. Surprisingly, obese, but not lean, mice inoculated with the replication-defective AdV-hACE2 lost significant weight, independent of SARS-CoV-2 infection, suggesting an obesity-dependent morbidity induced by the AdV-hACE2 vector. SARS-CoV-2 replication occurred in the lungs of both lean and obese mice, though, we did not observe differences in terms of disease outcomes, viral replication, or cytokine expression when normalized to mock-SARS-CoV-2-infected controls. Accordingly, due to the confounding influence of AdV-hACE2 on SARS-CoV-2 infection, we could not determine obesity's impact on SARS-CoV-2 infection. These data underscore the importance of selecting an appropriate model for nutrition studies and suggest that researchers should exercise caution when using the

AdV-hACE2 model to study SARS-CoV-2. Further studies using transgenic mice, mouse-adapted strains [28,29], or other animals such as hamsters [32,33] or ferrets [34,35] should be conducted to explore the relationship between COVID-19 and metabolic co-morbidities.

2.3 Materials and Methods

Cells and Viruses

Vero E6 cells were obtained from American Type Culture Collection (ATCC; CRL-1586) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1mg/mL gentamicin, 1% non-essential amino acids (NEAA) and 25 mM HEPES buffer in an incubator at 37°C with 5% CO₂.

The replication-defective adenovirus encoding human ACE2 (AdV-hACE2) was originally prepared by the University of Iowa and Washington University in St. Louis, as reported previously [3]. The viral stocks were propagated using 293A cells (ThermoFisher Catalog # R70507) and purified by cesium chloride gradient centrifugation prior to titering, also in 293A cells, as previously described [36]. SARS-CoV-2 strain 2019 n-CoV/USA_WA1/2020 was obtained from BEI Resources (Catalog # NR-52281) [37] and was propagated in Vero E6 cells to prepare stocks; infectious titers were determined by plaque assays on the same cell line.

Mice and Diets

C57BL/6N male and female mice were obtained from Charles River Laboratories at three to four weeks of age and allowed to acclimatize for a week before initiating diets. Mice were housed in groups of five per cage and maintained at ambient temperature with *ad libitum* supply of food and water, except for overnight food deprivation (12-14 hours) prior to blood glucose

measurements. All animal handling protocols were approved by the Institutional Animal Care and Use Committee (Protocol #20-060) at Virginia Tech.

All diets used for the study were obtained from Research Diets (New Brunswick, NJ, USA). Twenty mice (10 males and 10 females) were placed on a low-fat diet with 10% kcal fat (LFD; D12450K) and 20 (10 males and 10 females) on a high-fat diet with 60% kcal fat (HFD; D12492). Throughout the manuscript, we will refer to the groups as follows: lean (low fat diet) or obese (high fat diet). The mice were kept on these diets for 18-20 weeks before infections, and the same diets were continued until the end of the experiment. Table S1 displays the caloric information of these diets.

Fasting glucose levels

Two weeks prior to transduction by the adenovirus (16 weeks after diet initiation), mice were deprived of food overnight (12-14 hours), blood was collected by submandibular bleed and glucose levels were measured by Abox glucose monitoring kit.

Mouse infections

Mice were moved to a BSL-3 facility 24 hours prior to SARS-CoV-2 infections. For primary infections with the AdV-hACE2, 19-20-week-old mice were anesthetized with ketamine and xylazine (90 mg/kg and 5 mg/kg respectively) intraperitoneally and then inoculated intranasally with 10^8 plaque-forming units (PFUs) of the virus in 50 μ L of Roswell Park Memorial Institute (RPMI)-1640 media with no additives. Five days post-adenovirus inoculation, the mice received 10^5 PFUs of SARS-CoV-2 intranasally under ketamine and xylazine anesthesia, or RPMI-1640 media alone for mock-infected groups. Six infected and four control mice from each group were

sacrificed at days 4 and 10 after SARS-CoV-2 infection. Mice were weighed daily post-SARS-CoV-2 infection and monitored for visual symptoms of the disease. Blood was collected via submandibular bleeds (~200µL per mouse), serum was collected via centrifugation at 5000 x g for 10 minutes, transferred to fresh tubes and stored at -80°C.

Histopathology and organ titration

Tissues for viral titration were harvested aseptically in 2 mL tubes containing a 5 mm stainless steel bead, RPMI-1640, 10 mM HEPES and 1% FBS (herein referred to as the “tissue diluent”), to a final concentration of 10% weight by volume. (QIAGEN) at 30 cycles per second for 2 mins and then centrifuged at 5000 x g for 10 mins. Plaque assays were performed on the clarified supernatant. Briefly, Vero E6 cells at 100% confluency were inoculated with 50 µL of the serially diluted samples and incubated at 37°C in 5% CO₂ for 1 hour. Overlay media containing 0.6% tragacanth gum, 1x MEM (minimum essential media), 20 mM HEPES and 4% FBS were then added, and the plates were allowed to incubate for 2 days for plaque formation.

For histopathology, the tissues harvested from mice were fixed in 4% paraformaldehyde for at least 1 week. The Virginia Tech Animal Laboratory Services (ViTALS) performed paraffin embedding, sectioning and hematoxylin-eosin staining, and a board-certified pathologist scored the slides in a blinded manner.

RNA extraction

A section of the infected lung tissues (4 dpi) was collected in 0.5 mL of TRIzol LS reagent (ThermoFisher) in 2 mL tubes with a 5mm bead. Lung tissues were homogenized using a

TissueLyser II (QIAGEN) at 30 cycles per second for 2 mins and stored at -80°C. Samples were thawed and total RNA was extracted using the manufacturer's protocol for TRIzol extraction.

Reverse Transcription Quantitative PCR (RT-qPCR)

To quantify SARS-CoV-2 genomes, the 2019-nCoV RUO kit from Integrated DNA Technologies (IDT, Leuven, Belgium) was utilized. N2 combined primer-probe mix from the kit was used (Table S2) with Quantabio qScript XLT-One-Step RT-qPCR ToughMix (2X). The Bio-Rad CFX-96 (Hercules, CA, USA) was used for RT-qPCRs with the following conditions: 50°C for 10 mins for reverse transcription, 95°C for 3 mins for initial denaturation and polymerase activation, followed by 45 cycles of 95°C for 10 secs for denaturation, and 60°C for 30 secs for annealing/extension. For generating the standard curve, we used N gene RNA generated by *in vitro transcription* and used ten-fold serial dilutions to the point where no genome was detectable by qPCR. The virus concentration was calculated by fitting the C_q values of the samples to the standard curve and expressed in terms of N-gene copies/mL.

RT-qPCR was also performed on RNA extracted from the 4 dpi lung samples with NEB Luna Universal One-Step RT-qPCR Kit with SYBR-Green (NEB, Ipswich, MA, USA) to quantify the cytokines IL-6 and IFN- β . Primers were obtained from IDT and are listed in Table S2. The conditions for the reactions in a Bio-Rad CFX-96 were: 50°C for 10 mins for reverse transcription, 95°C for 1 min for initial denaturation and polymerase activation, followed by 45 cycles of 95°C for 10 secs for denaturation, and 60°C for 30 secs for annealing/extension, followed by a melt curve. The samples were calibrated with mouse GAPDH as the reference gene with respect to mock-infected groups as control. The relative expression/fold change was calculated using the Delta-Delta-Ct ($\Delta\Delta C_t$) method of relative quantification [38].

Statistical Analysis

Statistical analysis was done using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Details of the statistical technique used for analyses are provided in the figure legends. The level of significance has been determined by the following p-values: $p=0.1234$ (ns); $p = 0.0332$ (*), $p = 0.0021$ (**), $p = 0.0002$ (***), $p<0.0001$ (****). The error bars represent standard deviation (SD) from the mean, in all the figures and the dotted lines denote the limit of detection (L.O.D.). All statistical analyses were performed on data that passed the normality test using the Shapiro-Wilk test.

2.4 Results and Discussion

Wild-type C57BL/6N mice fed a high-fat diet had increased weight gain and blood glucose levels.

The proportion of COVID-19 patients requiring intensive care was previously reported to be directly proportional to the body mass index (BMI), being highest in patients with $BMI \geq 35$ (morbidly obese) [39]. Obesity has also been correlated with severe outcomes during the H1N1 influenza-A epidemic [40,41,42,17]. Furthermore, diet-induced obese mice had significantly higher mortality rates when infected with influenza virus [20] and more severe disease following chikungunya virus (CHIKV) [43] and dengue virus (DENV) infection [44]. We sought to use a similar diet-induced obese mouse model to investigate whether obese mice infected with SARS-CoV-2 exhibit worse disease outcomes as compared to their lean counterparts.

We observed a significant weight gain in high-fat diet (HFD)-fed mice compared to the low-fat diet (LFD)-fed groups starting 7 weeks after the initiation of diets ($p\text{-value} < 0.0001$)

(Fig. 1A). Weights were analyzed on the combined data for males and females on LFD and HFD. Separate analysis for males and females is presented in supplementary figure S1. To assess other metabolic parameters, we measured overnight fasting blood glucose levels after 9 and 16 weeks of the initiation of diets. We tested only 10 mice per group at 9 weeks to reduce stress on the mice. Hyperglycemia, defined as overnight fasting glucose levels of ≥ 150 mg/dL, was not significantly different between the lean and obese groups at 9 weeks (p-value=0.2344) (Fig. 1B). By 16 weeks, however, there was a significant difference between HFD and LFD groups (p-value=0.0001) (Fig. 1C). This 15 to 20-week diet regimen produced a mouse model that mirrored obesity and hyperglycemia in humans and, therefore, was appropriate for infection studies.

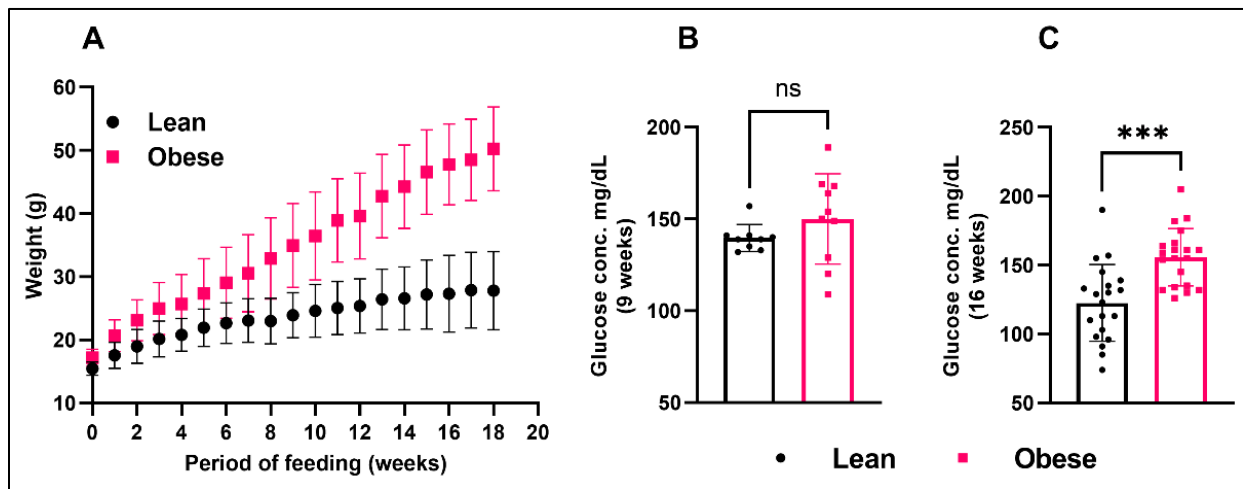


Fig.1: Metabolic characterization of mice fed low-fat (lean) and high-fat diets (obese).

A) Four-week-old C57BL/6N mice (20/group) were fed a high-fat diet (60% fat) or a low-fat diet (10% fat) for 15-20 weeks. Weights were measured weekly after initiating the diet. Statistical analysis was done using 2way-ANOVA with Sidak's multiple comparisons test. **B)** Blood glucose levels were measured following nine weeks of feeding **C)** Glucose levels were measured

after 16 weeks of feeding. Statistical analysis was done using Mann-Whitney test and a significantly high level of glucose was observed in obese mice compared to lean ones after 16 weeks of diet-initiation (p-value=0.0001). The error bars indicate standard deviation (SD) from mean.

Adenovirus-transduction caused morbidity in obese mice, independent of SARS-CoV-2

infection. Since mouse ACE2 receptors do not allow for WT SARS-CoV-2 infection, we first transduced mice with a replication-defective adenovirus encoding for human ACE2 (AdV-hACE2) intranasally to achieve hACE2 expression in the lungs and render mice susceptible to infection. We then inoculated the mice with SARS-CoV-2 or diluent five days post-transduction, as previously described [30,31] (Fig. 2A). Surprisingly, the obese mice inoculated only with AdV-hACE2 showed significant weight loss, similar to those infected with both AdV-hACE2 and SARS-CoV-2 (Fig. 2B). No weight loss was observed in lean mice treated with AdV-hACE2 or AdV-hACE2 and SARS-CoV-2. We found a significant weight loss in obese-AdV only mice compared to the lean-AdV only group starting from days 5 (p-value=0.0010) through day 9 post-AdV transduction (p-value=0.0242). The weights of the mice starting from the day of AdV inoculation in both percent weight loss and grams are presented in supplementary figure S2. Collectively, these data suggested that the AdV-hACE2 vector produced morbidity specifically in obese mice. However, we proceeded to test other parameters to determine if differences could be found between lean and obese mice in terms of viral replication, immune response, or lung pathology characteristic of SARS-CoV-2 infection.

To measure viral replication, a subset of the mice was euthanized on days 4 and 10 post-SARS-CoV-2 inoculation and infectious virus was quantified in the lung homogenates. As

expected, mice receiving only AdV-hACE2 tested negative for SARS-CoV-2 (Fig. 2C). Infectious lung viral titers between lean and obese infected groups were similar (p -value=0.7377) at 4 days post-SARS-CoV-2 infection, and 1 lean and 2 obese mice were negative for infectious virus. We next used RT-qPCR against the N-gene as a more sensitive means to detect viral RNA to determine if the mice became infected with SARS-CoV-2. At 4 days post-SARS-CoV-2 inoculation, all lean mice were positive for the N-gene, while both infectious-virus-negative obese mice were negative, indicating a lack of established infection in these obese mice. Accordingly, we have removed the 2 negative obese mice from Figure 2B-D because they were likely not infected. In Figure 2E, we present RT-qPCR data for the N-gene for lungs collected at 10 days post-SARS-CoV-2 infection; all 6 lean mice, but only 5 obese mice, tested positive for SARS-CoV-2 N-gene by RT-qPCR, and all the mice were negative for infectious virus tested by plaque assays. We present all the data, including the negatives, in supplementary figure S3. No differences were observed between lean and obese groups in N-gene copies/mL at either timepoint. This finding is consistent with previous studies that found no significant difference in infectious viral titers at peak viral replication between lean and obese mice infected with influenza virus [20], several alphaviruses [43] or dengue virus [44].

Severe COVID-19 disease is characterized by low levels of type I interferons (IFN- α and β) and over-production of inflammatory cytokines like interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α) [45,46,47,48,49]. Moreover, invasive mechanical ventilation (IMV) was associated with severe obesity in patients infected with SARS-CoV-2, irrespective of age, sex, diabetes, and hypertension [39]. Experiments using obese mice to study the pathogenesis of influenza A virus, another important respiratory virus, showed reduced expression of type I

interferons (IFNs), delayed expression of pro-inflammatory cytokines and chemokines [20], severe lung pathology, and increased viral spread, leading to increased mortality [16]. We thus sought to determine the contribution of obesity to cytokine expression and lung pathology following SARS-CoV-2 infection. For cytokine testing, RNA was extracted from lungs collected 4 days post-SARS-CoV-2 infection, and the expression of cytokines IL-6 and IFN- β was measured by RT-qPCR. The relative expression of these cytokines was calculated by the $\Delta\Delta C_t$ method, with hACE-AdV infected lean groups as control and mouse GAPDH as the reference gene. No significant difference in the relative fold-expression of the cytokines above was observed between hACE2-AdV+SARS-CoV-2 infected lean and obese groups at either 4 or 10 dpi (Fig. 3A).

We also examined lungs, liver, and heart for histopathological lesions. Lungs were evaluated and scored based on evidence of interstitial inflammation, intra-alveolar hemorrhage, and peribronchiolar and perivascular lymphoid hyperplasia. There was no evidence of interstitial or alveolar septal necrosis or hyaline membrane formation. Livers were evaluated for evidence of lipidosis and single cell necrosis. The hearts were evaluated for evidence of myocardial necrosis and inflammation. Representative lung images collected at 4 dpi are presented in Fig. 3B. The lung histopathology scores were similar for obese mice inoculated with AdV+SARS-CoV-2 compared to their AdV-only transduced counterparts at 4 dpi but became significantly higher for the SARS-CoV-2 infected mice at 10 dpi (Fig. 3C). We could hypothesize that this difference at the later timepoint was due to additional replication of SARS-CoV-2 after 4 dpi and the AdV subsequently being cleared. No differences in the histopathology scores for heart and liver were observed between the groups. Taken together, no major differences were observed between the

two groups; however, the weight loss data suggest that the AdV-hACE2 transduction caused an obesity-specific morbidity, thus confounding the study.

The mechanisms underlying the morbidity observed in AdV-hACE2 transduced obese mice are currently unclear. Future work using a control AdV will enable parsing out of vector-related mechanisms vs. ectopic expression of hACE2 will be informative. Replication-defective adenoviruses have been used in many gene therapy applications [50,51] and are known to activate both innate and adaptive immune responses in humans [52,53,54,55]. Further, they have been shown to cause hepatotoxicity in mice due to induction of TNF- α in the liver [56,57] and dose-dependent morbidity in non-human primates [58]. Accordingly, we posit that obese hosts may be more permissive to disease following transduction, possibly due to an overzealous immune response to the vector. The data presented here suggest caution should be taken when using adenovirus vectors in broader populations. Notably, the race for developing a vaccine against SARS-CoV-2 at “pandemic speed” [59] has renewed the interest of researchers in using adenovirus vectors, which makes our findings worth considering.

2.5 Limitations of the study

The major limitation of our study was the use of the adenovirus vector for transduction of mice with the human ACE2 receptor. The use of adenovirus vector was an additional variable that induced morbidity in the obese host, confounding our ability to assess obesity’s impact on SARS-CoV-2 infection in mice, the main objective of our study. Another limitation of this study was the lack of a true mock-treated group, with no AdV or SARS-CoV-2 infection, in the absence of which we cannot quantify the impact of the adenovirus vector versus hACE2 transgene on the obese host. Moreover, these differences could be specific to C57BL/6N mice

and we might observe different results with other mouse strains. Despite these constraints, this study provides useful information to the field relating to SARS-CoV-2 mouse models and advises caution when considering the AdV-hACE2 model for nutrition studies.

2.6 Conclusion

The increased risk of grave illness and fatalities in obese people post-SARS-CoV-2 infection underscores the importance of an effective animal model to study the mechanisms underlying worsened disease outcomes. Here, we generated diet-induced obese (DIO) mice and used a previously established adenoviral-transduction model to render them susceptible to SARS-CoV-2 infection. Our results demonstrate that although adenovirus-transduction sensitized obese and lean mice to SARS-CoV-2 infection in their lungs, it also induced morbidity specifically in obese mice. Therefore, this model was not appropriate for studying the relationship between underlying co-morbidities like obesity or diabetes and severe COVID-19. Future studies would benefit from using another mouse model, for example, hACE2 transgenic mice or a mouse-adapted strain of SARS-CoV-2.

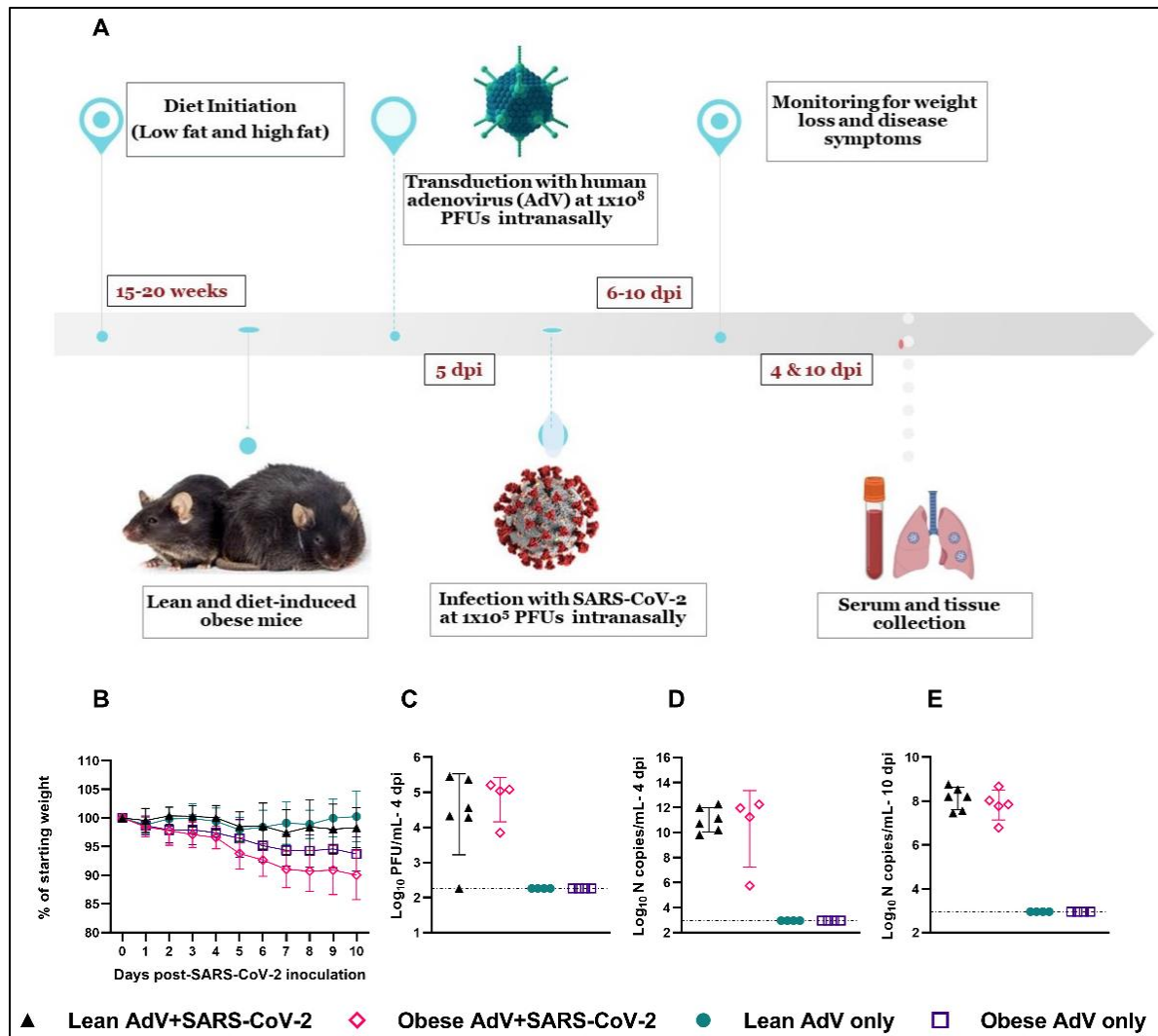


Fig.2: Timeline of the study and disease progression in lean and obese mice post-SARS-CoV-2 infection.

A) 18-20-week-old male and female C57BL/6N mice received 1×10^8 PFUs of hACE2-AdV intranasally (day -5) and then 1×10^5 PFUs SARS-CoV-2 or viral diluent intranasally (day 0). A subset of infected and control mice was euthanized at day 4 and the remaining at day 10 post-SARS-CoV-2 infection and blood and organs were collected for viral titers and histopathology.

B) Weights were measured daily after SARS-CoV-2 infection and statistical analysis was done

on the percent weight change compared to the weight on the day of infection, using 2way-ANOVA with Dunnett's multiple comparisons test. No significant difference was observed between only adenovirus (AdV) transduced groups and their AdV+SARS-CoV-2 infected counterparts. **C)** Infectious viral titer in the lungs was determined at four dpi. Statistical analysis was done on log transformed data, using Ordinary One-way ANOVA. No significant difference in lung viral titers was observed between lean and obese infected groups (p-value=0.7377). The assay Limit of Detection (L.O.D) was set at 2.255 Log₁₀PFUs/mL. **D)** Viral N-copies/mL from RNA extracted from lungs of mice at four dpi, was measured by RT-qPCR. **E)** Viral N-copies/mL from RNA extracted from lungs of mice 10 dpi measured by RT-qPCR. Statistical analysis was done on log transformed data using Ordinary One-way ANOVA. No significant difference in "N" gene copies was observed between lean and obese infected groups at four dpi (p-value=0.8241) or 10 dpi (p-value=0.6052). N copies and L.O.D. were calculated using the standard curve generated from "N-gene RNA standard". L.O.D. was at a Cq value of 38 or 2.947 Log₁₀ N copies/mL.

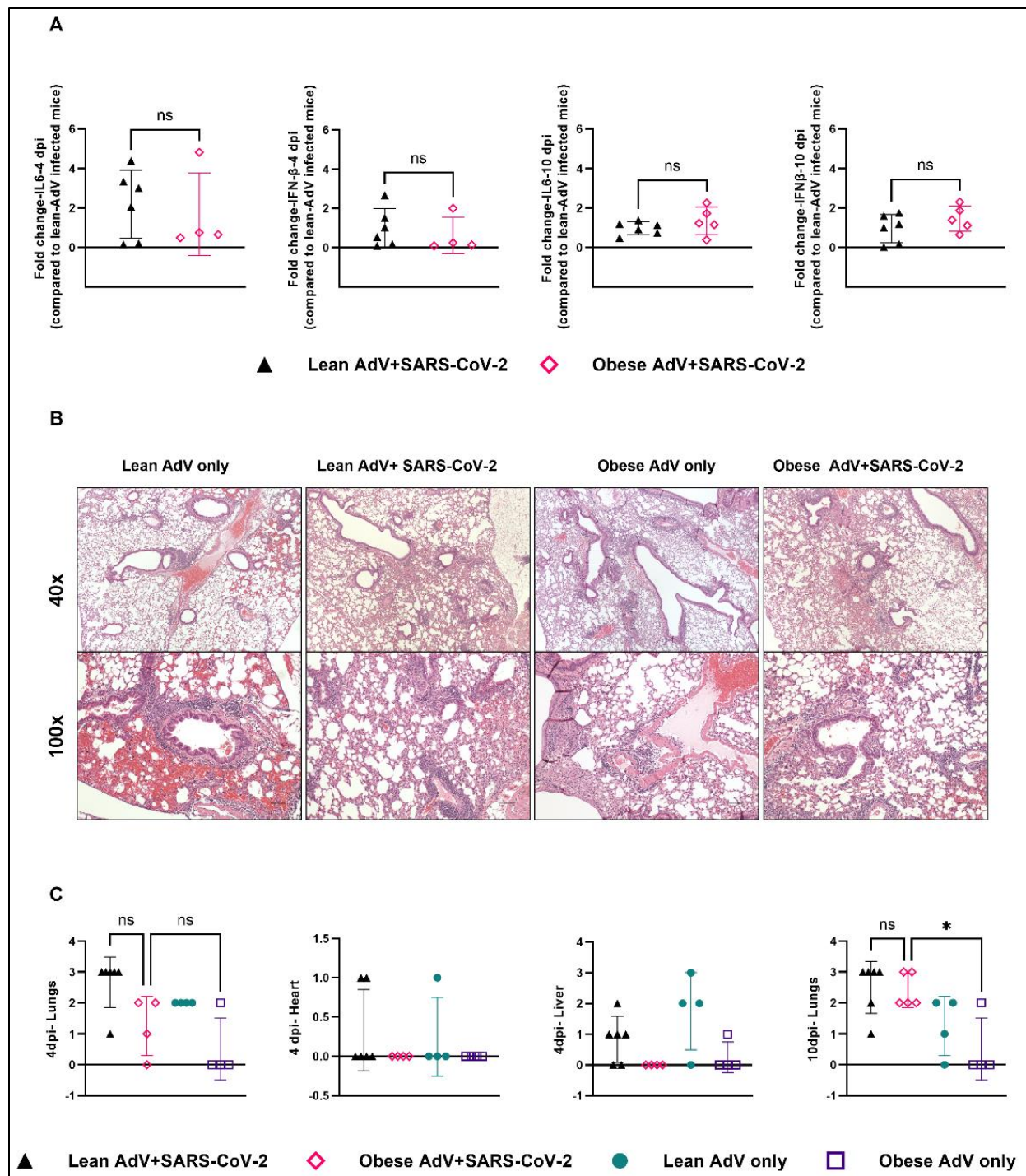


Fig.3: Cytokine mRNA expression and tissue histopathology.

A) Fold change in gene expression of cytokines, IL-6 and IFN- β at four- and 10-days post-SARS-CoV-2 infection, determined by RT-qPCR, compared to mock-infected mice, and

normalized to GAPDH. Statistical analysis was performed by Mann-Whitney test and no significant difference in gene expression was observed between lean and obese groups. **B)** Histopathology slides of the lungs of mice transduced only with AdV-hACE2 and those infected with AdV+SARS-CoV-2 at D4 post-SARS-CoV-2 infection. Images at the top row show a lower magnification (40x) and the bottom row shows medium magnification (100x). No gross pathological differences were observed in lung histopathology between different groups. **C)** Histopathology scores of lungs, heart, and liver four-days post-SARS-CoV-2 infection and for lungs at 10 dpi also. Statistical analysis was done using Ordinary one-way ANOVA with Tukey's multiple comparisons test. No difference in lung pathology was observed in lean groups infected with AdV+SARS-CoV-2 compared to their obese counterparts at four or 10 dpi (p-value=0.0695 and 0.9971 respectively). The difference between obese mice transduced only with AdV-hACE2 and those inoculated with AdV+SARS-CoV-2 was non-significant at four dpi (p-value=0.5679) but became significant at 10 dpi (p-value=0.0187). The error bars denote SD. No significant difference was observed between the groups in terms of histopathology scores of heart and liver.

Abbreviations

COVID-19: Coronavirus disease 2019; DIO: diet-induced obesity; ACE2: Angiotensin-converting enzyme 2; SARS-CoV: severe acute respiratory syndrome coronavirus; SARS-CoV-2: severe acute respiratory syndrome coronavirus-2; MERS: Middle East respiratory syndrome coronavirus; hACE2: human angiotensin-converting enzyme; hACE2-Tg: transgenic mice expressing human ACE2; AdV-hACE2: adenovirus vectors expressing hACE2; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; NEAA: non-essential amino

acids; LFD: low-fat diet; HFD: high-fat diet; BSL-3: biosafety level-3; PFUs: plaque forming units; RPMI: Roswell Park Memorial Institute; ViTALS: Virginia Tech Animal Laboratory Services; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; IL-6: interleukin-6; IFN- β : interferon- β ; L.O.D.: limit of detection; BMI: body-mass index; CHIKV: chikungunya virus; DENV: dengue virus; IFNs: interferons; TNF- α : tumor necrosis factor- α .

2.7 Supplementary Information

Figure S1: Weight gain analysis in males and females on LFD and HFD, over time.

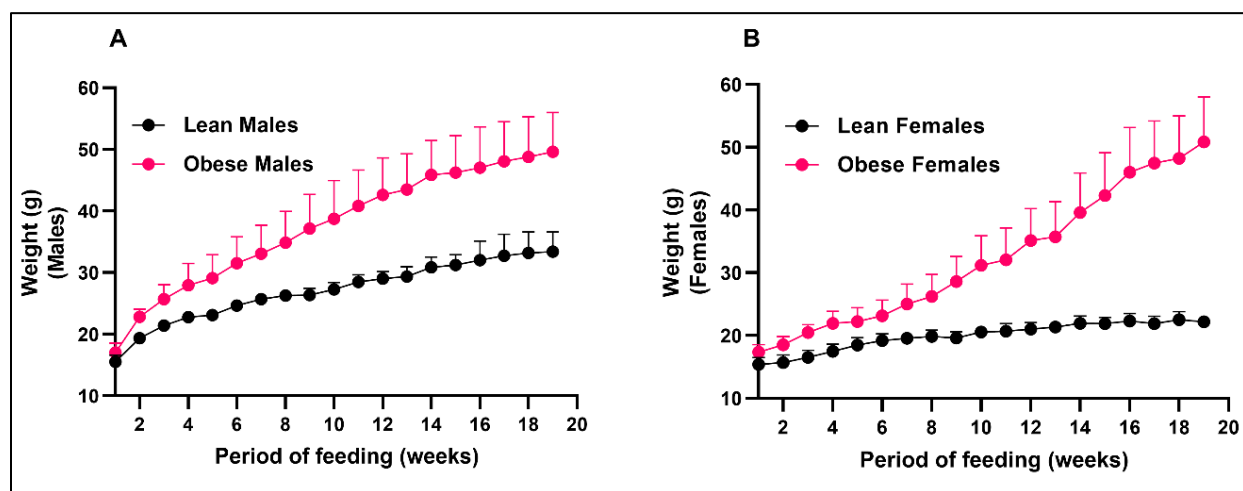


Fig.S1: Weight change in males and females on low and high-fat diet.

A) Weights of C57BL/6N male mice (10/group) fed a high-fat diet (60% fat) or a low-fat diet (10% fat) for 15-20 weeks. Weights were measured weekly after initiating the diets. Statistical analysis was done using 2way-ANOVA with Sidak's multiple comparisons test. Males on a HFD started showing significant weight gain from four weeks of diet initiation (p -value=0.0114) and gradually became more significant (p -value<0.0001) from seven week onwards. **B)** Weights of C57BL/6N female mice (10/group) fed a high-fat diet (60% fat) or a low-fat diet (10% fat) for 15-20 weeks. Weights were measured weekly after initiating the diets. Statistical analysis was

done using 2way-ANOVA with Sidak's multiple comparisons test. Females on a HFD started showing significant weight gain from six weeks of diet initiation (p-value=0.0104) and gradually became more significant (p-value<0.0001) from eight week onwards.

Figure S2: Actual weights and percent weight loss post-AdV inoculation.

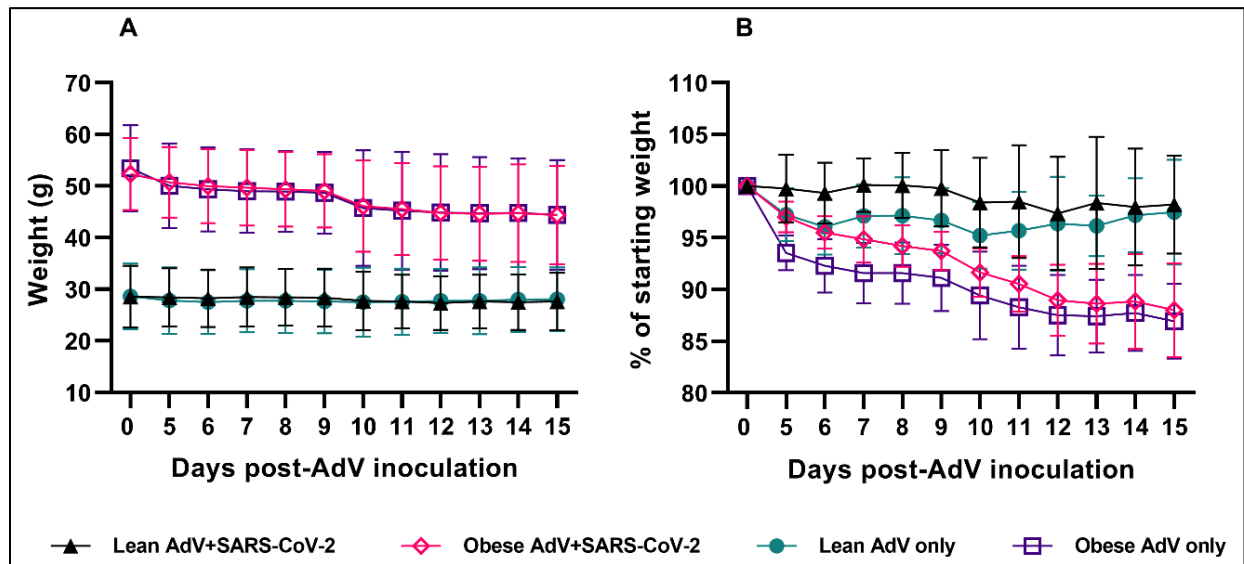


Fig.S2: Weight change (actual) and as percent weight loss post-AdV inoculation.

A) Actual weights of C57BL/6N mice (20/group) post-AdV inoculation (D0), followed by SARS-CoV-2 infection (D5) till the end of experiment (D15). **B)** Weight changes in lean and obese mice inoculated only with AdV vector compared to the ones transduced with the AdV vector and infected with SARS-CoV-2. Statistical analysis was done on the baseline corrected weights, using a 2 way-ANOVA with Dunnett's multiple comparisons test. A significant decrease in weights was observed in the AdV-only obese mice compared to the lean transduced (only) groups starting at seven dpi (p-value=0.0021) and became highly significant by the end of the experiment (p-value<0.0001).

Figure S3: Viral quantification in terms of PFUs/mL and N-genes/mL with all the data points.

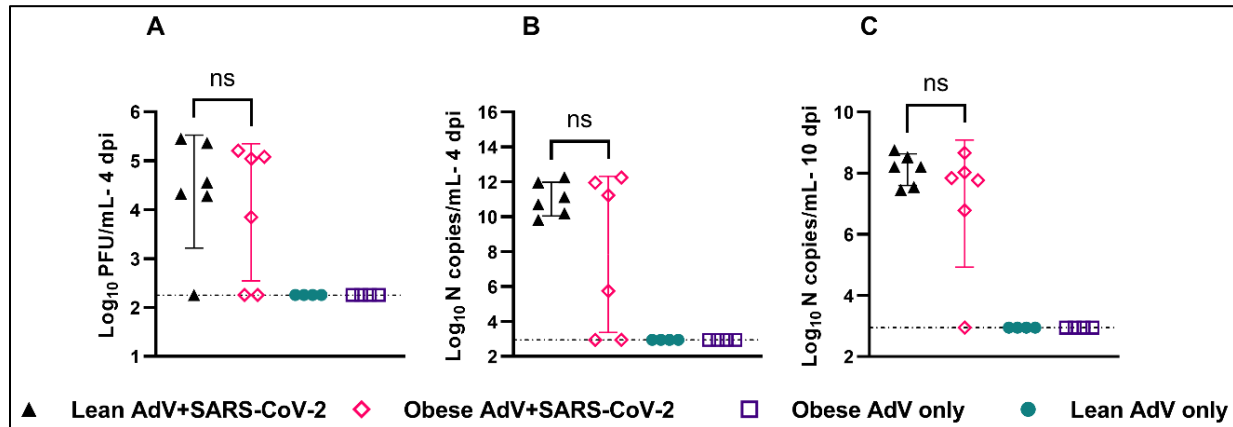


Fig.S3: Viral plaque assay titers and N-gene quantification values for all infected mice.

A) Infectious viral titer in the lungs was determined at four dpi. Statistical analysis was done on log transformed data, using Ordinary One-way ANOVA. No significant difference in lung viral titers was observed between lean and obese infected groups (p-value=0.8224). Samples that tested positive by RT-qPCR, but negative by plaque assays were assigned the L.O.D (2.255 Log₁₀PFUs/mL). **B)** Viral N-copies/mL from RNA extracted from lungs of mice at four dpi, was measured by RT-qPCR. **C)** Viral N-copies/mL from RNA extracted from lungs of mice 10 dpi measured by RT-qPCR. Samples with no Cq value were assigned the L.O.D. value (2.947 Log₁₀ N copies/mL). Statistical analysis was done on log transformed data using Ordinary One-way ANOVA. No significant difference in “N” gene copies was observed between lean and obese infected groups at four dpi (p-value=0.1198) or 10 dpi (p-value=0.2962). N copies and L.O.D. were calculated using the standard curve generated from “N-gene RNA standard”. L.O.D. was at a Cq value of 38 or 2.947 Log₁₀ N copies/mL.

Table S1: Caloric information of diets used for the feeding experiments.

NAME	HIGH FAT DIET (HFD)	LOW FAT DIET (LFD)
CODE	D12492	D12450K
Protein:	20 % Kcal	20 % Kcal
Fat:	60 % Kcal	10 % Kcal
Carbohydrate:	20 % Kcal	70 % Kcal
Energy density:	5.21 Kcal/g	3.82 Kcal/g

Table S2: Primers used for RT-qPCR.

Name	5' → 3' sequence
2019-nCoV_N2 Forward Primer*	TTA CAA ACA TTG GCC GCA AA
2019-nCoV_N2 Reverse Primer*	GCG CGA CAT TCC GAA GAA
2019-nCoV_N2 Probe*	FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-3IABkF
JW589- <i>Mus musculus</i> GAPDH For.	AGGTCGGTGTGAACGGATTG
JW590- <i>Mus musculus</i> GAPDH Rev.	TGTAGACCATGTAGTTGAGGTCA
JW591- <i>Mus musculus</i> IL-6 For.	TAGTCCTTCCTACCCCAATTTCC
JW592- <i>Mus musculus</i> IL-6 Rev.	TTGGTCCTTAGCCACTCCTTC
JW593- <i>Mus musculus</i> IFN- β For.	CAGCTCCAAGAAAGGACGAAC
JW594- <i>Mus musculus</i> IFN- β Rev.	GGCAGTGTA ACTCTTCTGCAT

*Source : <https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html>

2.8 Authors' Contributions

Study concept and design: JWL, PR. Data curation: PR, CC. Formal analysis: PR, JWL. Funding acquisition: JWL. Investigation: PR, CC. Methodology: PR, CC, JWL. Drafting of the manuscript: PR, JWL. Critical revision of the manuscript: all the authors. Statistical analysis: PR, JWL. Histopathology analysis: TLR. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

All animal handling protocols were approved by the Institutional Animal Care and Use Committee (Protocol #20-060) at Virginia Tech. Consent to participate “not applicable” for this study.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Chapter 3: Obesity fosters severe disease outcomes in a mouse model of coronavirus infection associated with transcriptomic abnormalities

Pallavi Rai^{1,2}, Jeffrey M Marano^{2,3}, Lin Kang^{1,4,5}, Sheryl Coutermarsh-Ott¹, Andrea R Daamen⁶,
Peter E Lipsky⁶ and James Weger-Lucarelli^{1,2*}

¹Department of Biomedical Sciences and Pathobiology, Virginia Tech, VA-MD College of Veterinary Medicine, Blacksburg, VA, USA.

²Center for Emerging, Zoonotic, and Arthropod-borne Pathogens, Virginia Tech, Blacksburg, VA, USA.

³Translational Biology, Medicine, and Health Graduate Program, Virginia Tech, Blacksburg, VA, USA.

⁴Biomedical Research, Edward Via College of Osteopathic Medicine, Monroe, LA, USA.

⁵College of Pharmacy, University of Louisiana Monroe, Monroe, LA, USA.

⁶AMPEL BioSolutions LLC, Charlottesville, VA, USA.

Author Note

Pallavi Rai  <https://orcid.org/0000-0001-7109-6132>

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Correspondence concerning this article should be addressed to James Weger-Lucarelli, Ph.D., Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, VA 24060. Email: weger@vt.edu

3.1 Abstract

Obesity has been identified as an independent risk factor for severe outcomes in humans with coronavirus disease 2019 (COVID-19) and other infectious diseases. Here, we established a mouse model of COVID-19 using the murine betacoronavirus, mouse hepatitis virus 1 (MHV-1). C57BL/6 and C3H/HeJ mice exposed to MHV-1 developed mild and severe disease, respectively. Obese C57BL/6 mice developed clinical manifestations similar to those of lean controls. In contrast, all obese C3H/HeJ mice succumbed by 8 days post-infection, compared to a 50% mortality rate in lean controls. Notably, both lean and obese C3H/HeJ mice exposed to MHV-1 developed lung lesions consistent with severe human COVID-19, with marked evidence of diffuse alveolar damage (DAD). To identify early predictive biomarkers of worsened disease outcomes in obese C3H/HeJ mice, we sequenced RNA from whole blood 2 days post-infection and assessed changes in gene and pathway expression. Many pathways uniquely altered in obese C3H/HeJ mice post-infection aligned with those found in humans with severe COVID-19. Furthermore, we observed altered gene expression related to the unfolded protein response and lipid metabolism in infected obese mice compared to their lean counterparts, suggesting a role in the severity of disease outcomes. This study presents a novel model for studying COVID-19 and elucidating the mechanisms underlying severe disease outcomes in obese and other hosts.

Keywords: Diet-induced obesity, mouse hepatitis virus 1, COVID-19, RNA sequencing, differential gene expression, biomarkers.

3.2 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for COVID-19, remains a global epidemiological concern as it continues to give rise to new variants exhibiting increased transmissibility and disease-causing potential^{1,2}. As of 14th January 2024, SARS-CoV-2 was responsible for ~774 million confirmed cases and ~7 million deaths globally and ~103 million confirmed cases and 1.2 million deaths in the U.S. alone³. The clinical manifestations range from mild respiratory symptoms to pneumonia, and in most severe instances, multi-organ failure and death⁴. Several epidemiological studies have shown strong associations between disease severity and co-morbidities, such as hypertension, diabetes, old age, cardiovascular diseases, and obesity⁵⁻¹¹. More severe cases and deaths related to COVID-19 have been associated with macrophage activation¹², caused by a dysregulated host immune response¹³.

Obesity has been identified as an independent risk factor for severe COVID-19¹⁴⁻¹⁸ and has been associated with chronic systemic inflammation^{19,20}. However, most of these inferences are correlative and are based either on data from epidemiological studies^{7,8,11,15,17} or from studies performed in non-natural hosts of SARS-CoV-2^{21,22}. Therefore, a relevant model of coronavirus infection, which mimics SARS-CoV-2 infection in humans, is needed to study the impact of obesity on coronavirus disease outcome. We hypothesized that this model would be useful for identifying biomarkers associated with disease severity in COVID-19 patients, which could have prognostic value for risk stratification of patients with COVID-19.

To this end, we used C57BL/6N and C3H/HeJ mouse models of diet-induced obesity and inoculated them with mouse hepatitis virus 1 (MHV-1), a pneumotropic betacoronavirus which has been previously used to study SARS-CoV-1 infection²³⁻²⁵. We observed more severe disease

in obese C3H/HeJ mice exposed to MHV-1 including increased weight loss, mortality, and tissue pathology. Moreover, RNA sequencing (RNAseq) analysis of whole blood RNA showed commonly altered genes and pathways between MHV-1 infected obese C3H/HeJ mice and humans with severe COVID-19 suggesting the relevance of our mouse model in identifying biomarkers of severe COVID-19 disease outcomes.

3.3 Materials and Methods

Mice and diets

Male and female C57BL/6N mice from Charles River Laboratories (Boston, MA, USA) and C3H/HeJ mice from The Jackson Laboratory (Bar Harbor, ME, USA) were obtained at three to four weeks of age and allowed to acclimatize for a week before initiating diets. Mice were housed in groups of five per cage and maintained at ambient temperature with *ad libitum* supply of food and water. All animal handling protocols were approved by the Institutional Animal Care and Use Committee (Protocol #20-060) at Virginia Tech.

All diets used for the study were obtained from Research Diets (New Brunswick, NJ, USA). Males and females of both strains were randomly divided and half of them were given a low-fat diet with 10% kcal fat (LFD; D12450K), while the other half were given a high-fat diet with 60% kcal fat (HFD; D12492). Throughout the manuscript, we will refer to the groups as follows: lean (low fat diet) or obese (high fat diet). The mice were kept on these diets for 17-18 weeks before infections, and the same diets were continued until the end of the experiment.

Glucose levels

Two weeks prior to MHV-1 inoculations (16 weeks after diet initiation), mice bled via submandibular bleeds and non-fasting glucose levels were measured at 9-10 am using the Abox glucose monitoring kit.

Mouse infections and health monitoring

Mice were transferred to disposable cages from Innovive (San Diego, CA, USA) and moved to a separate room, 3-4 days before MHV-1 infection. MHV-1 was a kind gift from Dr. Susan Compton and was propagated in 17Cl1 cells; infectious titers were determined by plaque assays on L2 Percy cells. Mice were anesthetized with ketamine and xylazine (90 mg/kg and 5 mg/kg respectively) intraperitoneally and then inoculated intranasally with 3×10^4 plaque-forming units (PFUs) of the virus in 50 μ L of Roswell Park Memorial Institute (RPMI)-1640 media with no additives or only 50 μ L of RPMI-1640 (mock-infection). Mice were monitored for visual symptoms of disease like inactivity, weight loss and mortality. They were euthanized at 8 dpi or when they crossed the threshold of weight loss (20% of weights at 0 dpi), by injecting ketamine and xylazine (90 mg/kg and 10 mg/kg respectively) intraperitoneally followed by cervical dislocation. For histopathology, the left lung lobe was perfused with and stored in 4% formaldehyde for at least a week. We also collected sections of the liver, spleen, and small intestine for histopathological analyses. The Virginia Tech Animal Laboratory Services (ViTALS) performed paraffin embedding, sectioning and hematoxylin-eosin staining, and a board-certified pathologist scored the slides in a blinded manner.

RNA extraction from whole blood

Mice were anesthetized with ketamine and xylazine (90 mg/kg and 5 mg/kg respectively) intraperitoneally and 100 μ L of whole blood was collected via submandibular cheek bleeds, in EDTA coated tubes. We added 300 μ L of TRIzol LS reagent (ThermoFisher) to each tube and total RNA was extracted using the manufacturer's protocol and stored at -80°C until use.

RNA sequencing and pathway enrichment data analysis

RNA from the whole blood of 16 MHV-1- and 16 mock-infected C3H/HeJ mice were submitted to BGI (Cambridge, MA, USA) to perform library prep and sequencing using their DNBseq platform. RNA quality was assessed using Nanodrop 260/280 and 260/230 ratios and raw reads were generated as Fastq files. We performed quality control on the raw reads using FastQC²⁶ and trimmed them using BBduk²⁷. The mouse reference genome (GRCm39) and corresponding annotations were downloaded from National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). The clean reads were mapped to the reference genome using the Spliced Transcripts Alignment to a Reference (STAR) with default parameters²⁸. HT-Seq (htseq-count) within the STAR package was used to estimate gene counts²⁹. Differential gene expression analysis was conducted using DESeq2³⁰, and Gene Set Enrichment Analysis (GSEA) was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We employed the integrated differential expression and pathway analysis tool (iDEP 0.96, South Dakota University, SD, USA)³¹ for these analyses, setting the default parameters to a p-adjusted value of <0.1 and fold change of >2 for identifying differentially expressed genes. The expression patterns of identified biomarker genes were plotted with gene plot feature in iDEP 2.0 using raw counts and standard deviation.

Gene set variation analysis (GSVA)

The R/Bioconductor software package GSVA³² was used as a non-parametric, unsupervised method to estimate enrichment of immune cell and pathway gene sets in RNAseq data as previously described^{12,33,34}. Gene sets used as input for GSVA can be found in Supplementary Table 2.

Statistical analysis

Statistical and regression analyses were done using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Details of the statistical technique used for the analyses are provided in the figure legends. The level of significance has been determined by the following p-values: $p \geq 0.05$ (ns); $p = 0.05$ to 0.01 (*); $p = 0.01$ to 0.001 (**); $p = 0.001$ to 0.0001 (***); $p < 0.0001$ (****). The error bars represent standard deviation from the mean and the dotted lines denote the limit of detection (L.O.D.). All statistical analyses were performed on data after testing them for normality using the Shapiro-Wilk test.

3.4 Results and Discussion

C3H/HeJ mice fed a high fat diet develop diet-induced obesity (DIO) similar to C57BL/6N mice. Based on previous literature and preliminary studies, we identified C57BL/6N and C3H/HeJ mice as resistant and susceptible models, respectively, to mouse hepatitis virus 1 (MHV-1)^{35,36}. C57BL/6N mice are an established model of diet-induced obesity (DIO)³⁷ and experience mild disease following MHV-1 infection³⁸. Numerous studies have demonstrated that C3H/HeJ mice—which experience severe disease following MHV-1 exposure—exhibit resistance to DIO³⁹⁻⁴¹. Conversely, other research has indicated that they exhibit dietary responses similar to C57BL/6N mice⁴². Therefore, to establish a model of obesity for studying its impact on MHV-1 infection, we

used both mouse strains. C57BL/6N and C3H/HeJ mice fed a high-fat diet (HFD) had significantly higher weights compared to those on a low-fat diet (LFD) from 2-weeks-post-diet-initiation (Fig.1A & B, $**p=0.0010$ and $**p=0.0030$ respectively), which gradually became more significant by 18 weeks (Fig.1A & B, $****p<0.0001$). Non-fasting glucose levels at 16-weeks-post-diet-initiation were significantly higher in obese mice compared to their lean counterparts in both, C57BL/6N and C3H/HeJ mice (Fig.1C & D, $***p<0.0001$ and $***p=0.0005$). This 18-week diet regimen produced C57BL/6N and C3H/HeJ mice that mirrored obesity and hyperglycemia in humans and, therefore, were appropriate to study the effect of obesity on coronavirus infection.

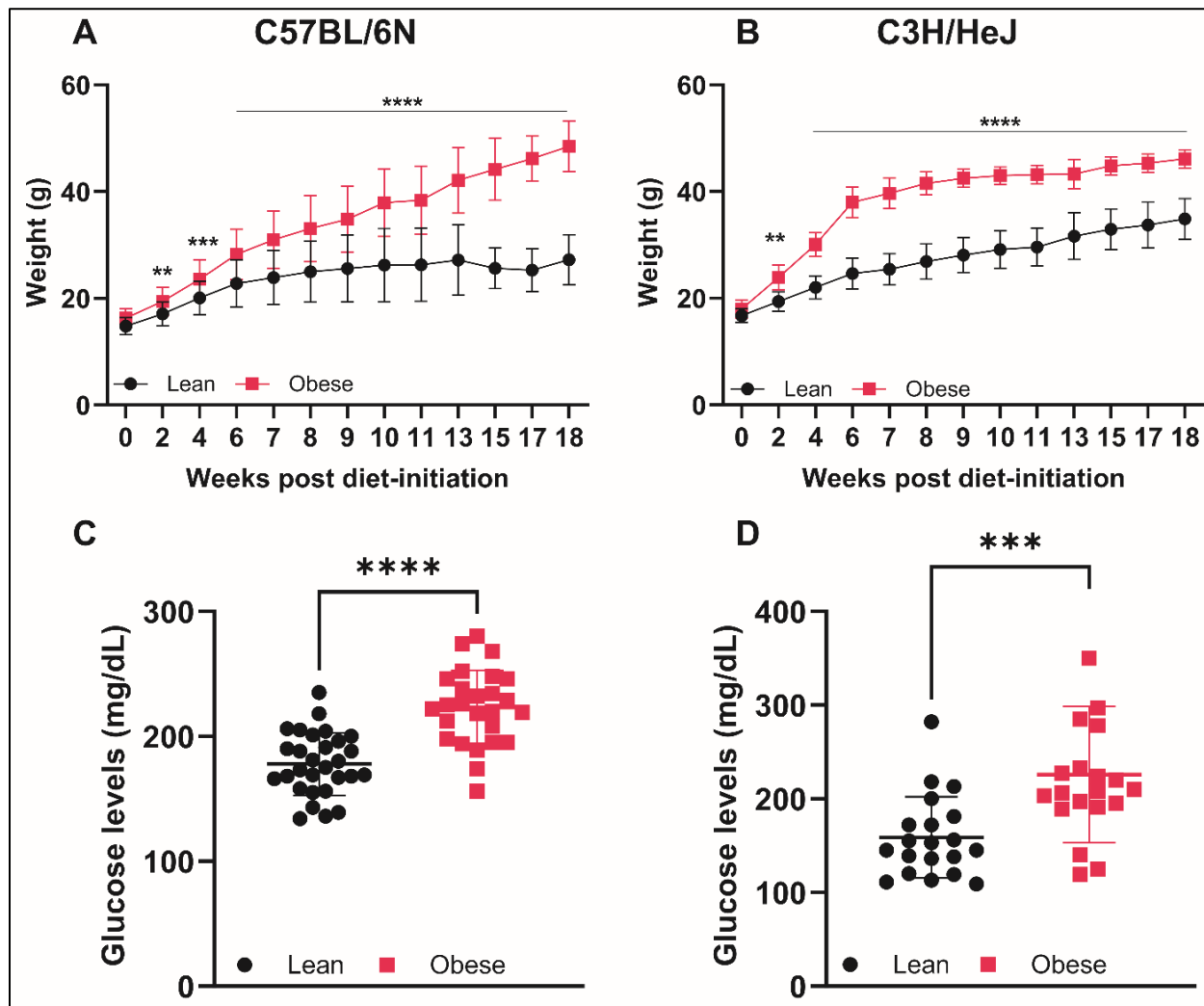


Fig.1: C57BL/6N and C3H/HeJ mice on a high fat diet develop diet-induced obesity (DIO).

Four-week-old C57BL/6N (n=22-23) and C3H/HeJ (n=14-20) were fed a HFD (60% fat) or a LFD (10% fat) for 18 weeks. **(A & B) Weight gain in lean and obese C57BL/6N and C3H/HeJ mice respectively.** Weights were measured throughout the feeding period and statistical analysis was carried out using mixed effects analysis with Sidak's multiple comparisons test. **(C & D) Glucose levels in lean and obese C57BL/6N and C3H/HeJ mice respectively.** Two weeks before infection (16-weeks-post-diet-initiation), blood was collected by submandibular cheek bleeds and non-fasting glucose levels were measured. Statistical analysis was done using an unpaired t-test.

The level of significance is represented as follows: $p < 0.001$ (**); $p = 0.001$ to 0.0001 (***); $p < 0.0001$ (****). The error bars indicate the standard deviation (SD) of the mean.

Obese C3H/HeJ mice exposed to MHV-1 experience more severe disease outcomes compared to C57BL/6N mice. Since its outbreak in 2019, severe COVID-19 outcomes have been strongly and independently correlated with obesity⁴³, with severe obesity leading to 3.6 times more ICU admissions¹⁷. Obesity significantly increased the risk of mortality in young COVID-19 patients¹¹ and has been shown in COVID-19 patients across the world⁴⁴⁻⁴⁶. To determine the impact of obesity on disease severity and identify an appropriate mouse model for studying coronavirus pathogenesis and biomarker discovery, lean and obese C57BL/6N and C3H/HeJ mice were inoculated intranasally with 3×10^4 PFUs of MHV-1 and monitored for weight loss, mortality, and other signs of illness until 8 days-post-infection (dpi) (Fig.2A) when mice were euthanized to collect tissues for histopathology.

Obese C57BL/6N mice inoculated with MHV-1 showed significantly greater weight loss compared to their lean counterparts at 2 and 8 dpi (Fig.2B, $**p=0.0029$; $*p=0.0483$, respectively). More strikingly, obese C3H/HeJ mice showed significantly higher weight loss at 1, 2, 6 and 7 dpi (Fig.2C, $***p=0.0004$, $**p=0.0018$, $*p=0.0472$ and $*p=0.0290$, respectively). There was no difference in the survival up to 8 dpi between lean and obese C57BL/6N mice (Fig.2D); in contrast, obese C3H/HeJ mice had 100% mortality by 8 dpi (Fig.2E, $***p=0.0002$) compared to 50% mortality in lean mice. The significant weight loss and mortality rates in our obese C3H/HeJ mice inoculated with MHV-1, therefore, mirror the morbidity and mortality observed in obese humans

suffering from COVID-19, suggesting the relevance of our infection model to study COVID-19 in the context of obesity.

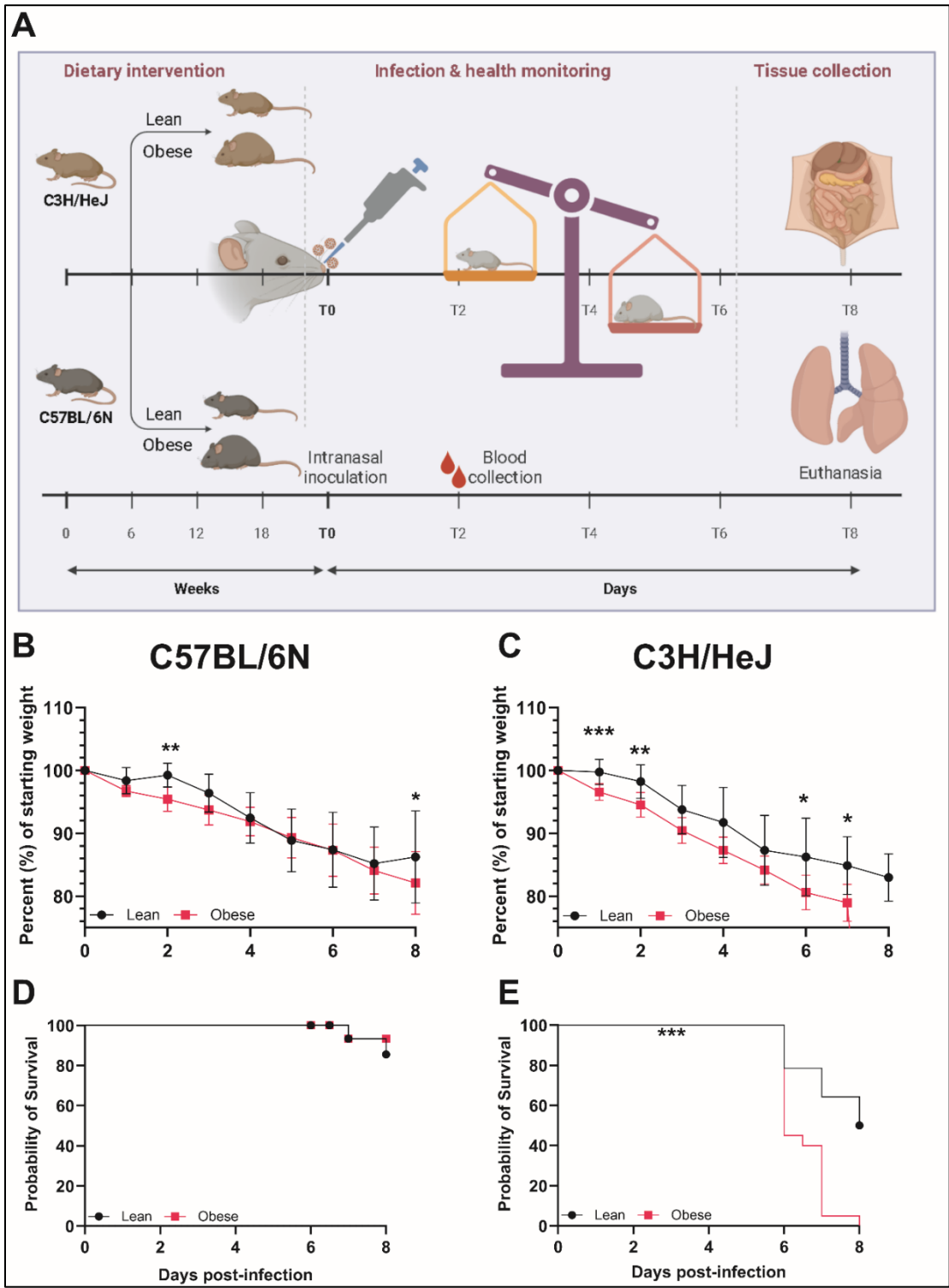


Fig.2: Obese C3H/HeJ mice exposed to MHV-1 have worse disease outcomes compared to C57BL/6N mice.

(A) Experimental design. Lean and obese C57BL/6N (n=20-25) and C3H/HeJ (n=15-20) mice were intranasally inoculated with 3×10^4 PFUs of MHV-1 at 18-weeks-post-diet-initiation (day 0). The mice were bled at 2 dpi and monitored for signs of illness, weight loss and mortality until 8 dpi. At 8 dpi, the remaining mice were euthanized, and tissues were collected for histopathology.

(B & C) Weight changes in lean and obese C57BL/6N (B) and C3H/HeJ (C) mice. Weights were measured daily, and statistical analysis was done on baseline corrected weights using mixed effects analysis with Sidak's multiple comparisons test.

(D & E) Survival rates in lean and obese C57BL/6N (D) and C3H/HeJ (E) mice. Infected lean and obese mice were monitored until 8 dpi. Mortality was recorded if a mouse succumbed to infection or reached criteria for humane euthanasia (20% weight loss compared to weights at 0 dpi). Statistical analysis was done using Log-rank (Mantel-Cox) test. The level of significance is represented as follows: $p < 0.05$ (*); $p < 0.001$ (**); $p = 0.001$ to 0.0001 (***); $p < 0.0001$ (****). The error bars indicate standard deviation (SD) of the mean. The studies were carried out as two independent replicates each for C3H/HeJ and C57BL/6N mice.

We next sought to determine the impact of MHV-1 infection on tissue pathology by examining the lungs and liver of C57BL/6N and C3H/HeJ mice that had been euthanized with more than 20% weight loss or at 8 dpi when the study was terminated (Fig.2A). Lung damage was assessed by identifying and semi-quantitatively scoring inflammation involving the alveolar septa, alveolar space, and surrounding vessels as well as the presence and degree of type II pneumocyte

hyperplasia. Liver damage was assessed by identifying and semi-quantitatively scoring hepatocyte vacuolation, hepatocyte necrosis, and overall liver inflammation. The individual parameters were then summed to obtain a composite histopathologic score for each organ. In C57BL/6N mice, no differences were observed between infected lean and obese mice in either tissue (Fig.3A & B). In C3H/HeJ mice, no differences were observed in the lungs of infected obese and lean mice (Fig.3C), whereas the composite liver scores were significantly higher in obese compared to lean mice (Fig.3D, * $p=0.0260$).

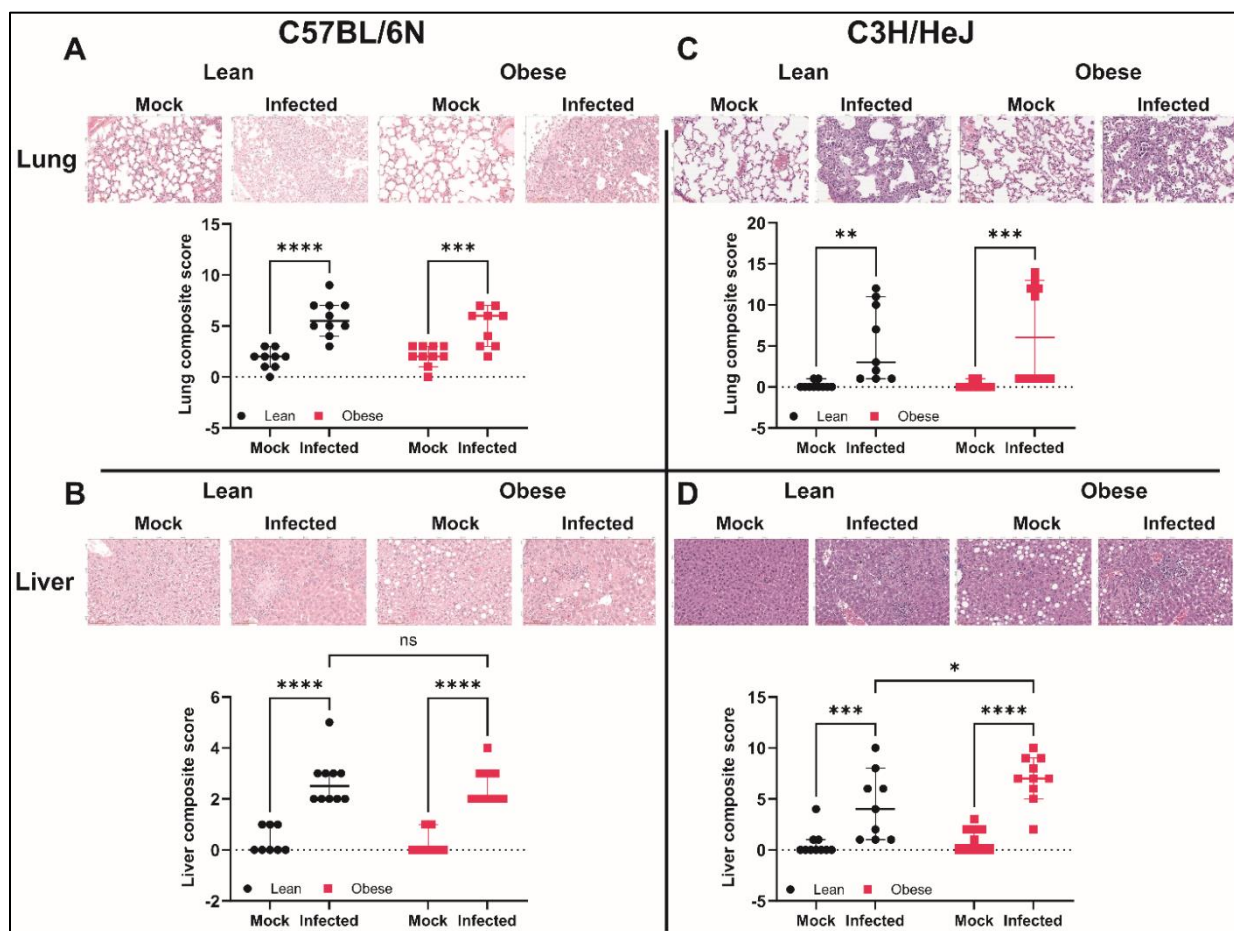


Fig.3: MHV-1 infection causes severe liver pathology in obese C3H/HeJ mice.

Lean and obese C57BL/6N and C3H/HeJ mice exposed to MHV-1 were euthanized at 8 dpi or when they crossed the threshold of weight loss (20% of the baseline), and tissues were collected

for histopathological analysis. H&E-stained images were captured at 20X, scale bar = 100 μ m.

Lung (A) & liver (B) histopathology of C57BL/6N mice. Lungs (top image) and livers (bottom image) from mock and MHV-1 infected lean and obese C57BL/6N mice, showed no significant difference in histopathological lesions between both the groups. **Lung (C) & liver (D) histopathology of C3H/HeJ mice.** Lungs (top image) and livers (bottom image) from mock and MHV-1 infected lean and obese C3H/HeJ mice were examined. Statistical analysis was carried out by 2way ANOVA. The level of significance is represented as follows: $p < 0.05$ (*); $p < 0.001$ (**); $p = 0.001$ to 0.0001 (***); $p < 0.0001$ (****), ns = non-significant. The error bars indicate SD of the mean.

In severe cases of COVID-19, lung damage is a driving factor responsible for most patient fatalities⁴⁷ and diffuse alveolar damage (DAD) is a common histological finding in patients with severe COVID-19⁴⁸⁻⁵¹. DAD represents injury to type I pneumocytes or endothelial cells in the alveolar septa and is characterized by inflammation in the alveolus and alveolar septa and type II pneumocyte hyperplasia⁵²⁻⁵⁴. Although we observed no differences in composite lung pathology between lean and obese infected groups (Fig.3A & C), we did identify type II pneumocyte hyperplasia and inflammation of alveoli and alveolar septa in C57BL/6N and C3H/HeJ (Fig.4) mice, consistent with DAD, confirming the validity of our MHV-1 infection models for studying COVID-19 pathogenesis.

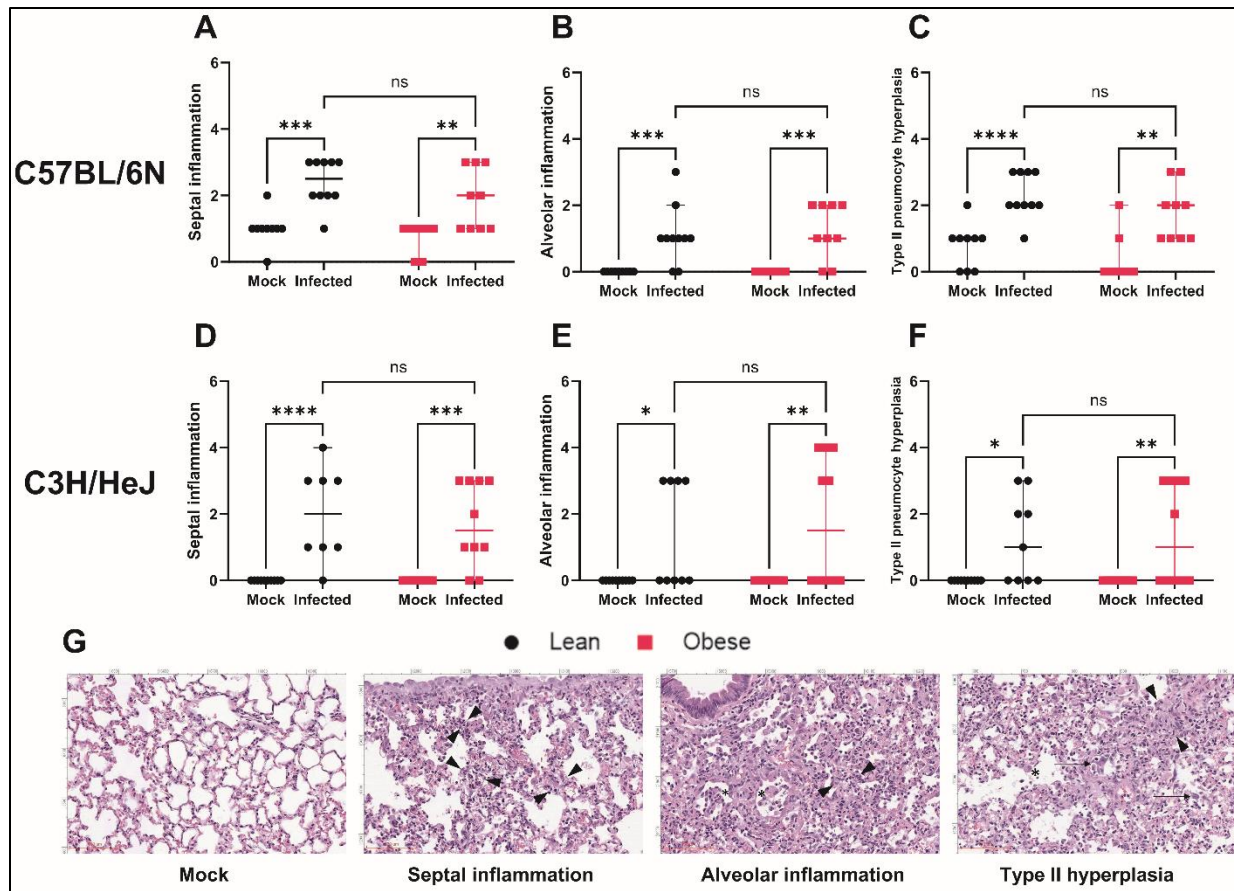


Fig.4: MHV-1 infection causes diffuse alveolar damage (DAD) in the lungs of C57BL/6N and C3H/HeJ mice.

Lungs of MHV-1 and mock-infected C3H/HeJ were collected for histopathological analysis. H&E-stained images were captured at 20X, scale bar = 100 um. Semi-quantitative histopathology scores showed significant inflammation of the septa and alveoli, along with the presence of type II pneumocyte hyperplasia, in MHV-1 infected compared to mock-infected C57BL/6N (A-C) and C3H/HeJ (D-F) mice. Statistical analysis was carried out by 2way ANOVA. **(G) Representative lung histopathology images from C3H/HeJ mice depicting diffuse alveolar lung damage (DAD).** Lungs of MHV-1 infected mice show alveolar and septal inflammation, along with type II pneumocyte hyperplasia compared to mock-infected mice. The level of significance is

represented as follows: $p < 0.05$ (*); $p < 0.001$ (**); $p = 0.001$ to 0.0001 (***); $p < 0.0001$ (****), ns = non-significant. The error bars indicate SD of the mean.

Pathways and genes altered in obese C3H/HeJ mice exposed to MHV-1 are similar to those identified in severe COVID-19 patients. COVID-19 is a complex multisystem disorder involving vast interactions of immune and inflammatory pathways⁵⁵. Identifying potential disease-associated biomarkers may be useful to predict the outcome of COVID-19 in humans to assess risk levels to enable timely medical intervention. In this study, we hypothesized that blood would be an easily accessible clinical sample to monitor the complex transcriptional changes occurring early during the course of COVID-19. Therefore, we carried out RNAseq on blood from MHV-1 and mock-infected lean and obese C3H/HeJ mice at 2 dpi.

First, we carried out principal component analysis (PCA) to assess the clustering of the samples. We observed a clear separation between MHV-1 and mock-infected groups in lean and obese C3H/HeJ mice; in contrast, we observed no obvious clustering for lean and obese groups (Fig.5A). Differential gene expression (DEG) analysis revealed 2635 upregulated and 863 downregulated genes in MHV-1 vs mock-infected lean mice (Supplementary Fig.S1-A) and 2089 upregulated and 681 downregulated genes in MHV-1 vs mock-infected obese mice (Supplementary Fig.S1-B). Pathway analysis was initially carried out by Gene Set Enrichment Analysis (GSEA) using Kyoto Encyclopedia of Genes and Genomes (KEGG) for identifying canonical pathways that were up or downregulated in MHV-1 infected vs mock lean (Supplementary Fig.S1-C) and obese mice (Supplementary Fig.S1-D). To account for the effect of mock infection on gene expression, we removed the genes expressed in mock-infected lean or

obese mice from their MHV-1 infected counterparts and then identified unique DEGs in MHV-1 infected obese vs lean mice. There were 177 upregulated and 334 downregulated genes that were unique to MHV-1 infected obese mice compared to the infected lean mice, after adjusting for the DEG expression in their respective mocks and this list was used for generating pathways using the ShinyGO 0.77 analysis tool. These pathways were then compared to those identified in severe vs mild cases of COVID-19 (generated from data obtained from Wang et al.⁵⁶) and we found numerous upregulated (Fig.5B) and downregulated (Fig.5C) pathways shared between MHV-1 infected obese vs lean C3H/HeJ mice and human patients with severe vs mild COVID-19. The common upregulated pathways included Tumor necrosis factor (TNF) signaling, NOD-like receptor (NLR) signaling, platelet activation, Neutrophil extracellular trap (NET) formation, and Rap1 signaling pathways; while the common downregulated pathways included T cell receptor signaling pathway, Th1, Th2, Th17 cell-differentiation, inflammatory bowel disease, hematopoietic cell lineage, Natural Killer (NK) cell mediated cytotoxicity and primary immunodeficiency pathways.

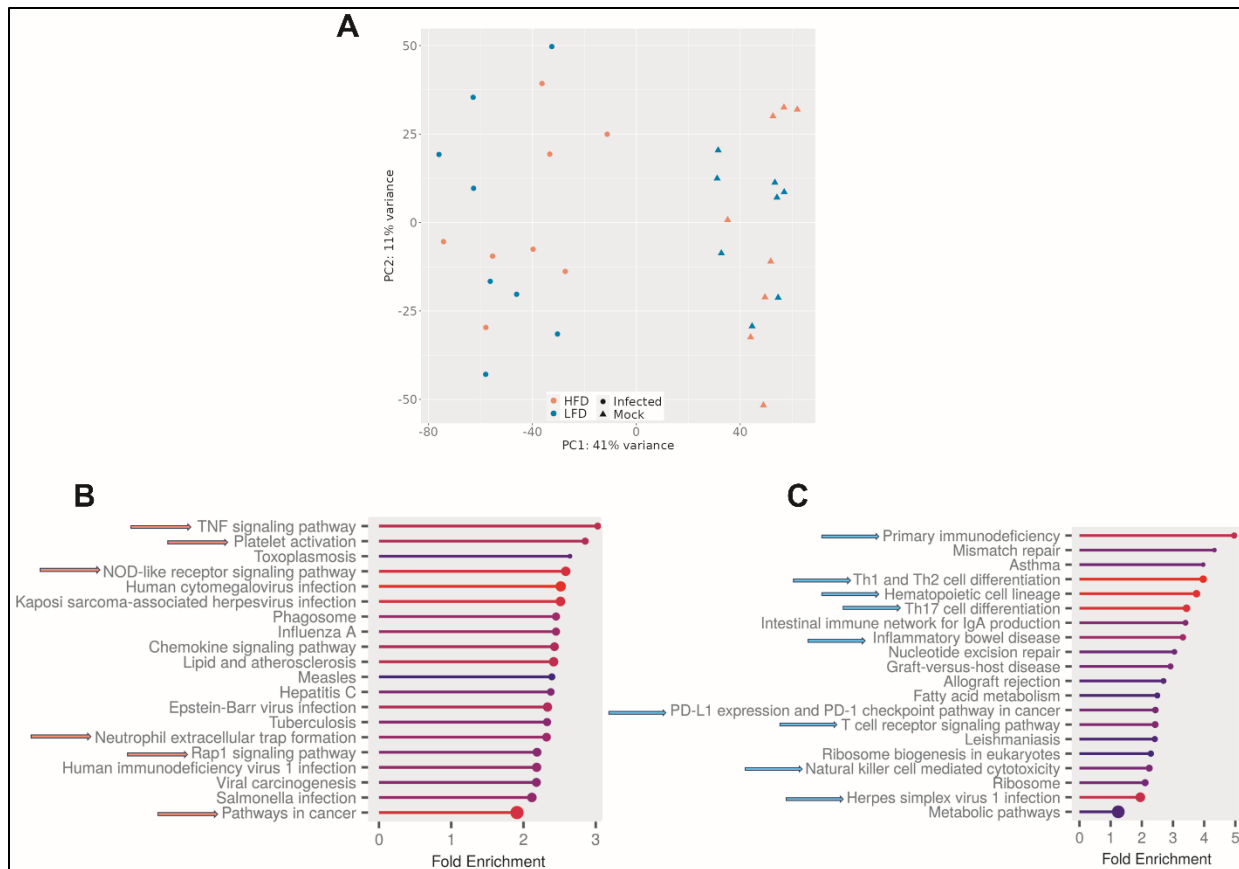


Fig.5: Similarities in gene expression and pathways in MHV-1 infected obese vs lean mice and severe vs mild COVID-19 patients.

RNAseq was performed on the RNA collected from blood of MHV-1 and mock-infected lean and obese C3H/HeJ mice (n = 8 per group) at 2 dpi. **(A) Principal component analysis (PCA) of MHV-1 and mock-infected lean and obese mice.** PC1 and PC2 were plotted to identify clustering of the MHV-1 and mock-infected samples. For identification of DEGs, genes with p-adjusted values < 0.1 and $\log_2\text{FoldChange} > 1$, were selected by integrated Differential Expression and Pathway (iDEP .96) analysis tool. **Upregulated (B) and downregulated (C) pathways unique to MHV-1 infected obese vs lean mice.** For this comparison, gene expression in mock-infected lean or obese mice was removed from their MHV-1-infected counterparts and the resulting data was compared between obese and lean mice to identify DEGs unique to obese infected mice. For

pathway analysis, DEGs with p-adjusted values < 0.1 and $\log_2\text{FoldChange} > 1$ were selected and pathways were generated using the ShinyGO 0.77 analysis tool. The pathways obtained were then compared to data from the blood of COVID-19 patients with severe disease; orange arrows indicate common upregulated and blue arrows indicate common downregulated pathways between MHV-1 infected obese vs lean mice and severe vs mild COVID-19 patients.

TNF has been shown to damage the respiratory epithelium and stimulate the synthesis of fibrin and collagen in the respiratory system^{57,58}. Although the direct role of TNF in severe COVID-19 has not yet been established, it has been associated with heart failure⁵⁹, blood clotting⁶⁰⁻⁶³, and is elevated in patients with obesity⁶⁴ and hypertension⁶⁵, all of which are risk factors for severe COVID-19. The NLR signaling pathway could potentially be linked to COVID-19 related multiple sclerosis (MS)⁶⁶ considering that the NLRP3 inflammasome has been implicated in COVID-19 pathogenesis⁶⁷. Platelet abnormalities in COVID-19 patients have been associated with disease severity and mortality⁶⁸ related to organ failure⁶⁹. The underlying mechanisms leading to severe COVID-19 symptoms, such as acute respiratory distress syndrome (ARDS), cytokine storm and thrombotic events, have been associated with neutrophils, particularly increased formation of neutrophil extracellular traps (NETs)⁷⁰. Excessive NET formation in SARS-CoV-2 infection has been associated with the onset of acute lung injury (ALI)⁷¹. The Rap1 signaling pathway has been implicated in the pathogenesis of respiratory, cardiovascular, and nervous system and some studies have shown that activation of the Rap1 signaling pathway can improve the symptoms of pulmonary fibrosis⁷².

T cells are recognized as potent immune modulators against various infections by activating cytotoxic or humoral immune-mediated reactions and are mainly divided into T helper

type 1 (Th1), T helper type 2 (Th2), T helper type 17 (Th17) and regulatory T (Treg) cells⁷³. The equilibrium between Th1 and Th2 responses has been linked to COVID-19 outcomes, with an adequate Th1 response being associated with good prognosis and Th2 activation being linked to worse disease outcomes^{74,75}. Th17 has been associated with chronic inflammation and autoimmune diseases and may be involved in SARS-CoV-2 induced pneumonia⁷⁶. Similarly, the other downregulated pathways involved hematopoietic cell lineage, NK cell-mediated cytotoxicity and primary immunodeficiency, suggesting lowered lymphocyte immune responses in MHV-1-infected mice and COVID-19 patients.

We also compared expression of individual DEGs unique to MHV-1 infected obese vs. lean mice with expression patterns in severe vs mild COVID-19 patients and found 20 upregulated and 23 downregulated genes shared between MHV-1 and COVID-19 (Supplementary Table 1). In particular, MHV-1 infected obese mice exhibited upregulation of *B4galt5*⁷⁷, *Flt1*⁷⁸, *Stx3*⁷⁹, and *Ass1*⁸⁰ and downregulation of *Crip2*⁷⁷ and *Rora*⁸¹ changes (Supplementary Fig.S2) which have also been associated with severe disease outcomes in COVID-19 patients. Strikingly, the similarities observed in the immune pathways and gene expression patterns in our MHV-1 infected obese vs lean mice suggest that our coronavirus infection model mimics COVID-19 in humans and could be a potentially relevant model to identify biomarkers of severe disease outcome in SARS-CoV-2 infected individuals. However, future mechanistic studies are needed to elucidate the precise roles of these biomarkers in coronavirus disease outcomes.

Since MHV-1 infection produced DAD in the lungs of C3H/HeJ mice associated with severe mortality (Fig.4), we next sought to determine the differential gene expression patterns between MHV-1- and mock-infected C3H/HeJ mice. We found 3135 upregulated and 872

downregulated genes in MHV-1 compared to mock-infected mice (Fig.S3). A recent study found high levels of fibroblast markers, such as collagen type 1 alpha 1 (Col1a1) and collagen type 1 alpha 2 (Col1a2), associated with organizing pneumonia (OP) in the lungs of patients with severe COVID-19 compared to healthy individuals⁸². Consistent with this, MHV-1-infected C3H/HeJ mice showed significantly upregulated Col1a1, Col1a2, along with other variants of collagen like Col4a3, Col6a2, Col6a3, Col12a1, Col13a1, Col9a1, Col9a3, Col24a1, Col25a1, and Col26a1 (Supplementary file 1) compared to mock-infected controls. Upregulation of Col1a1 and Col1a2 was associated with excessive fibrosis in the lungs of COVID-19 patients,⁸² which is a characteristic feature of the proliferative or 2nd stage of DAD⁸³ and similar findings in our MHV-1 infection model further support the relevance of our infection model for biomarker discovery.

Gene Set Variation Analysis (GSVA) showed significant differences between MHV-1 infected lean and obese C3H/HeJ mice. We further utilized our RNAseq data to carry out Gene Set Variation Analysis (GSVA) and compared the impact of MHV-1 infection and obesity on differential enrichment of disease-relevant immune cell, inflammatory, and cellular pathway gene signatures^{12,33,84} (Fig.6A & B). Several immune cell type signatures associated with COVID-19 patient disease pathology were also changed in MHV-1 infected mice, including enrichment of inflammatory neutrophils, low density granulocytes (LDGs), monocytes, and activated B cells as well as de-enrichment of T cells (Fig.6A). Pathway gene signatures representing response to inflammatory cytokines, such as interferons (IFN) and TNF, as well as metabolic pathways, including glycolysis and lipid metabolism were also elevated in infected mice (Fig.6B). Gene expression changes associated with obesity in the context of infection were much more subtle. However, we did find that the unfolded protein and lipid metabolism gene signatures were significantly increased in obese mice (Fig.6B). Unfolded protein response (UPR) has been linked

to high endoplasmic reticulum (ER) stress induced by significantly higher replication rates, with SARS-CoV-2 and MHV⁸⁵. Activated ER stress and UPR trigger endothelial dysfunction⁸⁶ which, in turn, has been implicated in the development of severe COVID-19 manifestations including ARDS⁸⁷ and pulmonary fibrosis⁸⁸. Dysregulated lipid metabolism, particularly triglycerides, were associated with severe COVID-19 outcomes⁸⁹. Lipids are important in coronavirus replication cycle by providing double membrane vesicles (DMVs)^{90,91} which are critical for its replication. Activation of UPR and lipid metabolism in obese C3H/HeJ mice in response to MHV-1 mimics similar responses observed in severe COVID-19, suggesting that these pathways might be crucial in COVID-19 pathogenesis.

In order to relate disease pathology with changes in gene expression, we carried out linear regression analysis using the inflammatory neutrophil gene signature, which was derived from gene expression studies of severe COVID-19 patients⁹², and clinical features. This revealed a significant correlation between the inflammatory neutrophil GSVA enrichment score and weight loss as well as lung and liver tissue pathology scores (Fig.6C-E) suggesting inflammatory neutrophils may also be contributing to disease severity in MHV-1 infected mice. In contrast, the activated B cell gene signature was significantly correlated with overall weight loss (Fig.6F), but not tissue pathology scores (Fig.6G-H) indicating that inflammatory neutrophils and not B cells were associated with tissue damage in infected mice. Overall, we have demonstrated the utility of MHV-1 infected C3H/HeJ mice as a model for human COVID-19 as well as the effect of diet-induced obesity on disease pathogenesis.

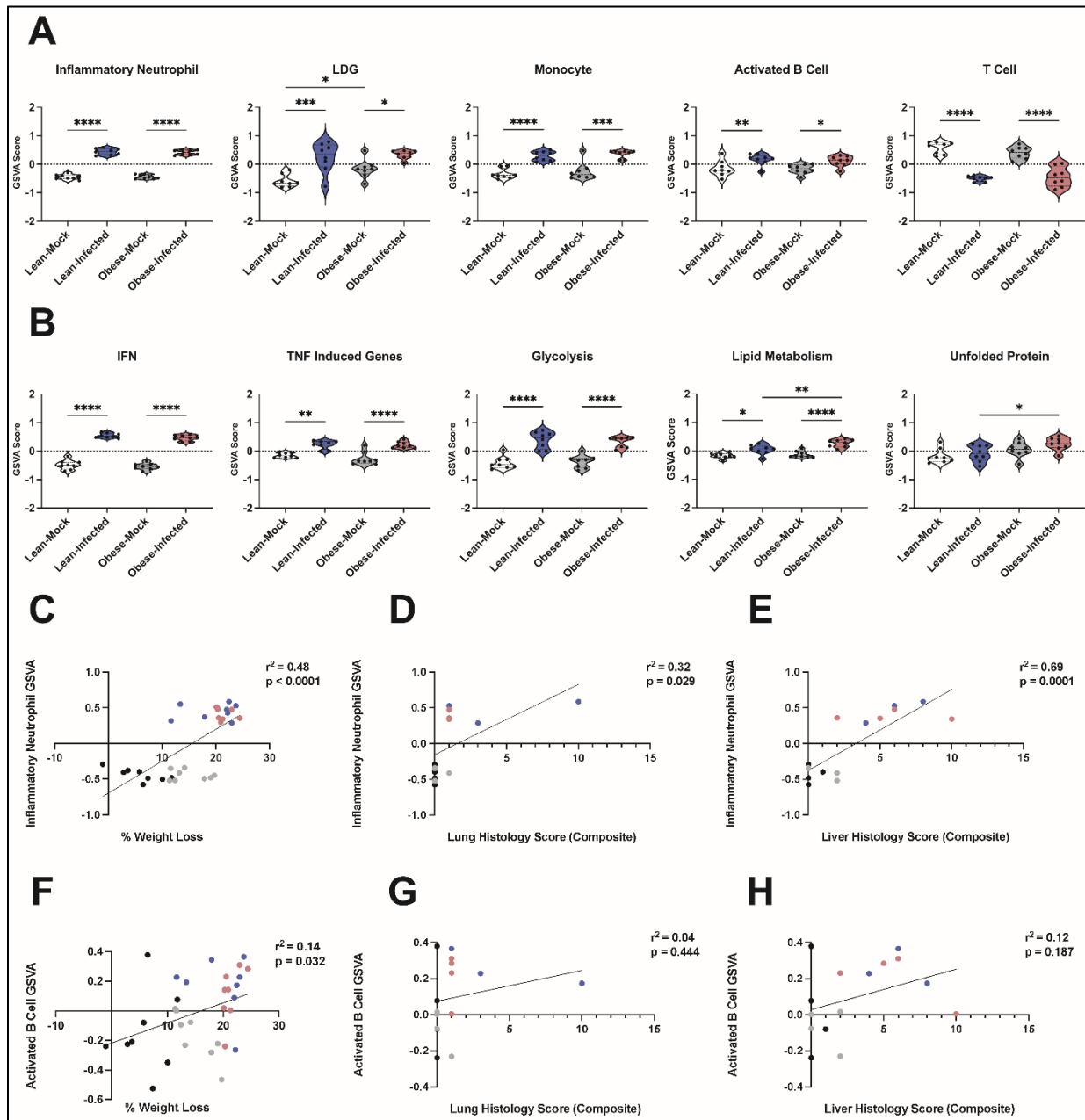


Fig.6: GSVA showed significant correlation between inflammatory neutrophils and measures of disease severity.

RNAseq data from MHV-1 and mock-infected lean and obese C3H/HeJ mice was analyzed by GSVA using immune cell (A) and inflammatory and cellular pathway (B) gene signatures.

Linear regression analysis between inflammatory neutrophil GSVA scores and weight loss (C), lung histopathology (D) and liver histopathology (E). Linear regression analysis between activated B cell GSVA scores and weight loss (F), lung histopathology (G) and liver histopathology (H). The correlation for each comparison is displayed as the r^2 value and the significance of the correlation is displayed as the p-value. Statistical and regression analysis was done using GraphPad Prism. The level of significance is represented as follows: $p < 0.05$ (*); $p < 0.001$ (**); $p = 0.001$ to 0.0001 (***) ; $p < 0.0001$ (****). The error bars indicate SD of the mean.

3.5 Limitations of the study

The major objective of this study was to identify a relevant animal model to recapitulate the effect of host immunity on disease outcomes observed in severe COVID-19 patients, particularly in the context of obesity. However, one limitation of the study was our focus on diet-induced obesity, which may not capture the obesity spectrum, including other associated comorbidities, such as diabetes, insulin resistance, and cardiovascular disease. Obesity alters the metabolic and immune status of an individual and has altered expression of cytokines, all of which might impact the disease outcome. To isolate diet-induced obesity as the only variable, we used inbred mice that were treated identically in all respects except diet. Despite these limitations, this study has successfully demonstrated that obesity worsens coronavirus disease outcome and established a model that resembles severe COVID-19 in terms of lung pathology and gene signatures. However, future studies utilizing other relevant animal models of SARS-CoV-2 pathogenesis are needed to

further confirm the validity of these studies and dissect the role of other components of obesity on COVID-19 severity and use these results to identify robust biomarkers.

3.6 Conclusions

In summary, we have identified a relevant mouse model that mimics COVID-19 in humans. We have leveraged this model to explore the complex relationship between obesity and coronavirus disease severity. Our findings suggest that obesity contributes to increased susceptibility to severe coronavirus disease and may modulate tissue-specific responses. The results obtained from this study can be used to validate the effectiveness of identified biomarkers by testing them in other animal models of COVID-19. However, future mechanistic studies are needed to elucidate the precise roles of these biomarkers in coronavirus disease outcomes.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

FUNDING SOURCES

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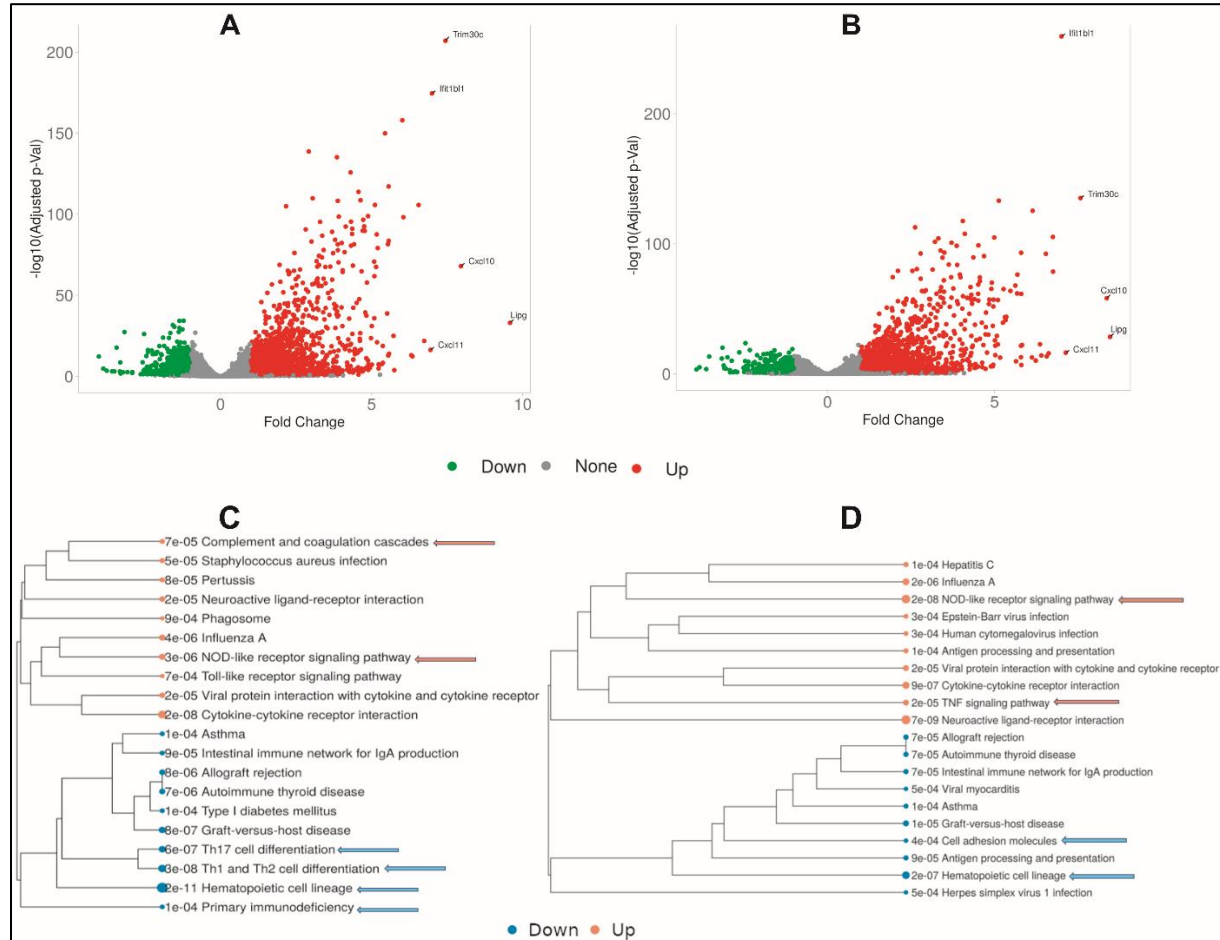
The funders had no role in the research design, collection and analysis of data or preparation and submission of the manuscript.

3.7 Author Contributions

Pallavi Rai: Conceptualization; data curation; investigation; methodology; validation; formal analysis; writing– original draft; visualization. Jeffrey M. Marano: Methodology; data curation; investigation; writing– review and editing. Lin Kang: Software; data curation; writing– review and editing. Sheryl Coutermarsh-Ott: formal analysis; data curation; writing– review and editing. Andrea R Daamen: Software; formal analysis, data curation; writing– review and editing. Peter E Lipsky: Software; data curation; writing– review and editing. James Weger-Lucarelli: Conceptualization; formal analysis; supervision; project administration; funding acquisition; resources; writing– review and editing; writing– original draft; visualization.

3.8 Supplementary Figures

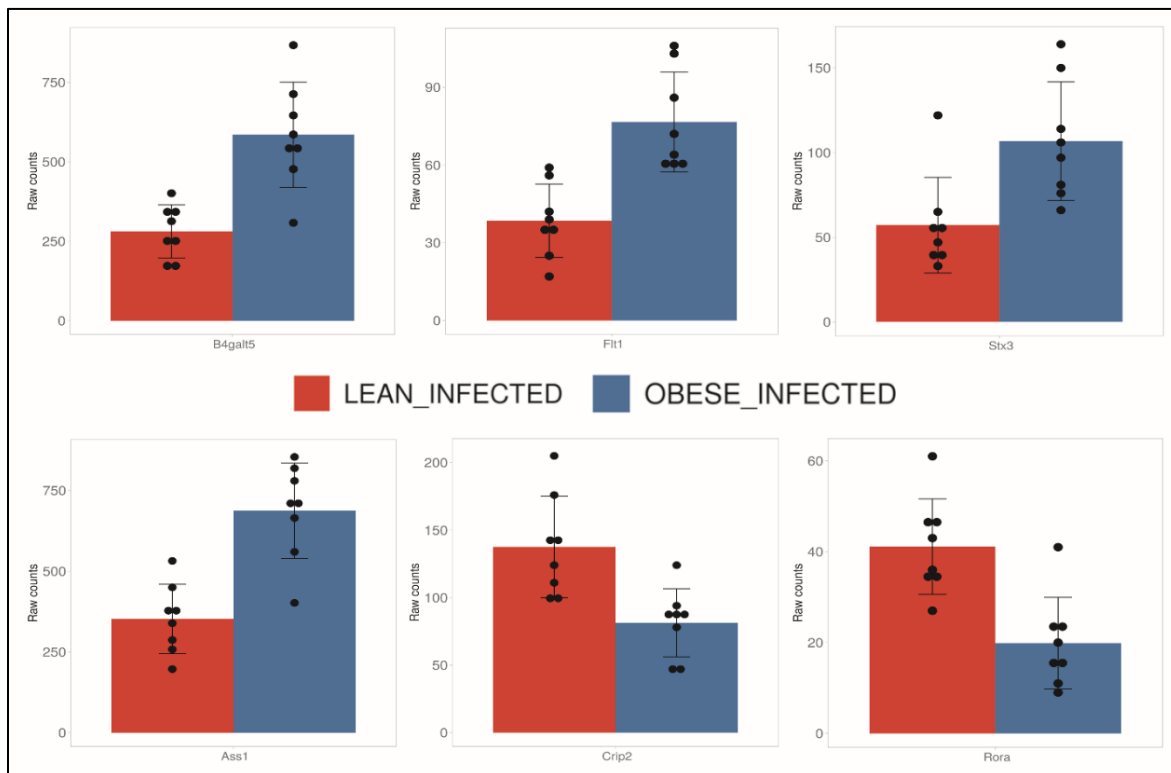
Fig.S1: Differential gene expression and pathway analysis in MHV-1 vs mock-infected lean and obese C3H/HeJ mice.



RNAseq was performed on the RNA collected from blood of MHV-1 and mock-infected lean and obese C3H/HeJ mice at 2 dpi. **(A & B) Volcano plot of differentially expressed genes (DEGs) for lean (A) and obese (B) mice.** For identification of DEGs, genes with p-adjusted values < 0.1 and $\log_2\text{FoldChange} > 1$, were selected by integrated Differential Expression and Pathway (iDEP .96) analysis tool. **(C & D) Pathway analysis for lean (C) and obese (D) mice.** Pathways were generated using Gene Set Enrichment Analysis (GSEA) using the Kyoto Encyclopedia of Genes and Genomes (KEGG). All the pathway analysis data were compared to data from the blood of

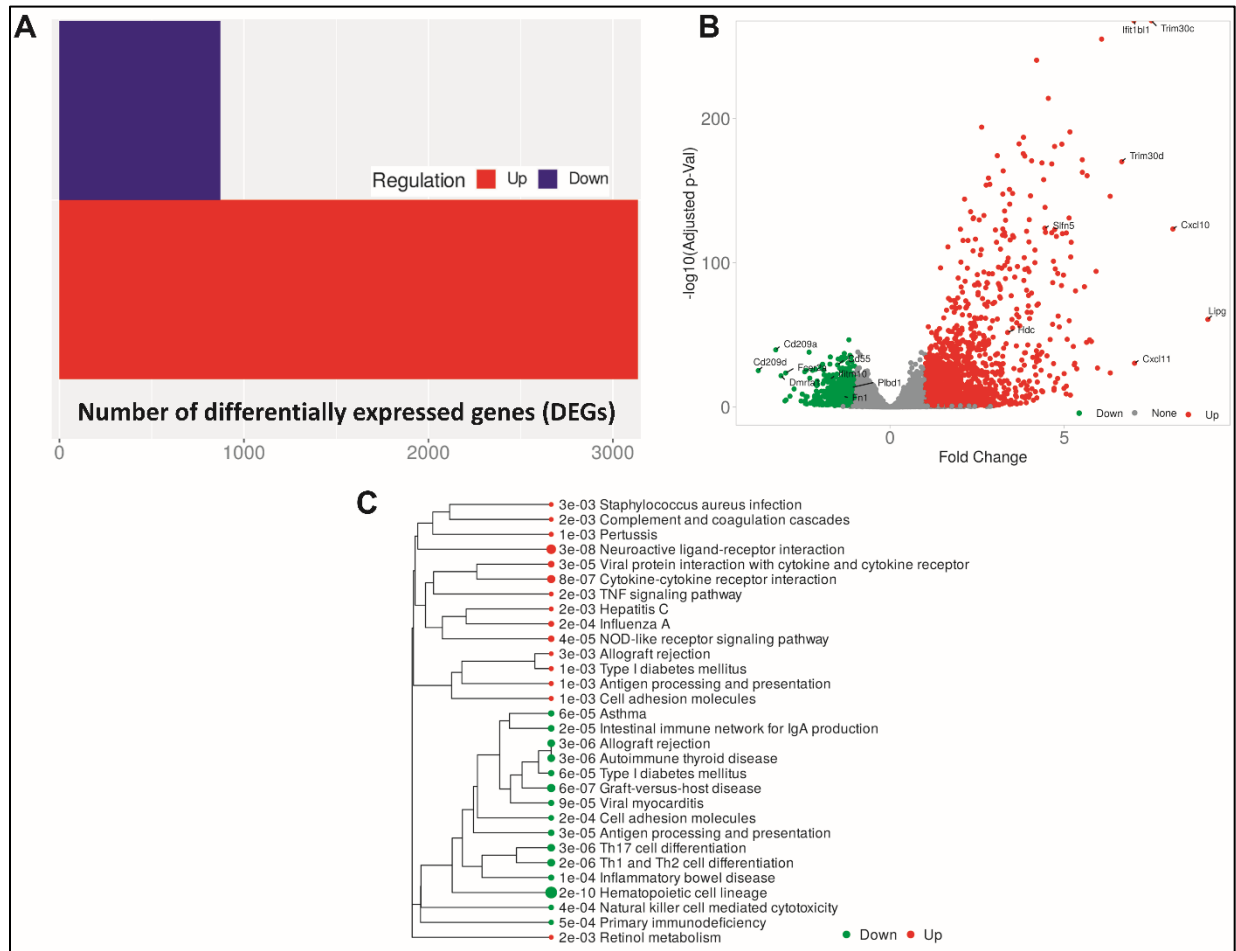
COVID-19 patients with severe disease; orange arrows indicate common upregulated and blue arrows indicate common downregulated pathways between MHV-1 infected vs mock and severe vs mild COVID-19 patients and the common up or downregulated pathways are represented by arrows.

Fig.S2: Expression patterns of genes in MHV-1 infected obese vs lean C3H/HeJ mice, based on raw counts data.



Biomarker genes were identified after comparing the mouse RNAseq data to severe vs mild COVID-19 patient data. Then iDEP 2.0 was used to perform gene plots on raw counts, with standard deviation, for the identified genes between MHV-1 infected obese and lean mice. B4galt5, Flt1, Stx3 and Ass1 showed a trend towards upregulation, while Crip2 and Rora showed a trend towards downregulation in obese vs lean mice.

Fig.S3: RNAseq analysis of blood RNA from MHV-1 and mock-infected C3H/HeJ mice.



RNAseq was performed on the RNA collected from blood of MHV-1 and mock-infected mice at 2 dpi. **(A) Number of differentially expressed genes in MHV-1 vs mock-infected C3H/HeJ mice.** Red barplot indicates number of upregulated and blue indicates downregulated DEGs. **(B) Volcano plot of differentially expressed genes (DEGs).** For identification of DEGs, genes with $p\text{-adjusted values} < 0.1$ and $\log_2\text{FoldChange} > 1$, were selected by integrated Differential Expression and Pathway (iDEP 1.1) analysis tool. **(C) Pathway analysis.** Pathways were generated using Gene Set Enrichment Analysis (GSEA) using the Kyoto Encyclopedia of Genes and Genomes (KEGG).

3.9 Supplementary Tables

Supplementary Table 1. List of up and downregulated DEGs, with log₂ Fold Change values, unique to MHV-1 infected obese vs lean C3H/HeJ mice with similar expression as severe vs mild COVID-19 patients.

Symbol	Log ₂ Fold Change		Regulation
	MHV-1	COVID-19	
Srgap1	5.593313745	1.288614	Up
Scn2b	4.760590346	0.8709768	Up
Hmcn1	4.674269052	0.77173757	Up
Lvrn	4.525300694	0.9098423	Up
Flt1	4.464887932	1.431504705	Up
Ticrr	3.146043378	1.533416	Up
Pdss1	1.891486116	0.6600827	Up
Pole2	1.725676487	0.7632403	Up
Arhgef37	1.697521173	1.362466202	Up
Myo10	1.640562322	1.429666	Up
Cc2d2b	1.45096369	1.862170706	Up
B4galt5	1.383974831	1.306495789	Up
Dnajc13	1.357049965	0.607348669	Up
Stx3	1.327024085	0.9542184	Up
Asph	1.283336276	2.192180081	Up
Ass1	1.174287366	0.665236365	Up

Slc8a1	1.069356564	1.531484	Up
Esam	1.046268861	1.319889762	Up
Tifa	1.044719796	0.8567695	Up
Trim9	1.348867313	1.061524	Up
Dtx3	-1.142670519	-1.290120726	Down
Kif3a	-1.100795517	-0.9768302	Down
Matk	-1.17576259	-1.677993	Down
Cdc25b	-1.366412368	-1.033647957	Down
Crip2	-1.246176575	-1.189354949	Down
Rora	-1.106910332	-1.600963	Down
Sh3yl1	-1.380657957	-0.7296559	Down
Kctd7	-1.243119704	-0.6274643	Down
Clic3	-2.546052451	-1.657180512	Down
Ctsf	-1.376541686	-0.830037696	Down
St8sia6	-1.070073644	-0.8505567	Down
Igsf8	-1.038611817	-0.8450607	Down
Egr2	-2.209850344	-0.776069905	Down
Syng1	-1.115853608	-0.7681419	Down
Ddb2	-1.397359623	-0.743174555	Down
Hspa12a	-2.842163756	-0.699411	Down
Akap7	-1.094982427	-0.65468549	Down
Zfp1	-1.569822542	-0.6469833	Down
Dusp8	-1.667002083	-0.624085255	Down

Reep6	-1.715118998	-0.6239955	Down
Pkd1l1	-1.611257667	-0.6195963	Down
Plxnd1	-1.196410739	-0.6095316	Down
Mars2	-1.332546191	-0.5980099	Down

Supplementary Table 2. Gene sets used as input for GSVA.

Gene Symbol	Entrez ID	Gene Set
Ada	11486	Activated B Cell
Batf	53314	Activated B Cell
Bcl3	12051	Activated B Cell
Cd28	12487	Activated B Cell
Cd69	12515	Activated B Cell
Cd80	12519	Activated B Cell
Cd86	12524	Activated B Cell
Mad2l2	71890	Activated B Cell
Nans	94181	Activated B Cell
Slc3a2	17254	Activated B Cell
Ung	22256	Activated B Cell
Xrcc4	108138	Activated B Cell
Aldoa	11674	Glycolysis
Aldob	230163	Glycolysis
Aldoc	11676	Glycolysis

Eno1	13806	Glycolysis
Gpi1	14751	Glycolysis
Hk2	15277	Glycolysis
Hkdc1	216019	Glycolysis
Ldha	16828	Glycolysis
Pfkfb3	170768	Glycolysis
Pfkip	56421	Glycolysis
Pgk1	18655	Glycolysis
Pkm	18746	Glycolysis
Slc2a1	20525	Glycolysis
Tpi1	21991	Glycolysis
Eif2ak2	19106	IFN
Gbp2	14469	IFN
Gbp3	55932	IFN
Herc6	67138	IFN
Ifi27	52668	IFN
Ifi30	65972	IFN
Ifi35	70110	IFN
Ifi44	99899	IFN
Ifi44l	15061	IFN
Ifit1	15957	IFN
Ifit2	15958	IFN
Ifit3	15959	IFN

Ifitm1	68713	IFN
Ifitm2	80876	IFN
Ifitm3	66141	IFN
Isg15	100038882	IFN
Isg20	57444	IFN
Mx1	17857	IFN
Mx2	17858	IFN
Oas1a	246730	IFN
Oas2	246728	IFN
Oas3	246727	IFN
Oasl1	231655	IFN
Rsad2	58185	IFN
Sp100	20684	IFN
Sp110	109032	IFN
1810037I17Rik	67704	Inflammatory_Neutrophil
Acsl1	14081	Inflammatory_Neutrophil
Adar	56417	Inflammatory_Neutrophil
Add3	27360	Inflammatory_Neutrophil
Adm	11535	Inflammatory_Neutrophil
Alox5ap	11690	Inflammatory_Neutrophil
Alpl	11647	Inflammatory_Neutrophil
Anxa1	16952	Inflammatory_Neutrophil
Anxa3	11745	Inflammatory_Neutrophil

Apol6	71939	Inflammatory_Neutrophil
Apol8	239552	Inflammatory_Neutrophil
B4galt5	56336	Inflammatory_Neutrophil
Baz1a	217578	Inflammatory_Neutrophil
Bri3	55950	Inflammatory_Neutrophil
Bst1	12182	Inflammatory_Neutrophil
C3ar1	12267	Inflammatory_Neutrophil
Capza1	12340	Inflammatory_Neutrophil
Casp1	12362	Inflammatory_Neutrophil
Casp1	12362	Inflammatory_Neutrophil
Casp4	12363	Inflammatory_Neutrophil
Cast	12380	Inflammatory_Neutrophil
Ccr1	12768	Inflammatory_Neutrophil
Cd177	68891	Inflammatory_Neutrophil
Cd37	12493	Inflammatory_Neutrophil
Cd44	12505	Inflammatory_Neutrophil
Cd53	12508	Inflammatory_Neutrophil
Cd55	13136	Inflammatory_Neutrophil
Cd55b	13137	Inflammatory_Neutrophil
Cd63	12512	Inflammatory_Neutrophil
Cd82	12521	Inflammatory_Neutrophil
Cdkn2d	12581	Inflammatory_Neutrophil
Ceacam1	26365	Inflammatory_Neutrophil

Cfl1	12631	Inflammatory_Neutrophil
Ckap4	216197	Inflammatory_Neutrophil
Clec4d	17474	Inflammatory_Neutrophil
Clec4e	56619	Inflammatory_Neutrophil
Cr1l	12946	Inflammatory_Neutrophil
Cst7	13011	Inflammatory_Neutrophil
Cystm1	66060	Inflammatory_Neutrophil
Ddx60	234311	Inflammatory_Neutrophil
Dysf	26903	Inflammatory_Neutrophil
Eif2ak2	19106	Inflammatory_Neutrophil
Emb	13723	Inflammatory_Neutrophil
Epsti1	108670	Inflammatory_Neutrophil
Fcer1g	14127	Inflammatory_Neutrophil
Fcgr1	14129	Inflammatory_Neutrophil
Ffar2	233079	Inflammatory_Neutrophil
Fgr	14191	Inflammatory_Neutrophil
Fkbp1a	14225	Inflammatory_Neutrophil
Fkbp5	14229	Inflammatory_Neutrophil
Flot1	14251	Inflammatory_Neutrophil
Fyb	23880	Inflammatory_Neutrophil
Gapdh	14433	Inflammatory_Neutrophil
Gbp2	14469	Inflammatory_Neutrophil
Gbp3	55932	Inflammatory_Neutrophil

Gbp5	229898	Inflammatory_Neutrophil
Gca	227960	Inflammatory_Neutrophil
Gimap4	107526	Inflammatory_Neutrophil
Glrx	93692	Inflammatory_Neutrophil
Gng5	14707	Inflammatory_Neutrophil
Grina	66168	Inflammatory_Neutrophil
Grn	14824	Inflammatory_Neutrophil
Gstk1	76263	Inflammatory_Neutrophil
Gyg	27357	Inflammatory_Neutrophil
H2-M3	14991	Inflammatory_Neutrophil
H2-Q1	15006	Inflammatory_Neutrophil
H2-Q2	15013	Inflammatory_Neutrophil
H2ac1	319163	Inflammatory_Neutrophil
H2ac23	665433	Inflammatory_Neutrophil
H2bc14	319186	Inflammatory_Neutrophil
Hif1a	15251	Inflammatory_Neutrophil
Hmgb2	97165	Inflammatory_Neutrophil
I830077J02Rik	433638	Inflammatory_Neutrophil
Ifi205	226695	Inflammatory_Neutrophil
Ifi44	99899	Inflammatory_Neutrophil
Ifi44l	15061	Inflammatory_Neutrophil
Ifih1	71586	Inflammatory_Neutrophil
Ifit1bl1	667373	Inflammatory_Neutrophil

Ifit2	15958	Inflammatory_Neutrophil
Ifit3	15959	Inflammatory_Neutrophil
Ifitm1	68713	Inflammatory_Neutrophil
Ifitm3	66141	Inflammatory_Neutrophil
Il1rn	16181	Inflammatory_Neutrophil
Il2rg	16186	Inflammatory_Neutrophil
Irf1	16362	Inflammatory_Neutrophil
Irf7	54123	Inflammatory_Neutrophil
Isg15	100038882	Inflammatory_Neutrophil
Isg20	57444	Inflammatory_Neutrophil
Itgam	16409	Inflammatory_Neutrophil
Jun	16476	Inflammatory_Neutrophil
Kcnj15	16516	Inflammatory_Neutrophil
Klf4	16600	Inflammatory_Neutrophil
Lap3	66988	Inflammatory_Neutrophil
Lgals9	16859	Inflammatory_Neutrophil
Lilra5	232801	Inflammatory_Neutrophil
Lilra6	18726	Inflammatory_Neutrophil
Limk2	16886	Inflammatory_Neutrophil
Lmnb1	16906	Inflammatory_Neutrophil
Lrg1	76905	Inflammatory_Neutrophil
Ly6e	17069	Inflammatory_Neutrophil
Ly96	17087	Inflammatory_Neutrophil

Mapk14	26416	Inflammatory_Neutrophil
Max	17187	Inflammatory_Neutrophil
Mcemp1	69189	Inflammatory_Neutrophil
Mettl9	59052	Inflammatory_Neutrophil
Mmp9	17395	Inflammatory_Neutrophil
Mob1a	232157	Inflammatory_Neutrophil
Msrb1	27361	Inflammatory_Neutrophil
Mt2	17750	Inflammatory_Neutrophil
Mtpn	14489	Inflammatory_Neutrophil
Mx2	17858	Inflammatory_Neutrophil
Myl12a	67268	Inflammatory_Neutrophil
Nbn	27354	Inflammatory_Neutrophil
Nfe2	18022	Inflammatory_Neutrophil
Nfil3	18030	Inflammatory_Neutrophil
Nfkbia	18035	Inflammatory_Neutrophil
Nmi	64685	Inflammatory_Neutrophil
Nt5c3	107569	Inflammatory_Neutrophil
Ntng2	171171	Inflammatory_Neutrophil
Nub1	53312	Inflammatory_Neutrophil
Nucb1	18220	Inflammatory_Neutrophil
Oas1a	246730	Inflammatory_Neutrophil
Oas1g	23960	Inflammatory_Neutrophil
Oas2	246728	Inflammatory_Neutrophil

Oas3	246727	Inflammatory_Neutrophil
Oasl1	231655	Inflammatory_Neutrophil
Parp14	547253	Inflammatory_Neutrophil
Parp9	80285	Inflammatory_Neutrophil
Pgd	110208	Inflammatory_Neutrophil
Phf11a	219131	Inflammatory_Neutrophil
Phf11b	236451	Inflammatory_Neutrophil
Phf11d	219132	Inflammatory_Neutrophil
Pik3ap1	83490	Inflammatory_Neutrophil
Pim1	18712	Inflammatory_Neutrophil
Pira12	100038909	Inflammatory_Neutrophil
Pira13	100041146	Inflammatory_Neutrophil
Pira2	18725	Inflammatory_Neutrophil
Pirb	18733	Inflammatory_Neutrophil
Plac8	231507	Inflammatory_Neutrophil
Plbd1	66857	Inflammatory_Neutrophil
Plek	56193	Inflammatory_Neutrophil
Plp2	18824	Inflammatory_Neutrophil
Plscr1	22038	Inflammatory_Neutrophil
Pml	18854	Inflammatory_Neutrophil
Prok2	50501	Inflammatory_Neutrophil
Prr13	66151	Inflammatory_Neutrophil
Psmb9	16912	Inflammatory_Neutrophil

Pstpip2	19201	Inflammatory_Neutrophil
Pten	19211	Inflammatory_Neutrophil
Rac2	19354	Inflammatory_Neutrophil
Rbck1	24105	Inflammatory_Neutrophil
Rbms1	56878	Inflammatory_Neutrophil
Rgs19	56470	Inflammatory_Neutrophil
Rhog	56212	Inflammatory_Neutrophil
Rnf10	50849	Inflammatory_Neutrophil
Rnf213	672511	Inflammatory_Neutrophil
Rpl28	19943	Inflammatory_Neutrophil
Rsad2	58185	Inflammatory_Neutrophil
S100a6	20200	Inflammatory_Neutrophil
Samd9l	209086	Inflammatory_Neutrophil
Samhd1	56045	Inflammatory_Neutrophil
Samsn1	67742	Inflammatory_Neutrophil
Sat1	20229	Inflammatory_Neutrophil
Sectm1a	209588	Inflammatory_Neutrophil
Sell	20343	Inflammatory_Neutrophil
Serpina1b	20701	Inflammatory_Neutrophil
Serpina1e	20704	Inflammatory_Neutrophil
Serpinb1a	66222	Inflammatory_Neutrophil
Serping1	12258	Inflammatory_Neutrophil
Sh3glb1	54673	Inflammatory_Neutrophil

Shisa5	66940	Inflammatory_Neutrophil
Slfn5	327978	Inflammatory_Neutrophil
Snx3	54198	Inflammatory_Neutrophil
Sp100	20684	Inflammatory_Neutrophil
Sp110	109032	Inflammatory_Neutrophil
Spi1	20375	Inflammatory_Neutrophil
Sptlc2	20773	Inflammatory_Neutrophil
Stat1	20846	Inflammatory_Neutrophil
Stat2	20847	Inflammatory_Neutrophil
Stxbp2	20911	Inflammatory_Neutrophil
Tap1	21354	Inflammatory_Neutrophil
Tmem123	71929	Inflammatory_Neutrophil
Tmsb10	19240	Inflammatory_Neutrophil
Tnfaip6	21930	Inflammatory_Neutrophil
Tnfsf10	22035	Inflammatory_Neutrophil
Tnfsf13b	24099	Inflammatory_Neutrophil
Tpm3	59069	Inflammatory_Neutrophil
Trim38	214158	Inflammatory_Neutrophil
Txn1	22166	Inflammatory_Neutrophil
Ube2j1	56228	Inflammatory_Neutrophil
Ube2l6	56791	Inflammatory_Neutrophil
Vim	22352	Inflammatory_Neutrophil
Wsb1	78889	Inflammatory_Neutrophil

Xaf1	327959	Inflammatory_Neutrophil
Xrn1	24127	Inflammatory_Neutrophil
Zbp1	58203	Inflammatory_Neutrophil
Zcchc2	227449	Inflammatory_Neutrophil
Zdhhc19	245308	Inflammatory_Neutrophil
Zyx	22793	Inflammatory_Neutrophil
Ctsg	13035	LDG
Elane	50701	LDG
Lcn2	16819	LDG
Mpo	17523	LDG
Osm	18413	LDG
Aadac	67758	Lipid Metabolism
Abhd6	6082	Lipid Metabolism
Acly	104112	Lipid Metabolism
Acsf5	433256	Lipid Metabolism
Angptl3	30924	Lipid Metabolism
Angptl4	57875	Lipid Metabolism
Apoa4	11808	Lipid Metabolism
Apoa5	66113	Lipid Metabolism
ApoE	11816	Lipid Metabolism
ApoH	11818	Lipid Metabolism
Ccdc3	74186	Lipid Metabolism
Cneph1r1	382030	Lipid Metabolism

Dbi	13167	Lipid Metabolism
Dgat1	13350	Lipid Metabolism
Dgat2	67800	Lipid Metabolism
Disp3	242748	Lipid Metabolism
Fabp3	14077	Lipid Metabolism
Gpam	14732	Lipid Metabolism
Gpld1	14756	Lipid Metabolism
Hsd17b13	243168	Lipid Metabolism
Ldlr	16835	Lipid Metabolism
Mid1ip1	68041	Lipid Metabolism
Nr1h2	22260	Lipid Metabolism
Nr1h3	22259	Lipid Metabolism
Pcx	18563	Lipid Metabolism
Pnpla2	66853	Lipid Metabolism
Ppara	19013	Lipid Metabolism
Slc27a1	26457	Lipid Metabolism
Srebf1	20787	Lipid Metabolism
Bst1	12182	Monocyte
C1qa	12259	Monocyte
C1qb	12260	Monocyte
C1qc	12262	Monocyte
C1rl	232371	Monocyte
Ccl8	20307	Monocyte

Cd14	12475	Monocyte
Cd163	93671	Monocyte
Cd68	12514	Monocyte
Clec10a	17312	Monocyte
Clec12a	232413	Monocyte
Clec4d	17474	Monocyte
Clec4e	56619	Monocyte
Csf1r	12978	Monocyte
Cxcl1	14825	Monocyte
Cxcl10	15945	Monocyte
Fcgr1	14129	Monocyte
Fcgr4	246256	Monocyte
Ifi211	381308	Monocyte
Il1b	16176	Monocyte
Il1rn	16181	Monocyte
Lilra5	232801	Monocyte
Mrc1	17533	Monocyte
Oscar	232790	Monocyte
P2rx5	94045	Monocyte
Pirb	18733	Monocyte
Sema4a	20351	Monocyte
Siglec1	20612	Monocyte
Thbd	21824	Monocyte

Tnf	21926	Monocyte
Cd247	12503	T Cell
Cd28	12487	T Cell
Cd3d	12500	T Cell
Cd3e	12501	T Cell
Cd3g	12502	T Cell
Cd4	12504	T Cell
Cd5	12507	T Cell
Cd8a	12525	T Cell
Cd8b1	12526	T Cell
Ets1	23871	T Cell
Gata3	14462	T Cell
Grap2	17444	T Cell
Lef1	16842	T Cell
Sh2d1a	20400	T Cell
Trac	100101484	T Cell
Trbc1	100125262	T Cell
TRDC	100123473	T Cell
Bcl2l10	12049	TNF Induced Genes
Ccl5	20304	TNF Induced Genes
Cd6	12511	TNF Induced Genes
Cd68	12514	TNF Induced Genes
Cp	12870	TNF Induced Genes

Csf2	12981	TNF Induced Genes
Csf3	12985	TNF Induced Genes
Dcn	13179	TNF Induced Genes
Gbp2	14469	TNF Induced Genes
Gdf15	23886	TNF Induced Genes
Gstt1	14871	TNF Induced Genes
H2-D1	14964	TNF Induced Genes
H2-K2	630499	TNF Induced Genes
H2-T23	15040	TNF Induced Genes
Hp	15439	TNF Induced Genes
Igdcc3	19289	TNF Induced Genes
Il6	16193	TNF Induced Genes
Jun	16476	TNF Induced Genes
Krt35	53617	TNF Induced Genes
Limk1	16885	TNF Induced Genes
Madcam1	17123	TNF Induced Genes
Mmp13	17386	TNF Induced Genes
Mmp3	17392	TNF Induced Genes
Nfkbia	18035	TNF Induced Genes
Notch3	18131	TNF Induced Genes
Saa3	20210	TNF Induced Genes
Sele	20339	TNF Induced Genes
Selp	20344	TNF Induced Genes

Serpina3n	20716	TNF Induced Genes
Sod2	20656	TNF Induced Genes
Tap2	21355	TNF Induced Genes
Tnfaip3	21929	TNF Induced Genes
Traf1	22029	TNF Induced Genes
Vcam1	22329	TNF Induced Genes
Yy1	22632	TNF Induced Genes
Bpnt2	242291	Unfolded Protein
Calr	12317	Unfolded Protein
Calu	12321	Unfolded Protein
Canx	12330	Unfolded Protein
Cds2	110911	Unfolded Protein
Derl1	67819	Unfolded Protein
Derl2	116891	Unfolded Protein
Dnajc3	100037258	Unfolded Protein
Edem2	108687	Unfolded Protein
Edem3	66967	Unfolded Protein
Erap1	80898	Unfolded Protein
Ergic2	67456	Unfolded Protein
Ero1l	50527	Unfolded Protein
Ext1	14042	Unfolded Protein
Galnt2	108148	Unfolded Protein
Golt1b	66964	Unfolded Protein

Herpud1	64209	Unfolded Protein
Hyou1	12282	Unfolded Protein
Kdelr2	66913	Unfolded Protein
Lman2	66890	Unfolded Protein
Lpgat1	226856	Unfolded Protein
Man1a	17155	Unfolded Protein
Manea	242362	Unfolded Protein
Manf	74840	Unfolded Protein
Pdia4	12304	Unfolded Protein
Pdia6	71853	Unfolded Protein
Pigk	329777	Unfolded Protein
Poglut2	72050	Unfolded Protein
Ppib	19035	Unfolded Protein
Sec24d	69608	Unfolded Protein
Sec61g	20335	Unfolded Protein
Spcs3	76687	Unfolded Protein
Ssr1	107513	Unfolded Protein
Ssr3	67437	Unfolded Protein
Tram1	72265	Unfolded Protein
Tram2	170829	Unfolded Protein
Uggt1	320011	Unfolded Protein
Xbp1	22433	Unfolded Protein

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Chapter 4: Insulin reduces the transmission potential of chikungunya virus and activates the Toll pathway in *Aedes aegypti* mosquitoes

Pallavi Rai^{1,2}, Emily M. Webb^{2,3}, Lin Kang^{1,4}, and James Weger-Lucarelli^{1,2*}

¹Department of Biomedical Sciences and Pathobiology, Virginia Tech, VA-MD College of Veterinary Medicine, Blacksburg, VA, USA.

²Center for Emerging, Zoonotic, and Arthropod-borne Pathogens, Virginia Tech, Blacksburg, VA, USA.

³Department of Entomology, Fralin Life Sciences Institute, Virginia Tech, Blacksburg, VA, USA.

⁴Biomedical Affairs & Research, Edward Via College of Osteopathic Medicine, Monroe, LA, USA.

***Correspondence:** weger@vt.edu

4.1 Abstract

Chikungunya virus (CHIKV) is an alphavirus which has re-emerged globally over the last two decades and has the potential to become endemic in the U.S. due to the presence of competent mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. CHIK disease is characterized by fever, rash, and joint pain, and causes chronic debilitating joint pain and swelling in >50% of infected individuals. Given the disease severity caused by CHIKV and the global presence of vectors to facilitate its spread, strategies to reduce viral transmission are desperately needed; however, the human biological factors driving CHIKV transmission are poorly understood. Towards that end, we have previously shown that mosquitoes fed on alphavirus infected obese mice have reduced infection and transmission rates compared to those fed on infected lean mice despite similar viremia in lean and obese mice. One of the many host factors that increase in obese hosts is insulin, which was previously shown to impact the infection of mosquitoes by several flaviviruses. However, insulin's impact on alphavirus infection of live mosquitoes is unknown and whether insulin influences mosquito-borne virus transmission has not been tested. To test this, we exposed *Ae. aegypti* mosquitoes to bloodmeals with CHIKV in the presence or absence of physiologically relevant levels of insulin and found that insulin significantly lowered both infection and transmission rates. RNA sequencing analysis on mosquito midguts isolated at 1-day-post-infectious-bloodmeal (dpbm) showed enrichment in genes in the Toll immune pathway in the presence of insulin, which was validated by reverse transcription quantitative polymerase chain reaction (RT-qPCR). We then sought to determine if the Toll pathway plays a role in CHIKV infection of *Ae. aegypti* mosquitoes; therefore, we knocked down *Myd88*, a critical immune adaptor molecule for the Toll pathway, in live mosquitoes, and found increased CHIKV infection

compared to the mock knockdown control group. Overall, these data demonstrate that insulin reduces CHIKV transmission by *Ae. aegypti* and activates the Toll pathway in the mosquitoes, suggesting that conditions resulting in higher serum insulin concentrations may reduce alphavirus transmission. Finally, these studies suggest that strategies to activate insulin or Toll signaling in mosquitoes may be an effective control strategy against medically relevant alphaviruses.

Keywords: mosquito-borne alphaviruses, insulin, chikungunya virus, toll pathway, gene knockdown.

4.2 Introduction

Chikungunya virus (CHIKV), a mosquito-borne alphavirus, has become a public health threat of grave concern. Over the past two decades, CHIKV infections have been reported in >100 countries^{1,2}. During its emergence in the Americas from 2013-2017, it infected ~3 million people³ and has the potential to spread further due to the widespread presence of competent vectors, *Aedes aegypti* and *Ae. albopictus*⁴. CHIK disease is characterized by fever, rash and severe joint and muscle pain and swelling that can last for years and occurs in >50% of infected people⁵. It is listed as a US National Institute of Allergy and Infectious Diseases (NIAID) category B priority pathogen⁶.

CHIKV is maintained in an urban transmission cycle involving humans and mosquitoes⁷. However, the human biological factors influencing CHIKV transmission are poorly understood. We have previously shown that *Ae. aegypti* mosquitoes fed on alphavirus-infected obese mice had significantly lower infection and transmission rates than those fed on healthy weight mice (hereinafter referred to as lean) despite ingesting similar viral titers⁸, suggesting some factor(s) associated with obesity influences mosquito infection. Obesity has assumed the status of a pandemic⁹, affecting 13% of adults globally and 43% of adults in the US¹⁰, with a prediction that 50% adults in the US will be obese by 2030¹¹. Obesity alters the homeostasis of various metabolic factors in the body like carbohydrates, lipids, cytokines, and insulin¹². High levels of insulin, or hyperinsulinemia has not only been associated with obesity and diabetes¹³, but has also increased remarkably in nondiabetic US adults¹⁴. Importantly, previous studies have shown that insulin activates insect immune pathways and reduces alphavirus and flavivirus replication *in vitro*¹⁵⁻¹⁷, possibly through the activation of Janus kinase/signal transducer and activator of transcription

(JAK/STAT) through extracellular-signal related kinase (ERK) signaling^{15,17}. Although these studies established insulin's antiviral role and its impact on mosquito immune pathways in the context of flavivirus infection, insulin's effect in live mosquitoes on alphavirus infection or mosquito transmission potential for any virus is still unknown.

In this study, we show that insulin at concentrations found in CHIKV-infected obese mice inhibits infection and transmission of CHIKV by *Ae. aegypti* mosquitoes and activates the Toll pathway in mosquito midguts in the context of CHIKV infection. RNA sequencing (RNAseq) analysis performed on these midguts showed upregulation of the Toll pathway in mosquitoes exposed to CHIKV in the presence of insulin. Furthermore, silencing of *Myd88*, an adaptor molecule in the Toll pathway, in live mosquitoes exposed to CHIKV significantly increased the infection rates compared to a mock knockdown control group, suggesting the Toll pathway is important for mosquito control of CHIKV infection. Finally, we found that insulin also significantly reduced infection rates at lower levels of 5 and 10 µg/L, but not at 2 µg/L. These results demonstrate that insulin, at physiologically relevant human concentrations, inhibits CHIKV infection of mosquitoes. Activating insulin or Toll signaling in the mosquito vector may represent an effective transmission control strategy against various mosquito-borne viruses.

4.3 Results

To determine a physiologically relevant insulin concentration for *in vivo* mosquito studies, we used an established mouse model of diet-induced obesity where mice kept on a high fat (60%) diet developed significantly higher insulin levels after 18 weeks compared to those kept on a low fat (10%) diet¹⁸. We then inoculated the mice with CHIKV and tested the serum of lean and obese mice for viremia (Fig.1A) and insulin levels (Fig.1B). We found that while the pre-infection serum insulin trended higher in obese mice, they were not statistically different from each other ($p=0.14$), however, the insulin levels increased drastically at peak viremia (3 dpi) in obese mice ($***p=0.0008$). Therefore, the mean serum insulin concentration observed in CHIKV-infected obese mice at peak viremia (27.7 $\mu\text{g/L}$) was used for downstream *in vivo* studies with mosquitoes.

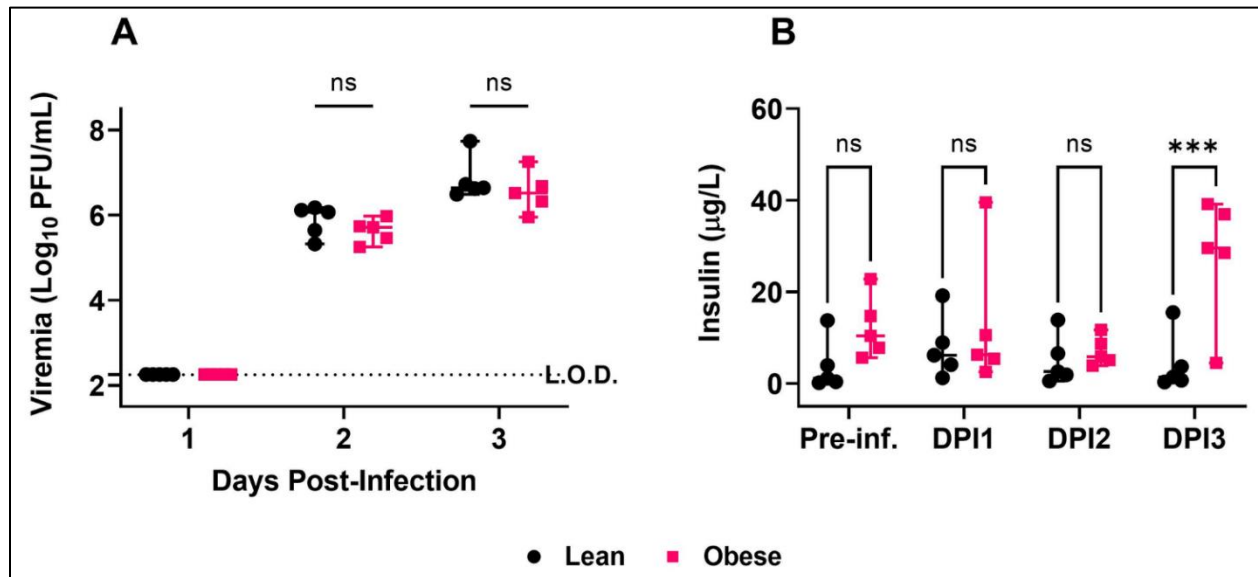


Fig.1: Insulin levels spike in obese mice inoculated with CHIKV at peak viremia. Lean and obese mice (n=5) were inoculated with 100 PFUs of CHIKV and serum was tested for viremia and insulin levels. **(A)** Viremia was measured in the serum by plaque assay from 1 to 3 days-post-

infection (dpi). **(B)** Insulin concentrations were measured in the serum samples pre-infection (pre-inf.) and from 1 to 3 dpi by ELISA. Statistical analysis was done using an unpaired t-test with Holm-Sidak correction for multiple comparisons, on samples tested for normality and outliers. Limit of detection (L.O.D.) is 2.255 log₁₀ PFU/mL. Data are presented as median with 95% CI; ns=non-significant ; ***p-value=0.0008.

To test insulin's impact on CHIKV infection and transmission, *Ae. aegypti* mosquitoes were exposed to CHIKV in the absence or presence of 27.7 µg/L human insulin. At 9-days-post-infectious-bloodmeal (dpbm), we tested mosquito bodies, legs/wings, and saliva for infectious virus to evaluate infection, dissemination, and transmission potential, respectively. Mosquitoes exposed to CHIKV in the presence of insulin had significantly lower infection (*p=0.0205), dissemination (*p=0.0115), and transmission rates (**p=0.0040), compared to those without insulin (Fig.2A), although no significant difference in viral titers was observed in the bodies (p=0.8882) (Fig.2B), legs/wings (p=0.9997) (Fig.2C) or saliva (p=0.9832) (Fig.2D) of the mosquitoes with detectable virus. Thus, while insulin reduces mosquito infection, dissemination, and transmission rates, the mosquitoes that become productively infected still allow for CHIKV replication.

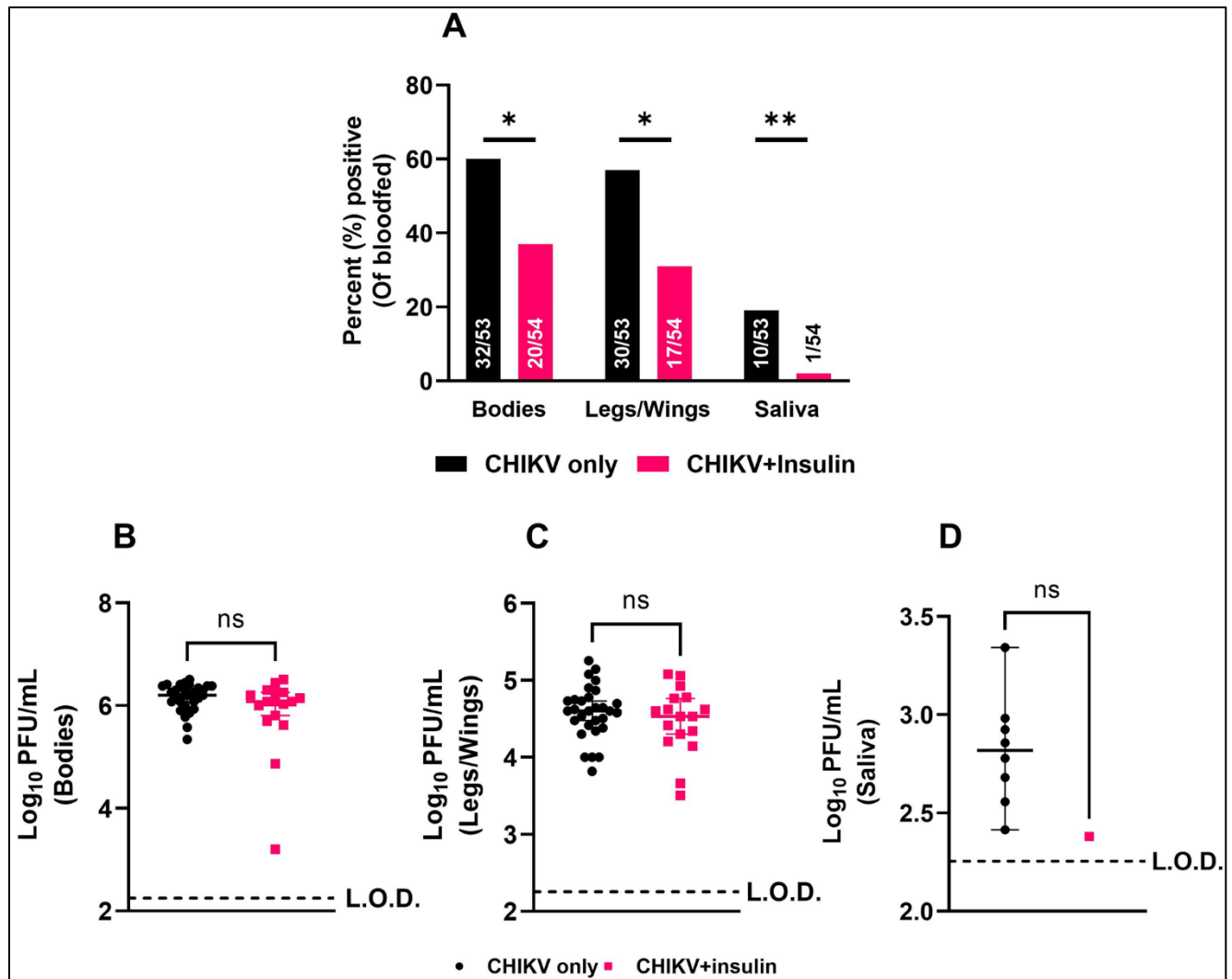


Fig.2. Insulin's effect on the vector competence of *Aedes aegypti* mosquitoes for chikungunya virus (CHIKV). *Ae. aegypti* mosquitoes were exposed to an infectious bloodmeal containing 7×10^7 PFUs/mL of CHIKV in the absence or presence of 27.7 $\mu\text{g/L}$ of insulin. Fully engorged mosquitoes were sorted and after 9 days, their bodies, legs/wings and saliva were collected to test for infection, dissemination, and transmission, respectively. **(A) Analysis of vector competence rates.** Infection, dissemination, and transmission rates were calculated as a percentage of the samples positive for infectious virus in bodies, legs/wings, or saliva, respectively to the total number of bloodfed

mosquitoes represented as n/X ; where n denotes the number of mosquitoes positive for infectious virus and X denotes the total number of bloodfed mosquitoes tested. Statistical analysis was done using a two-sided Fisher's exact test; * $p=0.0205$, * $p=0.0115$, ** $p=0.0040$, respectively. **(B), (C) & (D) Effect on CHIKV titers in bodies, legs/wings, or saliva.** Infectious virus was quantified by plaque assay. Statistical analysis was done using Mann-Whitney or unpaired t-test on log transformed titers tested for normality and outliers; ns-non-significant. Limit of detection (L.O.D.) is $2.255 \log_{10}$ PFU/mL of virus. Data are presented as the median with 95% confidence interval (CI). Data from two independent experiments were combined for these figures.

Potentially antiviral genes are upregulated in the midgut of mosquitoes exposed to CHIKV in the presence of insulin. After ingesting blood from an infected vertebrate host, the virus enters the mosquito midgut, the initial site of arbovirus infection¹⁹ and the first line of defense in mosquitoes²⁰. RNA interference (RNAi), JAK/STAT, Toll, and immune deficiency (IMD) are major immune pathways shown to impact midgut infection of mosquitoes^{15,21-23}. To identify insulin's impact on gene expression following exposure to CHIKV, we performed RNAseq on mosquito midguts exposed to CHIKV in the presence or absence of insulin at 1 dpi (Fig.3A). We performed pathway enrichment analysis on the differentially expressed genes (DEGs) identified by RNAseq and found enrichment of genes involved in the Toll and IMD pathway (Fig.3B, Supplementary Table 1, Supplementary File 1) in the presence of insulin. In contrast, genes involved in longevity regulation, phototransduction, and protein processing and endocytosis were downregulated (Supplementary Table 1, Supplementary File 1, Supplementary Fig.S1).

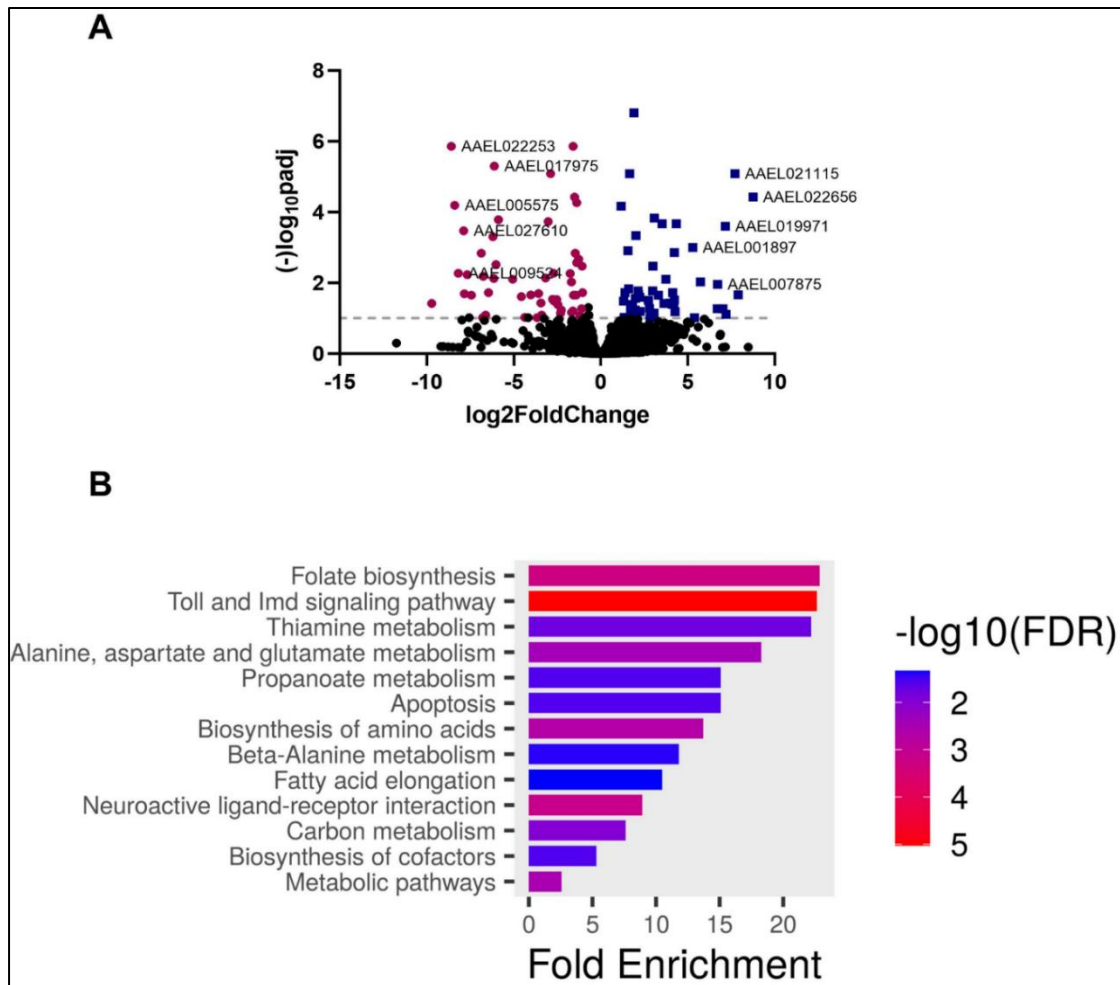


Fig.3. Differential gene expression in mosquito midguts exposed to CHIKV in the presence of insulin. (A) Volcano plot of differentially expressed genes (DEGs) in mosquitoes exposed to CHIKV in the presence or absence of insulin at 1-day-post-infectious-bloodmeal (dpbm). Blue squares represent upregulated DEGs while maroon dots represent downregulated DEGs. From each group, 3 individual midguts were sent for RNA sequencing. (B) **Upregulated pathways.** For pathway analysis, DEGs with p-adjusted values < 0.1 and $\log_2\text{FoldChange} > 1$, were selected by integrated Differential Expression and Pathway analysis (iDEP.96) and the pathways were generated using the ShinyGO 0.77 analysis tool.

To validate our RNAseq data, we performed reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis on the midgut RNA samples. We tested the samples for genes in the Toll and IMD pathways since these were enriched in our pathway analysis (Fig.3B). For the IMD pathway, Relish 2 (*REL2*), a transcription factor, and *IMD*, one of the proteins essential for phosphorylating REL2 were tested²⁴ (Fig.4A). For the Toll pathway, *Myd88*, an adaptor that connects the Toll receptor to the Pelle kinase²⁵, and *Cactus*, a negative regulator of the pathway which binds to the transcription factor, Relish 1 (*REL1*) and prevents the production of AMPs, were tested²⁶. No difference in gene expression was observed between the groups for the IMD pathway. In contrast, a significant upregulation of *Myd88* (Fig.4B; **p=0.005) and downregulation of *Cactus* (Fig.4B; *p=0.026) was observed in the midguts exposed to CHIKV in the presence of insulin compared to those without insulin, suggesting activation of the Toll pathway in the presence of insulin. We found no significant differences for genes in the RNAi and JAK/STAT pathways by RT-qPCR or RNAseq (Supplementary Fig.S2). These data demonstrate that insulin activates the Toll pathway in mosquito midguts in the context of CHIKV exposure.

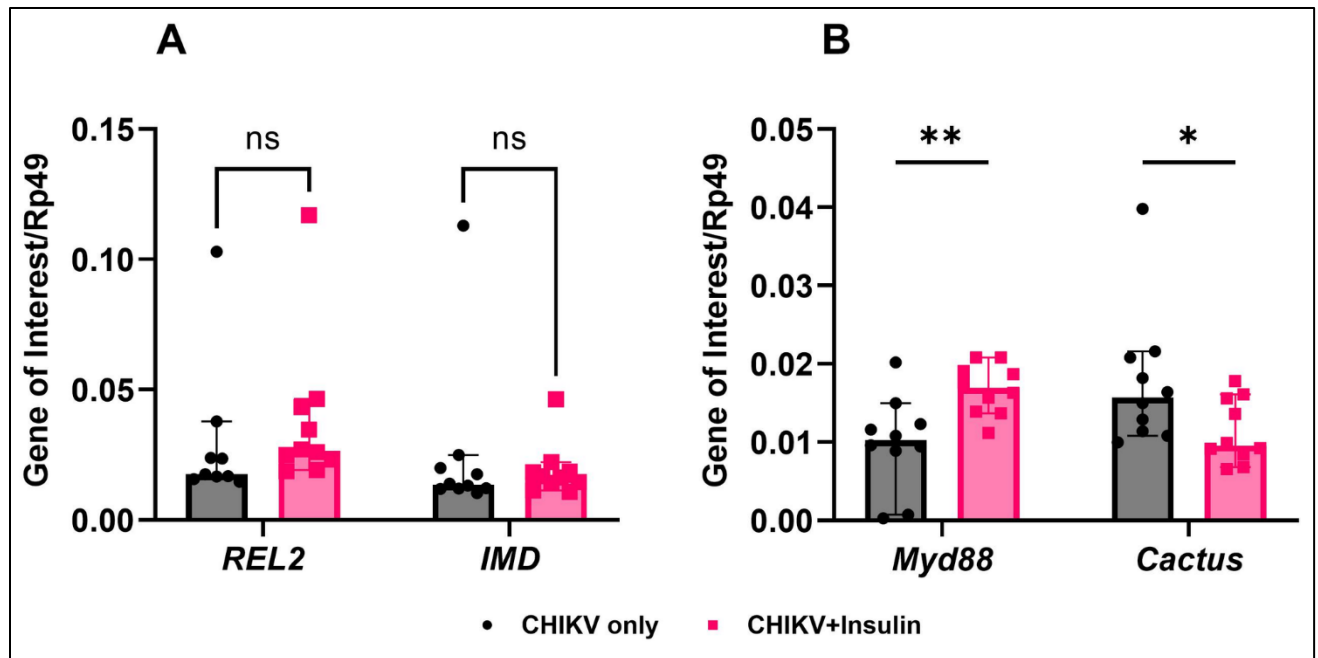


Fig.4: Insulin's effect on major immune pathways of *Aedes aegypti* mosquitoes infected with chikungunya virus (CHIKV). RNA extracted from mosquito midguts (n=10) exposed to chikungunya virus (CHIKV) in the presence or absence of insulin was tested by RT-qPCR. Values for the genes of interest (GOI) were normalized to the housekeeping gene, *Rp49*. **(A) IMD pathway.** Relish 2 (*REL2*), the transcription factor, and *IMD*, one of the proteins essential for phosphorylating *REL2*, were tested. **(B) Toll pathway.** *Myd88*, an adaptor for the Toll pathway, and *Cactus*, a negative regulator of the pathway, were tested. Statistical analysis was done using multiple unpaired t-tests on samples tested for normality and outliers; ns=non-significant, **p=0.005, *p=0.026. Data are presented as median with 95% confidence interval (CI). The studies were performed as two independent biological replicates.

Silencing of the Toll pathway adaptor protein, *Myd88*, enhances CHIKV infection rates in *Ae. aegypti* mosquitoes compared to mock. In the context of CHIKV infection, it has been

suggested that RNAi is the main antiviral pathway, while other pathways like JAK/STAT, Toll, and IMD have previously been thought to be less critical²⁷. However, we found that the Toll pathway was activated in the presence of insulin following CHIKV exposure, and thus hypothesized that the Toll pathway is important for CHIKV infection of mosquitoes. To test this, we used dsRNA to knockdown *Myd88* in live mosquitoes. We used dsRNA for green fluorescent protein (GFP) as a knockdown control. We observed a knockdown efficiency of ~90% 6-days-post-IT-injection (6-dpIT) of dsRNA (Fig.5A; ****p<0.0001). Surprisingly, mortality rates for the *Myd88* knockdown group were significantly higher than GFP knockdown controls 1-dpIT (Supplementary Fig.S3); however, survival rates from the remaining mosquitoes were similar at 6-dpIT injection when the mosquitoes were exposed to CHIKV. We then exposed mosquitoes to an infectious bloodmeal containing CHIKV and found significantly higher infection rates (Fig 4B; *p=0.0112) in mosquitoes depleted of *Myd88* compared to GFP controls, while the viral titers were similar (Fig.5C; p=0.6259). These data demonstrate that the Toll pathway, and *Myd88* in particular, limits CHIKV infection in *Ae. aegypti*.

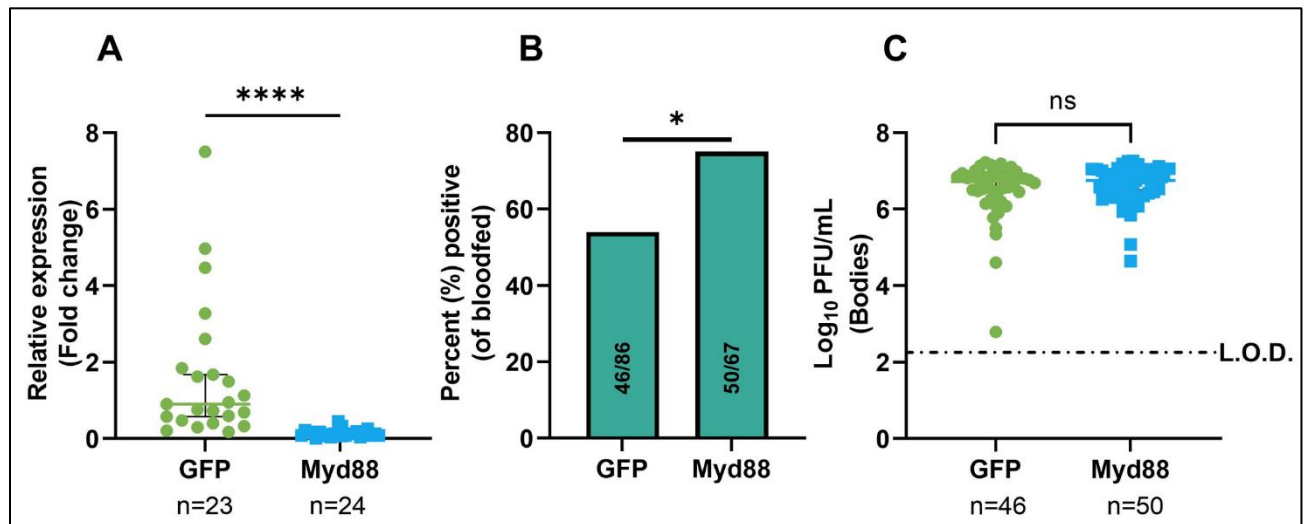


Fig.5: Silencing of Toll pathway adaptor, *Myd88*, reduces chikungunya virus (CHIKV) infection in *Ae. aegypti* mosquitoes. 1-day-old female *Ae. aegypti* mosquitoes were injected intrathoracically (IT) with 500 ng dsRNA for *Myd88* and green fluorescent protein (GFP) and 6 days later tested for KD efficiency and exposed to 7×10^7 PFUs/mL CHIKV. **(A) Knockdown efficiency.** RT-qPCR was performed on lysates collected from 5 whole mosquitoes at 6 days-post-IT injection. Ct values for *Myd88* were normalized to the housekeeping gene (*Rp49*), and *Myd88* expression was compared between GFP and *Myd88* silenced mosquitoes using delta delta Ct ($\Delta\Delta Ct$) method for relative gene expression. Statistical analysis was done by Mann Whitney test on samples tested for normality. Data are presented as median with 95% CI; **** $p < 0.0001$. **(B) Analysis of infection rates.** Mosquitoes were exposed to CHIKV 6 days-post-IT injection and 7 days later, infection rates were measured by taking the percentage of bodies positive for infectious virus to the total number of blood-fed mosquitoes. Statistical analysis was done using two-sided Fisher's exact test; * $p = 0.0112$. **(C) Quantification of infectious virus.** Infectious virus was quantified by plaque assay and compared between infected groups knocked down for *Myd88* (n=17) vs. GFP (n=16). Statistical analysis was done using unpaired t-test on log transformed titers tested for normality and outliers. Limit of detection (L.O.D.) is 2.255 logs of virus. Data are presented as median with 95% CI; ns=non-significant. The studies were performed as five independent biological replicates and combined for this analysis.

Insulin, at concentrations found in uninfected humans, inhibits CHIKV infection of *Ae. aegypti* mosquitoes. Insulin levels in humans range from 0.73 to 11 $\mu\text{g/L}$ depending on blood glucose levels^{28,29}, lower than the levels we found in obese mice (27.7 $\mu\text{g/L}$). Therefore, we next

tested whether lower insulin concentrations would similarly impact CHIKV infection of mosquitoes. We exposed mosquitoes to an artificial bloodmeal containing CHIKV with 0, 2, 5, or 10 $\mu\text{g/L}$ insulin and tested for infection 7 days later. To ensure that the defibrinated sheep blood used for artificial infections did not add additional insulin levels that would confound results, we quantified insulin in sheep blood and found that it was $\sim 0.31 \mu\text{g/L}$ (Supplementary Fig.S4). Compared to no insulin, we found similar infection rates at 2 $\mu\text{g/L}$ insulin, which corresponds to normal non-fasting insulin levels in healthy humans^{28,29}. In contrast, mosquitoes exposed to 5 $\mu\text{g/L}$ insulin trended towards reduced infection rates, while those exposed to 10 $\mu\text{g/L}$ insulin had significantly lower CHIKV infection rates compared to mosquitoes exposed to a bloodmeal with no added insulin (Fig.6). These results suggest that insulin, at concentrations found in uninfected humans, reduces CHIKV infection in *Ae. aegypti* mosquitoes.

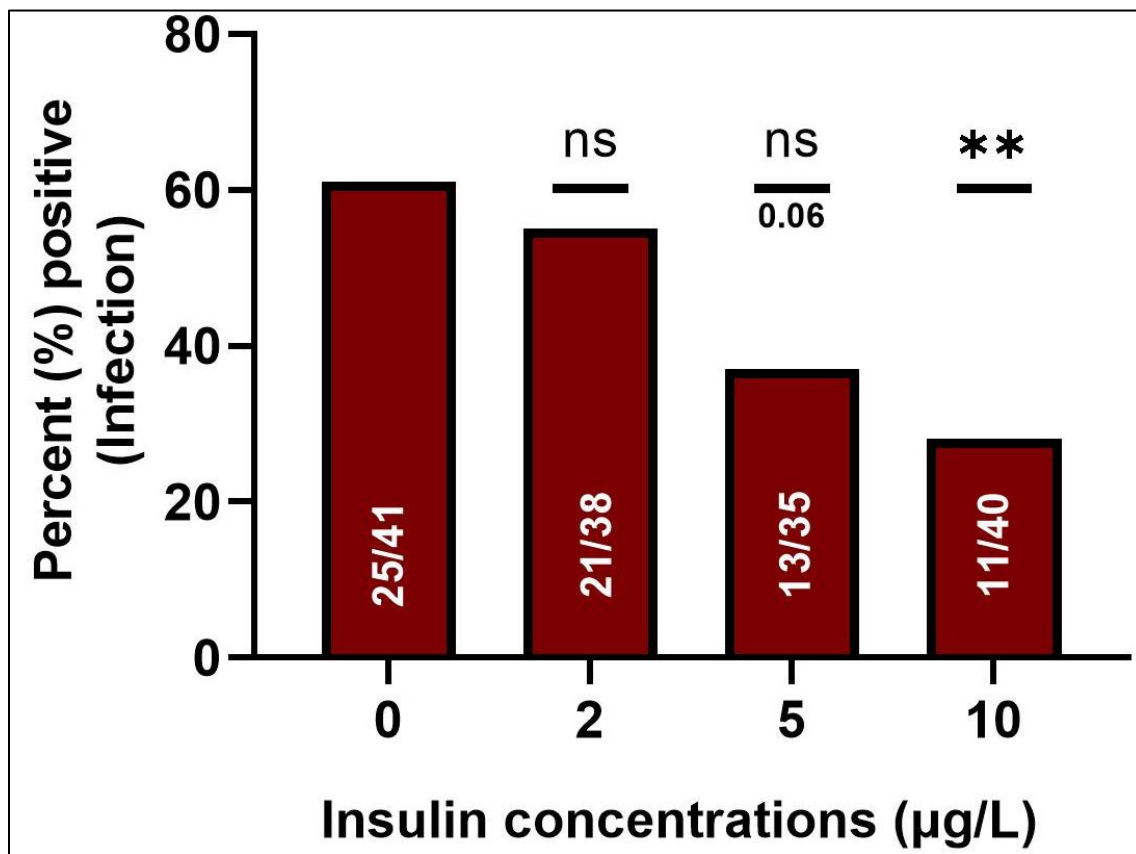


Fig.6: Impact of insulin, at concentrations found in humans, on chikungunya virus (CHIKV) infection of *Aedes aegypti* mosquitoes. *Ae. aegypti* mosquitoes were exposed to 7×10^7 PFUs/mL of CHIKV in the presence of varying insulin concentrations. Seven days later, whole bodies were collected and tested for infectious virus by plaque assay. Statistical analysis was done using two-sided Fisher's exact test; **p=0.0035, ns=non-significant. These studies were performed as two independent biological replicates, which have been combined for analysis.

4.4 Discussion

Vector-borne diseases contribute to ~17% of the overall infectious disease burden and ~700,000 deaths annually³⁰. *Aedes* spp. mosquitoes are important vectors of major human pathogens like dengue virus (DENV)³¹, Zika virus (ZIKV)³², and CHIKV³³ globally³⁴. However, few studies have interrogated the impact of human biological factors on mosquito-borne virus transmission, which limits our ability to develop control strategies. One exceedingly common biological factor in humans is obesity. In our previous studies, using a natural transmission cycle between mice and mosquitoes, we showed that *Ae. aegypti* mosquitoes fed on alphavirus-infected obese mice had significantly lower infection rates than those fed on lean mice, despite similar viremia in both groups⁸. Obesity alters numerous host factors including glucose, lipids, insulin, hormones, and cytokines³⁵. Insulin has been shown to have an antiviral effect on various flaviviruses¹⁵⁻¹⁷ in mosquito cells and in live mosquitoes and an alphavirus, Sindbis virus (SINV)¹⁷ *in vitro* and in flies, but the extent to which insulin impacts mosquito-borne virus transmission potential remains unknown.

Here we show that mosquitoes exposed to insulin, at concentrations found in CHIKV infected obese mice at peak viremia (27.7 μ g/L), have significantly lower infection and

transmission rates than those with no insulin exposure. However, since natural CHIKV transmission occurs between humans and mosquitoes, we also determined the impact of insulin at concentrations found in humans on CHIKV infection of *Ae. aegypti* mosquitoes. In uninfected humans, non-fasting insulin levels range from 0.73 to 11 $\mu\text{g/L}$ ^{28,29}, therefore, we exposed *Ae. aegypti* mosquitoes to an infectious bloodmeal containing CHIKV and varying levels of insulin (0, 2, 5, or 10 $\mu\text{g/L}$) and found that insulin trended to reduce CHIKV infection of mosquitoes at 5 and 10, but not 2 $\mu\text{g/L}$ (Fig.6). Importantly, the insulin levels present in humans with obesity following alphavirus infection are unknown and may be higher than the concentrations tested here. Consistent with this possibility, a dramatic increase in insulin following infection in obese, but not lean mice, has been previously reported with influenza virus³⁶ and murine cytomegalovirus (MCMV)³⁷ and in humans³⁷. Future research should assess insulin dynamics during alphavirus infection in humans. These results suggest that physiologically relevant levels of insulin inhibit CHIKV infection and transmission potential of *Ae. aegypti* mosquitoes.

Previous research has shown that insulin activates the JAK/STAT pathway and inhibits flavivirus infection in insects and mosquitoes¹⁵. Other studies have implicated insulin's antiviral effect to insulin-signaling dependent activation of ERK^{16,17} through secretion of anti-microbial peptides (AMPs)¹⁶, which are important components of the Toll and IMD pathway in insects³⁸. The Toll pathway has been implicated in controlling dengue virus (DENV) infection of *Ae. aegypti* mosquitoes^{39,40} through the secretion of various AMPs⁴¹, however its role in CHIKV infection is unknown. We performed RNAseq on midgut RNA from mosquitoes exposed to CHIKV in the presence or absence of insulin and found that genes present in the Toll and IMD pathways were enriched. We then validated these results by assessing expression of genes in the RNAi, JAK/STAT, Toll, IMD pathways, and found no differences in gene expression except for those in

the Toll pathway. Specifically, we found that the adaptor, *Myd88*, was upregulated while the negative regulator of the pathway, *Cactus*, was downregulated, suggesting Toll pathway activation in the presence of insulin. These data suggested the Toll pathway may be important for insulin's reduction in CHIKV infection in mosquitoes.

The importance of the Toll pathway in controlling CHIKV infection of mosquitoes was shown by knocking down *Myd88*, which increased infection rates in mosquitoes compared to a mock knockdown group. Interestingly, we also found that mosquitoes knocked down for *Myd88* had significantly lower survival rates compared to GFP knockdown controls at 1-dpIT (Supplementary Fig.S3). A possible explanation for this is the Toll pathway's impact on melanization in *Drosophila*⁴²; given that melanin helps in thermoregulation and maintaining gut microbiota in insects⁴³, its disruption might contribute to the high mortality rates in *Myd88* knockdown mosquitoes. Consistent with our findings, previous studies have shown that Toll pathway knockdown was lethal to insects at each developmental stage⁴⁴. In *Drosophila melanogaster*, the Toll pathway has been shown to protect against bacteria⁴⁵ and knockdown of this pathway might have increased the susceptibility of mosquitoes to bacterial infections leading to high mortality rates. We also found downregulation of pathways involved in longevity regulation and protein processing in the endoplasmic reticulum, in the presence of insulin. In *Drosophila*, various AMPs like cecropins, attacins, and defensins were present at higher levels in ageing flies^{46,47}. Therefore, the Toll pathway might be responsible for downregulating the longevity-regulating pathways. In the CHIKV life cycle, the structural polyprotein is cleaved into pE2 (precursor of E2 and E3), 6K and E1 in the endoplasmic reticulum (ER)⁴⁸, therefore, downregulation of protein processing in the ER might affect viral protein maturation. These results suggest that insulin's inhibitory effect on CHIKV infection might be due to several processes and

activation of the Toll pathway in the presence of insulin might be one such factor responsible for reducing CHIKV infection in *Ae. aegypti* mosquitoes, which needs to be validated by future studies.

In conclusion, we have shown that insulin, at physiologically relevant concentrations, inhibits CHIKV infection and transmission potential of *Ae. aegypti* mosquitoes, which may be mediated by the Toll pathway. Notably, this study establishes only an associative link between obesity, insulin, the Toll pathway, and the vector competence of *Ae. aegypti* mosquitoes, which needs to be examined in future studies. A thorough understanding of the interplay between human biological factors (e.g., metabolic status, age, sex, etc.) and the vector's immune response could serve as a foundation for novel control strategies against mosquito-borne viruses.

4.5 Experimental Procedures

Biohazard considerations

All experiments using CHIKV were performed in a BSL-3 facility in compliance with all CDC and NIH guidelines. These studies were approved by the Institutional Animal Care and Use Committee (IACUC#21-084) and Institutional Biosafety Committee (IBC#21-084) at Virginia Tech. All mosquito studies were performed under ACL3 conditions.

Cells and Viruses

Vero cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1 mg/mL gentamicin, 1% non-essential amino acids (NEAA) and 25 mM HEPES buffer in an incubator at 37°C with 5% CO₂. CHIKV SL-15649 (ECSA lineage), rescued

from an infectious clone, was a kind gift from Dr. Mark Heise^(Morrison et al., 2011) (the University of North Carolina at Chapel Hill).

Mice and Diets

C57BL/6N mice were obtained from Charles River Laboratories at three to four weeks of age and allowed to acclimatize for a week before initiating diets. Mice were housed in groups of five per cage and maintained at ambient temperature with *ad libitum* supply of food and water. All diets used for the study were obtained from Research Diets (New Brunswick, NJ, USA). Twenty mice (10 males and 10 females) were placed on a low-fat diet with 10% kcal fat (LFD; D12450K) and 20 (10 males and 10 females) on a high-fat diet with 60% kcal fat (HFD; D12492). Throughout the manuscript, we refer to the groups as lean (low fat diet) or obese (high fat diet). The mice were kept on these diets for 18-20 weeks before infections, and the same diets were continued until the end of the experiment.

Mosquito rearing and infections

Ae. aegypti females belonging to the 20th generation of a colony derived from wild mosquitoes collected from Guerrero, Mexico were used for these studies. The mosquitoes were maintained at 28°C, 70% relative humidity (RH) and a 12 h light:12 h dark cycle and fed with 10% sucrose solution *ad libitum*. Human insulin was obtained from MedChemExpress (NJ, USA; Catalog #HY-P0035). For infections, 3-5-day-old mosquitoes were separated into cartons and exposed to an artificial bloodmeal containing defibrinated sheep's blood and 7E+07 PFUs/mL CHIKV⁵⁰, with or without insulin using a Hemotek membrane feeder. For artificial infections, the infectious bloodmeal comprised of 30% infectious virus added to 70% of defibrinated sheep blood. Fully

engorged mosquitoes were sorted and maintained in an environmental chamber under the conditions mentioned above.

After 9 days, the mosquitoes were cold anesthetized and bodies, legs/wings, and saliva were collected to assess infection, dissemination, and transmission, respectively. Bodies and legs/wings were homogenized using Qiagen's TissueLyzer II at 25 cycles per minutes for 2 minutes in 2 mL tubes containing a metal bead and RPMI media with 2% FBS, 10 mM HEPES, 50 µg/mL of gentamicin sulphate and 2.5 µg/mL of Amphotericin B (herein referred to as mosquito diluent). Saliva was collected by placing the proboscis in a 20 µL barrier tip containing 10 µL immersion oil for 45 minutes, after which the contents were pipetted in a tube containing 50 µL mosquito diluent. Plaque assays were performed on the saliva samples and the supernatant from bodies and legs/wings as previously described⁵¹.

RNA extraction from mosquito midguts

Mosquito midguts were dissected 1-day-post-infectious-bloodmeal (dpbm) and transferred to 1.7 mL tubes containing 200 µL of TRIzol reagent (ThermoFisher). RNA was extracted using manufacturer's protocol and stored at -80°C until use.

Double stranded RNA (dsRNA) synthesis

Design and synthesis of dsRNA used in knockdown assays has been described previously⁵². The genes of interest (GOI) were PCR amplified using gene-specific primers with the T7 promoter sequence at 5' end of both the primers. A GFP-containing plasmid was used as a template for GFP while RNA isolated from a pool of *Ae. Aegypti* mosquitoes was used to amplify *Myd88*. Purified PCR amplicons were used as a template for dsRNA synthesis using the MEGAscript T7 transcription kit (ThermoFisher).

Gene silencing in *Ae. aegypti* mosquitoes

RNAi-mediated gene silencing was performed as previously described⁵². Briefly, 1-day-old *Ae. aegypti* mosquitoes were cold anesthetized and injected with 500ng of dsRNA targeting *Myd88* or GFP using the Nanoject II (Drummond). After injection, mosquitoes were kept in an environmental chamber for 6 days at 28°C and 70-80% RH. Mosquitoes were sucrose and water starved on day 5 and exposed to an infectious bloodmeal containing CHIKV on day 6 post-IT injection.

Enzyme linked immunosorbent assay (ELISA)

Insulin levels were measured in mouse serum using Mouse Insulin ELISA kit (Mercodia, NC, USA) and in defibrinated sheep blood using Sheep Insulin ELISA kit (Cusabio, CA, USA) according to manufacturers' instructions. Standard curve was generated by using the optical density (OD) values of standards and used to calculate the insulin levels of the samples. Insulin concentrations are expressed in µg/L.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

All primers were ordered from Integrated DNA Technologies (IDT, Iowa, USA) and are listed in Supplementary Table 2. RT-qPCR was performed using the NEB Luna Universal One-Step RT-qPCR kit with SYBR-Green (NEB, MA, USA). The conditions for the reactions in a Bio-Rad CFX-96 were 55°C for 10 min for reverse transcription, 95°C for 1 min for initial denaturation and polymerase activation, followed by 45 cycles of 95°C for 10 s for denaturation, and 60°C for 30 s for annealing/extension. Finally, a melt curve was performed from 60°C to 95°C for 0.05 s with 0.5°C increments. Relative gene expression was compared between mosquito midguts exposed to CHIKV in the presence or absence of insulin by normalizing them to *Rp49*, which has

been used previously with *Drosophila*¹⁵, live mosquitoes⁵³, mosquito cells¹⁷ and other insects⁵⁴. For testing the knockdown efficiency, the delta delta Ct ($\Delta\Delta\text{Ct}$) method of relative quantification was used⁵⁵.

Analysis of RNAseq data and pathway enrichment analysis

Raw reads were quality-controlled using FastQC⁵⁶ and trimmed using BBDuk⁵⁷. The reference (AaegL5) gene and genome sequences and corresponding annotations were downloaded from ensemble. The clean reads were mapped to the reference genome using STAR⁵⁸ with default parameters. Gene counts were estimated using HT-Seq (htseq-count)⁵⁹. Differentially expressed genes were identified by DESeq2 using integrated Differential Expression and Pathway analysis tool (iDEP.96, South Dakota State University, SD), using p-adjusted value < 0.1 and fold change > 2 ⁶⁰. Pathway enrichment analysis was done using ShinyGO 0.77 (South Dakota State University, SD).

Statistical analysis

Statistical analysis was done using GraphPad Prism 9 (GraphPad Software, CA, USA). Details of the statistical technique used for analyses are provided in the figure legends. The level of significance has been determined by the following p-values: $p \geq 0.05$ (ns); $p = 0.05$ to 0.01 (*); $p = 0.01$ to 0.001 (**); $p = 0.001$ to 0.0001 (***); $p < 0.0001$ (****). The error bars represent standard deviation from the mean and the dotted lines denote the limit of detection (L.O.D.). All statistical analyses were performed on data after testing them for normality using the Shapiro-Wilk test.

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DECLARATION OF INTERESTS

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Chapter 5: Obesity's unexpected influence: reduced alphavirus transmission and enhanced immune activation in the vector

Pallavi Rai^{1,2}, Emily M. Webb^{2,3}, Sally L. Paulson³, Lin Kang^{1,4} and James Weger-Lucarelli^{1,2*}

¹Department of Biomedical Sciences and Pathobiology, Virginia Tech

²Center for Emerging, Zoonotic, and Arthropod-borne Pathogens, Virginia Tech

³Department of Entomology, Fralin Life Sciences Institute, Virginia Tech

⁴Biomedical Affairs & Research, Edward Via College of Osteopathic Medicine, Monroe,
Louisiana, USA

Author Note

Pallavi Rai  <https://orcid.org/0000-0001-7109-6132>

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Correspondence concerning this article should be addressed to James Weger-Lucarelli, PhD, Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, VA 24060. Email: weger@vt.edu

5.1 Abstract

Chikungunya virus (CHIKV) and Mayaro virus (MAYV) are emerging/re-emerging alphaviruses of global public health concern, transmitted by *Aedes spp.* mosquitoes and responsible for recent disease outbreaks in the Americas. The capacity of these viruses to cause epidemics is frequently associated with heightened mosquito transmission, which in turn is governed by interactions of the virus with the vertebrate host and the vector. While numerous studies have looked at virus-vector interactions driving their epidemic potential, there is a considerable gap in understanding the impact of vertebrate host factors on alphavirus transmission by mosquitoes. Obesity is a ubiquitous biological factor, which we have shown reduces alphavirus transmission to and by mosquitoes. We hypothesized that alphavirus-infected obese bloodmeal activated mosquito immune genes and/or pathways, blocking their transmission by the mosquitoes. To test this, we fed mosquitoes on alphavirus-infected lean and obese mice and performed RNA sequencing (RNAseq) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) on the midgut RNA to identify potentially antiviral genes and/or pathways altered in the mosquitoes in response to an infected obese bloodmeal. Here, we confirmed that feeding on infected obese blood reduced the transmission potential of alphaviruses by *Ae. aegypti* mosquitoes. We found upregulation of the Toll pathway and downregulation of several metabolic and other genes in the mosquitoes fed on alphavirus-infected obese bloodmeal. Knockdown of two downregulated genes—AAEL009965 and fatty acid synthase (FASN)—reduced CHIKV and MAYV infection rates or titers in artificially infected mosquitoes, suggesting their possible role in reducing transmission rates. Through this study, we have used obesity to identify factors associated with alphavirus transmission by mosquitoes. This approach can be used to design broadly effective antiviral

measures against mosquito-borne viruses like releasing transgenic mosquitoes deficient in the identified genes.

5.2 Introduction

Chikungunya virus (CHIKV) and Mayaro virus (MAYV) are two emerging/re-emerging mosquito-borne alphaviruses that have caused recent outbreaks of acute febrile illness and chronic debilitating polyarthritides¹⁻³ in the Americas. The Pan American Health Organization (PAHO) reported ~136,000 cases of CHIKV in the Americas in the first three months of 2024, with the initial instances of transmission recorded in Argentina and Uruguay⁴. First reported in Trinidad and Tobago in 1954⁵, MAYV is now spreading throughout the Americas⁶, but its true prevalence may be underreported due to the absence of specific diagnostics and overlapping clinical symptoms⁷ with other important mosquito-borne diseases like dengue, malaria, and chikungunya. The absence of widely available vaccines and treatments, in conjunction with the widespread distribution of competent vectors, *Aedes aegypti* and *Aedes albopictus*⁸⁻¹⁰, underscores the substantial public health risk and epidemic potential posed by CHIKV and MAYV.

The epidemic potential of these viruses is frequently related to their enhanced transmission¹¹ by the mosquito vector, which, in turn, is influenced by vectorial capacity¹². Vectorial capacity measures the transmission potential of a vector-borne pathogen in a susceptible population¹³ and depends on various factors like the environment, vector, and vertebrate host. Vector competence—the ability of a vector to successfully transmit a pathogen—is an essential component of vectorial capacity¹³. Although extensive research has been done to demonstrate the influence of tissue barriers and antiviral responses within mosquitoes on their vector competence for various mosquito-borne viruses¹⁴⁻²², studies to investigate the impact of vertebrate host factors

on the vector and their role in influencing the transmission potential of various viruses, are limited. Obesity is a crucial host factor that has assumed the status of an ongoing pandemic²³ affecting 13% of adults globally and 42% of adults in the US²⁴ and has been shown to worsen the disease outcome of various important human pathogens like SARS-CoV-2²⁵, influenza²⁶, and as we have shown, various mosquito-borne viruses like CHIKV, MAYV²⁷ and dengue virus (DENV)²⁸. Interestingly, we found that *Ae. aegypti* mosquitoes fed on alphavirus-infected diet-induced obese (hereinafter referred to as obese) mice had reduced infection and transmission rates compared to those fed on healthy weight (hereinafter referred to as lean) mice, despite similar viremia between both the groups²⁷. However, the mechanism by which alphavirus-infected obese bloodmeal impacts their transmission by mosquitoes is still unknown.

In this study, we used a natural transmission cycle using mosquitoes and alphavirus-infected lean and obese mice and confirmed our previous finding that feeding on alphavirus-infected obese bloodmeal reduced their transmission by the mosquitoes. To determine the impact of alphavirus-infected obese bloodmeal on mosquito gene expression, we performed RNA sequencing (RNAseq) on midguts collected from mosquitoes fed on CHIKV- or MAYV-infected lean or obese mice at 1- and 4-days-post-infectious-bloodmeal (dpbm). We identified activation of the Toll pathway and downregulation of several metabolic and other genes at 1 dpbm in mosquitoes fed on CHIKV- or MAYV-infected obese mice compared to those fed on their lean counterparts. These results not only identify potentially immune-related genes in mosquitoes but also validate their relevance for alphavirus infection through knockdown studies. Therefore, these findings could potentially revolutionize the development of broadly effective antiviral strategies against major mosquito-borne viruses.

5.3 Methods

Biohazard considerations

All experiments involving MAYV were conducted in a BSL-2 facility, while CHIKV studies were carried out in a BSL-3 facility, in compliance with all CDC and NIH guidelines. These studies were approved by the Institutional Animal Care and Use Committee (IACUC#21-041) and Institutional Biosafety Committee (IBC#21-084) at Virginia Tech. All mosquito studies were conducted under ACL-3 conditions.

Cells and viruses

Vero cells (CCL-81) purchased from American Type Culture Collection (ATCC; Manassas, VA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1 mg/mL gentamicin, 1% non-essential amino acids (NEAAs) and 25 mM HEPES buffer in an incubator at 37 °C with 5% CO₂. CHIKV SL-15649 (ECSA lineage), derived from an infectious clone, was kindly provided by Dr. Mark Heise²⁹ (the University of North Carolina at Chapel Hill). MAYV strain TRVL4675 used for this study was derived from an infectious clone and has been described previously³⁰. Infectious viral titers were determined by plaque assay as previously described³¹.

Mice and diets

C57BL/6N mice, obtained from Charles River Laboratories at 3-4 weeks of age, were allowed a one-week acclimation period before initiating diets. Mice were housed in groups of 3-5 per cage and maintained at ambient temperature with access to food and water *ad libitum*. All diets used for the study were obtained from Research Diets (New Brunswick, NJ, USA). Twenty-eight mice (18 males and 10 females) were placed on a low-fat diet with 10% kcal fat (LFD; D12450K) and 28

(18 males and 10 females) on a high-fat diet with 60% kcal fat (HFD; D12492). Throughout the manuscript, we refer to the groups as lean (low-fat diet) or obese (high-fat diet). The mice were placed on their respective diets for 16-18 weeks before infections and remained on the diets until the end of the experiments.

Glucose and insulin measurements

Two weeks before infections (16 weeks after diet initiation), mice were bled via the submandibular vein, and non-fasting glucose levels were measured at 9-10 am using the Abox glucose monitoring kit. Serum insulin levels were quantified using the Mouse Insulin ELISA kit (Mercodia, NC, USA) according to the manufacturer's instructions. A standard curve was generated using the optical density (OD) values of standards and used to calculate the insulin levels of each sample, expressed in $\mu\text{g/L}$.

Mouse infections

Mice were inoculated subcutaneously in the left hind footpads, as described previously³². For CHIKV and MAYV infections, 10^2 and 10^4 plaque-forming units (PFUs) of the virus in 50 μL of Roswell Park Memorial Institute (RPMI)-1640 media with no additives was used, respectively. Mice were weighed daily and monitored for weight loss. Blood was collected via the submandibular vein, and serum was separated by centrifugation at $5000 \times g$ for 10 min, transferred to fresh tubes, and stored at -80°C . For infections and blood collections, mice were anesthetized using isoflurane inhalation. Mice were treated with 0.1 mg in 100 μL of IFNAR1 blocking antibody (MAR1-5A3, Leinco Technologies, Inc., Missouri, USA) via the intraperitoneal route one day before CHIKV infection to increase their susceptibility to infection. Viremia was measured in all studies by plaque assay as previously described.

Mosquito transmission studies

Ae. aegypti females belonging to the 20th-24th generation of a colony derived from wild mosquitoes collected from Guerrero, Mexico³³ were used for these studies. The mosquitoes were maintained on a 12 h light:12 h dark cycle, at 28 °C with 70% relative humidity (RH) and fed with 10% sucrose solution *ad libitum*. When the mice had high viremia—2 days for MAYV and 2 or 3 days for CHIKV—the mice were anesthetized with ketamine and xylazine (90 mg/kg and 5 mg/kg respectively) intraperitoneally, and the mosquitoes were allowed to feed for 45 minutes. Fully engorged mosquitoes were sorted into new cages and maintained in an environmental chamber under the conditions mentioned above. Immediately after the bloodmeal, 10-15 fully engorged mosquitoes were placed individually in 2 mL tubes containing a metal bead and mosquito diluent (RPMI media with 2% FBS, 10 mM HEPES, 50 µg/mL of gentamicin sulfate and 2.5 µg/mL of Amphotericin B) for comparing viral titers in mosquitoes fed on infected lean and obese mice as described below.

After 9 days, the remaining mosquitoes were cold anesthetized and bodies, legs/wings, and saliva were collected to assess infection, dissemination, and transmission, respectively. As previously described, bodies and legs/wings were homogenized using Qiagen's TissueLyzer II³⁴. Saliva was collected by placing the proboscis in a 20 µL barrier tip containing 10 µL immersion oil for 45 minutes, after which the contents were pipetted into a tube containing 50 µL mosquito diluent. Saliva samples and the supernatant from bodies and legs/wings were tested for infectious virus by plaque assays³¹.

RNA extraction from mosquito midguts

Mosquito midguts were dissected 1- and 4-days-post-infectious bloodmeal (dpbm) and stored in 1.7 mL tubes containing 200 μ L of TRIzol reagent (ThermoFisher). RNA was extracted as per the manufacturer's protocol and stored at -80°C until use.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed on midgut RNA isolated from mosquitoes fed on CHIKV- or MAYV-infected lean and obese mice using the NEB Luna Universal One-Step-RT-qPCR kit with SYBR Green (NEB, MA, USA). The reaction conditions in a Biorad CFX-96 were reverse transcription at 55 °C for 10 minutes and initial denaturation and reverse transcriptase deactivation at 95°C for 1 minute, followed by 44 cycles of denaturation at 95 °C for 10 seconds and 60 °C for 30 seconds for annealing/extension. A melt curve was generated from 60 °C to 95 °C with 0.5 °C increments at an interval of 0.05 seconds. For comparing relative gene expression, the expression of the gene of interest (GOI) was normalized to *Rp49*, a housekeeping gene that has been used for *Drosophila*³⁵, live mosquitoes³⁶, and other insects³⁷.

Analysis of RNAseq data and pathway enrichment analysis

RNA from the midguts of mosquitoes fed on CHIKV- or MAYV-infected lean and obese mice were submitted to Novogene Corporation Inc. (Sacramento, CA, USA) to perform library prep and RNA sequencing. The average sequencing bases per sample were 9667.37 Mbp with Q30 of 96.38%. Raw reads were quality-controlled using FastQC³⁸ and trimmed using BBDuk³⁹. The reference gene (AaegL5), genome sequences, and corresponding annotations were downloaded from Vectorbase. The clean reads were mapped to the reference genome using STAR⁴⁰ with default parameters. Gene counts were estimated using HT-Seq (htseq-count)⁴¹. Differentially

expressed genes (DEGs) with fold change $> 3^{42}$ were identified by DESeq2, and pathway analysis was done on these DEGs using the integrated differential expression and pathway analysis tool (iDEP.96, South Dakota State University, SD).

Double-stranded RNA (dsRNA) synthesis

dsRNAs for performing gene knockdown studies were designed and synthesized as described previously⁴³. The genes of interest (GOI) were PCR amplified using gene-specific primers with the T7 promoter sequence at the 5' end of both primers (**Supplementary Table S1**). dsRNA targeting the green fluorescent protein (GFP) was used as a control. The template for GFP was a GFP-containing plasmid, while RNA isolated from a pool of *Ae. aegypti* mosquitoes was used as a template for GOI. Purified PCR amplicons were used as a template for dsRNA synthesis using the MEGAscript T7 transcription kit (ThermoFisher) according to manufacturer guidelines.

Gene silencing and infections post-silencing in *Ae. aegypti* mosquitoes

RNAi-mediated gene silencing was performed as previously described⁴³. Briefly, 1-2-day-old *Ae. aegypti* mosquitoes were cold anesthetized and then intrathoracically (IT) injected with 500 ng of dsRNA targeting either the gene of interest (GOI) or GFP using the Nanoject II system (Drummond). After injection, mosquitoes were kept in an environmental chamber for 3 days at 28°C and 70–80% RH and fed with 10% sucrose solution *ad libitum*. Knockdown efficiency was tested by the delta-delta Ct ($\Delta\Delta Ct$) relative quantification method⁴⁴, with average GFP values being used as control and Rp49 as the housekeeping gene.

Mosquitoes were sucrose and water-starved on day 2-post-IT injections (dpIT) and exposed to an infectious blood meal containing CHIKV or MAYV at 3 dpIT. For infections, mosquitoes were exposed to an artificial blood meal containing defibrinated sheep's blood and 7E+07

PFUs/mL of CHIKV⁴⁵ or MAYV using a Hemotek membrane feeder. For artificial infections, the infectious blood meal comprised 30-50% infectious virus mixed with defibrinated sheep blood. Fully engorged mosquitoes were sorted and maintained in an environmental chamber under the conditions mentioned above. After 9 days, the mosquitoes were cold anesthetized and bodies, legs/wings, and saliva were collected and tested for infection, dissemination, and transmission, respectively, as described above.

Statistical analysis

Statistical and regression analyses were done using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Details of the statistical technique used for the analyses are provided in the figure legends. The level of significance has been determined by the following p-values: $p \geq 0.05$ (ns); $p = 0.05$ to 0.01 (*); $p = 0.01$ to 0.001 (**); $p = 0.001$ to 0.0001 (***); $p < 0.0001$ (****). The error bars represent the standard deviation from the mean and the dotted lines denote the limit of detection (L.O.D.). All statistical analyses were performed on data after tested for normality using the Shapiro-Wilk test and outliers using the ROUT method.

5.4 Results and Discussion

C57BL/6N mice fed a high-fat diet developed hyperglycemia and hyperinsulinemia resembling obesity in humans. We have previously established that obesity worsens the disease outcome after infection with various alphaviruses; however, it also reduces alphavirus infection of mosquitoes²⁷. To determine the mechanism by which obesity was driving reduced alphavirus transmission by the vector, we used previously established mouse models of diet-induced obesity⁴⁶. Briefly, 4-week-old C57BL/6N mice were fed a low (LFD) or high-fat diet (HFD) for 18 weeks, at which time, they were inoculated subcutaneously with CHIKV or MAYV via footpad

injections. At peak viremia, mosquitoes were allowed to feed on these mice, midgut dissections were done at 1- and 4-days-post-infectious bloodmeal (dpbm), and vector competence dissections were performed at 9 dpbm (**Fig.1A**). As expected, mice fed a HFD weighed more than mice fed LFD fed controls from 2 weeks post-diet-initiation until the day of infection (**Fig.1B, ****p < 0.0001**). Obesity is associated with increased glucose levels (hyperglycemia)⁴⁷ and elevated fasting and non-fasting insulin levels (hyperinsulinemia)⁴⁸. Therefore, we measured glucose and insulin levels in our lean and obese mice at 16 weeks post-diet-initiation, which were significantly higher in obese compared to lean mice (**Fig.1C & D; ****p < 0.0001**). Thus, our mouse model of diet-induced obesity (DIO) mimics human obesity in terms of high weight gain, hyperglycemia, and hyperinsulinemia.

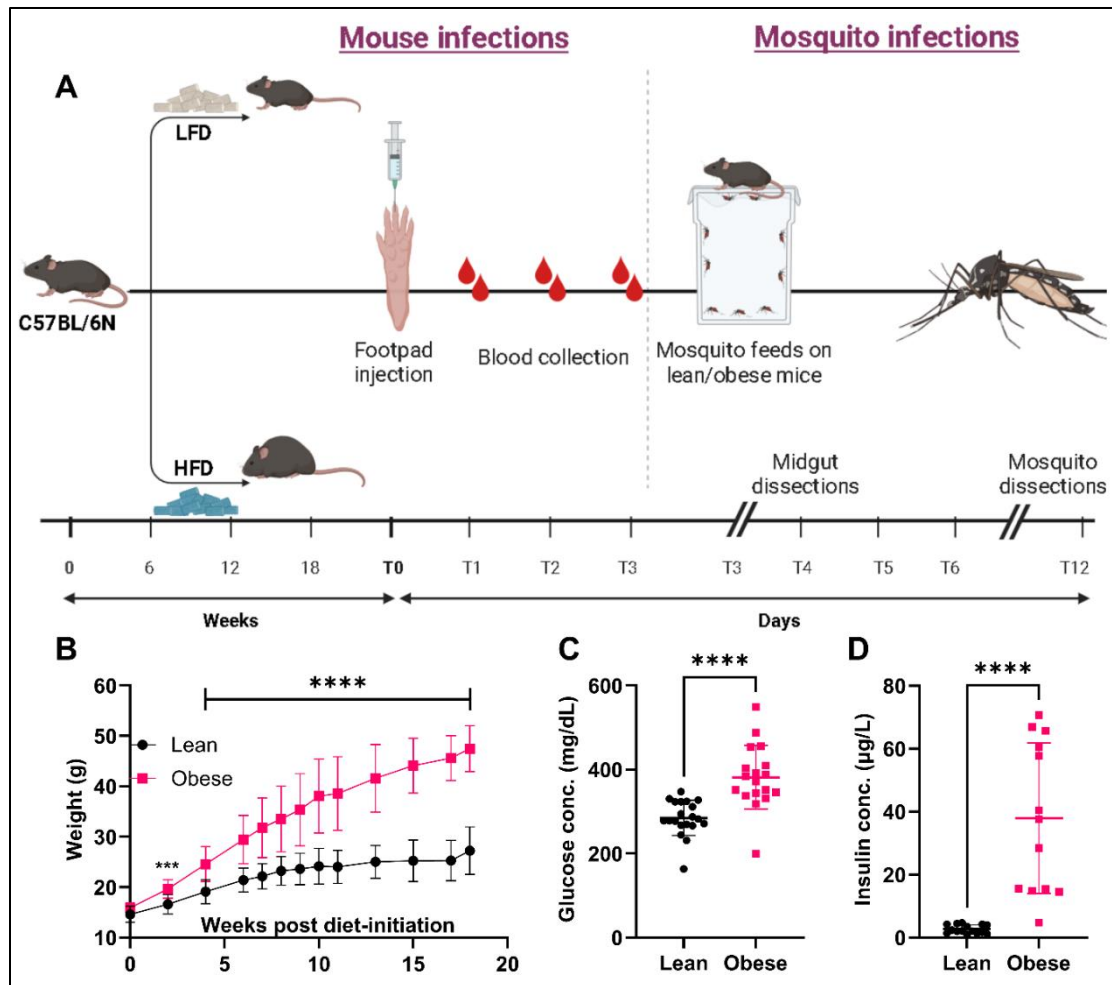


Fig.1: C57BL/6N mice fed a high-fat diet develop diet-induced obesity (DIO) with hyperglycemia and hyperinsulinemia resembling humans with obesity.

(A) Experimental design. Four-week-old C57BL/6N were fed a high (60% fat) or a low (10% fat) diet for 18 weeks. Lean and obese C57BL/6N mice (**n=8-10/group**) were inoculated with either 10^2 PFUs of CHIKV or 10^4 PFUs of MAYV via footpad inoculation. For CHIKV infections, the mice were intraperitoneally injected with 0.1 mg of MAR-1, a type I IFN receptor-blocking antibody, 12 hours before infections to make them susceptible to CHIKV. During high viremia (2 dpi for MAYV and 3 dpi for CHIKV), 3-4 days old *Ae. aegypti* mosquitoes were allowed to feed

on these mice. Midgut dissections were done on 1- and 4-days-post-infectious bloodmeal (dpbm), and dissections for vector competence were done at 9 dpbm. **(B)** Weights were measured throughout the feeding period at 1-2-week intervals, and statistical analysis was conducted using mixed-effects analysis with Sidak's multiple comparisons test. **(C)** Glucose and **(D)** insulin levels were measured two weeks before infection (16-weeks-post-diet-initiation). Statistical analysis was done using an unpaired t-test or Mann-Whitney test; *** $p = 0.001$ to 0.0001 ; **** $p < 0.0001$. The error bars indicate the standard deviation (SD) of the mean.

Ae. aegypti* fed on alphavirus-infected obese mice have reduced transmission potential despite ingesting similar amounts of virus.** We previously showed that mosquitoes fed on MAYV-infected obese mice have reduced infection and transmission rates compared to those fed on lean mice²⁷. Here, we used a natural transmission cycle between mice and mosquitoes **(Fig.1A)** and found that mosquitoes fed on infected obese mice had significantly lower infection and transmission rates compared to those fed on lean mice for CHIKV **(Fig.2A, * $p < 0.0001$; ** $p = 0.0011$ respectively)** and MAYV **(Fig.2B, **** $p < 0.0001$; ** $p = 0.0080$ respectively)**. To assess whether the mosquitoes were exposed to similar amounts of virus, we measured viral titers in the serum of alphavirus-infected lean and obese mice immediately before the mosquito feeding and found no difference between the groups for CHIKV or MAYV **(Fig.2C)**. Additionally, we collected mosquito bodies immediately after they fed on CHIKV- and MAYV-infected mice and quantified infectious virus and found that they had similar viral titers **(Fig.2D)**, suggesting that difference in viremia was not contributing to the reduced transmission potential of these mosquitoes for CHIKV and MAYV.

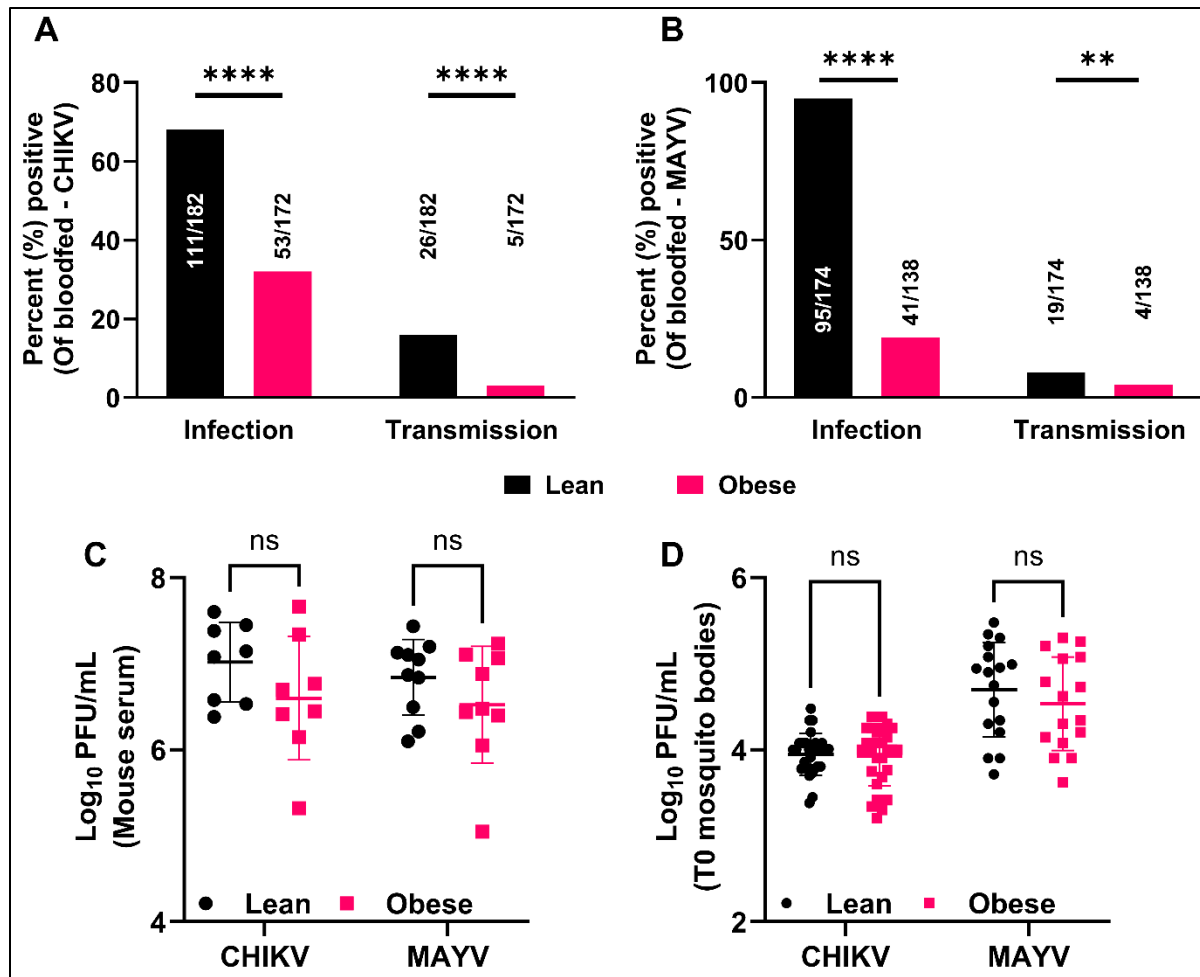


Fig.2: *Ae. aegypti* mosquitoes fed on alphavirus-infected obese mice have reduced transmission potential.

Ae. aegypti mosquitoes (n=50-60/mouse) were allowed to feed on CHIKV- or MAYV-infected lean and obese mice at peak viremia. (A & B) Infection and transmission potential of *Ae. aegypti* mosquitoes fed on alphavirus-infected lean and obese mice for CHIKV (A) and MAYV (B). Infection and transmission rates were calculated as a percentage of samples positive for infectious virus in the bodies and saliva respectively, compared to the total number of bloodfed mosquitoes. Statistical analysis was conducted using a two-sided Fisher's exact test. (C) Viremia in lean and obese mice before mosquito feeds. On the day of peak viremia, mice were anesthetized with

ketamine and xylazine (90 mg/kg and 5 mg/kg, respectively) intraperitoneally, blood was collected immediately before the mosquito feeds, and infectious virus was quantified by plaque assay. Statistical analysis was performed on log-transformed values using 2way ANOVA. **(D)** Viral titers in mosquito bodies immediately after the blood meal. Mosquitoes were allowed to feed on infected and anesthetized mice for 45 minutes, after which fully engorged mosquitoes were sorted. 2 to 3 fully engorged mosquitoes were collected from each mouse immediately after the bloodmeals in 2 mL tubes with metal beads and viral diluent. The bodies were homogenized, and infectious viral titers were determined in the supernatant of 20-30 mosquitoes using plaque assays. Statistical analysis was performed on log-transformed values using unpaired t-test or Mann-Whitney test; **p = 0.0080 ****p < 0.0001; ns – non-significant. The error bars indicate the standard deviation (SD) of the mean.

The Toll pathway is activated in mosquito midguts in response to alphavirus-infected obese bloodmeal. Based on the result that mosquitoes fed on alphavirus-infected lean or obese mice were exposed to similar amounts of virus, we hypothesized that alphavirus-infected obese bloodmeal potentially modulates the expression of immune-related genes and/or pathways in the vector, leading to their reduced transmission. Since the midgut is the initial site of arbovirus infection and one of the major transmission barriers, we evaluated midguts at 1- and 4-day-post-infectious bloodmeal (dpbm) for the major antiviral immune pathways in *Ae. aegypti*: RNA interference (RNAi), Janus-kinase signal transducer and activator of transcription (JAK/STAT), Toll and immunodeficiency (IMD) pathways. We sought to evaluate the vector's transcriptional response at the earliest time of midgut infection, and when the virus has escaped the midgut; therefore, 1 and 4 dpbm samples were collected^{42,49}. For the RNAi pathway, we tested for the expression of Argonaute-2 (Ago-2), an RNA nuclease critical for RNAi-mediated antiviral

responses and found no difference between the groups (**Supplementary Fig.S1**). For the JAK/STAT pathway, we tested the expression of the transcription factor, STAT, and the receptor, Domeless (Dome), and found no difference between mosquitoes fed on CHIKV (**Fig.3A**) or MAYV (**Fig.3B**) infected lean or obese mice. For the IMD pathway, no difference in the expression of the receptor protein, Imd, or the transcription factor, Rel2, was observed between mosquitoes fed on CHIKV (**Fig.3C**) or MAYV (**Fig.3D**) infected lean or obese mice. For the Toll pathway, we evaluated the expression of the adaptor protein, Myeloid differentiation primary response (Myd88), and the negative regulator of the Toll pathway, Cactus. We found significant downregulation of Cactus (**Fig.3E; *p=0.0478**) while no significant difference in Myd88 expression (**Fig.3E**) in mosquitoes fed on CHIKV-infected obese vs lean mice. Similarly, mosquitoes fed on MAYV-infected obese mice showed a significant downregulation of Cactus (**Fig.3F; **p=0.0011**) and an upregulation of Myd88 (**Fig.3F; **p=0.0048**) compared to mosquitoes fed on MAYV-infected lean mice. These results suggest that alphavirus-infected obese bloodmeal activates the Toll pathway in the mosquitoes, which might be one of the factors responsible for reducing infection rates and the transmission potential of mosquitoes for alphaviruses fed on obese vs lean mice. These results are consistent with our previous study where activation of the Toll pathway in the presence of insulin reduced CHIKV infection of *Ae. aegypti* mosquitoes³⁴. However, the role of the Toll pathway in reducing the vector competence of mosquitoes in the context of obesity needs to be tested by performing a knockdown of the Toll pathway components in the mosquitoes and then assessing its impact on their vector competence by feeding them on alphavirus-infected lean and obese mice.

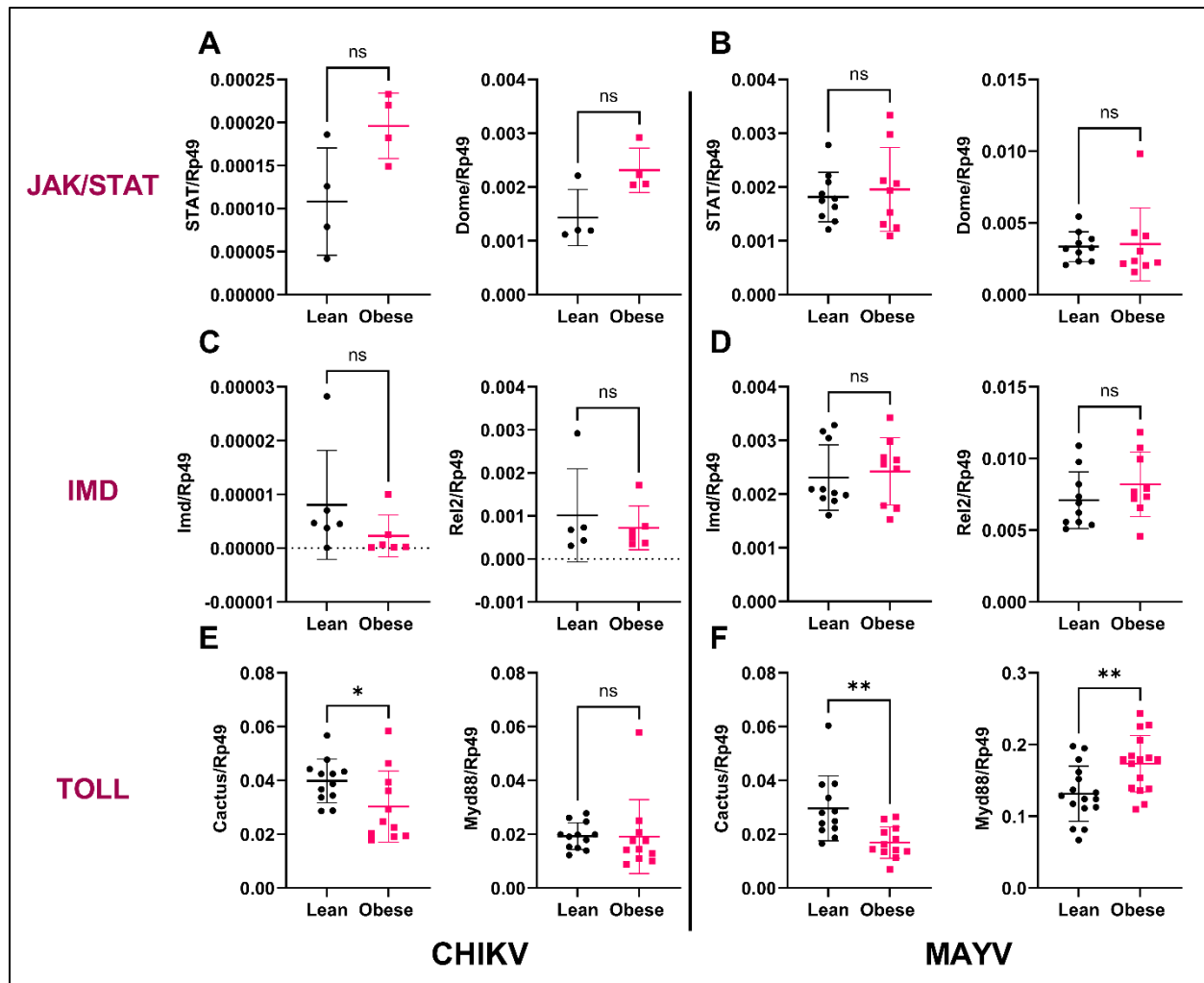


Fig.3: The Toll pathway is activated in the midguts of *Ae. aegypti* mosquitoes in response to alphavirus-infected obese bloodmeal.

Ae. aegypti mosquitoes (n=50-60/mouse) were allowed to feed on CHIKV- or MAYV-infected lean and obese mice, at peak viremia. Fully engorged mosquitoes were sorted and maintained in an environmental chamber at 28°C with 70-80% relative humidity, for 9 days. Midguts were dissected 1 day later, RNA was extracted, and gene expression was assessed by reverse-transcription quantitative PCR (RT-qPCR). Expression of genes of interest was normalized to the housekeeping gene, Rp49, and then compared between mosquitoes fed on CHIKV- or MAYV-

infected lean or obese mice. **(A & B) JAK/STAT pathway.** The transcription factor, STAT, and the pathway receptor, Domeless (Dome) were tested for mosquitoes (5-10 mosquitoes/group) fed on CHIKV (A) or MAYV (B) infected lean or obese mice. **(C & D) IMD pathway.** The receptor protein, Imd, and the transcription factor, Rel2 were tested for mosquitoes (5-10 mosquitoes/group) fed on CHIKV (C) or MAYV (D) infected lean or obese mice. **(E & F)** Expression of the adaptor protein, Myeloid differentiation primary response (Myd88), and the negative regulator of Toll pathway, Cactus were tested for mosquitoes (11-12 mosquitoes/group) fed on CHIKV (E) or MAYV (F) infected lean or obese mice. Statistical analysis was done by unpaired t-test or Mann-Whitney test; * $p < 0.01$; ** $p = 0.01$ to 0.001 ; ns – non-significant. The error bars indicate the standard deviation (SD) of the mean.

***Ae. aegypti* mosquitoes exposed to alphavirus-infected obese bloodmeal show altered gene and pathway expression which differ depending on the virus and time post-exposure.** We next sought to identify common genes and/or pathways with putative immune-related functions altered in mosquitoes in response to both CHIKV- and MAYV-infected obese bloodmeal. Thus, we performed RNA sequencing (RNAseq) analysis on midguts collected from mosquitoes fed on CHIKV- or MAYV-infected lean or obese mice at 1 dpbm, representing the initial midgut infection or early time-point⁵⁰ and at 4 dpbm representing the late time-point during which the virus would be disseminated from the midgut⁴⁹.

Differentially expressed genes (DEGs) with fold change > 3 ⁵⁰ were identified at 1 and 4 dpbm in mosquitoes fed on obese vs lean mice for both CHIKV and MAYV. We found 244 upregulated and 110 downregulated genes for CHIKV at 1 dpbm, while at 4 dpbm, there were 9 upregulated and 6 downregulated genes. For MAYV, 138 genes were upregulated and 455

downregulated at 1 dpbm, while at 4 dpbm there were 38 upregulated and 102 downregulated genes. We next identified genes that were similarly up or downregulated at the same time point for both CHIKV and MAYV. We found 7 downregulated genes in mosquitoes fed on both CHIKV- and MAYV-infected obese vs lean mice at 1 dpbm but no common upregulated genes (**Fig.4C & Table 1**). These genes were selected to validate their role in the infection of *Ae. aegypti* mosquitoes with alphaviruses. We then sought to broaden our search by identifying pathways altered in the same direction and same time point in response to both CHIKV- and MAYV-infected obese bloodmeal, but there were no common pathways in response to both the viruses (**Supplementary Figure S2**).

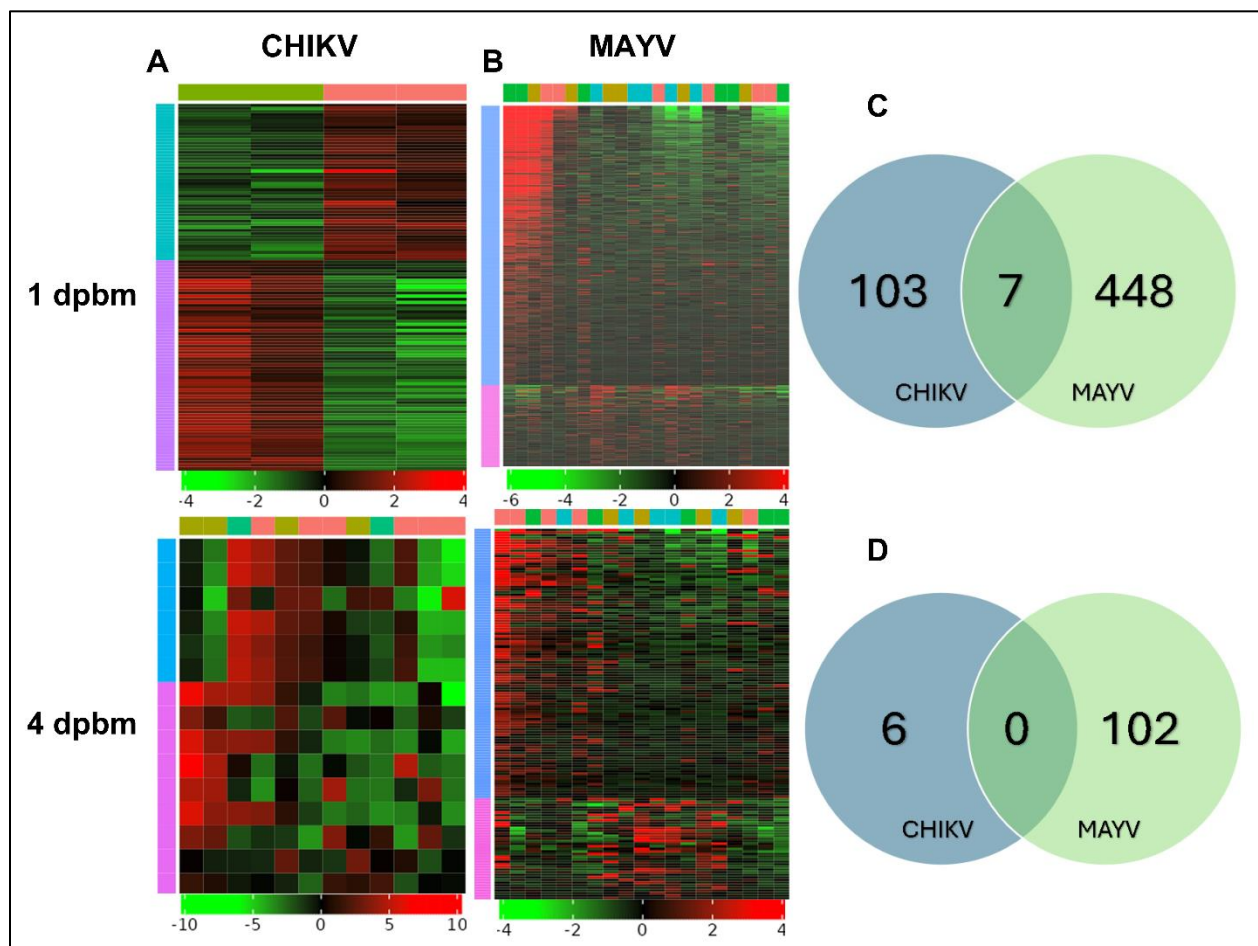


Fig.4: Identification of genes commonly expressed in mosquitoes fed on CHIKV- or MAYV-infected obese vs lean mice at 1- and 4 days-post-infectious bloodmeals (dpbm).

RNA sequencing (RNAseq) analysis was performed on the RNA extracted from mosquito midguts fed on CHIKV- or MAYV-infected lean or obese mice at 1 and 4 dpbm. Differentially expressed genes (DEGs) in obese vs lean-fed mosquitoes with fold change > 3 were generated by integrated Differential Expression and Pathway (iDEP 1.1) analysis tool and selected for downstream analyses. **Heatmaps showing differential gene expression** between mosquitoes fed on obese vs lean mice for CHIKV at 1 dpbm (**A, top panel**) and 4 dpbm (**A, bottom panel**) and MAYV at 1 dpbm (**B, top panel**) and 4 dpbm (**B, bottom panel**). Venn diagrams were generated using Venny 2.1 for commonly altered genes expressed between mosquitoes fed on obese vs lean mice for CHIKV and MAYV at 1 dpbm (**C**) and 4 dpbm (**D**).

An unspecified protein in *Ae. aegypti*, AAEL009965, and fatty acid synthase (FASN) are potentially proviral for CHIKV and MAYV. We next sought to confirm the impact of the 7 commonly downregulated genes on the infection of *Ae. aegypti* mosquitoes with CHIKV and MAYV by performing gene knockdowns, followed by artificial infections with CHIKV or MAYV (**Fig.5A**). Because they were downregulated in obese mice, we hypothesized that silencing of these genes would reduce the infection rates and/or viral titers compared to the control group. To test this, we generated dsRNA for the identified genes and green fluorescent protein (GFP) as a knockdown control. The mosquitoes were then infected with CHIKV 3-days-post-intrathoracic (dpIT) injection. Knockdown of AAEL009965, an uncharacterized protein, and fatty acid synthase (FASN) showed reduced infection with CHIKV, which followed the trend in our RNAseq data, while knockdown of AAEL007283, AAEL017536, and AAEL022809 showed a trend towards

increased CHIKV infections. We could not amplify AAEL010150 and AAEL026093 from mosquito RNA, therefore these genes were not tested. We then silenced AAEL009965 (labeled as 9965 in Fig.5) and FASN before exposure to an artificial bloodmeal containing MAYV. Knockdown of AAEL009965 showed reduced infection rates for MAYV (**Fig.5B; **p=0.0031**), while knocking down FASN showed no difference in MAYV infection rates compared to the control group (**Fig.5D**). We observed a knockdown efficiency of 60-70% for both the genes for both CHIKV and MAYV. We then sought to determine if these knockdowns would impact viral titers and found no difference between AAEL009965 or FASN and the control group for CHIKV (**Fig.5C & E**). However, with MAYV, we saw a trend towards reduced viral titers in the AAEL00965 group (**Fig.5C; p=0.0850**) and significantly lower titers for the FASN knocked down group (**Fig.6D; **p=0.0093**) compared to the control group. AAEL009965 is an ortholog of the tetratricopeptide repeat protein 12-like in *Ae. albopictus* C6/36 cell line⁵¹, which is involved in glycoprotein binding; however, its role in insect immunity has not been studied. FASN is a key component in the lipid metabolism pathway, and its silencing in insects was shown to alter various key genes involved in lipogenesis⁵². Lipids play a crucial role in various stages of the alphavirus life cycle⁵³, suggesting a possible mechanism by which FASN silencing reduces viral infection rates and titers. Therefore, these commonly identified genes could be used for alphavirus transmission control, although it would require rigorous testing with other medically relevant alphaviruses.

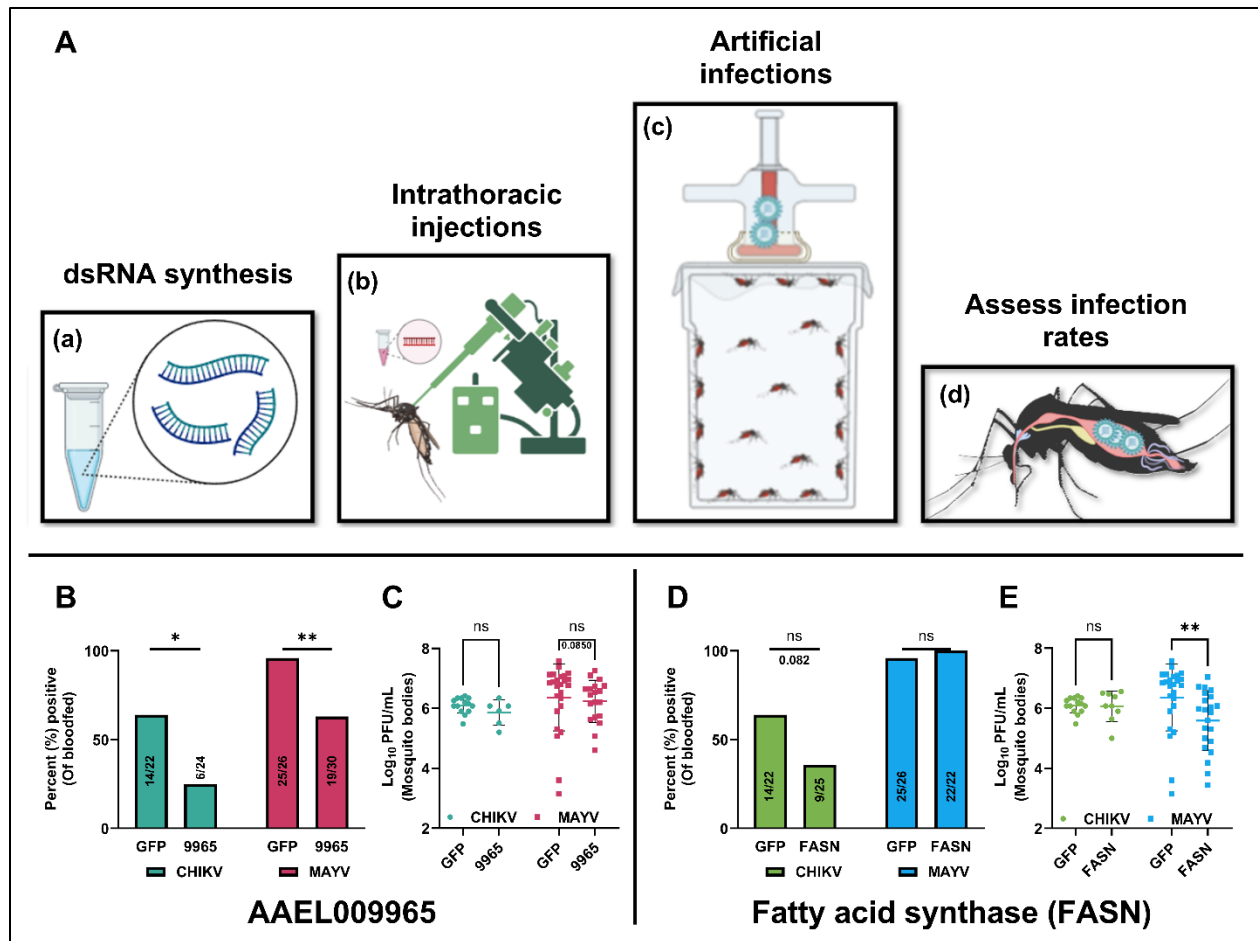


Fig.5: AAEL009965 and fatty acid synthase (FASN) are proviral for CHIKV and MAYV.

(A) Experimental design. dsRNA was synthesized for AAEL009965 and FASN as genes of interest (GOI) and green fluorescent protein (GFP) as control (a). 1-2-days-old *Ae. aegypti* females were cold anesthetized and intrathoracically (IT) injected with 500 ng dsRNA against GOI or control (b). 3-days-post-IT injections, mosquitoes were exposed to 7×10^7 PFUs/mL of CHIKV or MAYV using Hemotek feeders (c) and 7 days later, infection rates were calculated by calculating the percentage of bodies positive for CHIKV or MAYV compared to the total number of blood-fed mosquitoes (d). **Infection rates for CHIKV and MAYV after knockdown of AAEL009965 (B) or FASN (D) compared to GFP control.** Statistical analysis was conducted using a two-sided

Fisher's exact test. **Viral titers for CHIKV and MAYV after knockdown of AAEL009965 (C) or FASN (E).** Statistical analysis was done by unpaired t-test or Mann-Whitney test on log-transformed titers tested for normality and outliers. The level of significance is represented as follows: * $p < 0.01$; ** $p = 0.01$ to 0.001 ; ns – non-significant. The error bars indicate the standard deviation (SD) of the mean. These studies were performed as two independent biological replicates, which have been combined for analysis.

5.5 Conclusion

Mosquito-borne viruses cause significant public health impacts and economic losses worldwide. Most of these viruses are RNA viruses with high mutation rates that enable them to adapt to multiple hosts⁵⁴ and evade host immunity, enhancing their zoonotic potential⁵⁵. Moreover, the requirement for mosquito-borne viruses to alternate between a vertebrate host and a vector imposes bottlenecks that further impact their adaptability and transmission potential^{56,57}. Understanding the interactions between the virus, vector, and vertebrate host can provide useful insights for designing transmission control strategies. Here we utilized obesity as a host biological variable to identify its impact on mosquito immune response and identify genes that can be used to control alphavirus infection of mosquitoes. Through this study, we have shown that obesity reduces the transmission potential of *Ae. aegypti* mosquitoes for CHIKV and MAYV, by activating the Toll pathway and inhibiting certain proviral genes identified in mosquito midguts. The release of transgenic mosquitoes overexpressing genes leading to the activation of the Toll pathway or those deficient in the identified proviral genes could be a feasible strategy to control the spread of mosquito-borne viral diseases.

5.6 Tables

Table 1: Downregulated genes common between mosquitoes exposed to either CHIKV- or MAYV-infected obese bloodmeal at 1-day-post-bloodmeal (dpbm).

<i>Gene ID</i>	Gene Name	Log₂ Fold Change	
		CHIKV	MAYV
<i>AAEL007283</i>	Acetyl CoA synthetase	-2.290126868	-1.88926
<i>AAEL009965</i>	Unspecified product	-2.184179696	-2.48433
<i>AAEL010150</i>	Unspecified product	-3.280313661	-2.02079
<i>AAEL017536</i>	Holotricin glycine-rich repeat protein anti-microbial peptide	-1.856474924	-1.68861
<i>AAEL022809</i>	Unspecified product	-2.605221966	-2.66772
<i>AAEL026093</i>	Unspecified product	-5.955726837	-2.84262
<i>AAEL029069</i>	Fatty acid synthase	-4.356104755	-2.45794

5.7 Supplementary Tables

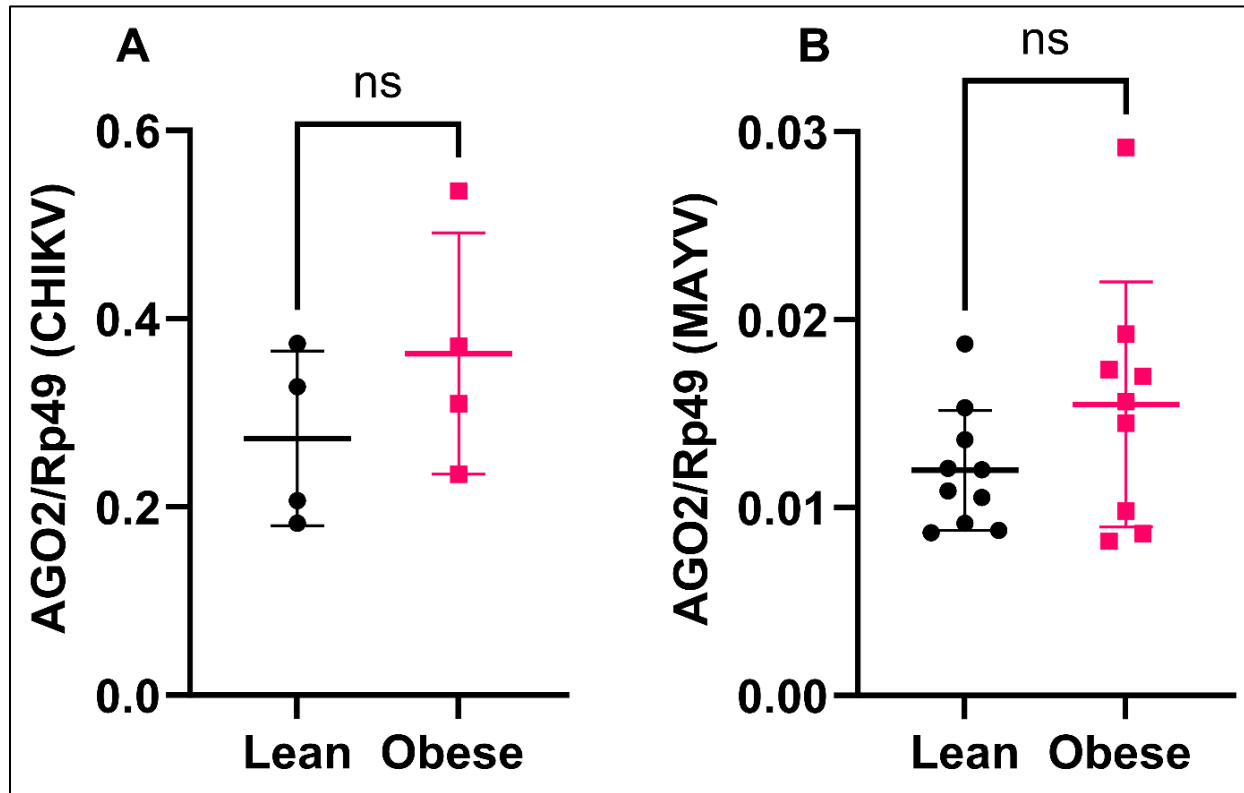
Supplementary Table S1: List of *Ae. aegypti* primers used for this study.

Primers	Sequence	Reference
AAEL009965 T7-F ¹	TAATACGACTCACTATAGGGGAACCAGGAGCGAACCGTTA	This study
AAEL009965 T7-R ²	TAATACGACTCACTATAGGGTTGTCGTCGCATCCTTCCC	This study
AAEL029069 T7-F	TAATACGACTCACTATAGGGGCAAGTGCTGTACCAATCCG	This study
AAEL029069 T7-R	TAATACGACTCACTATAGGGCAGCGGGTTCAGGAGTTCTT	This study
AAEL009965 qPCR-F	CCAGTGGCGATAAGGGCAA	This study
AAEL009965 qPCR-R	TTCTCCAGGTCCTTCTTGTC	This study
AAEL029069 qPCR-F	GCAGCCTACTCCTGGTCATC	This study
AAEL029069 qPCR-R	GCAACCACAGGAATGGCATC	This study
Rp49-qPCR-F	AAGAAGCGGACGAAGAAGT	Rai et al., 2023
Rp49-qPCR-R	CCGTAACCGATGTTTGGC	

**F¹ stands for forward primer; R² stands for reverse primer.

5.8 Supplementary Figures

Figure S1: Testing mosquito midguts for RNA interference in response to alphavirus-infected obese bloodmeal.



Ae. aegypti mosquitoes (n=50-60/mouse) were allowed to feed on CHIKV- or MAYV-infected lean and obese mice, at peak viremia. Fully engorged mosquitoes were sorted and maintained in an environmental chamber at 28°C with 70-80% relative humidity, for 9 days. Midguts were dissected 1 day later, RNA was extracted, and gene expression was assessed by reverse-transcription quantitative PCR (RT-qPCR). Expression of genes of interest was normalized to the housekeeping gene, Rp49, and then compared between mosquitoes fed on CHIKV- or MAYV-infected lean or obese mice. **(A & B) RNAi pathway.** Ago2, a critical component of the RNA-induced silencing complex (RISC) in the RNAi pathway was tested for mosquitoes fed on CHIKV

(A) or MAYV (B) infected lean or obese mice. Statistical analysis was done by unpaired t-test or Mann-Whitney test. The level of significance is represented as follows: ns – non-significant. The error bars indicate the standard deviation (SD) of the mean.

Figure S2: Pathways altered in mosquitoes fed on CHIKV- or MAYV-infected obese vs lean mice at 1- and 4-days-post-infectious bloodmeals (dpbm).



Differentially expressed genes (DEGs) in obese vs lean-fed mosquitoes with fold change > 3 were selected and pathways were generated by Gene Set Enrichment Analysis (GSEA) using Kyoto

Encyclopedia of Genes and Genomes (KEGG) in iDEP 1.1. **(A & B) Pathway analysis at 1 day-post-infectious bloodmeal (dpbm)** for CHIKV (A) and MAYV (B) respectively. **(C & D) Pathway analysis at 4 dpbm** for CHIKV (C) and MAYV (D) respectively. Green dots indicate downregulated pathways and red dots indicate upregulated pathways in mosquitoes fed on CHIKV- or MAYV-infected obese vs lean mice.

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Chapter 6: Conclusion

6.1 Conclusion

Obesity has emerged as a major health issue, transitioning from an issue affecting predominantly affluent nations to a significant health concern impacting populations globally¹. Numerous clinical and epidemiological studies have associated severe obesity (BMI ≥ 40) with various metabolic conditions like increased lipid levels (hyperlipidemia), increased glucose levels (hyperglycemia), increased leptin levels (hyperleptinemia), and increased insulin levels (hyperinsulinemia), along with a range of comorbidities like diabetes mellitus, hypertension, and cardiovascular diseases²⁻⁴. Altered host factors in an obese host predispose them to worse disease outcomes following infection with various pathogens, including respiratory viruses like influenza⁵ and SARS-CoV-2^{6,7} and mosquito-borne viruses like WNV⁸, DENV⁹, CHIKV, and MAYV¹⁰

The 21st century has witnessed many viral disease outbreaks including the SARS-CoV-1 outbreak in 2003¹¹, the CHIKV outbreak in 2005-06¹², the H1N1 influenza pandemic in 2009¹³, the Middle East Respiratory syndrome coronavirus (MERS-CoV) outbreak in 2012¹⁴, the Ebola virus outbreak in 2013¹⁵ and the ongoing SARS-CoV-2 pandemic since 2019¹⁶- all of which are RNA viruses. RNA viruses have rapid but error-prone replication, large population sizes and short generation times^{17,18} making them highly adaptable to multiple hosts. Even a single mutation can modify viruses to enhance their host range- as seen during the La Reunion outbreak of CHIKV in 2005-06, where mutations in the E1 (fusion) and E2 (receptor binding) proteins^{19,20} enhanced their fitness in *Ae. aegypti* mosquitoes- or increase their transmissibility and virulence as seen with SARS-CoV-2 variants of concern²¹⁻²³. Thus, genetic plasticity allows RNA viruses

to adapt to new environments and cause epidemics; however, the impact of host biological factors in driving the pathogenesis and transmission of these viruses is poorly understood. Given the increasing prevalence of obesity, this study aimed to investigate obesity as a potential risk factor for the pathogenesis and transmission of SARS-CoV-2 and emerging alphaviruses, specifically CHIKV and MAYV. The broader goal was to leverage these findings to mitigate risks and implement transmission control strategies within vulnerable populations.

Obesity stands out as a significant, independent risk factor for severe COVID-19 outcomes, as highlighted through various epidemiological studies²⁴⁻²⁶. Various possible mechanisms have been linked to obesity and severe COVID-19. Individuals with obesity often experience a dysregulated immune response, chronic inflammation, and impaired respiratory function, which predispose them to severe respiratory complications when infected with SARS-CoV-2²⁷. Moreover, obesity is frequently associated with co-morbidities such as diabetes, hypertension, and cardiovascular diseases²⁻⁴, further amplifying the risk of adverse outcomes. The excess adipose tissue in obese individuals can also serve as a reservoir for the virus, prolonging viral shedding and transmission²⁸. Additionally, obesity-related factors like reduced lung capacity and compromised ventilation-perfusion ratios exacerbate the progression of respiratory distress, leading to the characteristic feature of severe COVID-19, acute respiratory distress syndrome (ARDS)²⁹. The ongoing COVID-19 pandemic underscores the critical importance of research into effective countermeasures which require ongoing refinement, particularly in response to emerging variants and the diverse spectrum of disease manifestations in response to the virus. Patients exposed to SARS-CoV-2 manifest disease symptoms ranging from mild, such as fever and flu-like symptoms^{30,31}, to severe complications like acute respiratory distress syndrome (ARDS)³², septic shock, and death³³. Thus, a comprehensive

understanding of viral pathogenesis is essential for developing effective therapeutics and vaccines, for which the use of suitable animal models is indispensable. The selection of animal models for SARS-CoV-2 research should mimic the key aspects of COVID-19 to facilitate thorough and accurate investigations. Severe COVID-19 is primarily attributed to dysregulated host immune response³⁴, but the infection models used to study COVID-19 pathogenesis often involve modifications to either the host or the virus³⁵, thereby ignoring the natural host immune response towards the virus. Moreover, these models suffer from other drawbacks like developing only a mild form of the disease or causing fatal encephalitis³⁶, thereby not representing the important aspects of COVID-19. Through our research, we aimed to identify a natural coronavirus infection model that accurately reflects host immune responses against the virus, specifically focusing on obesity's impact on disease severity. Additionally, we wanted to leverage this model to identify potential biomarkers capable of predicting disease outcomes post-exposure to SARS-CoV-2.

Based on previous studies with SARS-CoV-1 and our preliminary studies, we identified C57BL/6N and C3H/HeJ mice as resistant and susceptible models respectively³⁷ to a mouse betacoronavirus, mouse hepatitis virus 1 (MHV-1)³⁸. Here we established a mouse model of COVID-19 and showed that the disease was more severe in obese C3H/HeJ mice, with a 100% mortality by 8 days-post-infection (dpi), while the disease manifestations in obese C57BL/6N mice were similar to those observed in lean ones infected with MHV-1. Our MHV-1-infected C3H/HeJ mice showed diffuse alveolar damage (DAD) in the lungs and severe liver pathology, resembling severe manifestations of COVID-19 in humans. We then used the C3H/HeJ mouse model to identify early predictive biomarkers of severe disease outcome by performing RNA sequencing (RNAseq) on whole blood RNA samples collected from MHV-1 infected lean and

obese mice at 2 dpi and assessed for changes in gene and pathway expression. We found various genes and pathways commonly shared between our MHV-1-infected obese mice and patients with severe COVID-19, suggesting the relevance of our mouse model for studying COVID-19 and identifying risk factors for severe disease outcomes.

As previously mentioned, obesity rates in low and middle-income countries (LMICs) are approaching the levels observed in developed nations^{39,40}. These countries face substantial risks from mosquito-borne viral diseases, resulting in significant losses both in terms of mortality and financial burdens^{41,42}. Alphaviruses, particularly CHIKV and MAYV are of grave public health concern due to their recent emergence in the Americas^{43,44} and the chronic debilitating disease⁴⁵ caused by them. Alphavirus-infected obese mice had more severe disease outcomes and infected mosquitoes at significantly lower rates than their lean counterparts¹⁰; however, the mechanisms involved in driving reduced transmission rates by ingesting alphavirus-infected obese bloodmeal were not identified. Using a natural transmission cycle between lean and obese mice and mosquitoes, we confirmed that mosquitoes fed on CHIKV- or MAYV-infected obese mice had reduced transmission potential compared to those fed on their lean counterparts. We hypothesized that the obese bloodmeal activated the vector's immune response leading to reduced transmission of the virus, and to test this, we dissected mosquito midguts and tested them for differential gene expression between mosquitoes fed on alphavirus-infected obese bloodmeal compared to those fed on lean ones. We identified activation of the Toll pathway and downregulation of potentially proviral genes, AAEL009965 and fatty acid synthase (FASN), through RT-qPCR and RNAseq respectively, in the mosquito midguts in response to both CHIKV- and MAYV-infected obese bloodmeal. The antiviral activity of the Toll pathway and proviral activity of AAEL009965 and FASN were further confirmed by knocking them down in

mosquitoes and infecting them with CHIKV or MAYV. We also identified insulin as a component of obese blood and showed it to have an antiviral effect on CHIKV infection of mosquitoes⁴⁶.

Through these studies, we wanted to determine obesity's impact on viral pathogenesis and transmission, particularly in the context of SARS-CoV-2 and alphaviruses. We used mouse models of diet-induced obesity and since severe COVID-19 outcomes have been attributed to the host immune response against SARS-CoV-2, we used a mouse coronavirus to account for the host's immune response to the virus in disease progression. Here we have shown that obesity worsens coronavirus disease outcomes and reduces alphavirus transmission by *Ae. aegypti* mosquitoes. Moreover, the altered gene expression observed in obese mice can be used to design effective preventive or protective measures by predicting the disease outcomes or designing effective transmission control strategies against these viruses.

6.2 References

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