HOST-PATHOGEN INTERACTIONS DURING ARBOVIRAL INFECTIONS

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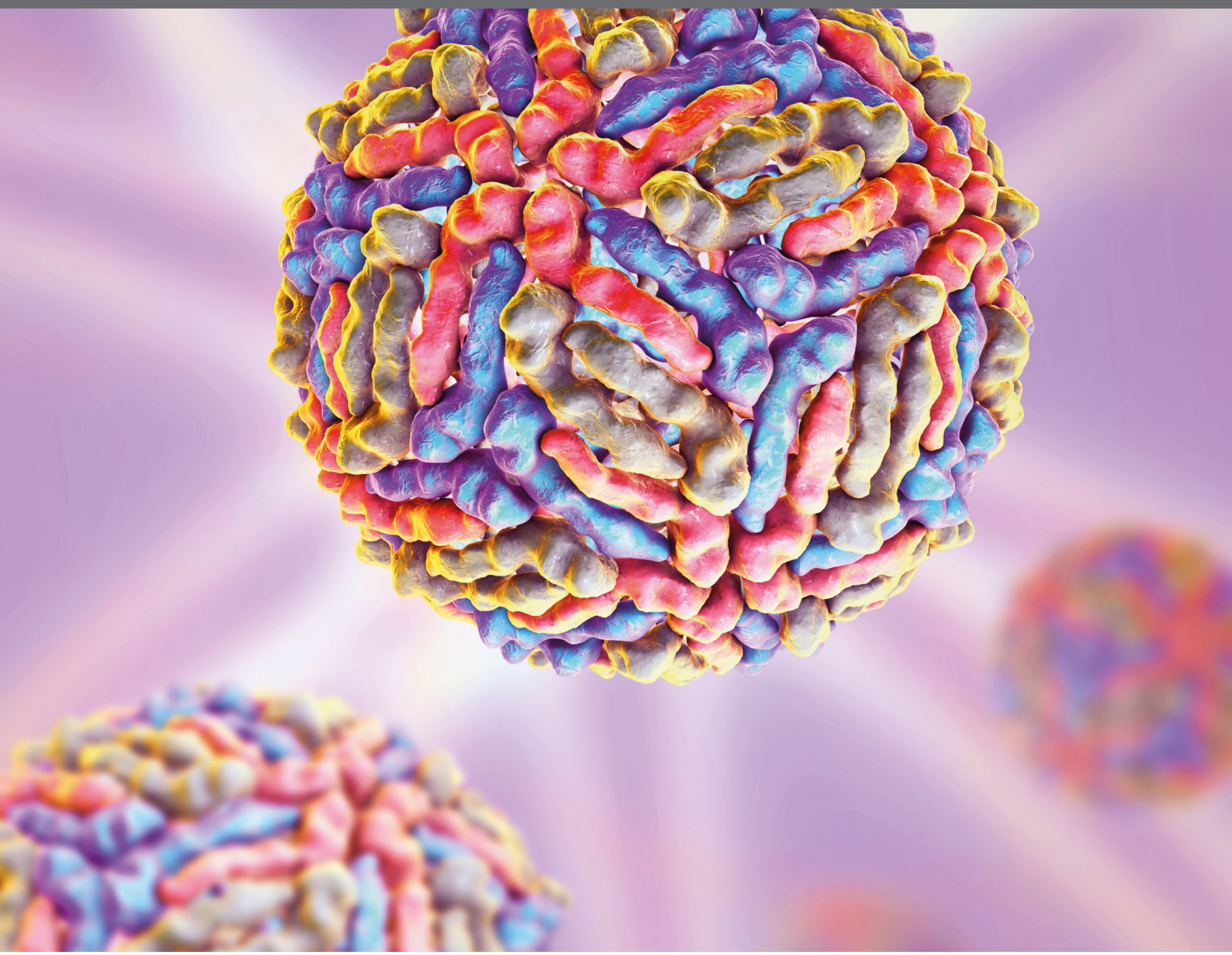

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HOST-PATHOGEN INTERACTIONS DURING ARBOVIRAL INFECTIONS

Topic Editors:
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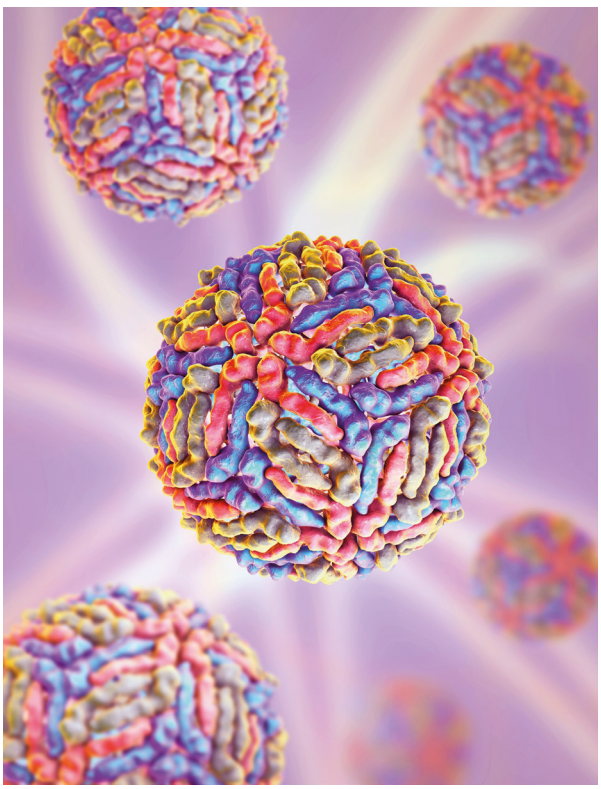

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Arboviruses, or arthropod-borne viruses, such as West Nile virus, Dengue virus, and Zika virus, pose a serious threat to public health worldwide. West Nile virus was introduced to the United States in 1999, and is now endemic, causing over 2,000 domestically acquired cases in the U.S. annually in mammals. Similarly, dengue and Zika viruses are endemic in the Americas and pose novel threats to the human population. Tick-borne viruses such as Powassan virus and Heartland virus are increasing in frequency in the U.S. At this time, there is no commercially available vaccine or therapeutic to treat these viral infections. Because nearly every mammalian case of these virus infections originates from the insect vector, it is imperative that we also understand viral pathogenesis, transmission, and the immune response in insect models as well as pathogenesis and the host responses in infected mammals.

The development of methods to block arbovirus transmission from the vector may be effective at ceasing arthropod-to-human infection since the current recommendation to prevent these viral infections is to decrease contact with mosquitoes. Additionally, improving our understanding of epidemiology and ecology will also help develop measures to reduce virus spread. In order to better study mammalian and insect host responses to infection, a number of genetic tools are available, such as fly, mosquito, and tick models, recombinant viruses, mouse models, and bioinformatics tools.

The studies described in this eBook will illustrate host responses to infection using mammalian and insect models, identify critical signaling nodes of the innate immune response, and discuss virus transmission from insect to mammal or among infected hosts. Taken together, these studies will elucidate means of decreasing infections in human populations and provide potential targets for future study and therapeutic design.

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EDITORIAL
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Editorial: Host-Pathogen Interactions During Arboviral Infections

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Keywords: West Nile virus, dengue virus, chikungunya virus, tick-borne encephalitis virus, Japanese encephalitis virus, Zika virus, systems biology, immunity

Editorial on the Research Topic

Host-Pathogen Interactions During Arboviral Infections

Arboviruses (Arbo = ARthropod-BOrne) are a diverse group of viruses that are transmitted by arthropod vectors, most commonly by insects such as mosquitoes and blood-feeding flies, or arachnids such as ticks. With the exception of African swine fever virus, a DNA virus, all arboviruses are RNA viruses and belong to one of six viral families: *Bunyaviridae* (e.g., Rift Valley fever virus), *Reoviridae* (e.g., Bluetongue virus), *Orthomyxoviridae* (e.g., Bourbon virus), *Rhabdoviridae* (e.g., vesicular stomatitis virus), *Togaviridae* (e.g., equine encephalitis and chikungunya viruses), or *Flaviviridae* (e.g., tick-borne encephalitis, dengue, Japanese encephalitis, West Nile, Zika, and yellow fever viruses) (Hanley and Weaver, 2008). Flaviviruses pose a major human threat, and the world's population is at risk of infection with several flaviviruses: In the United States, West Nile virus (WNV) is the leading cause of arbovirus infections (Lindsey et al., 2014) and caused over 2,000 confirmed cases in 2017 (ArboNET, 2018). Zika virus (ZIKV), while previously considered a minimal threat to human health, is now present in over 30 countries (Tham et al., 2018). Additionally, over 40% of the world's population is at risk for dengue virus (DENV) infection (Jindal et al., 2014). All of these flaviviruses can cause severe complications in human patients: two-thirds of West Nile virus cases are classified as neuroinvasive, Zika virus is associated with Guillain-Barré syndrome and microcephaly, and dengue virus causes hemorrhagic fever in 500,000 patients annually. There are no post-exposure therapeutics available for any flavivirus and no approved vaccines other than for yellow fever and Japanese encephalitis viruses, indicating an unmet need in medicine. Moreover, diabetic individuals are at a higher risk for contracting West Nile virus disease (Nash et al., 2001). Flavivirus infection is only expected to increase, due to climate change affecting the geographical ranges of the mosquito vectors (Chen et al., 2013; Kraemer et al., 2015). Thus, it is imperative that current research investigates the both the immune response to and pathogenesis of these arboviral infections. The purpose of this research topic is to provide a platform for the dissemination of high-quality primary research articles, comprehensive reviews, and opinions that explore the host responses to arboviral infections. Serendipitously, each of the five review articles published in our research topic nicely introduces one of the five primary research articles, and our topic includes an opinion article that discusses flaviviral vaccine development, which suitably ties together the research topic.

An important topic covered is the growing threat of infection by tick-borne viral pathogens. Mlera and Bloom highlight the need for further research into the role of small-to-medium-sized mammals in tick-borne flavivirus (TBFV) biology. Rodents are a true reservoir of TBFVs, as these animals harbor the virus without showing clinical disease.

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While the research community agrees that rodents play a major role in TBFV transmission, there remains a need for better understanding of the host response to infection with these viruses. TBFV infections in humans are often neurotropic and can cause acute neuroinflammation or tick-borne encephalitis (TBE) disease. Cases of TBE are highest in the summer-autumn period, and Daniel et al. provide epidemiological analysis arguing that this is likely caused by increased viral replication at higher temperatures. Thus, bites during the summer-autumn period deliver a higher viral load to humans, and they call for these data to be used to forecast TBE risk.

Mosquito-borne viruses and the host responses that determine tropism and pathogenicity are also covered. As reviewed by Ahlers and Goodman, while *Culex* mosquitoes transmit WNV to dead-end mammalian hosts, birds are reservoirs for WNV. Each host species exhibits similar innate immune responses that function through detection of viral RNA and subsequent JAK/STAT pathway activation that leads to adaptive immunity in mammals. In humans, WNV begins replicating in the skin at the site of mosquito bite before traveling to the lymph node, which can lead to viremia and neuronal infections. Garcia et al. show that keratinocytes exhibit a type I and III interferon response and elevated pro-inflammatory cytokines when infected. Interestingly, the addition of mosquito saliva to keratinocytes reduced WNV-mediated inflammatory responses and lead to increased viral replication.

Key host cellular mechanisms of pathogenesis are explored, including the important role of host lipids in viral replication and downstream effects on specific host tissues. Cholesterol plays a fundamental role during flavivirus infection, from viral entry and innate immune responses to viral egress. Due to the dependence of flaviviruses on cholesterol, Osuna-Ramos et al. contend that FDA-approved cholesterol-lowering drugs could be repurposed to combat flaviviral infections. Cholesterol and other lipid molecules also play a major role in epidermal homeostasis and hair follicle regeneration (Driskell et al., 2014). Here, Wei et al. show that DENV infects dermal papilla cells, leading to reduced cell viability due to increased inflammatory cytokines and caspase activation. These results could mechanistically explain why some DENV-infected individuals display hair loss as a clinical manifestation.

Since the year 2000, computational and mathematical modeling of biological systems, also known as systems biology, has been widely used as a holistic approach to discover novel ways in which the virus and host interact. Here, Petit and Shah review how systems biology, particularly next-generation sequencing, mass spectrometry, and other "-omics"-based techniques, have been used in the study of arbovirus-vector interactions. For example, Chan et al. performed a kinase/phosphatase-wide RNAi screen to discover that checkpoint kinase 2 (CHK2), which regulates the cell cycle, is activated during Japanese encephalitis virus (JEV) infection to aid in its replication. As such, inhibition or knockdown of CHK2 reduced JEV infection. This study elegantly demonstrates how systems biology is used to identify new strategies to combat arboviral infections.

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne arbovirus that has caused several epidemics throughout the world in the last decade. Tanabe et al. stress the importance of fully understanding the host cellular response in order to discover new soluble markers of infection and therapeutic strategies, including new vaccines and monoclonal antibodies, to diagnose and combat viral disease. With regard to cellular markers and tropism of flaviviral infections, García-Nicolás et al. used porcine and human monocyte-derived dendritic cells to test flavivirus infectivity. They demonstrated that select viruses interfered with cytokine production and innate immunity in a species-dependent manner. Together, these articles emphasize the significance of cellular responses in realizing the zoonotic and pathogenic potential of arboviral infection.

Finally, Martín-Acebes et al. explore the phenomenon of antibody-dependent enhancement (ADE), which may lead to disease exacerbation during ZIKV infection. Specifically, the development of immunity to other flaviviral infections, particularly DENV, with which ZIKV co-circulates, or yellow fever virus, for which there is a vaccine, could facilitate ADE during ZIKV infection. However, more epidemiological and animal model data must be analyzed before fully understanding the role of ADE during ZIKV infection and pathogenesis. The findings from these studies could also help combat infection with each of the other arboviruses discussed in this research topic.

As ecological change drives the spread of vector species into new geographical regions and incidence increases, arboviral pathogens are a growing threat to public health. Research that characterizes host-pathogen interactions and mechanisms of pathogenesis is critically important to understanding the underlying biology and developing effective medical countermeasures. In conclusion, we want to thank all authors and reviewers for their valuable contributions and insight to this research topic. This topic is timely and we hope that it inspires increased research efforts and insight into arbovirus biology and pathogenicity to ultimately alleviate the burden of arboviral infections.

AUTHOR CONTRIBUTIONS

AG wrote the first draft of the manuscript. AR revised the manuscript.

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ORIGINAL RESEARCH
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Monocyte-Derived Dendritic Cells as Model to Evaluate Species Tropism of Mosquito-Borne Flaviviruses

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Several mosquito-borne Flaviviruses such as Japanese encephalitis virus (JEV), West Nile virus (WNV), Dengue Virus (DENV), and Zika virus (ZIKV) can cause severe clinical disease. Being zoonotic, Flaviviruses infect a wide variety of terrestrial vertebrates, which dependent of the virus-host interactions, can enhance ongoing epidemics and maintain the virus in the environment for prolonged periods. Targeted species can vary from amphibians, birds to various mammals, dependent on the virus. For many mosquito-borne flaviviruses the spectrum of targeted species is incompletely understood, in particular with respect to their contribution to the maintenance of virus in certain geographical regions. Furthermore, little is known about virus and host factors contributing to species tropism. The present study utilized human and porcine monocyte-derived dendritic cells (MoDC) as a cell culture model to better understand Flavivirus species tropism and innate immune responses. MoDC were selected based on their presence in the skin and their role as an early target cell for several Flaviviruses and their role as immune sentinels. While differences in viral infectivity and replication were minor when comparing porcine with human MoDC for some of the tested Flaviviruses, a particularly strong replication in human MoDC was found with USUV, while JEV appeared to have a stronger tropism for porcine MoDC. With respect to innate immune responses we found high induction of TNF and IFN-β in both human and porcine MoDC after infection with JEV, WNV, and USUV, but not with DENV, ZIKV, and Wesselsbron virus. Spondweni virus induced these cytokine responses only in porcine MoDC. Overall, innate immune responses correlated with early infectivity and cytokine production. In conclusion, we demonstrate Flavivirus-dependent differences in the interaction with MoDC. These may play a role in pathogenesis but appear to only partially reflect the expected species tropism.

Keywords: Flavivirus, monocyte-derived dendritic cells, *in vitro* model, infection, tropism, innate immune response

INTRODUCTION

Within the genus of Flavivirus more than 60 species are described, which are mostly transmitted by arthropods to vertebrates. 50% of these virus species are mosquito-born, 28% are transmitted by ticks, and for the rest the vector is unknown (Simmonds et al., 2017). Some Flaviviruses such as Japanese encephalitis virus (JEV) infect a broad range of vertebrate hosts varying from amphibians, to birds and various mammals, while others have restricted host affinity, such as Dengue virus (DENV) only known to infect primates (Go et al., 2014; Simmonds et al., 2017). The majority of Flaviviruses are kept in enzootic cycles between hematophagous arthropods and vertebrates, vectors, which are infected during the blood sucking on viremic hosts. More than half of the described Flaviviruses cause zoonotic diseases, ranging from febrile illness, to encephalitis-related disease or life-threatening hemorrhagic fever (Borio et al., 2002; Weissenböck et al., 2010; Go et al., 2014; Gould et al., 2017). The fact that several Flaviviruses have recently emerged, such as West Nile virus (WNV) and Zika virus (ZIKV) in the Americas, and WNV and Usutu virus (USUV) in Europe represents a serious warning that the current distribution of Flaviviruses could expand in the near future (Gould et al., 2017). The recent identification of human cases of DENV after local transmission in different countries of Europe (France and Spain) highlights the risk of the introduction of new Flaviviruses where competent mosquito vectors are present, such as *Aedes (A.) albopictus*. It is important to note that none of the recent Flavivirus outbreaks have been predicted and that factors contributing to emergences are not well understood. For many of the less studied Flaviviruses the potential contribution of various vertebrate species to the maintenance of the virus is incompletely understood (Go et al., 2014). In particular, the susceptibility of animals living in close proximity to man such as domestic animals and livestock are important.

Despite the relatively broad host range, Flaviviruses have preferential vertebrate hosts, which differ in a virus-specific manner demonstrating adaptation to particular vertebrates. Examples are the preferences of JEV serocomplex viruses for birds, and in the case of JEV also for pigs, in which the virus causes several days of viremia, making the pig an amplifying host during epidemics (Turtle and Solomon, 2018). It is also important to note that dependent on the species the outcome of vertebrate infections ranges from inapparent infection to severe disease. For example, WNV and JEV can cause particularly severe disease in horses. Furthermore, particular vertebrates play an important role in maintaining the virus in the environment and therefore play a role as a "reservoir" (Kuno et al., 2017). Despite the veterinary and public health threat caused by these viruses the knowledge on the viral and host factors responsible for species tropism and the above-mentioned pathogenic features of Flavivirus infection is very limited. A relevant cell culture system modeling early events in infection, such as replication and innate immune responses would be very valuable to address the above questions. Cell lines are problematic because differences in their susceptibility to infection and support of viral replication often depend more on the different cell lines rather than the species of origin, as documented for ZIKV (Chan et al., 2016). This may be due to de-differentiation during their generation.

For such *in vitro* studies, the present work employed monocyte-derived dendritic cell (MoDC). This was based on the rational that these and related cells are present at the site of entry in the skin, where they are believed to support the virus replication of several Flaviviruses early after infection, and participate in early innate immune responses (Wu et al., 2000; McCullough et al., 2009; Schmid and Harris, 2014; Schmid et al., 2014; Hamel et al., 2015; Bowen et al., 2017; Vielle et al., 2018). MoDC cells can be generated from any species if sufficient numbers of blood monocytes can be obtained and recombinant cytokines are available. Another major advantage is that with animals of sufficient size blood sampling does not require killing. Furthermore, during infection-caused skin inflammation monocytes will be attracted and differentiate into both MoDC and monocyte-derived macrophages (Tamoutounour et al., 2013), which are likely to be involved in innate immune responses against arboviruses as demonstrated for DENV (Schmid and Harris, 2014; Schmid et al., 2014).

Considering the importance of pigs for livestock in many parts of the world and its known role as amplifying host for JEV, we decided to investigate if and how porcine MoDC differ from their human counterparts in their interaction with a collection of Flaviviruses. Our aim was on one side to evaluate how well the MoDC culture model can reflect species tropism of flaviviruses, and on the other side to identify differences between Flaviviruses in infection, replication and innate immune responses. To this end, we selected viruses that have caused recent epidemics including JEV, WNV, USUV, DENV, and ZIKV. We also included Spondweni virus (SPOV) and Wesselsbron virus (WESSV), both currently circulating only in Sub-Saharan countries. SPOV was included as a virus being closely related to ZIKV, and WESSV as a virus having a tropism for domestic animals, in particular ruminants (Hubalek et al., 2014b). Our data demonstrate Flavivirus-dependent species differences in virus susceptibility, replication and innate responses, and thereby provides information on species tropism of emerging Flaviviruses.

MATERIALS AND METHODS

Ethics Statement

All procedures involving animals comply to the Animal Welfare Act (TSchG SR 455), the Animal Welfare Ordinance (TSchV SR 455.1), and the Animal Experimentation Ordinance (TVV SR 455.163) of Switzerland. All studies were reviewed by the ethical committee for animal experiments of the canton of Bern and approved by the cantonal veterinary authorities (Amt für Landwirtschaft und Natur LANAT, Veterinärdienst VeD, Bern, Switzerland). Specifically, porcine blood sampling was approved with the license #BE88/14. Human buffy coats were provided by the Swiss Transfusion SRC (Swiss Red Cross) Inc. (Regional transfusion blood service, Bern, Switzerland) which collected blood from anonymized healthy donors after ethical approval, and authorized use of human buffy coats given by the Swiss Transfusion SRC Institutional review board. All performed

experiments were done following protocols designed according to the guidelines of the institution.

Viruses

In this study we included different Flavivirus: JEV Laos strain (genotype 1; GenBank CNS769_Laos_2009; kindly provided by Prof. Remi Charrel, Aix-Marseille Université, Marseille, France), WNV NY99 (GenBank DQ211652.1; kindly donated by Prof. Martin Groschup, Friedrich-Loeffler-Institute, Germany), USUV SAAR-1776 strain (GenBank AY453412; kindly provided by Prof. Richard Hoop, University of Zürich, Zürich, Switzerland); DENV-3 VN32/96 strain, (serotype 3, GenBank EU482459, kindly provided by Dr. Katja Fink, Singapore Immunology Network, SIgN, Singapore), ZIKV strain PR-2015 (Asian lineage, PRVABC59; GenBank KX377337; obtained from Public Health England PHE); SPOV strain SM-6 V-1s (South Africa, GenBank DQ 859064.1 Originator: Oxford Institute of Virology, provided by EVAg, Marseille, France) and WESSV strain SAH-177 99871-2, passage 8 (South Africa, GenBank EU707555.1, Originator: UTMB collection, provided by EVAg, Marseille, France). All Flaviviruses were propagated in *A. albopictus* C6/36 cells (ATCC® CRL-1660™) in MEM (Gibco, Lucerne, Switzerland) supplemented with sodium pyruvate at 100 mM (Gibco), non-essential amino acids (MEM NEAA; Gibco) and 2% fetal bovine serum (FBS) (*v/v*) (Biochrome, Bioswisstec AG, Schaffhausen, Switzerland) at 28°C and in 5% CO_2 atmosphere conditions. Virus titrations were obtained using C6/36 cells as previously described (Ricklin et al., 2016). Infected cells were detected using immunoperoxidase monolayer assay (IPMA) with the anti-flavivirus E mAb 4G2 (ATCC, HB-112™). Titers were calculated and expressed as 50% tissue culture infective dose per ml ($TCID_{50}$/ml).

Porcine and Human Monocyte-Derived Dendritic Cells Differentiation

Porcine and human MoDC (pMoDC and hMoDC, respectively) were generated as previously described (Carrasco et al., 2001; Vielle et al., 2018). Briefly, porcine blood was collected from specific pathogen free (SPF) Swiss Large White pigs breed in our own facilities. Then peripheral blood mononuclear cells (PBMC) were isolated using ficoll-paque density centrifugation (1.077 g/L; GE Healthcare Life Sciences, Dübendorf, Switzerland). Human PBMC were isolated from buffy coats of anonymous healthy blood donors (Interregional blood transfusion SRC Ltd, Bern) by the same procedure described for porcine cells. Porcine monocytes were sorted as $CD172a^+$ cells using monoclonal antibody (mAb), clone 74-22-15A (hybridoma kindly provided by Dr. A. Saalmüller, Veterinary University of Vienna, Austria) and anti-Mouse IgG MicroBeads (Miltenyi Biotec, Germany). Human monocytes were sorted as $CD14^+$ cells using coated magnetic beads (human) according to the manufacturer's instructions (Miltenyi Biotec). For both species, we employed LS magnetic columns and the MACS system (Miltenyi Biotec). Then porcine monocytes were cultured at 5×10^5 cell/ml in Dulbecco's modified Eagle's medium (DMEM) containing Glutamax (ThermoFisher Scientific, Zug, Switzerland) supplemented with 10% of FBS (Gibco), porcine GM-CSF (Summerfield et al., 2003) and porcine IL-4 (100 U/ml, own production; Carrasco et al., 2001). Similarly, human monocytes were plated at 1×10^6 cells/ml in RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco), Glutamax (Gibco), penicillin-streptomycin (Gibco), human GM-CSF (100 ng/ml; Miltenyi Biotec), and human IL-4 (40 ng/ml; Miltenyi Biotec). Then, cells were incubated for 6 days at 39°C (for porcine cells) or 37°C (for the human cells) and 5% CO_2; on the third day of incubation fresh medium supplemented with cytokines was added to the cultures. MoDC differentiation was verified by microscopy and by flow cytometry. The culture resulted in over 95% DC-like cells defined as for porcine cells $CD172a^+CD80/86^+$ and $CD14^{low}$ expression (Carrasco et al., 2001) and as $CD11c^+CD14^-CD19^-CD3^-CD56^-$ cells (human) (Vielle et al., 2018).

Flavivirus Infection on pMoDC and hMoDC

Porcine MoDC and hMoDC were incubated for 1.5 h at 39 or 37°C (for pMoDC or hMoDC, respectively) in 5% CO_2 with the virus preparations at a multiplicity of infection (MOI) of 1 $TCID_{50}$ per cell. Then, the cells were washed 3 times with phosphate buffered saline (PBS), and fresh medium supplemented with 2% FBS and cytokines as described above were added to the cells. After 24 and 48 h post infection (hpi), supernatants were harvested and stored at −80°C. MoDC were harvested as cell suspensions with cold PBS/5xEDTA and fixed with 4% (*w/v*) paraformaldehyde (PFA) for 10 min at room temperature, then washed with PBS 0.1% (*w/v*) saponin, and immunolabelled with mAb 4G2 in 0.3% (*w/v*) saponin in PBS during 15 min on ice. After washing the cells with 0.1% (*w/v*) saponin in PBS, anti-mouse IgG2a conjugated with Alexa 647 fluorochrome (ThermoFisher) was added for 10 min on ice. The cells were acquired on a FACSCantoII (Becton Dickinson) and results analyzed with Flowjo V.9.1 software (Treestar, Inc., Ashland, OR, USA). For analyses doublets and cells with low forward/side scatter corresponding to debris and dead MoDC were excluded by electronic gating. For the quantification of dead cells in the cultures we also employed FSC/SSC gating previously shown to correspond to dead MoDC detected by propidium iodide staining (Garcia-Nicolas et al., 2014). Viral titers were determined in C6/36 cells for JEV, WNV, USUV, ZIKV, DENV, SPOV, and WESSV using IPMA as described above.

Cytokines Measurement

The porcine TNF and IL-1β, and the human TNF, IL-1β, and IFN-β expressions were quantified using commercial kits (R&D Systems, UK), with detection limits of 30 pg/ml for porcine and human TNF kit, 60 or 4 pg/ml for the porcine or human IL-1β ELISA, respectively, and 10 pg/ml for the human IFN-β test. Porcine IFN-β production was measured with reagents from Kingfisher with all incubations done at room temperature. Briefly, the polyclonal capture antibody against porcine IFN-β was diluted at 1 μg/ml to coat ELISA plates overnight at room temperature, then blocked with 4% FBS in PBS (also used as diluent for the other ELISA components) for 2 h. Samples (1:1 in diluent) and standards (2-fold-dilutions from

500 to 2 pg/ml of provided porcine IFN-β) were added at a final volume of 100 µl/well and incubated for another 2 h. After washing, the biotinylated detection antibody (0.5 µg/ml) was added for 2 h followed by Streptavidin-coupled horseradish peroxidase (DAKO) for 30 min, and the final addition of 3,3′,5,5′-tetramethylbenzidine (TMB; Dako) for the colorimetric reaction. After 30 min incubation in the dark, the reaction was stopped with 50 µl of 0.18 M sulfuric acid solution and absorbance was measured at 450 nm.

Statistics

Figures and data analyses employed GraphPad Prism 7 Software (GraphPad Software, Inc., San Diego, CA, USA). All experiments were independently performed between 3 and 9 times with cells from different donors, and each condition in triplicates. For viral titrations, differences between groups were assessed using a Kruskal–Wallis test, and for individual differences the Mann–Whitney *U*-test with Bonferroni correction as *post hoc* was employed. For group differences in the percentages of infected cells and levels of cytokines expression comparisons, we employed a one-way ANOVA test with Bonferroni correction as *post hoc*. Correlation analysis between infected cells, dead MoDC, viral titers, and expressed cytokines were calculated by Spearman's Rho analysis; a correlation between two different factors was considered relevant with $R^2 > 0.5$. For all data a p value lower than 0.05 was considered statistically significant. In the Figures * indicates $p \leq 0.05$, $^{**}p \leq 0.002$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

RESULTS

hMoDC and pMoDC Differ in Their Susceptibility to Flaviviruses

With the aim to investigate differences in the susceptibility of pMoDC and hMoDC to different Flaviviruses, the percentages of E protein expression were determined at 24 and 48 hpi (**Figure 1**). At 24 h pMoDC showed the highest susceptibility to USUV followed by JEV, WNV and SPOV, and the lowest infectivity was found with DENV-3 and ZIKV (**Figure 1A**). With hMoDC, highest susceptibilities were found with USUV and SPOV, followed by JEV, WNV, DENV-3, ZIKV, and WESSV (**Figure 1C**). Interestingly, for none of the viruses a statistically significant difference was found when infectivity was compared at 24 h (**Figure 1C**). At 48 h, infectivity in pMoDC was significantly increased for JEV and WESSV, but decreased for USUV, DENV-3, ZIKV, and SPOV (**Figures 1B,E**). This contrasted to hMoDC, in which for none of the viruses an increased infectivity was found. USUV and SPOV cause reduced levels of infection (**Figures 1C,D**). When the percentages of infected cells from different hosts were compared at 48 hpi, we observed species-dependent differences in the Flavivirus infectivity. JEV, WNV, and WESSV showed a higher preference for porcine cells, while ZIKV for hMoDC (**Figure 1H**).

Species-Dependent Differences in Flavivirus Replication in MoDC

Titrations of the supernatants from the experiments shown in **Figure 1** collected at 24 hpi, demonstrated that in pMoDC JEV and SPOV replicated to the highest titers, followed by WESSV, WNV, ZIKV, USUV, and finally DENV-3 (**Figure 2A**). In hMoDC at 24 h, high titers were found for JEV, WNV, USUV followed by SPOV, WESSV, ZIKV, and DENV-3 having again the lowest titers (**Figure 2C**). Species comparison at 24 h revealed higher titers of USUV but significantly lower levels of DENV-3, ZIKV, and SPOV when comparing hMoDC to pMoDC (**Figure 2G**). At 48 hpi in pMoDC, virus titers remained without significant changes with the exception of decreasing DENV-3 titers (**Figures 2B,E**). For hMoDC we found an increase for ZIKV and WESSV titers over time (**Figures 2D,F**). The species comparison of viral titers at 48 hpi revealed that again USUV had a clear advantage for replication in hMoDC. Surprisingly, SPOV and ZIKV better replicated in porcine cells (**Figure 2H**).

MoDC Death Induced by Flaviviruses

We next determined the impact of Flavivirus infection on MoDC viability. To this end we employed forward/side scatter plots, to determine the percentages of shrunken cells with increased granularity as shown in **Figure 3A**. At 24 hpi, pMoDC death was limited but significantly induced following challenge with USUV, SPOV followed by ZIKV and DENV (**Figure 3B**). In hMoDC, also low levels of dead cells were found at the early time point with significantly higher death induced by USUV (**Figure 3D**). This dramatically changed at 48 hpi when Flavivirus infection was associated with induction of death in pMoDC by most of the tested viruses with the exceptions of DENV-3 and ZIKV (**Figure 3C**). At 48 hpi in hMoDC, USUV infection induced the highest levels of cell death followed by JEV and WNV (**Figure 3E**). Interestingly, the correlation between cell death and infection (E protein expression) was higher for hMoDC when compared to pMoDC (**Figures 3F–I**).

Innate Immune Responses in MoDC

These analyses focused on IFN-β, TNF, or IL-1β secretion by MoDC. At none of the tested time points, we were able to detect virus-induced IL-1β with pMoDC and hMoDC. Overall, USUV induced higher secretion of IFN-β compared to the other viruses in both species and at any investigated time points (**Figures 4A–D**). Interestingly, in pMoDC SPOV induced strong IFN-β secretion (**Figures 4A,B**). In hMoDC, although at 24 hpi only USUV induced IFN-β, at 48 hpi also JEV and WNV induced significant levels of IFN-β secretion (**Figures 4C,D**). Interestingly, ZIKV and DENV never induced any IFN-β secretion in MoDC (**Figures 4A–D**).

Concerning TNF responses, similar results were obtained as for IFN-β. Both USUV and SPOV induced TNF production in porcine cells at 24 hpi. At 48 h, this cytokine was induced after challenge with JEV, WNV, USUV, and SPOV in pMoDC (**Figures 4E,F**). TNF responses in hMoDC were only observed with USUV at 24 h, and at 48 h after infection with the closely related viruses of the JEV serocomplex (JEV, WNV, and USUV; **Figures 4G,H**).

Considering the similarity of the IFN-β and TNF results we tested the correlation of these two parameters. Indeed, we found

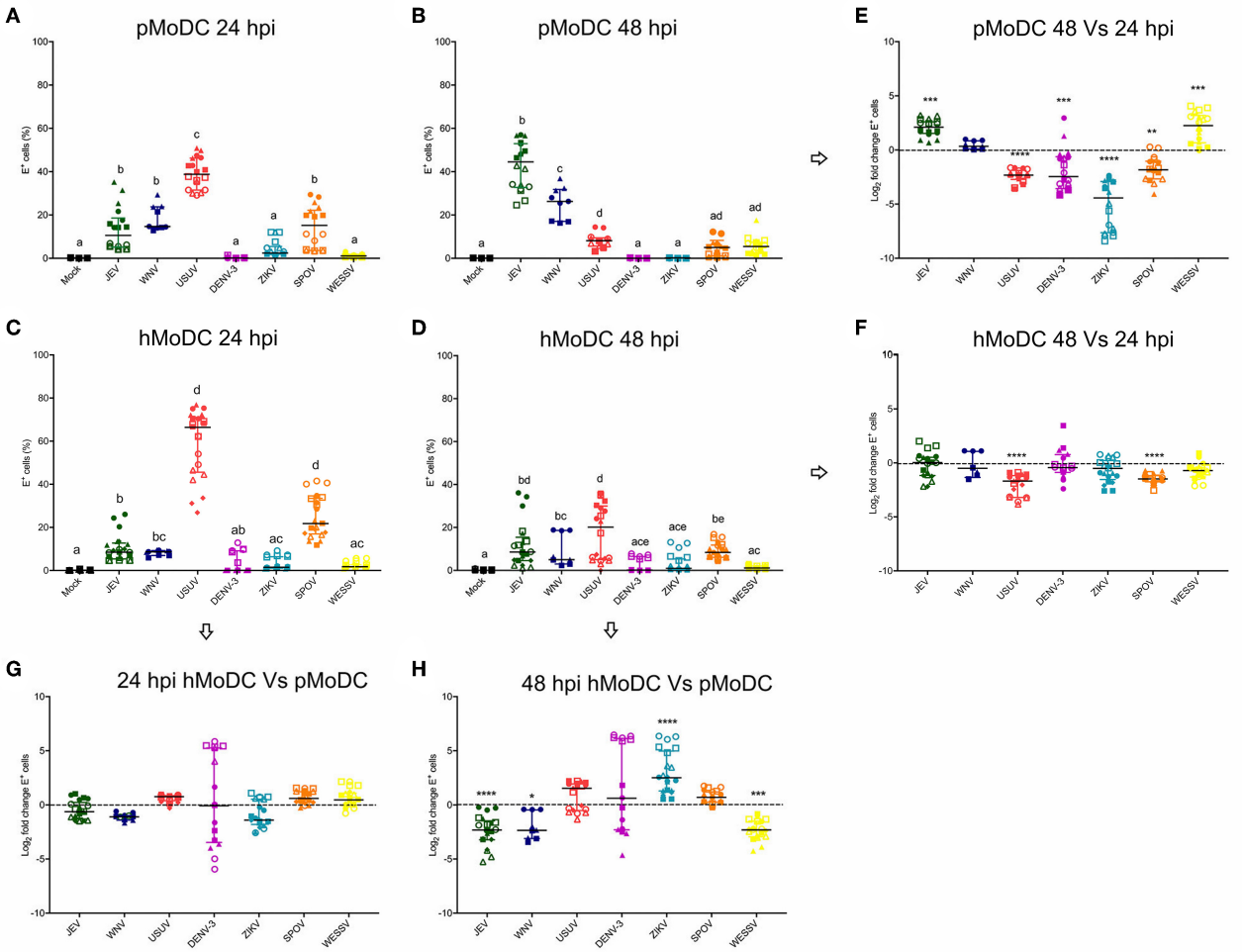

FIGURE 1 | Comparative susceptibility of pMoDC and hMoDC to selected Flaviviruses. **(A–D)** E protein expression in pMoDC and hMoDC after infection with different viruses at a MOI of 0.1 $TCID_{50}$ per cell. E protein expression was quantified after 24 **(A,C)** and 48 hpi **(B,D)** by flow cytometry. **(G,H)** Relative ability of Flaviviruses to infect hMoDC and pMoDC shown as fold change of infection (E protein positive cells) calculated at 24 **(G)** and 48 **(H)** hpi. **(E,F)** Fold change in infected cells between 24 and 48 hpi, shown for pMoDC and hMoDC, respectively. All experiments were performed in triplicates and repeated at least three, and up to seven times with cells from different donors. Each symbol represents a different blood donor. Results are presented as scatter plots showing the mean and all points. The different superscript letters in **(A–D)** indicate significant differences ($p \leq 0.05$) between the different viruses. Fold change infected cells results are expressed in logarithmic scale of base 2; for each infection condition significant differences between the calculated fold change and the reference level (equal to 0, dotted line) are indicated (*$p \leq 0.05$; **$p \leq 0.002$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

a strong correlation between the secretion of IFN-β and TNF in cells from both species at the tested time points (**Figures 4H–L**), indicating that both cytokines are triggered by similar innate pathways.

Correlation of Innate Immune Responses With Infection

As IFN-β and TNF are presumably induced by viral RNA, we analyzed the correlations of IFN-β and TNF to the percentage of virus-infected cells and also viral titers at the time of supernatant harvest. At 24 hpi the percentage of virus-infected pMoDC highly correlated with the secretion of IFN-β and TNF (R^2 values of 0.7 and 0.53, respectively), but not at 48 hpi (**Figures 5A,B,E,F**). With hMoDC we found a positive correlation for both time points between the number of infected cells and the secretion of cytokines (**Figures 5C,D,G,H**). On the other hand, cytokines secretion did not correlate with the viral titers for any of the selected host species, indicating a lack of association between both factors (**Supplementary Figure 1**). We also tested a possible association cell death and IFN-β/TNF secretion. While this correlation between was weak for pMoDC, a higher positive correlation was found for hMoDC (**Supplementary Figure 2**). Altogether, these results suggest that innate immune responses depend on the number of infected cells. In general, only viruses inducing relatively high levels of infection cause a detectable cytopathic effects. Importantly, for the viral titers we found neither a positive nor negative correlation with cytokine responses.

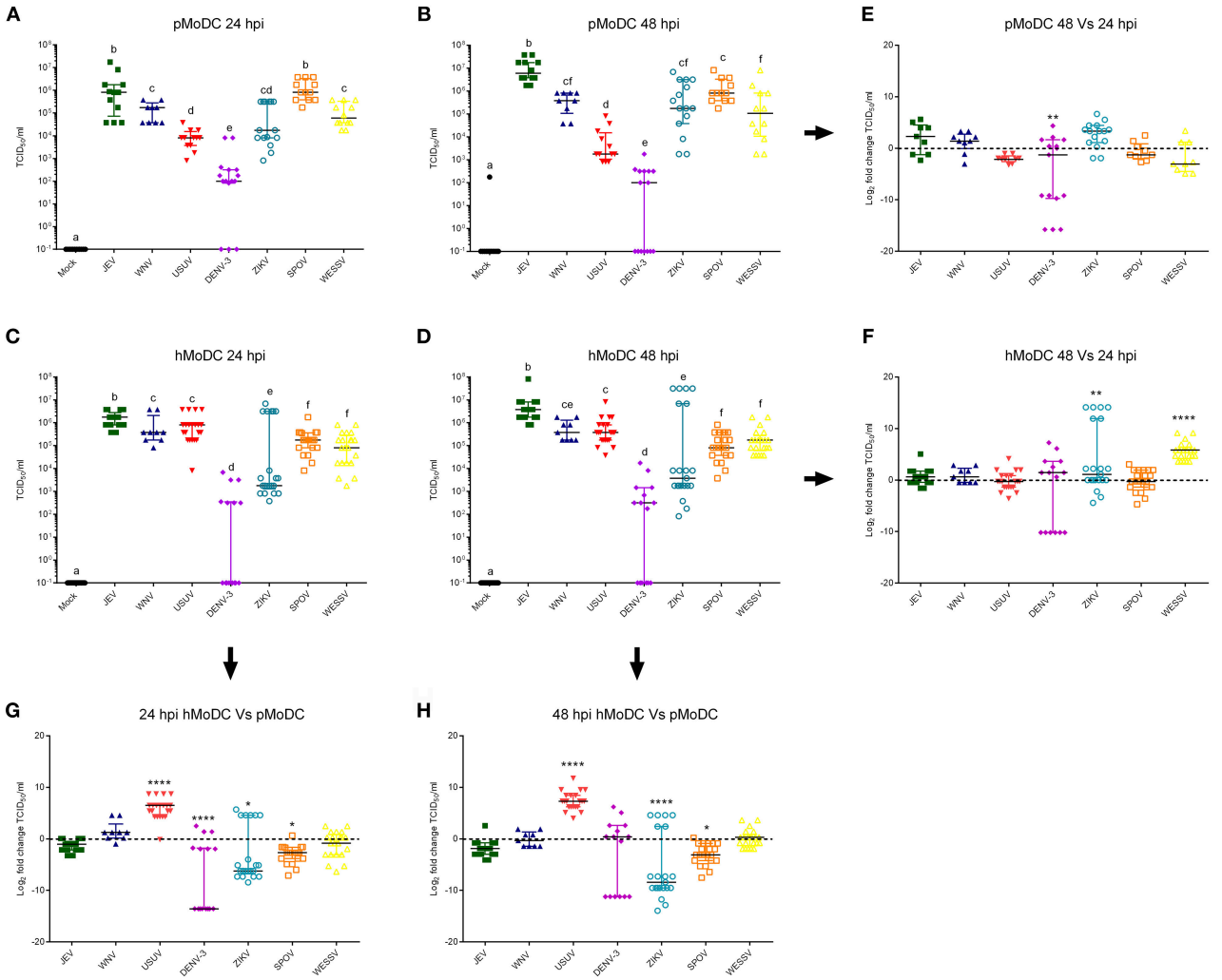

FIGURE 2 | Flavivirus shedding after infection of pMoDC and hMoDC. **(A–D)** Viral titers released from pMoDC and hMoDC after infection with the different Flaviviruses at a MOI of 0.1 $TCID_{50}$ per cell. Supernatants were collected at 24 **(A,C)** and 48 hpi **(D,B)**. All Flaviviruses titers were quantified using in C6/36 cells. **(G,H)** Relative ability of the viruses to replicate in human and porcine MoDC, shown as fold change of titers at 24 **(G)** and 48 **(H)** hpi. **(E,F)** Ratio of viral titers from at 48 vs. 24hpi for pMoDC and hMoDC, respectively. All experiments were performed in triplicates and repeated at least three, and up to seven times with cells from different donors. Each symbol represents a different blood donor. Results are presented as scatter plots showing the mean and all points. The different superscript letters in **(A–D)** indicate significant differences ($p \leq 0.05$) between the different viruses. Fold change infected cells results are expressed in a logarithmic scale of base 2; for each infection condition significant differences between the calculated fold change and the reference level (equal to 0, dotted line) are indicated (*$p \leq 0.05$; **$p \leq 0.002$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

DISCUSSION

Considering the dual host life with alternation between arthropods and vertebrates requiring adaptation to quite different hosts, it is not surprising that many mosquito-borne Flaviviruses, such as WNV and JEV can infect a wide range of vertebrate hosts. Nevertheless, there are clearly preferential vertebrate hosts, which differ in a viruses-specific manner demonstrating adaptation to particular vertebrates. The present study was initiated, considering that the knowledge on virus adaptation is incomplete in terms of the degree of adaptation of some of the newly emerged and some of the more locally restricted and neglected Flavivirus to particular vertebrate species is unclear. Our aim was to identify virological and immunological factors involved using MoDC as *in vitro* model. To validate the model, we selected human and pigs. Altogether, our data demonstrate prominent differences in the rate of infection, released infectious virus and innate immune responses, which were dependent on both the host and the virus species. Considering this, the crucial question is how well do these differences recapitulate what is known about species tropism. For JEV we observed that pMoDC were more permissive and replicated to the highest titers at 48 h compared to the rest of the selected Flaviviruses, which appears to be in accordance

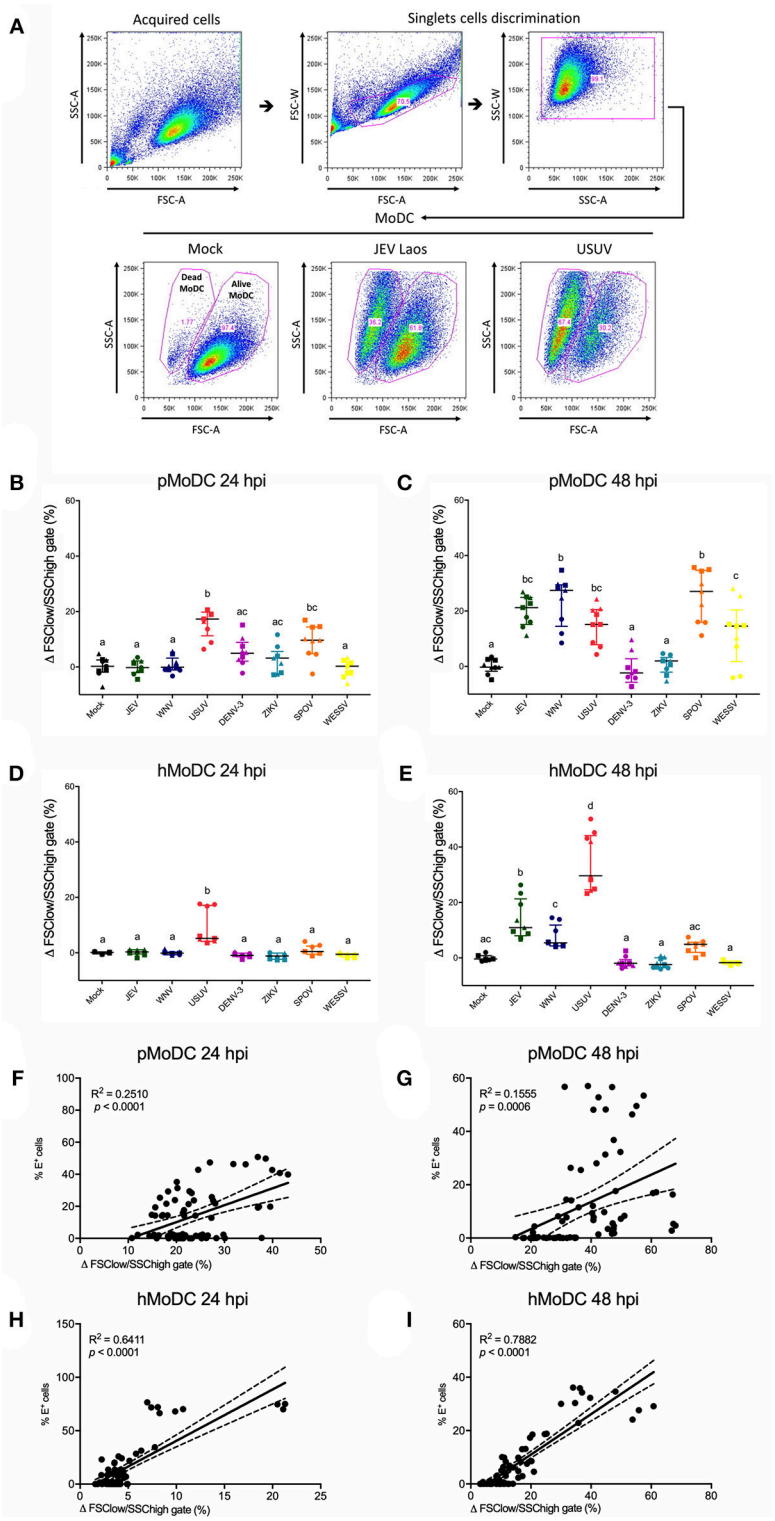

FIGURE 3 | Flavivirus induced cell death in pMoDC and hMoDC. MoDC were infected with different Flaviviruses at MOI of 0.1 $TCID_{50}$ per cell. **(A)** Gating strategy to eliminate doublets and quantify shrunken dead MoDC based on their forward/side scatter plots. Representative results are shown for Mock, JEV, and USUV. **(B–E)** Difference between the percentage of the average of shrunken in the mock with the virus-challenged cells was calculated and represented as Δ Dead MoDC (%) and represented at 24 and 48 hpi for pMoDC (**B,C**, respectively) and hMoDC (**D,E**, respectively). Each symbol represents a different blood donor. Results are presented as scatter plots showing the mean and all points. Different superscript letters indicate significant difference ($p \leq 0.05$) between the percentages of dead MoDC induced by distinct viruses. **(F–I)** Correlation analysis between the number of dead cells and E positive cells at 24 and 48 hpi for pMoDC **(F,G)** d hMoDC **(H,I)** calculated by Spearman's Rho analysis. Correlations are shown as linear regression, R^2 and p values are indicated for each analysis.

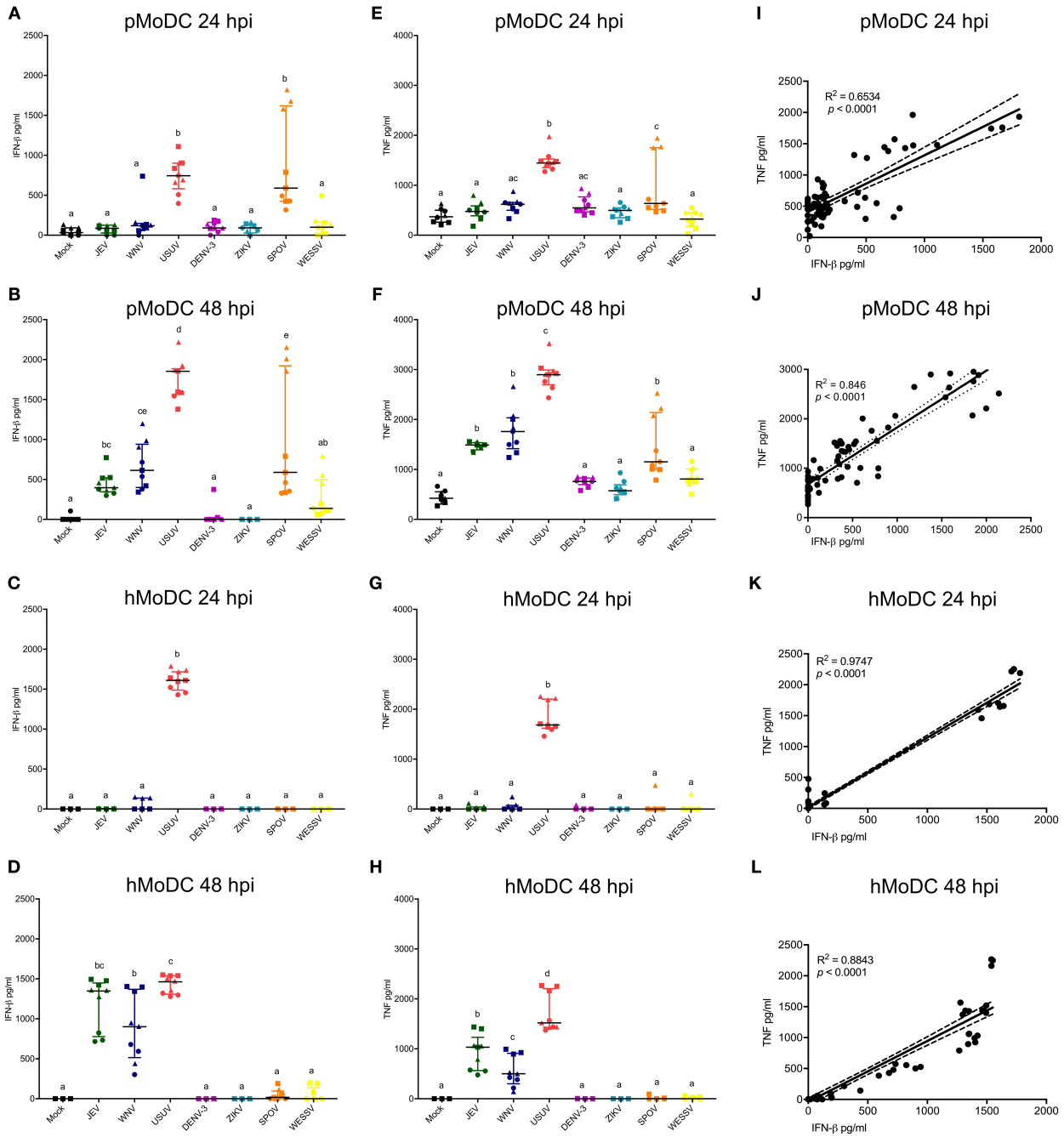

FIGURE 4 | Cytokine responses following infection of MoDC. **(A,B)** IFN-β levels in supernatants of pMoDC harvested at 24 and 48 hpi (**A,B**, respectively). **(C,D)** IFN-β levels in supernatants of hMoDC harvested at 24 and 48 hpi (**C,D**, respectively). **(E,F)** TNF levels in supernatants of pMoDC harvested at 24 and 48 hpi (**A,B**, respectively). **(G,H)** TNF levels in supernatants of hMoDC harvested at 24 and 48 hpi (**C,D**, respectively). Supernatants were derived from the cultures shown in **Figures 1**, **2**. TNF was quantified by ELISA. Each symbol represents a different blood donor. Results are presented as scatter plots showing the mean and all points. Different superscript letters indicate significant differences ($p \leq 0.05$) between the amount of TNF induced in MoDC by distinct Flaviviruses. **(I,J)** Correlation between IFN-β and TNF for pMoDC at 24 and 48 hpi. **(K,J)** Correlation between IFN-β and TNF for hMoDC at 24 and 48 hpi. For **I–L**, correlations were calculated by Spearman's Rho analysis and are shown as linear regression, R^2 and *p*-values are indicated for each analysis.

with an enormous amount of field and *in vivo* experimental data on pigs (Mansfield et al., 2017). Also, hMoDC supported high levels of JEV replication in accordance to work published by others (Cao et al., 2011; Gupta et al., 2014). The high seroconversion rate in endemic areas indicates that humans are indeed highly susceptible to JEV, although only few develop the

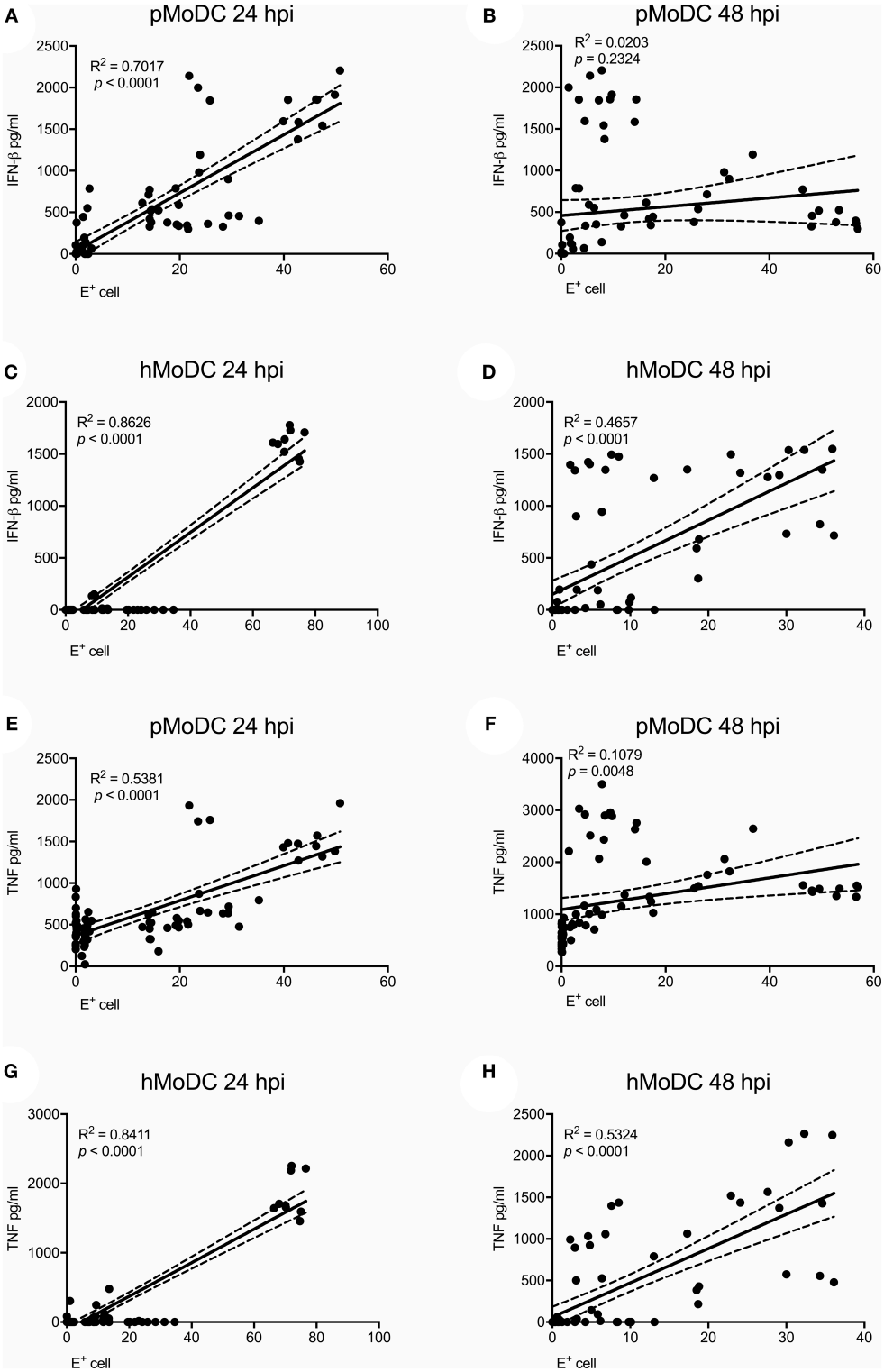

FIGURE 5 | Correlation analysis between E protein positive cells and pro-inflammatory cytokines. Correlations between E protein positive cells and IFN-β **(A–D)** or TNF **(E–H)** for pMoDC at 24 and 48 hpi (**A,E** and **B,F**; respectively) and for hMoDC at 24 and 48 hpi (**C,G** and **D,H**; respectively) were calculated by Spearman's Rho analysis. Correlations are shown as linear regression, R^2 and *p* values are indicated for each analysis.

disease. Nevertheless, human do not develop viremia of sufficient duration and intensity to re-infect feeding mosquitoes (Turtle and Solomon, 2018). A possible explanation could be that the strong IFN-β response identified in MoDC would limit further virus replication and dissemination in most infected individuals. Of note, it has been speculated that in those patients developing encephalitis, infected monocytic cells could be the Trojan horse mediating transport of the virus across the blood-brain barrier (Lannes et al., 2017).

For WNV we found that despite of low numbers of WNV-infected pMoDC, the virus efficiently replicated. Although some *in vivo* experiments suggest that pigs would not play an important role as amplifying host for WNV (Ilkal et al., 1994; Teehee et al., 2005), a seroconversion of pigs has been demonstrated in about of 22.5% of tested serum samples from feral pigs in the US, in 17.6% of wild boars in Europe and in 15.5% of farmed pigs also in Europe (Gibbs et al., 2006; Escribano-Romero et al., 2015). This clearly demonstrated that pigs are susceptible to WNV and a role of this species in the epidemiology cannot be excluded. Interestingly, our study showed that WNV infected and replicated to high titers in hMoDC without inducing early innate cytokine responses. These results which support previously published data (Rawle et al., 2015) could indicate that WNV has undergone a certain degree of adaptation to human cells.

During the last two decades, since USUV emerged in Europe, several outbreaks occurred affecting wild and domestic birds (Weissenböck et al., 2003; Hubalek et al., 2014a; Grottola et al., 2017; Michel et al., 2018). Our *in vitro* results demonstrate that although at 24 h many pMoDC are infected by USUV, the infection rate drops strongly at 48 h and gives rise only to relatively low viral titers. This is associated with high IFN-β and TNF responses, possibly indicating a poor adaptation of this virus to the pig. This would be in line with surveillance data for WNV and USUV. Although antibodies against both viruses were found in wild boars, in domestic pigs only WNV-specific antibodies were detected (Escribano-Romero et al., 2015). However, it is not known if wild boars differ from domestic pigs in their susceptibility to USUV. hMoDC were also highly susceptible to USUV but replicated to higher titers when compared to pMoDC. Nevertheless, similar to another report (Cacciotti et al., 2015), also in hMoDC high IFN-β and TNF responses were found which would be expected to limit virus spread *in vivo*. Field data demonstrate that humans are infected by USUV in endemic areas, although only in rare cases with severe clinical symptoms or even fatal outcome (Pecorari et al., 2009; Santini et al., 2015; Grottola et al., 2017).

The present study showed that pMoDC are resistant to infection by DENV-3, which is likely to be responsible for the lack of innate immune responses in pMoDC. These results are in line with previous field observations, supporting that DENV vertebrate hosts are mainly restricted to primates (Chen and Vasilakis, 2011). Nevertheless, some authors suggested that other vertebrates could be hosts for DENV in the rainforest, or that pigs could be used as an animal model (de Thoisy et al., 2004; Cassetti et al., 2010). With human cells, we observed a low susceptibility to infection by DENV-3, and an overall inefficient virus replication. This could be related to the strain used as previous reports indicate highly variable levels of infection which may depend on the serotype and virulence (Navarro-Sanchez et al., 2003; Silveira et al., 2011).

With respect to ZIKV, although the frequency of E protein expressing pMoDC was low, porcine cells supported ZIKV replication well, in absence of any detectable IFN-β and TNF response. These *in vitro* data could be in line with experimental studies showing that newborn piglets developed viremia between 3 and 5 days after intra-dermal inoculation with ZIKV (Darbellay et al., 2017a,b). In accordance to previously published work, our data show that ZIKV replicates in hMoDC in a donor dependent manner and that this infection did not induce effective innate immune responses (Bowen et al., 2017; Vielle et al., 2018).

Although SPOV is genetically closely related to ZIKV (Haddow et al., 2016), pMoDC were more susceptible to the infection by SPOV than ZIKV and the virus efficiently replicated in swine cells. The infection caused high amounts of IFN-β and TNF, possibly limiting prolonged virus infection. With respect to possible hosts that could maintain or amplify SPOV in the wildlife, previous reports demonstrated that neither birds nor rodents seem to act as reservoir for the virus, and experimental studies suggest non-human primates as possible target for the virus (Haddow et al., 2016). Our results suggest that serological field data from endemic areas should be performed to address a possible role of pigs in the viral life cycle. In hMoDC, SPOV showed comparable characteristics in terms of its efficient replication and the lack of IFN-β and TNF induction.

A considerable broad range of hosts have been described for WESSV, including human, sheep, goat, cattle, pigs, dogs, rats, and other wild life species (Coetzer and Barnard, 1977; Simpson et al., 1979; Coetzer and Theodoridis, 1982; Barnard, 1997; Hubalek et al., 2014b; Diagne et al., 2017). However, in neither human nor porcine MoDC we found high levels of infected cells but relatively good viral titers in the supernatants of the cell cultures, without evidence for induction of innate immune responses.

Another potentially interesting readout was virus induced cell death, which correlated well with the levels of infected cells, indicating a direct viral cytopathogenic effect. Nevertheless, we also found a high correlation of cell death with IFN-β and TNF responses. This may indicate that host response to infection may contribute to cell death.

Our results showing IFN-β and TNF following certain Flavivirus infections, are in accordance with previously published works indicating that hMoDC produce both TNF and IFN type I secretion during the infection by DENV or JEV (Sooryanarain et al., 2012; Schmid et al., 2014). Interestingly, in our *in vivo* experimental studies in pigs we could neither detected IFN-β nor TNF in serum samples (Ricklin et al., 2016; Garcia-Nicolas et al., 2017). This would suggest that the production of such cytokines by MoDC would be locally restricted. Porcine MoDC, secrete lower levels of IFN-β after JEV challenge than hMoDC, which is possibly due to a delayed exposure of JEV dsRNA in the cytosolic compartment of porcine MoDC as previously reported (Espada-Murao and Morita, 2011). The observation that certain of the selected Flaviviruses such as ZIKV,

SPOV, and WESSV did not induce innate immune responses despite good replication in hMoDC, indicates differences in the viruses' ability to evade innate immunity and raises the questions about the mechanisms. For instance, in ZIKV-infected cells the existence of subgenomic Flavivirus RNA has been related to the blocking of IFN type I transcription induced by RIG-I (Manokaran et al., 2015; Villordo et al., 2016). For other Flaviviruses, such as JEV, DENV or ZIKV, NS2A, NS4A, NS4B or NS5 proteins were demonstrated to participate in evasion of the IFN type I system (Miorin et al., 2017). Clearly, future studies are required to investigate how these different mechanisms of innate immune evasion contribute to the species tropism of distinct viruses.

In addition to differences in innate immune responses, the role of viral receptors for species tropism requires consideration. A difficulty could be the relative flexibility of *Flaviviruses* in receptor usage. For example, this can be glycosaminoglycans like heparan sulfate (Su et al., 2001), vimentin (Liang et al., 2011), laminin (Thongtan et al., 2012), CD4 (Thongtan et al., 2012), α5β3 integrins (Chu and Ng, 2004), CD209, and CLEC4G (Shimojima et al., 2014; Wang et al., 2016) for attachment of JEV to host cells.

When interpreting the results of the present work, it is important to note that for logistic reasons we were only able to include one strain for each virus species, and extrapolations to all viruses from the studied species should be done with care as strain differences can be important as mentioned above for DENV. Furthermore, in most cases it is impossible to directly correlate our *in vitro* data to *in vivo* experimental data. These of course are completely lacking for humans and are only partially available for pigs. Nevertheless, altogether some clear differences in Flavivirus-MoDC interactions were identified *in vitro*, indicating that such data can give valuable information on virus-host interactions. These can model certain aspects of *in vivo* infection, and can help to evaluate differences in innate immune responses against Flaviviruses. In this context, the biggest surprise was the high level of infection and replication of USUV in hMoDC, which may be interpreted as a warning concerning the zoonotic potential of USUV.

DATA AVAILABILITY

All datasets for this study are included in the manuscript. Raw flow cytometry and ELISA data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

OG-N, MR, and AS: conceptualization; OG-N, ML, MR, and AS: methodology; OG-N, ML, and MR: investigation; OG-N and AS: formal analysis; AS: supervision; OG-N and AS: writing–original draft; OG-N, ML, MR, and AS: writing–review and editing; AS: funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00005/full#supplementary-material

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CORRECTION
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Corrigendum: Monocyte-Derived Dendritic Cells as Model to Evaluate Species Tropism of Mosquito-Borne Flaviviruses

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Keywords: Flavivirus, monocyte-derived dendritic cells, *in vitro* model, infection, tropism, innate immune response

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A Corrigendum on

Monocyte-Derived Dendritic Cells as Model to Evaluate Species Tropism of Mosquito-Borne Flaviviruses

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In the original article, there was a mistake in **Figure 1.** The plots **C** and **F** in **Figure 1** were wrong and not from hMoDC as they should have been. The corrected Figure 1 appears below. The text of the Figure legends and the article remains unchanged as they describe the correct **Figure 1**. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

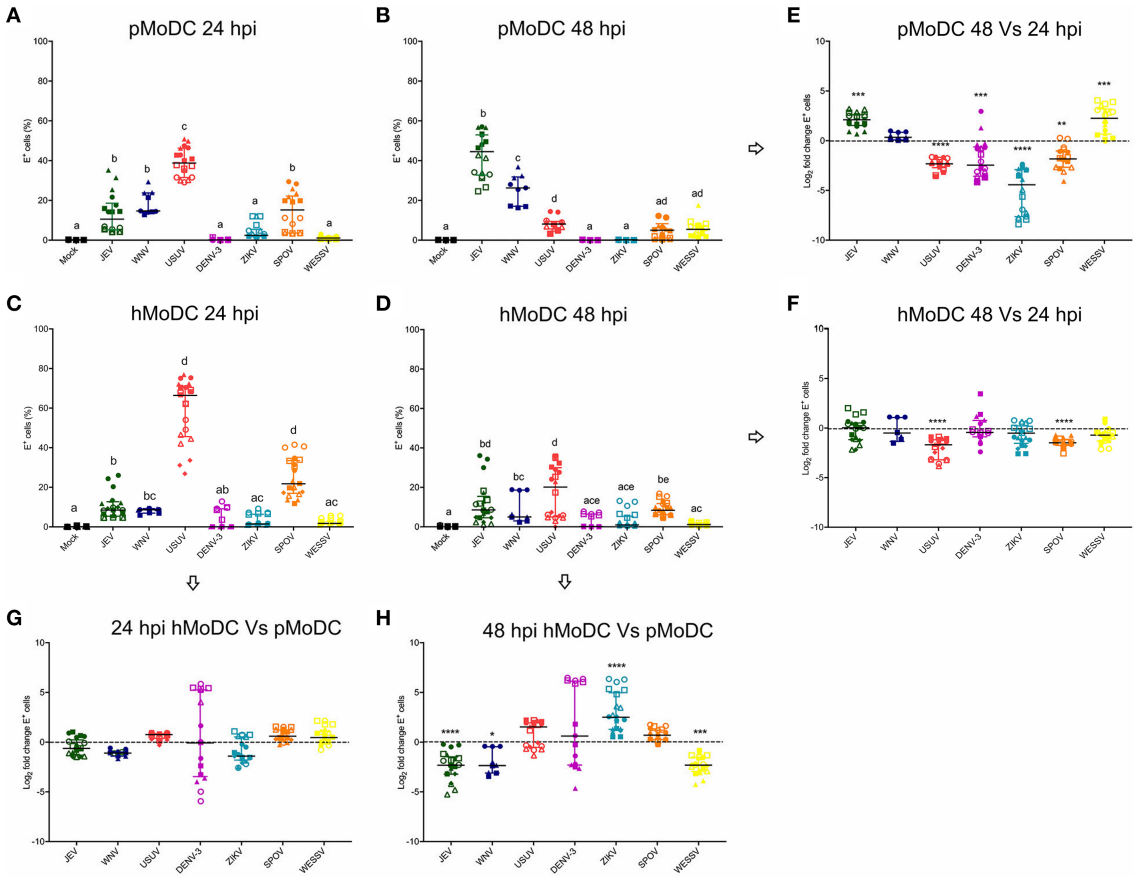

FIGURE 1 | Comparative susceptibility of pMoDC and hMoDC to selected Flaviviruses. **(A–D)** E protein expression in pMoDC and hMoDC after infection with different viruses at a MOI of 0.1 $TCID_{50}$ per cell. E protein expression was quantified after 24 **(A,C)** and 48 hpi **(B,D)** by flow cytometry. **(G,H)** Relative ability of Flaviviruses to infect hMoDC and pMoDC shown as fold change of infection (E protein positive cells) calculated at 24 **(G)** and 48 **(H)** hpi. **(E,F)** Fold change in infected cells between 24 and 48 hpi, shown for pMoDC and hMoDC, respectively. All experiments were performed in triplicates and repeated at least three, and up to seven times with cells from different donors. Each symbol represents a different blood donor. Results are presented as scatter plots showing the mean and all points. The different superscript letters in **(A–D)** indicate significant differences ($p \leq 0.05$) between the different viruses. Fold change infected cells results are expressed in logarithmic scale of base 2; for each infection condition significant differences between the calculated fold change and the reference level (equal to 0, dotted line) are indicated ($^{*}p \leq 0.05$; $^{**}p \leq 0.002$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$).

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Innate Immune Response of Primary Human Keratinocytes to West Nile Virus Infection and Its Modulation by Mosquito Saliva

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West Nile Virus (WNV) is a flavivirus involved in many human infections worldwide. This arthropod-borne virus is directly co-inoculated with mosquito saliva through the epidermis and the dermis during blood meal. WNV starts replicating in the skin before migrating to the draining lymph node, leading to widespread viremia and in some cases to neurological symptoms. Skin is a complex organ composed of different cell types that together perform essential functions such as pathogen sensing, barrier maintenance and immunity. Keratinocytes, which represent 90% of the cells of the epidermis, are the organism's first line of defense, initiating innate immune response by recognizing pathogens through their pattern recognition receptors. Although WNV was previously known to replicate in human primary keratinocytes, the induced inflammatory response remains unknown. The aim of this study was first to characterize the inflammatory response of human primary keratinocytes to WNV infection and then, to assess the potential role of co-inoculated mosquito saliva on the keratinocyte immune response and viral replication. A type I and III interferon inflammatory response associated with an increase of IRF7 but not IRF3 mRNA expression, and dependent on infectious dose, was observed during keratinocyte infection with WNV. Expression of several interferon-stimulated gene mRNA was also increased at 24 h post-infection (p.i.); they included CXCL10 and interferon-induced proteins with tetratricopeptide repeats (IFIT)-2 sustained up until 48 h p.i. Moreover, WNV infection of keratinocyte resulted in a significant increase of pro-inflammatory cytokines (TNFα, IL-6) and various chemokines (CXCL1, CXCL2, CXCL8 and CCL20) expression. The addition of *Aedes aegypti* or *Culex quinquefasciatus* mosquito saliva, two vectors of WNV infection, to infected keratinocytes led to a decrease of inflammatory response at 24 h p.i. However, only *Ae. Aegypti* saliva adjunction induced modulation of viral replication. In conclusion, this work describes for the first time the inflammatory response of human primary keratinocytes to WNV infection and its modulation in presence of vector mosquito saliva. The effects of mosquito saliva assessed in this work could be involved in the early steps of WNV replication in skin promoting viral spread through the body.

Keywords: West Nile virus, keratinocytes, mosquito, saliva, immunomodulation, innate immune response, viral replication, interferon-stimulated genes

INTRODUCTION

Since its discovery in 1937 from a febrile woman in Uganda, West Nile virus (WNV) has been involved in mild febrile disease outbreaks in Africa, Asia and Europe (Lim et al., 2011). It is now endemic on the North American continent after its introduction in 1999 (Lanciotti et al., 1999) and propagating southward. WNV is considered the first cause of viral encephalitis worldwide (Chancey et al., 2015; David and Abraham, 2016). In about 20% of human cases of WNV infection, a mild febrile disease appears after 2–14 days of incubation (Lindsey et al., 2012; Sejvar, 2016). Less than 1% of infected patients then develop a WNV neuroinvasive disease such as aseptic meningitis, encephalitis, or acute poliomyelitis-like syndrome that can be life-threatening (Campbell et al., 2002).

WNV is an arthropod-borne virus belonging to the *Flavivirus* genus, as do Dengue (DENV), Zika (ZIKV) or yellow fever viruses. WNV primary hosts are birds while mammals, particularly humans and horses, represent accidental and dead-end hosts infected through the inoculation by infected female mosquitoes (Kramer et al., 2008). *Culex* mosquitoes participate in an enzootic cycle between birds and represent a "bridge" vectors between the avian and mammalian hosts (Turell et al., 2005) because of their opportunistic feeding behavior.

Virus inoculation in the skin is a key step in the pathophysiology of WNV infection. Thereby, skin constitutes not only the first site of viral replication in host but also the initiation site of the antiviral immune response. Skin is organized in three successive layers, the epidermis, the most superficial, the dermis and the hypodermis, the deepest. The multi-layered epidermis consists mainly of keratinocytes that express pathogen recognition receptors (PRRs) involved in the recognition of highly conserved pathogen molecular patterns (PAMPs) among microorganisms (Lebre et al., 2007). Once activated by a PAMP, transmembranar toll-like receptors (TLRs) and/or retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) such as RIG-I and melanoma differentiation antigen 5 (MDA5) trigger downstream signaling pathways contributing to the induction of an innate immune response (Akira, 2006). During blood-feeding, WNV is co-inoculated with mosquito saliva predominantly in the extravascular space of the skin (Styer et al., 2007) and starts to replicate in keratinocytes (Lim et al., 2011). Mosquito saliva contains many proteins that modulate host hemostasis and immune response, facilitating blood feeding but also virus transmission (Ribeiro, 2000; Schneider and Higgs, 2008; Styer et al., 2011; Moser et al., 2016).

WNV is a positive-sense, single-stranded (ss) RNA virus transcribed in a complementary negative RNA, thereby constituting a double-stranded replicative form (ds-RF) (Brinton, 2013). The negative ssRNA is used in turn as a template for simultaneous synthesis of multiple positive ssRNA constituting partial double-stranded (ds) replicative intermediates (RI) (Brinton, 2013).

Thus, once injected in the skin and starting replicating, flaviviral RNAs are recognized as PAMPs in the cells either as ssRNA sensed by TLR7 and RIG-I or dsRNA recognized by TLR3, RIG-I and MDA5 (Westaway et al., 1999; Akira, 2006; Shipley et al., 2012). PRR activation by flaviviral PAMPs leads to expression of chemokines, cytokines, type I and type III interferons (IFNs) and interferon-stimulated genes (ISGs) by skin cells (Garcia et al., 2017). Nonetheless, although innate immune response of cutaneous cells to DENV or ZIKV has been investigated (Surasombatpattana et al., 2011; Hamel et al., 2015), the inflammatory response of WNV infected keratinocytes remains unknown. The aim of this study was to characterize the antiviral response of human primary keratinocytes during WNV infection. Moreover, as mosquito saliva can exert wide effects promoting blood-meal and infection, we investigated the effects of the saliva of two mosquito species on WNV replication and inflammatory response induced in these cells.

MATERIAL AND METHODS

Isolation and Culture of Normal Human Epidermal Keratinocytes From Skin Samples

The Ethics Committee of the Poitiers Hospital approved the use of human skin samples for research studies. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Normal abdominal or breast skin was obtained from patients undergoing plastic surgery. Small pieces of skin were thoroughly washed with phosphate-buffered saline solution free of calcium and magnesium (PBS; Gibco) after removal of fat. The skin was minced into fragments of about 125 mm^2 using scalpel blades. Skin samples were incubated overnight at 4°C in a dispase solution (25 U/mL; Life Technologies). Epidermal sheets were removed from the dermis, and keratinocytes were dissociated by trypsin digestion (trypsin-EDTA; Gibco) for 15 min at 37°C. The cell suspension was then filtered through a 280 µm sterile filter. Dulbecco's modified essential medium (DMEM; Gibco) supplemented with 10% of fetal bovine serum (FBS; Gibco) was added vol/vol and the suspension was centrifuged at 300 × g for 10 min. Keratinocytes were seeded at a density of 10^7 cells in 75-cm^2 tissue culture flask in Keratinocyte-Serum Free Medium (K-SFM) supplemented with bovine pituitary extract (25 µg/mL) and recombinant epidermal growth factor (EGF) (0.25 ng/mL; all were purchased from Invitrogen, Life Technologies). The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 until confluence and then stored frozen in liquid nitrogen until use. Finally, keratinocytes were seeded in sterile 24-well culture plates at a density of 4×10^4 cells/well in K-SFM supplemented with bovine pituitary extract and EGF and cultured to 80% confluence. Cells were then starved overnight in K-SFM alone before stimulation.

WNV Strain Production

A lineage 1 clinical strain of WNV was used in this study. The strain isolated from a human brain during the epidemic that occurred in Tunisia in 1997 was provided by Dr I. Leparc Goffart (French National Reference Center on Arboviruses, Marseille, France). The viral stock was produced on the *Ae. albopictus* clone C6/36 cells (ATCC® CRL-1660™). Cells were cultivated in Leibovitz's L-15 medium (Gibco) supplemented with 2 % of

tryptose-phosphate (Gibco) and 5 % of FBS in 75-cm^2 tissue culture flask at 28°C until 50% of confluency and then infected at a Multiplicity Of Infection (MOI) of 0.01 for 72 h. Cell supernatant of infected cells and uninfected C6/36 cell used for control, were clarified by centrifugation in 50 ml tubes for 15 min at 1,500 × g. Then, the viral suspension and the supernatant from uninfected C6/36 suspension were ultrafiltrated in amicon ultra-4 centrifugal filter units 100 kD (Dutscher) for 5 min at 3,000 × g. The viral suspension and the supernatant from uninfected C6/36 suspension were finally frozen at −80°C in cryotubes containing 500 µL of Leibovitz's L-15 medium supplemented with 0.5 M sucrose and 50 mM HEPES. The final viral titer was $10^{7.97}$ $TCID_{50}$ (Tissue Culture Infection Dose) per mL as determined by plaque assays on Vero cell monolayers.

Mosquito Saliva

Saliva were obtained from 7-day-old adult females *Aedes aegypti* (Bora-Bora strain) and *Culex quinquefasciatus* (Slab strain) mosquitoes grown in insectary at 27°C under 70 ± 8% relative humidity and 12:12 light and dark photoperiod. On the hatching day, larvae were equally seeded into plastic trays containing water. Larvae were fed *ad libitum* with a mixture of rabbit and fish-food whilst adults were fed with 10% sucrose solution [w/v]. Salivation was performed on individual mosquitoes according to an adapted protocol from (Dubrulle et al., 2009). Each individual was chilled to remove legs and wings. The mosquito's proboscis was then inserted into a micropipette tip containing 10 µL of DMEM with protease inhibitor (Gibco). After 30 min, the saliva-containing DMEM was expelled and collected into an 1.5 ml tube.

Viral Infection

Human primary keratinocyte cultures (60–80% of confluency) from 6 to 8 different patients were infected at MOI of 0.1, 1, and 10 and incubated for 24, 48, and 72 h at 37°C in 5% CO_2 in K-SFM medium. Mocked-infected keratinocytes incubated with uninfected C6/36 cell supernatant were used as control. For experiments assessing the role of saliva, keratinocytes were infected as previously described in presence of 0.5 µg/L of mosquito saliva for 24 or 48 h.

Cell culture supernatants and cell monolayers were collected at each point of the time course infection in order to perform viral quantification by RT-qPCR and transcriptomic analysis of inflammatory marker expression as described below.

RNA Extraction, Reverse Transcription and Real-Time PCR Analysis

RNA Extraction

For viral RNA quantification in cell supernatant, Total DNA/RNA of 200 µL of keratinocyte supernatant was extracted on NucliSENS easyMAG® automated system (bioMérieux) according to manufacturer's recommendations. For intracellular viral RNA quantification and evaluation of the host inflammatory response, total RNA extraction from keratinocyte monolayer was performed using the Nucleo-Spin XS RNA extraction kit according to the manufacturer's instructions (Macherey-Nagel). RNA concentrations and purity were determined using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

Viral Quantification by RT-qPCR

Viral quantification in cell supernatants and keratinocytes was performed using a one-step real time RT-PCR assay from 5 µL of total RNA in 96-well plates on Applied Biosystems 7500 thermocyler. Reaction mixtures consisted of 12.5 µL of Master Mix (Invitrogen), 0.5 µL (0.2 µM) of forward (5′-3′ GTGCGGTCTACGATCAGTTT) and reverse primers (5′-3′ CACTAAGGTCCACACCATTCTC), and 0.25 µL (0.1 µM) of 5′FAM and 3′Dark Quencher probe (5′-3′ AATGTGGGAAGCAGTGAAGGACGA), 0.5 µL of SuperScript III reverse transcriptase (Invitrogen) and DNA polymerase platinium Taq (Invitrogen), 0.5 µL of RNase out (Invitrogen) and 5.25 µL of water. The calibration range was performed using a transcript produced using a plasmid containing WNV genome deleted from genes coding structural proteins provided by Dr P.W. Mason (Microbiology and Immunology department, Texas University, Galveston, USA). The transcripts were diluted in order to get a calibration range allowing the quantification of viral load from 10^2 to 10^7 RNA copies/mL. Each standard of the calibration curve was added in duplicate to each analysis.

Transcriptomic Analysis of the Innate Antiviral Immune Response in Keratinocytes

Total RNA (1 µg) was reverse transcribed using SuperScript II kit (Invitrogen). Quantitative real time PCR was performed in 96-well plates using LightCycler-FastStart DNA Master^{Plus}SYBR GREEN I kit (Roche) on LightCycler 480 (Roche). Reaction mixtures consisted of 1X DNA Master Mix, 1 µM forward and reverse primers designed using Primer 3 software and 12.5 ng of cDNA template in a total volume of 10 µl. PCR conditions were as follows: 5 min at 95°C, 40 amplification cycles comprising 20 s at 95°C, 15 s at 64°C, and 20 s at 72°C. Samples were normalized with regard to two independent control housekeeping genes (Glyceraldehyde-Phospho-Dehydrogenase and 28S rRNA gene)

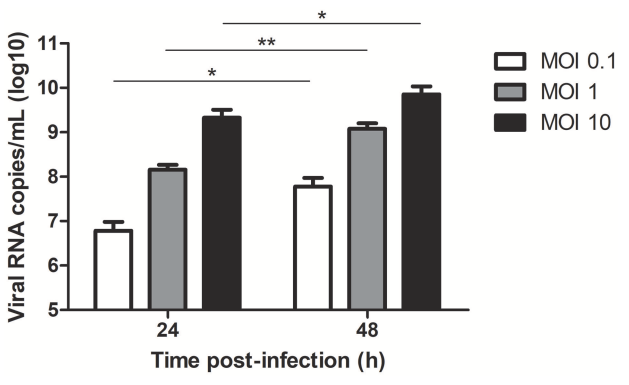

FIGURE 1 | WNV amplification in RNA copies/ml in 24 and 48 h-infected keratinocyte supernatants at MOI of 0.1, 1, and 10. Data are represented as mean + SEM of 6–8 independent experiments. *$p < 0.05$ and **$p < 0.01$.

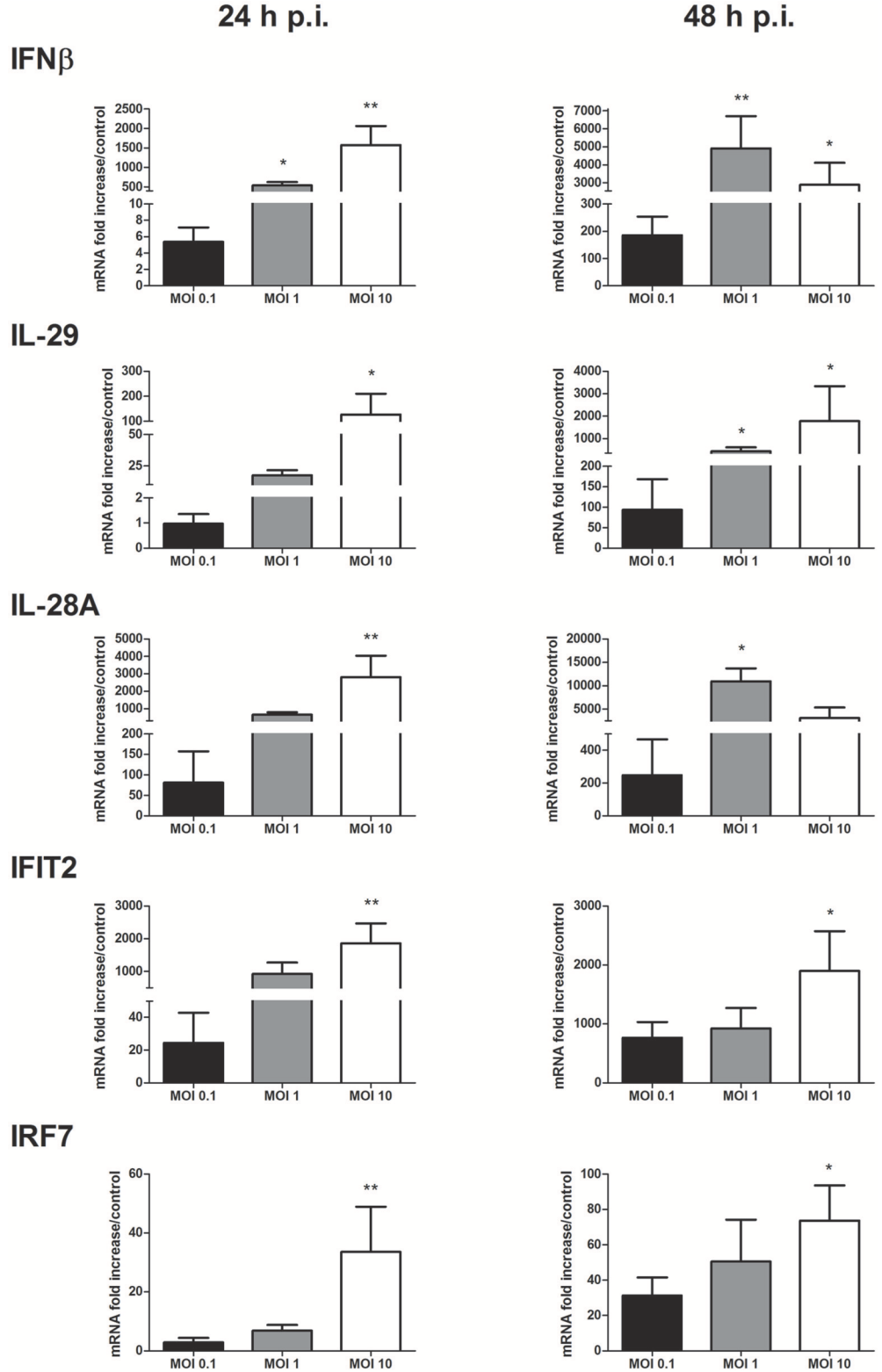

FIGURE 2 | IFN and ISG mRNA expression in response to keratinocyte infection by WNV. IFNβ, IL-29, IL-28A, IFIT2, and IRF7 mRNA expression in human primary keratinocytes infected with WNV for 24 and 48 h, at MOI of 0.1, 1, and 10. mRNA expression levels are expressed as the fold increase above mock-infected cultures. Data are represented as mean + SEM of four independent experiments. *$p < 0.05$ and **$p < 0.01$.

and reported according to the ΔΔCT method as RNA fold increase: $2^{\Delta\Delta}\ CT = 2^{\Delta CTsample - \Delta CTreference}$.

Viral Quantification by End-Point Dilution Assay

Vero cells were seeded, in 96-well plates, the day before titration at the rate of 4×10^3 cells/well in DMEM (Gibco) supplemented with 2% SVF. The suspension was successively diluted from 10^{-1} to 10^{-9} in DMEM medium supplemented with 2% SVF. Then, 100 μL of each dilution were deposited in a row of 6 wells. A reading was performed after 96 h of incubation at 37°C in an atmosphere containing 5% CO_2. The wells in which the cells had a cytopathic effect were considered positive for viral infection. The titer of the viral suspension was then determined using the Kärber's method for assessing the 50% tissue culture infective dose (TCID50).

Enzyme-Linked Immunosorbant Assay (ELISA)

Cell culture supernatants were UV inactivated at 2 joules/cm^2 for 10 min using a Bio-Link crosslinker in order to inactivate virus. Levels of IFNβ and CCL20 in cell culture supernatants were determined for each sample using human ELISA kits (R&D systems for IFNβ; PeproTech for CXCL10) in accordance with the manufacturers' specifications.

Statistical Analysis

Results were analyzed by GraphPad Prism version 5. The statistical significance of the difference between two groups was evaluated by the Wilcoxon's test. Differences were considered to be significant at $p < 0.05$.

RESULTS

Primary Human Keratinocytes Are Permissive to WNV Infection

In a first step, the capacity of WNV to replicate in epidermal keratinocytes from different patients was evaluated at MOI of 0.1, 1, and 10.

The viral quantification of WNV in keratinocyte supernatant using RT-PCR analysis showed an increase of WNV viral load of about 1 log per 24 h during the 48 first hours of keratinocyte infection for all the MOI tested (**Figure 1**). At 72 h p.i., WNV RNA production increased by 2-fold only for the MOI of 0.1 and 1, the MOI of 10 resulting in massive lysis of keratinocytes (data not shown).

Interferon and Interferon Stimulated-Gene mRNA Expression in Keratinocytes Infected by WNV

The IFN antiviral response was then characterized in keratinocytes infected with WNV at MOI of 0.1, 1, and 10. A significant induction of type I and III IFN expression such as IFNβ, IFNλ1 (interleukin (IL)-29) and λ2 (IL-28A) was observed as soon as 24 h p.i. and sustained at 48 h p.i. in a MOI-dependent manner (**Figure 2**). IFNβ and IL-28A mRNA expression was induced at 24 h p.i. from 5 to 1,570 times and from 80 to 2,800 times, respectively. In a consistent way, ISG such as IFIT1 (data not shown), IFIT2, IFIT3 (data not shown) and IRF7 mRNA expression was induced (**Figure 2**). IFIT2 mRNA expression was

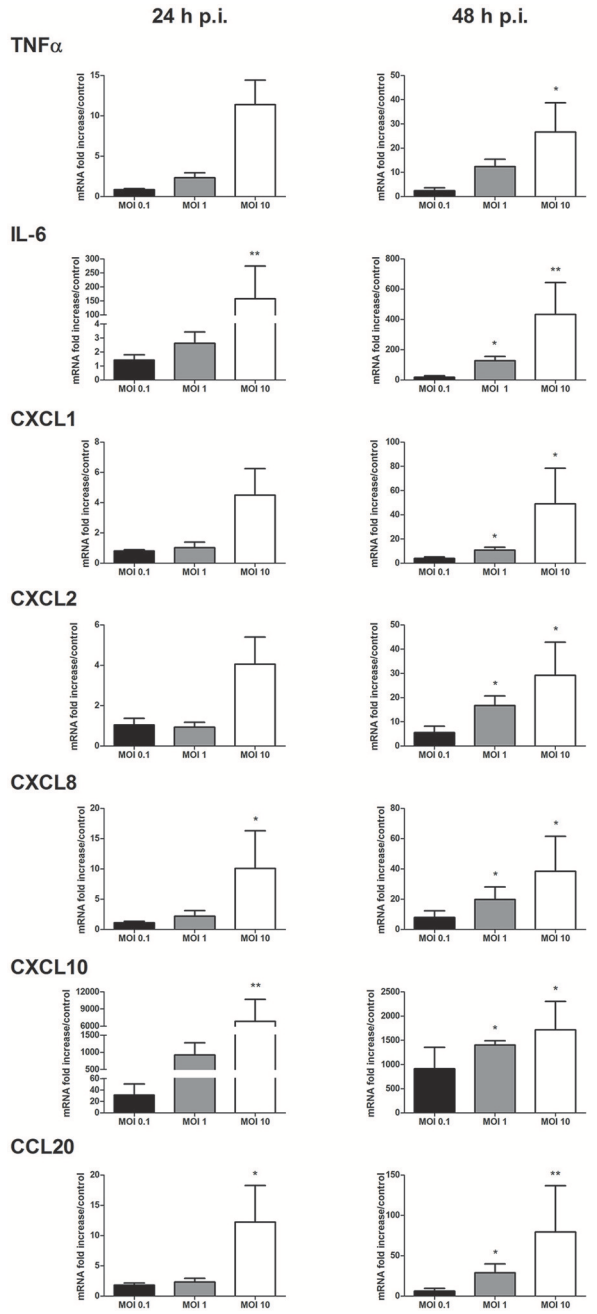

FIGURE 3 | Cytokine and chemokine mRNA expression in response to keratinocyte infection by WNV. TNFα, IL-6, CXCL1, CXCL2, CXCL8, CXCL10, and CCL20 mRNA expression by human primary keratinocytes infected with WNV for 24 and 48 h, at MOI of 0.1, 1, and 10. mRNA expression levels are expressed as the fold increase above mock-infected cultures. Data are represented as mean + SEM of four independent experiments. *$p < 0.05$ and **$p < 0.01$.

increased from 20 to 1,860 times at 24 h p.i. at MOI of 0.1 and 10, respectively. On the other side, IRF3 mRNA levels were not modulated in comparison to mock-infected keratinocytes (data not shown).

Cytokine and Chemokine mRNA Expression in Response to Keratinocyte Infection With WNV

The profile of inflammatory mediators induced by WNV infection was completed by focusing on cytokines and chemokines known to be expressed by keratinocytes. Messenger RNA expression of pro-inflammatory cytokines such as TNFα, IL-6 and chemokines such as CXCL1, CXCL2 CXCL8, CXCL10, and CCL20 was MOI- and time-dependently increased (**Figure 3**). Among them, CXCL10 was the most induced marker with mRNA levels 922 to 6,826-fold higher in WNV-infected cells compared to mock-infected cells at 24 h p.i.

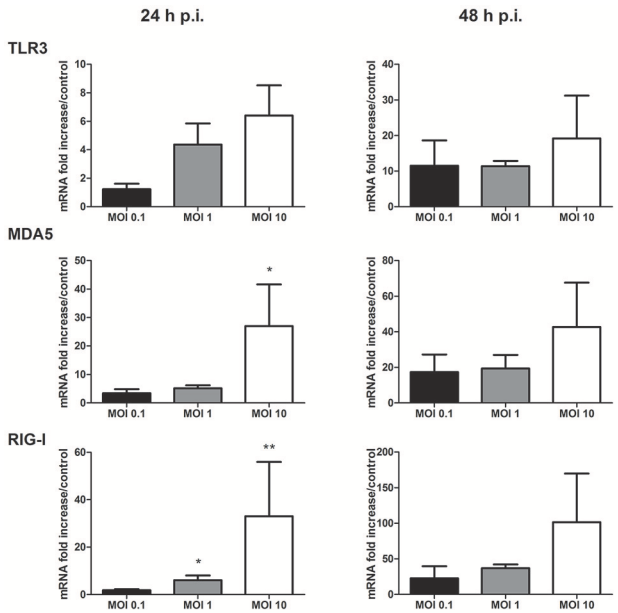

FIGURE 4 | TLR3, MDA5, and RIG-I mRNA expression by human primary keratinocytes infected with WNV for 24 and 48 h, at MOI of 0.1, 1, and 10. mRNA expression levels are expressed as the fold increase above mock-infected cultures. Data are represented as mean + SEM of four independent experiments. *$p < 0.05$ and **$p < 0.01$.

PRR Expression in Infected Keratinocytes

As PRR signaling is crucial to initiate innate antiviral and proinflammatory responses following flaviviral infection, modulation of their expression during WNV replication was studied. The transmembranar TLR3, and the cytosolic helicases MDA5 and RIG-I PRR mRNA expression was increased in infected keratinocytes after 24 and 48 h of infection (**Figure 4**). PRR mRNA level increase was related to the infectious dose used, the stronger induction being observed at the MOI of 10 (**Figure 4**). TLR7 mRNA expression was not detected in infected as well as in mock-infected human primary keratinocytes (data not shown).

Effect of *Ae. Aegypti* Saliva on WNV Replication and the Inflammatory Response of Infected Keratinocytes

As WNV is a virus transmitted through the bite of a hematophagous arthropod, we wanted to assess the potential role of saliva from two distinct mosquito species on viral replication in human primary keratinocytes and on the inflammatory response induced. Saliva from *Ae. aegypti* or *Cx. quinquefasciatus* mosquitoes was thereby co-inoculated with WNV for keratinocyte infection, at a protein concentration of 0.5 μg/l that is consistent with physiological conditions of mosquito bites (Wasserman et al., 2004).

Keratinocyte infection with WNV in presence of *Ae. aegypti* saliva resulted in a decrease of WNV viral load assessed by RT-qPCR after 24 h of infection in cell supernatant as well as in cell lysate. Whereas at 48 h of infection, a significant increase of viral replication in cells infected in presence of saliva was noted in comparison to cells infected without saliva (**Figure 5**). A similar trend was observed with infective viral particle quantification of the supernatants by end-point dilution assay (**Supplementary Figure 1**).

As a parallel to the viral replication, a significant decrease of inflammatory mediator mRNA levels such as IL-28A, IFIT2, CXCL10, and CCL20 was observed at 24 h p.i. in

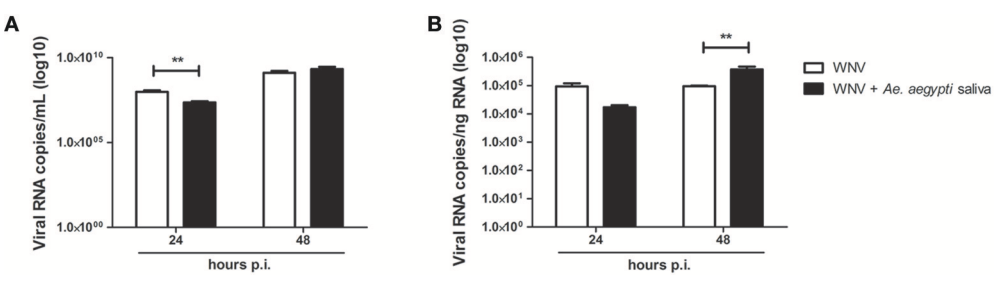

FIGURE 5 | Effect of 0.5 μg/L of *Aedes aegypti* saliva on WNV replication. Viral loads were determined in cell supernatant (**A**, in log10 viral RNA copies/mL) and in cell lysates (**B**, in log10 viral RNA copies/ng RNA) at 24 and 48 h post-infection. Data are represented as mean + SEM of three independent experiments performed in duplicate. **$p < 0.01$.

cells treated with *Ae. aegypti* saliva in comparison to cells infected without saliva (**Figure 6**). In presence of saliva, mRNA expression of macrophage colony-stimulating factor (M-CSF) was slighty reduced at 24 h and 48 h p.i. (**Figure 6** and **Supplementary Figure 2A**). PRR mRNA expression tended also to decrease in presence of saliva at 24 h p.i. (**Figure 6**). This decrease also concerned more specific ISGs with previously described antiflaviviral activities such as viperin, 2′-5′-oligoadenylate synthetase 1 (OAS1), MX1 and ISG20 mRNA expression at early steps of infection (**Figure 6**). At the protein level, secretion of IFNβ was weakly reduced while that of CXCL10 was significantly inhibited in cells infected with *Ae. aegypti* saliva (**Supplementary Figure 3**).

Interestingly, *Ae. aegypti* saliva alone exerted an immunomodulatory effect on uninfected human primary keratinocytes after 24 h of stimulation by tending to inhibit the basal mRNA expression levels of MX1, OAS1, viperin, CCL20 and the PRRs (**Supplementary Figure 4**).

Effect of *Cx. quinquefasciatus* Saliva on WNV Replication and the Inflammatory Response of Infected Keratinocytes

Adjunction of *Cx. quinquefasciatus* saliva to the viral inoculum during keratinocyte infection did not significantly modulate viral load after 24 or 48 h of infection in cell supernatant or in cell lysate (**Figure 7**).

Nonetheless, a significant decrease of inflammatory mediator expression such as IFNβ, IL-28A, IFIT2, M-CSF and the PRRs TLR3 and RIG-I was observed at 24 h p.i. and tend to be sustained at 48 h p.i. (**Figure 8** and **Supplementary Figure 2B**). Messenger RNA levels of known antiflaviviral ISGs such as viperin, OAS1, MX1, and ISG20 also significantly decreased in presence of *Culex* saliva at 24 h p.i. (**Figure 8**). Finally, *Culex* saliva exert a significant inhibitory effect on the secreted levels of IFNβ and CXCL10 by WNV-infected keratinocytes (**Supplementary Figure 5**).

Contrary to *Ae. aegypti* saliva, *Cx. quinquefasciatus* saliva alone did not induce a decrease of ISG mRNA expression but tended to increase CCL20, IFNβ and IL-28A mRNA expression in uninfected human primary keratinocytes after 24 h of stimulation (**Supplementary Figure 6**).

DISCUSSION

WNV is transmitted through the inoculation by a mosquito bite mainly in the extravascular compartment of the dermis and the epidermis (Styer et al., 2007). Our study confirmed that primary human keratinocytes, the main cell type of the epidermis, were permissive to WNV as previously described by Lim et al, with an increase of viral load occurring mostly during the 48 first hours of infection (Lim et al., 2011). Dengue and Zika viruses, two other arboviruses belonging to the *Flavivirus* genus, were also shown to replicate in human keratinocytes so as in other skin cell types such as fibroblasts and dendritic cells (Hamel et al., 2015; Duangkhae et al., 2018). Unpublished data from our group demonstrate

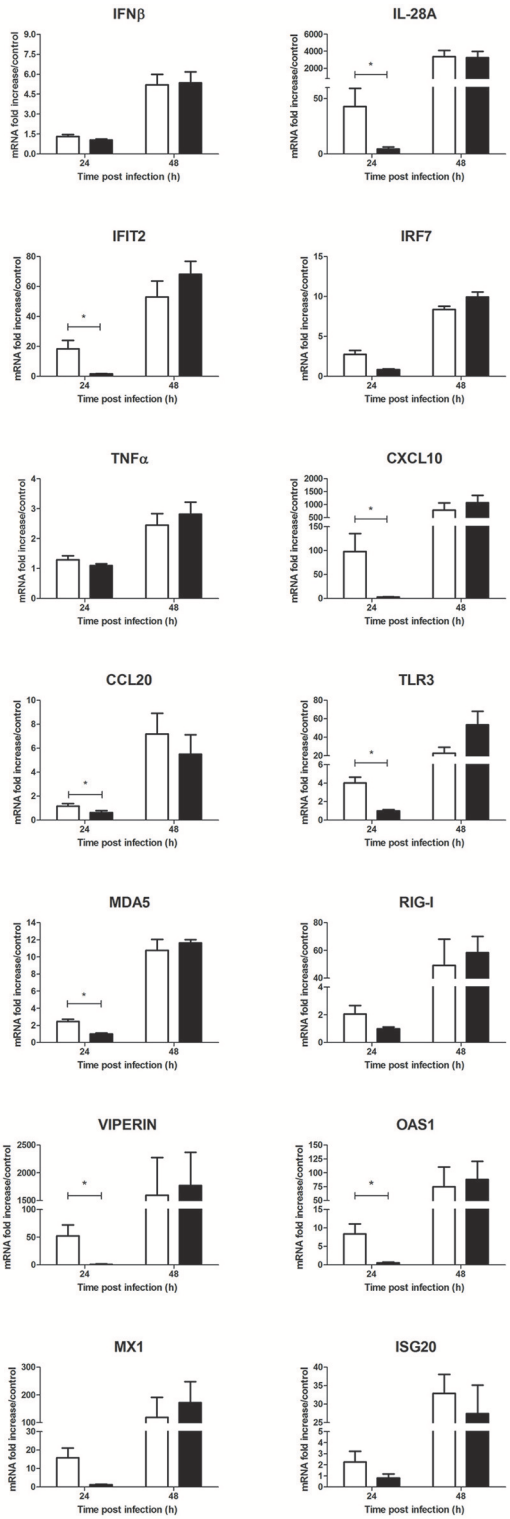

FIGURE 6 | Effect of 0.5 μg/L of *Aedes aegypti* saliva on the WNV-induced inflammatory response during human primary keratinocyte infection. IFNβ, IL-28A, IFIT2, IRF7, TNFα, CXCL10, CCL20, TLR3, MDA5, RIG-I, viperin, OAS1, MX1, and ISG20 mRNA expression by keratinocytes infected with WNV at MOI of 1 for 24 and 48 h. Data are represented as mean + SEM of three independent experiments performed in duplicate. *$p < 0.05$.

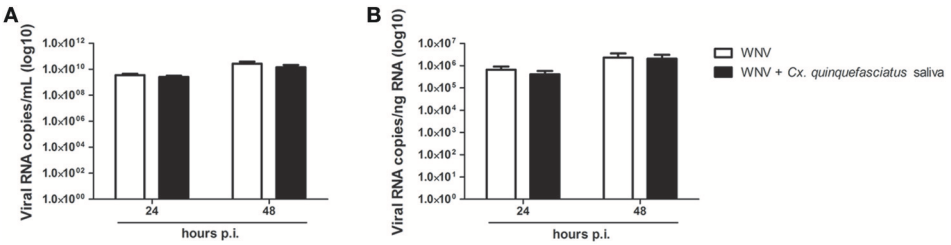

FIGURE 7 | Effect of 0.5 μg/L of *Culex quinquefasciatus* saliva on WNV replication. Viral loads were determined in cell supernatant (**A**, in log10 viral RNA copies/mL) and in cell lysates (**B**, in log10 Viral RNA copies/ng RNA) from infected keratinocytes. Data are represented as mean + SEM of three independent experiments performed in duplicate.

that WNV is also able to replicate in dermal fibroblasts and endothelial cells. Skin, thus, constitutes not only the first inoculation site but also the first replication site of WNV during infection. Moreover, in several cases, acute WNV infection is characterized only by dermatological manifestations, such as non-pruritic macular erythema, suggesting a tropism of the virus for the skin following viremic spread (Del Giudice et al., 2005).

The inflammatory response of human primary keratinocytes to WNV infection was described for the first time in this work. Keratinocytes are resident skin cells with innate immune functions, harboring numerous PRRs involved in the detection of PAMPs in order to initiate an inflammatory response to microbial infection (Briant et al., 2014). Hence, these epidermal cells are part of the first line of defense against WNV, as well as against other arboviruses, and act as sentinels thanks to their pathogen-sensing capacities and their privileged location in the largest interface of our body with the environment. A MOI-dependent inflammatory response was observed in keratinocytes at 24 h p.i. mainly involving pleiotropic cytokines, such as TNFα and IL-6, various chemokines, type I and III IFNs, and ISGs such as IFIT proteins. IFNs and IFN-dependent mediators are known to be strongly induced during viral infection and exert potent antiviral activities (Lazear et al., 2011; Lazear and Diamond, 2015). Type I IFN is thought to control WNV infection by (i) the induction of cell-intrinsic antiviral effectors, known as ISGs, restricting different steps of virus replication and (ii) by activating the adaptive immune (Lazear et al., 2011; Lazear and Diamond, 2015). It has been shown that cell IFN-treatment before infection or addition of IFN to WNV already infected cells, reduced viral replication (Isaacs and Westwood, 1959; Samuel and Diamond, 2005) and that in mice lacking type I IFN receptor, WNV infection spread faster than in wild type mice leading to 100% of mortality (Samuel and Diamond, 2005). Type III IFNs are also members of IFN family with antiflaviviral properties (Lazear et al., 2015; Douam et al., 2017). However, IFNλ exerts a weaker antiviral effect against WNV than type I IFN in two human cell lines (Ma et al., 2009). In our work, type I and III IFN expression was induced in human primary keratinocytes according to MOI and time of infection suggesting that they could play a role against WNV during cutaneous infection. Moreover, type I and III IFNs induce and regulate expression of ISGs. Induction of several ISG mRNA expression such as IFIT-1 to 3, viperin, OAS1, MX1 and ISG20, was observed in WNV-infected keratinocytes. It has been suggested that viperin and ISG20 inhibited steps in viral proteins and/or viral RNA biosynthesis during human embryonic kidney 293 cell infection with WNV (Jiang et al., 2010; Szretter et al., 2011), whereas OAS1 polymorphism was associated with WNV infection susceptibility in mice and humans (Mashimo et al., 2002; Kajaste-Rudnitski et al., 2006; Lim et al., 2009; Szretter et al., 2011). As regards IFIT proteins, they own a broad-spectrum antiviral activity helping to control viral replication and pathogenesis. They have been demonstrated to regulate protein translation through several mechanisms such as by interacting with eIF3 (Hui et al., 2005; Terenzi et al., 2006), binding of uncapped or incompletely capped viral RNA, and sequestering viral RNA or proteins in the cytoplasm (Diamond and Farzan, 2013). They might also interplay in regulation of intrinsic and extrinsic cell immune responses (Diamond and Farzan, 2013). *In vivo*, a tissue-specific antiviral effect of IFIT2 has previously been described in a mouse model of central nervous system WNV infection (Cho et al., 2013). Nonetheless, flaviviruses such as WNV have developed strategies to subvert IFIT1 and IFIT2 functions using 2′-O methyltransferase activity (Daffis et al., 2010; Szretter et al., 2011). In conclusion, although human keratinocytes are permissive to WNV, they express specific antiviral proteins during infection to limit viral replication in the skin.

The innate antiviral cellular response involves IRF-family transcription factors as regulators of host defense by inducing production of IFNs and ISGs. In human keratinocytes, Kalali *et al.* reported a constitutive expression of IRF3 whereas expression of IRF1, IRF2, and IRF7 was inducible (Kalali et al., 2008). IRF3 and IRF7 have been described involved in protection against WNV infection in mouse models (Daffis et al., 2007, 2008). Our results showed that WNV infection of primary keratinocytes induced an increase of IRF7 expression without modulating IRF3 mRNA levels. This has been previously reported in human skin cells infected with the other flaviviruses, DENV or ZIKV (Surasombatpattana et al., 2011; Hamel et al., 2015). These results could suggest a major role of IRF7 in host skin immune response even if we cannot exclude a role of IRF3 as its expression is constitutive.

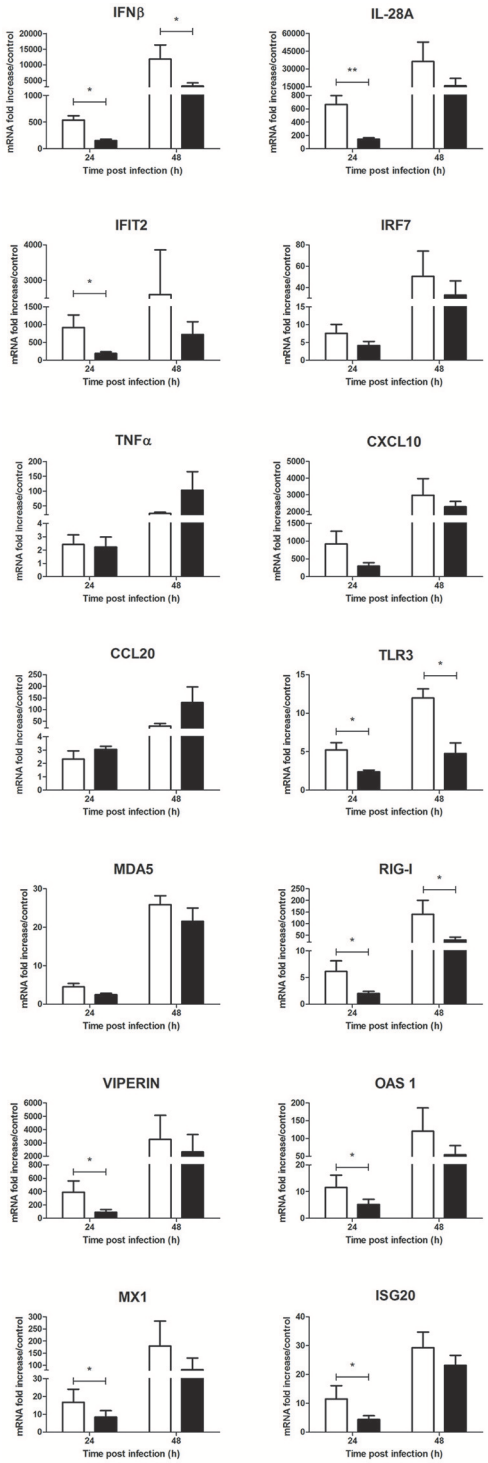

FIGURE 8 | Effect of 0.5 μg/L of *Culex quinquefasciatus* saliva on the WNV-induced inflammatory response during human primary keratinocyte infection. IFNβ, IL-28A, IFIT2, IRF7, TNFα, CXCL10, CCL20, TLR3, MDA5, RIG-I, viperin, OAS1, MX1, and ISG20 mRNA expression by keratinocytes infected with WNV at MOI of 1 for 24 and 48 h. Data are represented as mean + SEM of three independent experiments performed in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$.

IRF3 and IRF7 are phosphorylated after recruitment of the adaptor molecule interferon promoter-stimulating factor 1 (IPS-1) by dsRNA-activated RLRs, MDA5 and RIG-I (Sato et al., 2000; Kato et al., 2006). Keratinocyte infection with WNV was also associated with an increase of TLR3, RIG-I and MDA5 mRNA expression, this phenomenon may contribute to an amplification loop facilitating antiviral ISG expression. If the role of TLR3 in WNV sensing has been controversial (Fredericksen et al., 2004, 2008; Wang et al., 2004; Fredericksen and Gale, 2006; Daffis et al., 2008), MDA5 and RIG-I have been described cooperating in WNV sensing in a time-dependent manner, involving first RIG-I and then MDA5 (Fredericksen et al., 2004, 2008; Fredericksen and Gale, 2006). Moreover, the role of TLR7 in WNV sensing has been reported *in vitro*, considering a higher viral replication and weaker cytokine expression in keratinocytes from TLR7$^{-/-}$ mice than in those from wild-type mice (Welte et al., 2009). TLR7 response following cutaneous WNV infection has also been suggested to promote Langerhans cell migration from the skin to the draining lymph nodes (Welte et al., 2009). Nonetheless,*in vivo*, no difference has been observed regarding susceptibility to WNV encephalitis and in blood or brain RNA viral loads, between wild-type and TLR7$^{-/-}$ mice (Welte et al., 2009). In unstimulated primary human keratinocytes, TLR7 has been reported to be unexpressed but its expression was induced following stimulation with synthetic viral ds RNA analog poly(I:C) (Kalali et al., 2008). In our primary cultures, TLR7 was neither constitutively expressed, nor induced following WNV infection, suggesting that this receptor is not involved in WNV sensing by keratinocytes from the basal layer of the epidermis. However, we cannot exclude a role of TLR7 during WNV infection of the whole epidermis as TLR7 has been described as being constitutively expressed in the human epidermal layers with the exception of the basal layer in normal skin biopsies (Donetti et al., 2017).

The inflammatory response induced in infected human primary keratinocytes also consisted in mRNA level increase of the pleiotropic cytokines TNFα and IL-6 as well as chemokines, such as CXCL1, CXCL2, CXCL8, CXCL10, and CCL20. CCL20 plays an important role in the homing of lymphocytes and dendritic cells to inflammation site, and CXCL1, CXCL2, and CXCL8 are polymorphonuclear (PMN) leucocytes attracting chemokines. CXCL1 mRNA expression has been described to be induced by WNV infection *in vitro* (Quick et al., 2014) and *in vivo* (Kumar et al., 2014). PMN leucocytes have been suggested to play a biphasic role, deleterious first and then beneficial, in pathophysiology of WNV infection (Bai et al., 2010). CXCL10 is induced in several models of WNV brain infection as well as in WNV-infected blood donors (Cheeran et al., 2005; Klein et al., 2005; Garcia-Tapia et al., 2007; Tobler et al., 2008; Qian et al., 2011; Quick et al., 2014; Bielefeldt-Ohmann et al., 2017). This T-cell chemoattractant chemokine was the most strongly induced mediator during keratinocyte infection and it was reported that the plasmatic level of this protein was higher among WNV-infected individuals with a better outcome (Hoffman et al., 2016). CXCL10 as well as TNFα are involved in CD8 (+) T cell traffic playing a major role in WNV clearance (Klein et al., 2005;

Shrestha et al., 2008) and protecting against WNV spread. TNFα also possesses widespread antiviral effects (Benedict, 2003).

Taken together, our results highlight the role of primary keratinocytes as innate immune cells able to establish a cutaneous antiviral response and attract different types of leukocytes, including PMN, T and dendritic cells, in the area of virus inoculation.

WNV contained in a mosquito's salivary glands is inoculated with saliva during mosquito blood meal after probing the skin (Styer et al., 2007; Choumet et al., 2012). Because of mosquito saliva wide properties and as skin is the first site of WNV replication, we assessed the potential role of mosquito saliva on WNV replication and inflammatory response induced in infected human primary keratinocytes.

On the one hand, adjunction of *Ae. aegypti* saliva resulted in a two-step phenomenon regarding WNV replication, a first inhibitory effect at an early time of infection and, later on, a proviral one. It is known that some identified *Ae. aegypti* saliva proteins such as aegyptin or D7 proteins can interfere with DENV replication (McCracken et al., 2014; Conway et al., 2016). Previously, during DENV and CHIKV infection of human primary keratinocytes and human fibroblasts, respectively, in presence of *Ae. aegypti* saliva product, a proviral effect was also reported (Surasombatpattana et al., 2014; Wichit et al., 2017).

On the other hand, adjunction of *Cx. quinquefasciatus* saliva did not have any consequence on WNV replication in keratinocytes. Styer et al. have demonstrated that *in vivo,* mosquito-infected chickens had higher WNV viremia and viral shedding as compared to those infected with needles in the absence of saliva (Styer et al., 2006). In a mouse model, animals previously exposed to uninfected-mosquito bites before being infected with WNV exhibited higher viremia, tissue-titers and neurological symptoms than those infected in the absence of mosquito saliva suggesting that *Culex* saliva could also exert a proviral effect in mammals (Styer et al., 2011). Nonetheless, at the inoculation site, there was no increase of viral titer in skin of mosquito-vs. needle-infected mice at any time of infection and even a decrease of viral titer at 24 h of infection (Styer et al., 2011) suggesting that *Culex* saliva is also not proviral in mice's skin. Congruently, our results suggest that human keratinocytes are not sensitive to the proviral effect of *Culex* saliva.

Concerning the effect of saliva on uninfected human primary keratinocytes, *Ae. aegypti* saliva tended to reduce the basal levels of antiviral ISGs at 24 and 48 h post-stimulation. Interestingly, this anti-inflammatory effect was not found using *Cx. quinquefasciatus* saliva. These results are consistent with a previous work of Wanasen *et al.* demonstrating that salivary gland extract (SGE) from *Ae. aegypti* induced a significant decrease of cell proliferation and cytokine production in murine splenocytes contrary to *Cx. quinquefasciatus* SGE (Wanasen et al., 2004). It has been suggested that according to the concentration of SGE delivered at the site of injection, the immunomodulatory effect observed could change, thereby creating a local and differential immunological environment according to the distance from the site of the bite (Wasserman et al., 2004; Styer et al., 2011). The differential immunomodulatory effect of *Culex* and *Aedes* saliva has been suggested as being related to vector's host preferences, as *Culex* are preferentially ornithophilic mosquitoes whereas *Aedes* more anthropophilic species (Wanasen et al., 2004; Schneider and Higgs, 2008). Therefore, we hypothesized that the immunomodulatory effect of *Culex* saliva would probably be more specific to bird species. Furthermore, *Aedes* and *Culex* mosquito saliva do not exhibit the same salivary protein composition and biological effects (Ribeiro, 2000). During WNV infection of human primary keratinocytes, the immunomodulatory effect of *Aedes* and *Culex* saliva was observed mainly at 24 h p.i. at the transcriptomic level and also at 48 h p.i. for IFNβ and CXCL10 secretion. Schneider & Higgs have hypothesized that mosquito saliva could impair immune response to arbovirus through downregulation of Th1 and antiviral cytokines (Schneider and Higgs, 2008). A few studies have assessed the effect of *Ae. aegypti* saliva on skin cell inflammatory response in the context of flaviviral infections. Decreased expression of type I IFN-responsive genes was observed in presence of *Ae. aegypti* saliva (Surasombatpattana et al., 2014; Wichit et al., 2017). In this study, decreased expression of IFNs and known antiflaviviral ISGs, including MX1, OAS1, ISG20 and viperin was observed in presence of both *Aedes* and *Culex* saliva after 24 h of keratinocyte infection. Inhibition of the keratinocyte antiviral response by mosquito saliva could favor the viral replication which may involve other skin cells surrounding keratinocytes such as Langerhans cells, melanocytes or deeper dermal mast cells and fibroblasts. Finally, a decrease of M-CSF mRNA, a cytokine involved in macrophage differentiation and proliferation, was noted during keratinocyte infection in presence of saliva. As macrophages are known to play a protective role against WNV infection (Ben-Nathan et al., 1996; Bryan et al., 2018), the inhibitory effect of mosquito saliva on M-CSF expression could also result in a lessening of the immune response favorable to virus propagation.

CONCLUSION

This work highlights for the first time the inflammatory response of human primary keratinocytes infected by WNV. These skin resident cells permissive to WNV, are able to sense viral PAMPs and initiate immune response through expression of various inflammatory mediators and antiviral effectors. This may lead to prompt induction of an antiviral state in infected as well as in uninfected neighboring cells, thereby helping to limit viral spread. We also showed that the saliva from two potential vectors of the infection exerts an inhibitory effect on the antiviral response of WNV-infected keratinocytes with adverse effects enhancing knowledge of WNV pathophysiology in the skin.

AUTHOR CONTRIBUTIONS

MG, NL, and CB conceived and designed the study. MG performed the experiments and wrote the draft. HA and MW provided *Culex* saliva. FD, MB, and DM provided *Aedes* saliva. AD performed some transcriptomic analysis. CB and

NL contributed to the revision of the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00387/full#supplementary-material

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Dengue Virus Infects Primary Human Hair Follicle Dermal Papilla Cells

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During the epidemic of the dengue virus (DENV) infection in Taiwan in 2014 and 2015, we observed an abnormally high frequency of increased scalp hair shedding in infected individuals that could not be explained by telogen effluvium. In this study, the mechanism of hair loss caused by DENV was explored. Human hair follicle dermal papilla cells (HFDPCs) are essential for hair follicle morphogenesis and cycling. Thus, we established an *in vitro* DENV infection model in HFDPCs. On immunofluorescence analysis, HFDPCs that were susceptible to DENV infection responded to type I interferon (IFN) treatment, and the cells showed antibody-dependent enhancement (ADE) effect. The expression of the pro-inflammatory cytokines, interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α), revealed an inflammatory response in DENV-infected HFDPCs. In particular, DENV infection impaired cell viability, and it activated caspase-associated cell death signaling in HFDPCs. In conclusion, our data indicate that direct infection with DENV causes inflammation and cell death in HFDPCs, which is involved in the mechanisms of hair loss after DENV infection. The knowledge of DENV infection in an immune-privileged tissue, such as hair follicles, may suggest their use for further studies on post-dengue fatigue syndrome (PDFS).

Keywords: dengue virus, hair loss, human hair follicle dermal papilla cells, cell death, inflammation

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INTRODUCTION

South Taiwan experienced an epidemic of dengue virus (DENV) infection in 2014 and 2015, with more than 50,000 confirmed cases of dengue. Dengue virus type 1 (DENV-1) was the main causative agent in 2014, and in 2015 DENV type 2 was the causative agent (Wang et al., 2016). During the epidemic, we observed an abnormally high frequency of increased scalp hair shedding in infected individuals, without scarring or permanent hair loss. This type of hair loss occurred as early as the first month after acute dengue infection. However, the cellular reason for DENV-mediated hair loss was unknown.

Reports of hair loss with DENV infection are sporadic (Harn, 1989; Qiu et al., 1993; Jensenius et al., 1997; Tristão-Sá et al., 2012; Hitani et al., 2015; Chu and Yang, 2017). Our clinical observations agreed with these reports that the hair loss was not associated with the dengue infection severity (Tristão-Sá et al., 2012). Hair loss occurred in many victims as early as within the initial 2 weeks postinfection. More than two-thirds of the cases occurred within the first 2 months postinfection (Qiu et al., 1993; Jensenius et al., 1997; Tristão-Sá et al., 2012; Hitani et al., 2015; Chu and Yang, 2017). The timing of hair shedding, which occurs about 2–3 months after acute infection, could not

be fully explained by telogen effluvium. Telogen effluvium is a form of non-scarring alopecia characterized by diffuse hair shedding that is triggered when a physiological stress, such as systemic infection, causes large number of hairs to enter the telogen or the resting phase of the hair follicle at the same time. Massive hair loss is not observed until the new anagen hairs begin to grow. The emerging hairs help to force the resting hairs out of the follicle. Hence, the interval between the inciting event in telogen effluvium and the onset of shedding corresponds to the length of the telogen phase, which is about 3 months. From our experience and based on literature, it could be said that the onset of hair loss associated with DENV infection was too early to be explained well by telogen effluvium.

Previous studies focused on the role of hematopoietic-related cells and endothelium in DENV infection, and DENV has been found to infect, and replicate in, multiple cell types such as hematological lineage cells, liver, and endothelium (Noisakran et al., 2010, 2012). We speculated that DENV might cause hair loss by infecting hair follicles or by interfering with hair growth through infection of hair follicle-associated cells. However, the interaction between DENV and hair follicles has not been evaluated. The human hair follicle is composed of epidermal (epithelial) and dermal (mesenchymal) compartments (Millar, 2002). Human hair follicle dermal papilla cells (HFDPCs) are mesenchymal cells that can be isolated from the hair papilla of normal human scalp hair follicles. Hair papilla in the adult hair follicle plays a crucial role in the dermal-epidermal interactions that control hair production and the events of the hair growth cycle (Driskell et al., 2011). Since the deletion of specific genes in early dermal papilla has revealed a significant signaling impairment during the hair follicle morphogenesis (Ramos et al., 2013), it has been suggested that the dermal papilla is the center for signaling network.

Hence, we used HFDPCs to clarify whether DENV can infect such cells and to study the cellular responses, such as inflammation and cell death, of DENV-mediated hair loss. Antibody-dependent enhancement (ADE) occurs when non-neutralizing antiviral proteins facilitate virus entry into host cells, thereby leading to increased infectivity in cells (Martina et al., 2009). We also assayed for ADE in HFDPCs.

MATERIALS AND METHODS

HFDPCs

We used commercial primary HFDPCs, which are mesenchymal cells isolated from the hair papilla of normal human scalp hair follicles (Cat. #602-05a and Cat. #602t-05a, Cell Applications, Inc. San Diego, CA). HFDPCs were cultured in specific growth medium (Cell Applications, Inc.) supplemented with 12% fetal bovine serum (FBS) at 37°C and 5% CO_2.

Dengue Virus

The strains, DENV-1 (766733A) and DENV-2 (PL046) (GenBank accession no. AJ968413.1), were isolated from patients with dengue fever and were kindly provided by Yi-ling Lin (Academia Sinica, Taipei) (Lin et al., 1998). They were propagated in the mosquito cell line C6/36 (ATCC: CRL-1660) grown in RPMI 1640 medium containing 5% FBS. To determine virus titers, culture medium from DENV-2 infected C6/36 cells was harvested for plaque-forming assay. Various virus dilutions were added to 80% confluent baby hamster kidney (BHK-21) cells (BCRC: 60041, Hsinchu, Taiwan) and were incubated at 37°C for 2 h. After adsorption, cells were washed and overlaid with 1% agarose (SeaPlaque; FMC BioProducts, Philadelphia, PA) containing RPMI 1640 medium with 1% FBS. After 7 days of incubation, cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet.

Viral Infection

HFDPCs (4×10^4 cells/well in 12-well plates) were replaced with serum free medium, and then infected or not infected (untreated control) with DENV at a multiplicity of infection (MOI) of 10 or 50. In some experiments, DENV-2 with an MOI of 0.1–10 was used. After 4 h of adsorption, the virus soup was removed, and the cells were incubated with growth medium supplemented with 2% FBS for 4 days. In some cases, HFDPCs were pretreated or not pretreated (untreated control) with 500 IU/ml of recombinant human interferon alpha-2a (IFNα-2a, Cat. #Cyt-204, ProSpec-Tany TechnoGene Ltd, Ness-Ziona, Israel) for 16 h, and then the cells were infected with DENV-2. For ADE assay, HFDPCs were incubated with or without (untreated control) anti-envelope (E) protein antibody (α-E, mouse monoclonal antibody, #YH0025, 1:10,000, Yao-Hong Biotechnology, Taipei) and DENV-2 (MOI = 10 or 50).

Immunofluorescence Assay

Human hair follicle dermal papilla cells were fixed with 4% paraformaldehyde for 30 min, and then permeabilized with 0.5% Triton X-100 for 10 min. After two washes with phosphate buffered saline (PBS), cells were blocked with 10% skim milk in PBS. Infected cells were detected by incubation with the antibody targeting NS3 (#YH0034, 1:10,000, Yao-Hong Biotechnology), and then with the secondary anti-mouse Alexa Fluor 488 antibody (Thermo Fisher Scientific, Waltham, MA) (Wang et al., 2015). DAPI was used to stain nuclei. Fluorescence signals were observed by fluorescence microscopy (Zeiss, Axio Observer A1, Oberkochen Germany).

Cell Viability Assay

The WST-1 assay (Roche, Basel, Switzerland) was used to assess cell viability. HFDPCs were grown at a density of 5×10^3 cells/well in 96-well plates overnight, followed by infection with DENV (MOI = 10, MOI = 50). After 4 days of incubation, 10 μl of Cell Proliferation Reagent WST-1 was added directly to each well, and then was incubated again for 2 h at 37°C. Cell viability was quantified by multi-well spectrophotometry (Anthos, Biochrom, Cambridge, UK). Absorbance at 450 nm was tested, and the reference wavelength was set at 620 nm. The absorbance was related to the cell viability percentage using the formula [(OD of infection test/OD of untreated control) × 100].

Western Blot Analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 50 mM Tris-HCl [pH

8.0]) or SDS buffer (2% SDS, 50 mM Tris-HCl [pH 7.5]) containing protease inhibitor and phosphatase inhibitor cocktail (Roche). Harvested extracts were separated by 10% SDS-PAGE and transferred to PVDF membranes. Later, the membranes were incubated with primary antibody, and then with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) and were finally visualized using enhanced chemiluminescence (Thermo Fisher Scientific). Image acquisition and signal density measurements were carried out using the BioSpectrum Image System (UVP, Upland, CA). The following primary antibodies were used: anti-caspase 1 (GTX111630, GeneTex, Irvine, CA), anti-caspase 3 (#9665, Cell Signaling Technology, Danvers, MA), anti-caspase 7 (GTX1002337, GeneTex), anti-caspase 8 (#4790, Cell Signaling Technology), anti-bone morphogenetic protein 4 (BMP-4; GTX100875, GeneTex), anti-phospho-STAT1 (phospho-Tyr701, #9167 Cell signaling), anti-STAT1 (#14994, Cell Signaling), anti-phospho-STAT2 (phospho-Tyr690, GTX50721, GeneTex), anti-STAT2 (#14994, Cell Signaling), anti-DENV NS3 (GTX124252, GeneTex), and anti-GAPDH (#60004-1-Ig, Proteintech Group, Rosemont, IL).

RT^2 Profiler PCR Array and RT-qPCR

Total RNA was extracted from HFDPCs by using Trizol reagent (Thermo Fisher Scientific) as instructed in the protocol. Total RNA (1,000 ng) from each sample underwent cDNA synthesis by using the RT^2 First Strand Kit (Qiagen, Valencia, CA). The resulting cDNA was mixed with H_2O plus SYBR green dye, and then dispensed into a 96-well RT^2 Profiler PCR array plate as described (Qiagen). DNA amplification was carried out using the Applied Biosystems StepONE Plus Real-Time PCR system (Thermo Fisher Scientific). The fold-change in gene expression in DENV-infected HFDPCs was relative to that of the untreated control. We considered a fold-change threshold of at least two-fold upregulation or downregulation.

The specific gene expression was also confirmed by RT-qPCR. cDNA was synthesized from 500 ng total RNA using Superscript III Reverse Transcriptase (Invitrogen). qPCR amplification was carried out with 3 ng of cDNA in 10 µl SYBR Green using the Applied Biosystems StepONE Plus Real-Time PCR system (Thermo Fisher Scientific). Transcript levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences for gene detection are provided in **Table S1**.

Statistical Analysis

Significant differences between the groups were determined by Student's *t*-test using GraphPad Prism (La Jolla, CA). Data are expressed as mean ± SD from 3∼4 independent experiments. $P < 0.05$ was considered to be statistically significant.

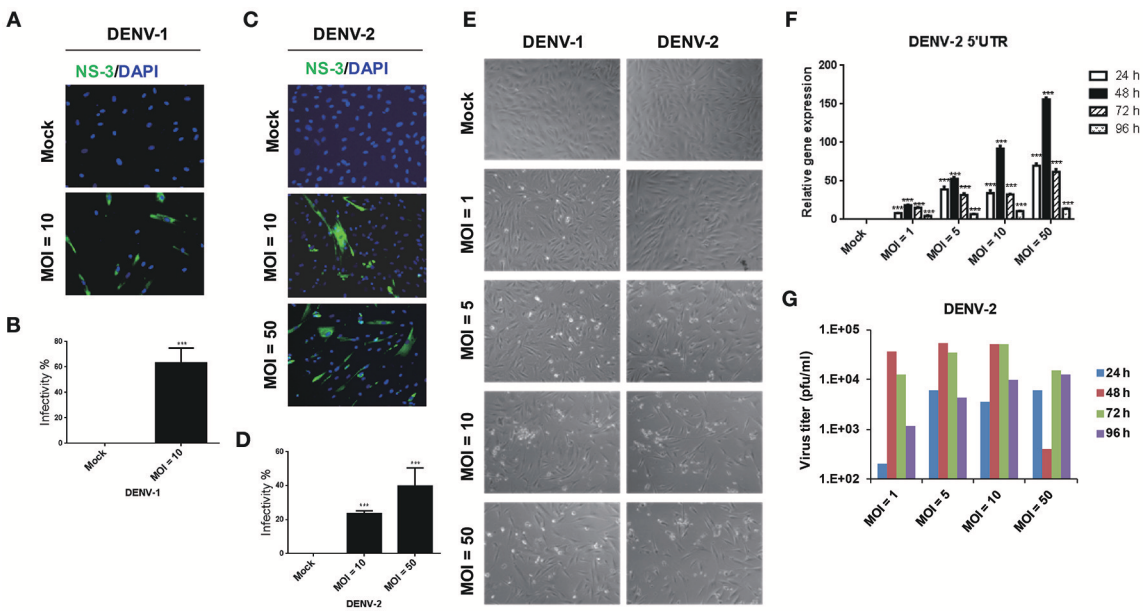

FIGURE 1 | Infective ability of dengue virus type 1 (DENV-1) and DENV-2 in hair follicle dermal papilla cells (HFDPCs). **(A,C)** Immunofluorescence assay of HFDPCs with DENV-1 (MOI = 10) and DENV-2 (MOI = 10 and 50) infection for 4 days. Shows NS3 protein signal (green fluorescence) of DENV infection and DAPI staining (blue fluorescence) for cell nuclei. **(B,D)** Quantification of the DENV-1 and DENV-2 infectivity in HFDPCs. Data are mean ± SD of three observation fields. ***$P < 0.005$ vs. untreated control. **(E)** The cell morphology of HFDPCs with DENV-2 infection (MOI = 1, 5, 10, and 50) for 72 h under brightfield microscope were observed and captured. **(F)** RT-qPCR of DENV-2 5′-UTR expression in HFDPCs infected with DENV-2 (MOI = 1, 5, 10, and 50) were performed at 24, 48, 72, and 96 h postinfection. The gene expression was normalized to GAPDH gene. Data are mean ± SD from three independent tests, ***$P < 0.005$ vs. untreated control. **(G)** Detection of DENV-2 virions in the growth medium of HFDPCs. The growth medium of HFDPCs with DENV infection (MOI = 1, 5, 10, and 50) were harvested at 24, 48, 72, and 96 h postinfection by virus titration plaque assay in BHK-21 cells.

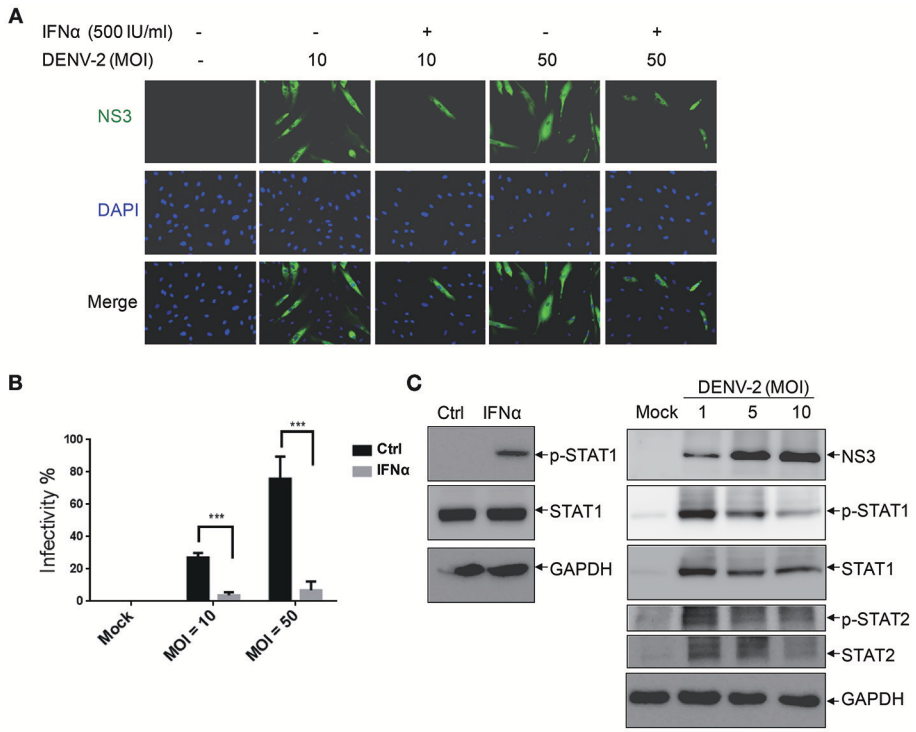

FIGURE 2 | Interferon α (IFNα) inhibits DENV-2 in HFDPCs. **(A)** HFDPCs (4×10^4 cells/well) were pretreated with IFNα (500 U/ml) before DENV-2 infection at MOI 10 and 50 for 4 days. Immunofluorescence assay of DENV-infected cells (green fluorescence) with anti-NS3 antibody. Cell nuclei are stained with DAPI (blue fluorescence). Merged images are also presented. **(B)** Quantification of DENV-2-infected cells. Data are mean ± SD of three observation fields. ***$P < 0.005$ vs. untreated control. **(C)** Left panels, HFDPCs (1×10^5) were treated with IFNα-2a (1,000 U/ml) for 6 h, the untreated cells are indicated as ctrl. Right panels, HFDPCs infected with untreated control or DENV2 at MOI = 1, 5, and 10 for 4 days. Immunoblotting of levels of phospho-STAT1/STAT2 and total STAT1/STAT2 in whole cell extracts. DENV-2 NS3 indicates viral infection and GAPDH is the loading control. The represented data are from the consistent results of two or three independent experiments.

RESULTS

DENV-1 and DENV-2 Infection of HFDPCs

During the dengue outbreak in Taiwan in 2014 and 2015, DENV-1 and DENV-2 were the most prevalent serotypes (Wang et al., 2016); therefore, we used these two serotypes in this study. Since HFDPCs are indispensable for regenerating new hair follicles, we investigated whether HFDPCs were susceptible to DENV infection. HFDPCs were infected with DENV-1 (MOI = 10) and DENV-2 (MOI = 10 and 50). After 4 days, DENV-infected cells were detected by immunofluorescence assay; the infectivity of DENV-1 was 63% (MOI = 10) (**Figures 1A,B**) and that of DENV-2 was 23 and 40% (MOI = 10 and 50), respectively (**Figures 1C,D**). Thus, HFDPCs were susceptible to infection with DENV, particularly DENV-1. Compared with the untreated infection control, the morphology of HFDPCs infected with DENV-1 and DENV-2 changed, and the cytopathic effect (CPE) was also observed (**Figure 1E**). Furthermore, the DENV-2 5′-untranslated region (UTR) gene replication was detected in HFDPCs with DENV-2 infection (MOI = 1, 5, 10, and 50). The viral RNA replication peak was detected at 48 h post-infection, but the peak decreased at 72 and 96 h, which suggested that the severe CPE could not support the replication of DENV in HFDPCs (**Figure 1F**). The virions were also detected in the culture medium of DENV-2-infected HFDPCs, and the titration assay showed a similar result with viral RNA detection, which indicated that DENV replicated in HFDPCs without CPE (**Figure 1G**).

IFNα Attenuates DENV-2 Infectivity in HFDPCs

The activation of the innate immune pathway and inflammatory pathway during dengue disease was revealed, which show a dual role in mediating both protection and exacerbation of disease (Costa et al., 2013). Type I IFN is an antiviral cytokine used against virus infection in host cells, our previous report showed that DENV-2 was able to induce IFNβ production in cells (Chang et al., 2006); however, its antiviral activity could be modulated by DENV (Yu et al., 2012). Thus, it would be important to understand whether the antiviral activity of IFNα is effective against DENV infection in HFDPCs. The cells were pretreated with IFNα before infection with DENV-2 (MOI = 10 and 50). Interferon-alpha treatment significantly decreased the DENV-2 infectivity from 27 to 3% at MOI = 10 and from 76 to 7% at MOI = 50 as compared with untreated control cells

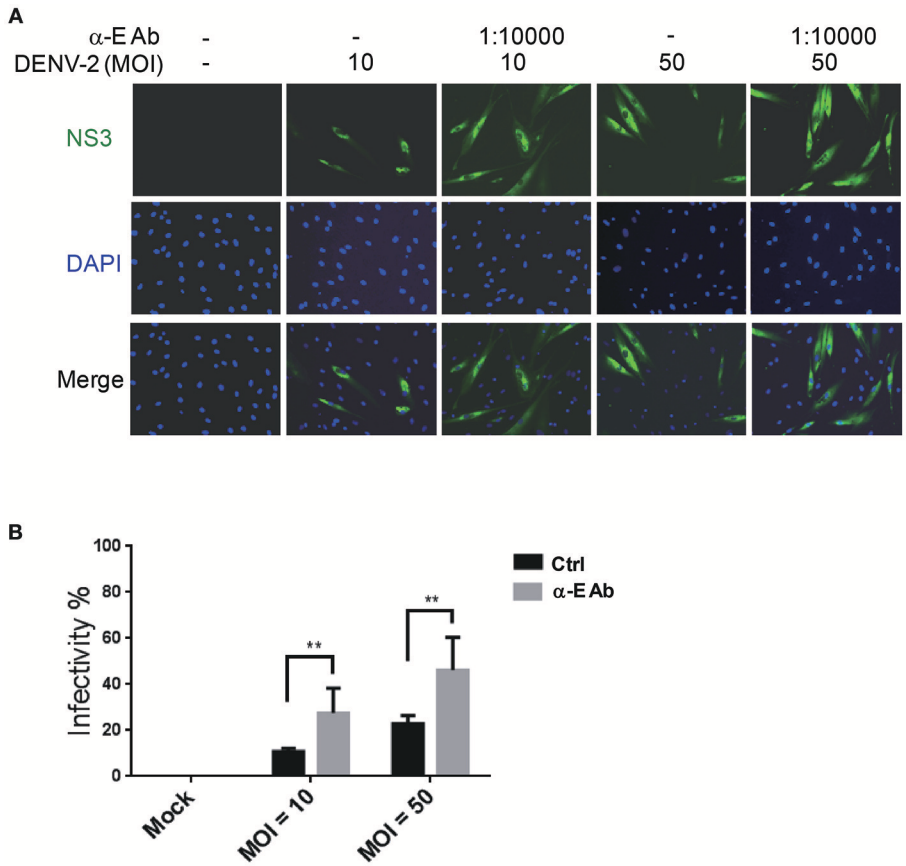

FIGURE 3 | Antibody-dependent enhancement increases DENV-2 infectivity in HFDPCs. **(A)** HFDPCs (4×10^4 cells/well) were infected with DENV-2 (MOI = 10 and 50) with α-E antibody (10,000-fold dilution). After 4 days of infection, HFDPCs were stained with anti-NS3 antibody and DAPI to detect DENV (green color) and cell nuclei (blue color), respectively. **(B)** The infectivity rate was quantified. Data are mean ± SD of three observation fields. $^{**}P < 0.01$ vs. untreated control.

(**Figures 2A,B**). These data provided evidence for IFNα mediated inhibition of DENV-2 infection in HFDPCs.

We revealed the effect of type I IFN against DENV-2-infected HFDPCs (**Figures 2A,B**). Therefore, we measured the level of Janus kinase (JAK)-signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2) activation in the type I IFN signaling pathway. The studies showed that IFNα-2a induced the expression of phosphorylated STAT1 in HPDPCs (**Figure 2C**, left panels). These data could explain that the pretreatment with type I IFN inhibited DENV-2 infection in HFDPCs. The immunoblots showed that the high level of STAT1/2 phosphorylation was induced by DENV-2 infection at MOI = 1, but it was decreased by DENV-2 infection at MOI = 5 and 10 in HPDPCs (**Figure 2C**, right panels), which indicated that a large amount of DENV-2 infection was able to downregulate the IFN signaling pathway.

ADE Increases the Infective Ability of DENV-2 in HFDPCs

Subneutralizing levels of antibodies have been shown to enhance DEN-V replication *in vitro* and disease severity in animal models (Zellweger et al., 2010). In addition, the ADE is also directly associated with the severity of secondary dengue disease in humans (Katzelnick et al., 2017). This is because ADE can enhance the entry of DEN-V into hematopoietic cells (Flipse et al., 2016b). We would like to understand whether the ADE effect accelerates the infection of DENV-2 in HFDPCs. Previous studies have demonstrated that the Fc receptors are functionally expressed in human keratinocytes and skin-derived human mast cells (Cauza et al., 2005; Zhao et al., 2006). Thus, we measured the FcγR mRNA expression in HFDPCs and in the control cells, HaCaT keratinocytes. When compared with HaCaT cells, HFDPCs expressed a higher mRNA level of Fc receptors, such as FcγRIIA, FcγIIB, and neonatal Fc receptor (FcRn) (**Figure S1A**). The FcRIIA/B protein expression in the surface of HFDPCs was also detected (**Figure S1B**). Then, we added anti-E protein antibody to cultures during virus adsorption. This treatment significantly increased DENV-2 infectivity from 11 to 28% at MOI = 10 and 23 to 46% at MOI = 50 as compared with untreated control cells (**Figures 3A,B**). These data suggest that ADE might play roles in the pathogenic infection of HFDPCs.

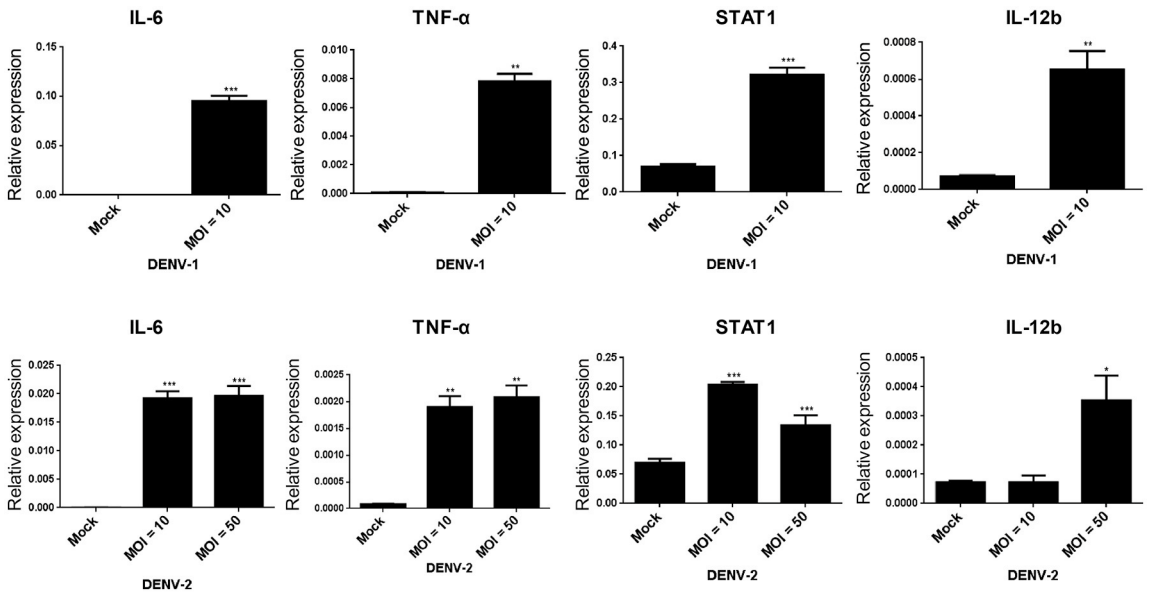

FIGURE 4 | DENV-1 and−2 induce interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), signal transducer, and activator of transcription 1 (STAT1), and IL-12b gene expression in HFDPCs. RT-qPCR of IL-6, TNF-α, STAT1, and IL-12b expression in HFDPCs infected with DENV-1 (MOI = 10) and DENV-2 (MOI = 10 and 50) for 4 days. The gene expression was normalized to GAPDH gene. Data are mean ± SD from three independent tests, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$ vs. untreated control.

DENV Induces Inflammatory Cytokine Expression in HFDPCs

On DENV infection, an appropriate inflammatory response would be activated in the host to clear the pathogen, thereby limiting the risk of disease. However, the inflammatory activity is also involved in the pathogenesis of DENV (Chaturvedi et al., 2000; Costa et al., 2013). The NF-κB activity mediated inflammatory response was evaluated in HFDPCs with lipopolysaccharide (LPS) challenge (Hill et al., 2012). So, it is important to understand whether DENV triggers pro-inflammatory cytokine expression in HFDPCs. Our data showed that DENV-1 and DENV-2 induced the expression of the pro-inflammatory cytokines interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), and IL-12b, as well as the downstream mediator STAT1 in HFDPCs (**Figure 4**). In addition, IL-1β, IL-6, and IL-8 expression and the DENV-2 5′-UTR region were induced by various MOI (0.1–10) of DENV-2 (**Figure S2**). Therefore, DENV infection triggered an inflammatory response in HFDPCs.

DENV Impairs the Cell Viability of HFDPC

Viral infection-mediated cell death is a strategy of host to restrict virus replication and spread (Okamoto et al., 2017). In **Figure 1E**, we observed the CPE in HFDPCs and detected lower levels of DENV-2 RNA and virions at late infection phase (**Figures 1F,G**). Again, by using the WST-1 cell proliferation assay, we detected that DENV-2 significantly decreased the cell viability after DENV-2 infection for 96 h (**Figure 5A**). Also, the levels of caspase 3, caspase 7, and caspase 8, and factors of apoptosis were increased in DENV-2-infected HFDPCs (**Figures 5B,C** and **Figure S3**), which suggested that the apoptosis-associated caspase cascade was activated by DENV-2. Bone morphogenetic protein signaling in HFDPCs is required for the growth and differentiation of hair follicles (Rendl et al., 2008; Solanas and Benitah, 2013). The protein level of BMP-4 was reduced with DENV-2 infection (**Figures 5B,C**). We also found that, DENV downregulated the level of dermal papilla signature genes, such as alkaline phosphatase (ALPL), noggin (NOG), lymphoid enhancer-binding factor 1 (LEF-1), and wingless-related MMTV integration sit 5A (WNT5A), in HFDPCs (**Figure S4**); these molecules are required in the maintenance of intrinsic dermal papilla properties (Ohyama et al., 2012). These data indicated a dysfunction of supporting hair growth in DENV-2-infected HFDPCs.

We used RT^2 Profiler PCR Array to assay the DENV-triggered cell death signaling pathways in HFDPCs, including genes in the pathways of autophagy (cathepsin S, FAS, and TNF), apoptosis (caspase 1, CD40, C-abl oncogene 1, and caspase 7), and necrosis (forkhead box I1). The genes' altered expression was shown in the scatter plot (**Figure S5**) and confirmed by RT-qPCR (**Table 1**) and suggested that DENV-2 infection activated cell damage/death signaling in HFDPCs.

DISCUSSION

In this study, we demonstrated that HDFPCs were susceptible to DENV infection, which could be related with the massive scalp hair loss observed with the epidemic of DENV infection in Taiwan in 2014 and 2015. The infection could be reduced by type I IFN treatment. The ADE effect, frequently seen in

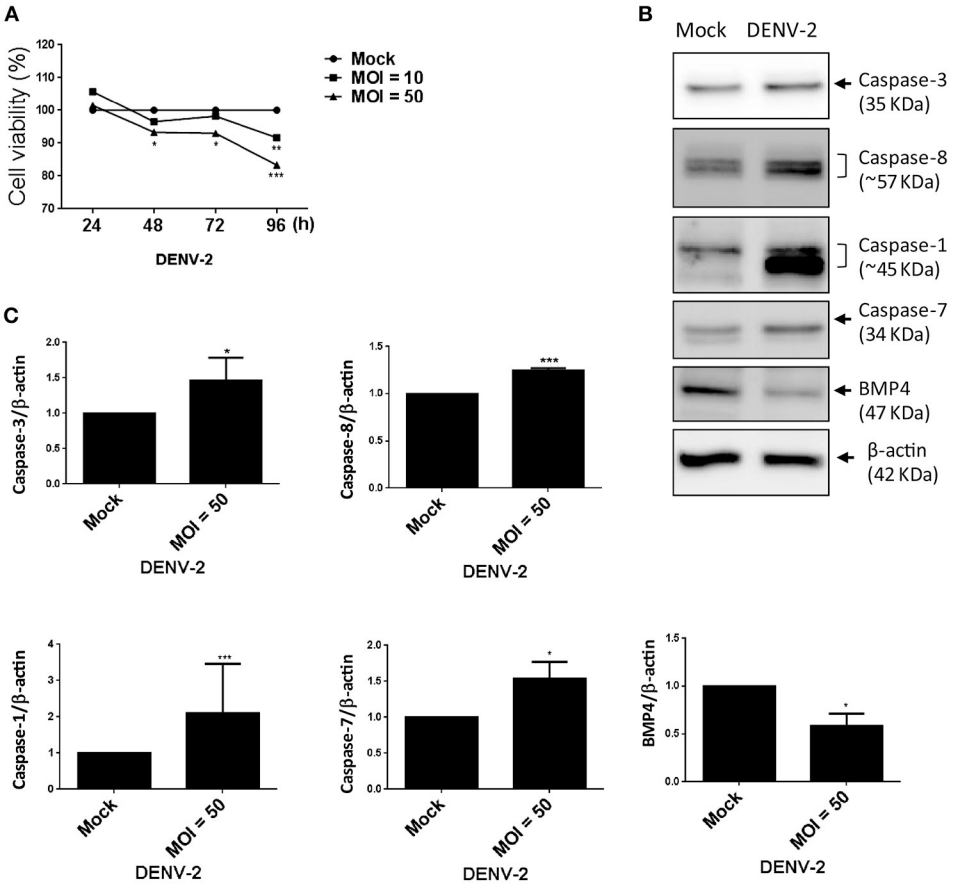

FIGURE 5 | DENV-2 induces cell-damage and apoptosis pathway in HFDPCs. **(A)** HFDPCs (4×10^4 cells/well) were infected with DENV-2 (MOI = 10 and 50), and cell viability was detected by WST-1 assay at 24, 48, 72, and 96 h postinfection. Data are mean ± SD from three independent tests. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. untreated control. **(B)** Western blot analysis of protein expression of caspase 3, caspase 8, caspase 1, and caspase 7, and BMP-4 in HFDPCs (2×10^5 cells/well) infected with DENV-2 (MOI = 50) for 4 days. β-actin expression was an internal loading control. The full membrane images are shown in **Figure S4**. **(C)** Quantification of protein levels (mean ± SD) from three independent assays. $^*P < 0.05$ and $^{***}P < 0.005$ vs. untreated control.

TABLE 1 | Changes in gene expression in dengue virus type 2 (DENV-2)-infected hair-follicle dermal papilla cells (HFDPCs).

Pathway	Gene	PCR array	qRT-PCR	*P*-value qRT-PCR
Autophagy	CTSS	404.06	131.78 ± 92.5	<0.001
	FAS	2.16	1.36 ± 0.26	<0.001
	TNF	2.68	2.21 ± 0.86	<0.001
Apoptosis	CASP1	54.90	31.72 ± 21.72	<0.001
	CD40	6.02	1.58 ± 0.41	<0.001
	ABL1	2.11	2.16 ± 0.82	<0.001
	CASP7	2.05	1.41 ± 0.30	<0.001
Necrosis	FOXI1	2.62	2.32 ± 0.94	<0.001

Data are represented as fold change (DENV-2 treated vs. untreated control). CTSS, cathepsin S; TNF, tumor necrosis factor; CASP1 and 7, caspase 1 and 7; ABL1, C-abl oncogene 1; FOXI1, forkhead box I1.

macrophages (Flipse et al., 2016a), was also found in HFDPCs. The expression of the pro-inflammatory cytokines IL-6 and TNF-α implied that there is an inflammatory response in HFDPCs after DENV infection. In particular, DENV caused cell death, activated caspase-associated cell death signaling, and reduced BMP-4 protein level. Direct DENV infection of HFDPCs might play roles in the post-dengue fatigue syndrome (PDFS), including hair loss. Our findings provide further evidences about the pathophysiology of hair loss after DENV infection.

Our data show that primary non-immortalized HFDPCs could be infected with DENV-1 and DENV-2 with various MOI of inoculation dose; this is the first evidence of DENV targeting HFDPCs (**Figure 1**). However, the virus infectivity was comparatively poorer with non-immortalized HFDPCs than with other immortalized cell lines we reported earlier, such as A549 lung carcinoma cells or WS1 skin fibroblastomas, in which effective DENV infection could be achieved at MOI = 1 with an infection period of 24 h (Wang et al., 2015). Since the information on DENV tissue tropism is limited, DENV localization was never detected in scalp tissue (Jessie et al., 2004; Balsitis et al., 2009). Thus, the discrepancy of DENV infectivity between HFDPCs and other cell types remains to be further explored.

Antibody-dependent enhancement is important in helping the virus invade hematopoietic cells and it plays critical roles in the pathophysiology of dengue hemorrhagic fever (Flipse et al., 2016b). The non-neutralizing antibody can help DENV to enter human hematopoietic lineage cells, because DENV-antibody complexes are targeted to Fcγ receptor (FcγR)-bearing cells (Flipse et al., 2016b). Upon interaction of the antibodies with FcγR, the virion is internalized in the cell (Martina et al., 2009). The polymorphism of Fc gamma receptors (FcγR) was found to be a genetic factor involved in the development of dengue disease (Loke et al., 2002).We evaluated the expression of different FcγR in HFDPCs, the results showed that the FcγRIIA expression level was higher than the other types of FcγR (**Figure S1**). Interestingly, ectopic expression of FcγRIIA (CD32) was found to enhance DENV immune complex infectivity more effectively than FcγRIA (CD64) in COS-7 cells (Rodrigo et al., 2006), this report may partly support our ADE analysis in HFDPCs. Since ADE has never been reported in hair follicle-associated tissue, ours is the first study which revealed that ADE facilitates the infectivity of DENV in HFDPCs (**Figure 3**), which suggests that the serotype cross-reactive antibody might be involved in hair loss with PDFS. However, it remains unclear whether secondary or more severe DENV infections cause higher frequency or degree of hair loss in patients. Further investigations would be needed to answer this issue.

Type I IFN plays a key role against virus infection in hosts (Chang et al., 2006; De La Cruz Hernandez et al., 2014; Palma-Ocampo et al., 2015), this study supports our finding that type I IFN efficiently inhibits DENV replication in HFDPCs (**Figures 2A,B**). We also found that STAT1/2 was highly activated in HFDPCs with low MOI of DENV-2 infection (MOI = 1), but this high level of STAT1/2 phosphorylation was decreased in cells with high MOI of virus infection (MOI = 5 and 10) (**Figure 2C**, right panels). The high MOI of DENV-2 infection might feature a type I IFN evasion machinery, which was described in other RNA virus infection models (Lin et al., 2004; Ho et al., 2005; Lu et al., 2012). Dengue virus-induced inflammatory cytokines such as TNF-α, IL-6, and IL-8 are critical in the pathogenesis of dengue fever and dengue hemorrhagic fever (Chaturvedi et al., 2000; Martina et al., 2009). We detected DENV-induced inflammation in HFDPCs as well (**Figure 4**). The hair follicle constitutes an area of immune privilege, lacking adaptive immunity (Christoph et al., 2000). Thus, inflammatory or cytokine expression in innate immunity might be an important response to invasive microbes or physical stress (Hill et al., 2012; Shin et al., 2016).

We showed that DENV infection induced HFDPC death, caspase cascade activation, and death-associated gene expression, which indicates pathogenic infection in HFDPCs (**Figure 5**). Dengue virus-caused hair loss may occur by direct infection or by killing hair follicle-associated cells. Furthermore, DENV may interfere with the normal orchestration of the hair cycle through mediators such as cytokines. The hair cycle is tightly controlled by a complex cross talk between hair follicles and the surrounding tissues (Driskell et al., 2011; Higgins et al., 2013). More specifically, hair growth is regulated by the epithelial-mesenchymal interaction of hair follicle cells and the adjacent tissue (dermal papilla and subcutaneous cell tissue), which involves various molecular signaling pathways, including BMP, the Wnt family, fibroblast growth factor, transforming growth factor β, and Hedgehog pathways (Yang and Cotsarelis, 2010). Most of these signals are not derived from hair follicle cells but from adjacent cells. Hair dermal papilla cells maintain bulge stem cells and germ cells in the quiescent state during telogen by producing BMP-4 and interfering with the balance of BMP-4 and BMP inhibitors and fibroblast growth factor (FGF) (Rendl et al., 2008; Solanas and Benitah, 2013). Our data confirms that DENV-infected dermal papilla cells exert an altered signal for hair cycle control by downregulating BMP-4 (**Figure 5**).

Not only BMP family (Rendl et al., 2008), but also Wnt/β-catenin and FGF have been known to play important roles in affecting HFPCs to regulate hair cycle (Kwack et al., 2013). Furthermore, there are many cellular factors reported to be involved in altering hair cycle, such as ALPL, NOG, LEF-1, and WNT5A (Ohyama et al., 2012; Higgins et al., 2013). Our supplementary data also showed the reduction of ALPL, NOG, LEF-1, and WNT5A genes in DENV-infected HFDPCs (**Figure S4**). This data suggests that DENV-mediated pathological hair shedding might be through modulation of the signaling activity in hair cycle.

Evidences of DENV infection in an immune-privileged tissue, such as hair follicles, would help to elucidate another clinical puzzle—the PDFS. Indeed, about one-quarter of dengue victims have prolonged chronic fatigue, major depression, and arthralgia for months after recovery, usually 3–6 months, or even up to 2 years (Seet et al., 2007; García et al., 2011; Shin et al., 2016). The mechanisms are still unknown, but autoimmune reaction (García et al., 2011) and continuous virus replication are the possible explanations. Our findings reinforce the possibility that PDFS is caused by the prolonged residence of virus in some immune-privileged tissues, such as hair follicles, where the immune system does not clear the virus; thus, there is still continuous viral shedding after the acute infection phase.

The hair follicle is a highly complex appendage of the skin containing multiple cell types. The follicle undergoes constant cycling throughout the life of the organism, including growth and resorption, with growth being dependent on different cell types. Although HFDPCs play a pivotal role in hair follicle morphogenesis and cycling, the effect of DENV infection in other cell types involved in hair growth should be further studied.

In conclusion, our study confirms that DENV can directly infect hair follicle-associated cells, causing molecular and cytopathic changes and leading to a disturbed hair cycle. Recognizing that DENV can infect hair follicles helps elucidate the pathophysiology of hair loss after DENV infection. Knowledge of DENV infection of immune-privileged tissues, such as hair follicles, may imply the need for further studies to identify their role in PDFS.

AUTHOR CONTRIBUTIONS

K-CW and T-HC conceived and designed the experiments, analyzed the data, contributed reagents, materials, analysis tools, and wrote the manuscript. M-SH performed the experiments and prepared the figures. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00268/full#supplementary-material

Table S1 | qPCR primer sequences used in this study.

Figure S1 | The expression of human Fc gamma receptors in HFDPCs.

Figure S2 | Induction of IL-1β, IL-6, and IL-8 expression and the DENV-2 5′-UTR by various MOI (0.1–10) of DENV-2.

Figure S3 | The full images of western blotting.

Figure S4 | Reduction of ALPL, LEF1, NOG, and WNT5A expression by DENV-1 and DENV-2.

Figure S5 | RT^2 profiler PCR Array analysis of DENV-triggered cell-death signaling pathway.

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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
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Human Kinase/Phosphatase-Wide RNAi Screening Identified Checkpoint Kinase 2 as a Cellular Factor Facilitating Japanese Encephalitis Virus Infection

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Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, causes acute encephalitis in humans with high mortality. Not much is known about the interactions between viral and cellular factors that regulate JEV infection. By using a kinase/phosphatase-wide RNAi screening approach, we identified a cell cycle-regulating molecule, checkpoint kinase 2 (CHK2), that plays a role in regulating JEV replication. JEV infection induced G1 arrest and activated CHK2. Inactivation of CHK2 and its upstream ataxia-telangiectasia mutated kinase in JEV-infected cells by using inhibitors reduced virus replication. Likewise, JEV replication was significantly decreased by knockdown of CHK2 expression with shRNA-producing lentiviral transduction. We identified CHK2 as a cellular factor participating in JEV replication, for a new strategy in addressing JEV infection.

Keywords: Japanese encephalitis virus (JEV), kinase/phosphatase-wide RNAi screening, checkpoint kinase 2 (CHK2), ataxia-telangiectasia mutated kinase (ATM), cell cycle G1 arrest

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INTRODUCTION

Japanese encephalitis virus (JEV) is the most important cause of viral encephalitis in Southeast Asia, with 30,000–50,000 cases reported annually (Hegde and Gore, 2017). JEV is transmitted in a zoonotic cycle between mosquitoes and vertebrate-amplifying hosts, mainly swine and wading birds (Mansfield et al., 2017). As a member of the *Flavivirus* genus, the JEV virion is enveloped and has a positive-sense single-stranded RNA genome. The initial steps of JEV infection include virus attachment to cell-surface receptors and entry via receptor-mediated endocytosis. Translation of the viral genome produces a polyprotein that is processed to structural core (C), precursor of membrane (prM), and envelope (E) proteins and the nonstructural proteins NS1∼NS5. Flaviviral genome replication occurs by the viral replicase complex via RNA-dependent RNA polymerization. The positive-sense genomic RNA is transcribed to a replication-intermediate negative-sense RNA, which is then used as a template to synthesize genomic RNA for subsequent translation and assembly of virion progeny (Tiroumourougane et al., 2002; Fields et al., 2007).

How a virus triggers DNA damage signaling is not fully understood, but previous reports have suggested that the cellular DNA repair machinery can recognize viral genetic materials, such as replicating nucleic acids and viral proteins, upon infection (Weitzman et al., 2004). Some viruses have been shown to interact with and/or affect components of the ATM DNA damage pathway

(Lilley et al., 2007; Bagga and Bouchard, 2014). DNA viruses, such as human cytomegalovirus (CMV) activate the ATM checkpoint pathway during DNA replication and inhibit DNA damage responses by mislocalizing checkpoint proteins from the nucleus to cytoplasm (Gaspar and Shenk, 2006). Herpes simplex virus (HSV) induces an ATM-damage response that is essential for viral replication (Lilley et al., 2005; Shirata et al., 2005). Inhibition of CHK2 kinase activity by the CHK2 inhibitor II significantly reduces the CPE and genome replication of HSV-1 in corneal epithelium (Alekseev et al., 2015). Hepatitis C virus (HCV), an RNA virus belonging to *Flaviviridae*, induces G2/M phase arrest (Wu et al., 2008) and activates the dsDNA damage response pathway by causing DSBs and enhancing the mutation of cellular genes (Machida et al., 2004). Knockdown of ATM or CHK2 suppresses RNA replication of HCV (Ariumi et al., 2008), and HCV NS3-NS4A interacts with ATM, whereas HCV NS5B interacts with both ATM and CHK2 (Lai et al., 2008). JEV is able to manipulate the cell cycle, which reduces the proliferation of neural progenitor cells (Das and Basu, 2008) and allows for persistent infection (Kim et al., 2015). JEV is also able to manipulate the cell cycle, which reduces the proliferation of neural progenitor cells (Das and Basu, 2008) and allows for persistent infection (Kim et al., 2015).

Only a few host proteins have been found involved in JEV infection, with the roles in JEV replication remaining largely unknown. In this study, we identified CHK2, which participates in cellular regulation of JEV infection, by using a kinase/phosphatase-wide siRNA screening strategy. Both CHK2 activation and G1 phase arrest were noted in JEV infected cells and the activation of CHK2 appeared to be beneficial for JEV replication.

MATERIALS AND METHODS

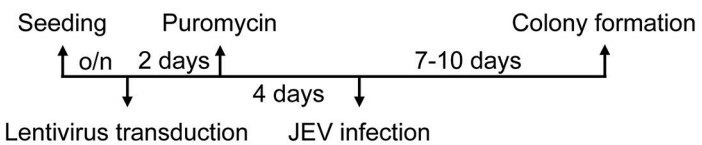
Cell Lines and Reagents

U87 cells (ATCC HTB-14), a human glioblastoma cell line, were cultured in minimal essential medium (Gibco) containing 10% fetal bovine serum (FBS), 1% sodium pyruvate, and 2 mM L-glutamine. A549 cells, a human lung carcinoma cell line, were cultured as previously described (Chang et al., 2006). BE(2)C (ATCC CRL68), a human neuroblastoma cell line were cultured in10% FBS and 2 mM L-glutamine containing RPMI 1640 (Gibco) medium. HEK293T, a human embryonic kidney cells, were cultured in 10% FBS and 2 mM L-glutamine containing Dulbecco's Modified Eagle's Medium (Gibco). Experiments were done using cells with passage 3–5. The ATM inhibitor (KU-55933) was from Calbiochem and the CHK2 inhibitor II was from Merck.

Viruses and Viral Infection

JEV strain RP-9 (Chen et al., 1996) was propagated in C6/36 cells. In Taiwan, JEV is classified as a BSL-2 pathogen according to the "Guidelines for research involving recombinant DNA molecules" issued by National Science Council, Taiwan (NSC., 2004). For viral infection, cells were adsorbed with viruses at the indicated multiplicity of infection (MOI) for 1 h at 37°C. Virus titers

A



B

	Gene symbol	Gene description	shRNA-encoding genes
1	CHEK2	checkpoint kinase 2	GAACAGATAAATACCGAACATCTCGAGAT GTTGGGTATTTATCTGTTC
2	HK1	hexokinase 1	CCTGGGAGATTTCATGGAGAACTCGAGTT CTCCATGAAATCTCCCAGG
3	PTPN4	protein tyrosne phosphatase, non-receptor type 4	GCTGTATATGATGTAGTGGAACTCGAGTT CCACTACATCATATACAGC
4	INPP1	inositol polyphosphate-1-phosphatase	CTTCTTAGCAAAGTCCTCAATCTCGAGAT TGAGGACTTTGCTAAGAAG
5	DCK	deoxycytidine kinase	CAGAGTGGACAATTTATCCTCGAGGATAA ATTGTCCACTCTGTC
6	PPP2R5A	protein phosphatase 2 regulatory subunit B' alpha	CACTGAATGAACTGGTTGAGTCTCGAGAC TCAACCAGTTCATTCAGTG
7	LILRB1	leukocyte immunoglobulin like receptor B1	GCTGTTACTATGGTAGCGACACTCGAGT GTCGCTACCATAGTAACAGC

FIGURE 1 | Establishing a human kinases/phophatases-wide RNAi screen system. **(A)** Overview of RNAi screening to genes involved in regulation of JEV infection. U87 cells transduced with lentiviruses expressing shRNAs targeting human kinases and phosphatase were selected with puromycin (10 μg/ml) for 4 days and infected JEV at an MOI of 10. **(B)** Cells survived from JEV infection were identified for candidate genes.

(PFU/ml) were determined by plaque-forming assays by using BHK-21 cells as described (Wu et al., 2002).

Human Kinase/Phosphatase-Wide RNAi Screening

U87 cells were transduced with VSV-G pseudotyped lentiviruses expressing shRNAs targeting 1,260 human kinases and phosphatases (obtained from the National RNAi Core Facility, Taiwan). After puromycin selection, cells were infected with JEV and surviving cell colonies were cultured. Genomic DNAs from surviving cells were extracted by using the QIAamp DNA Mini and Blood Mini Kit (Qiagen). The DNA regions containing shRNAs were amplified by PCR with primers (5′-TAATTTCTTGGGTAGTTTGCAGTT-3′ and 5′-CCCCAATCCCCCCTTTTC-3′) and cloned into pcDNA3.1/V5-His-TOPO (Invitrogen). The genes targeted

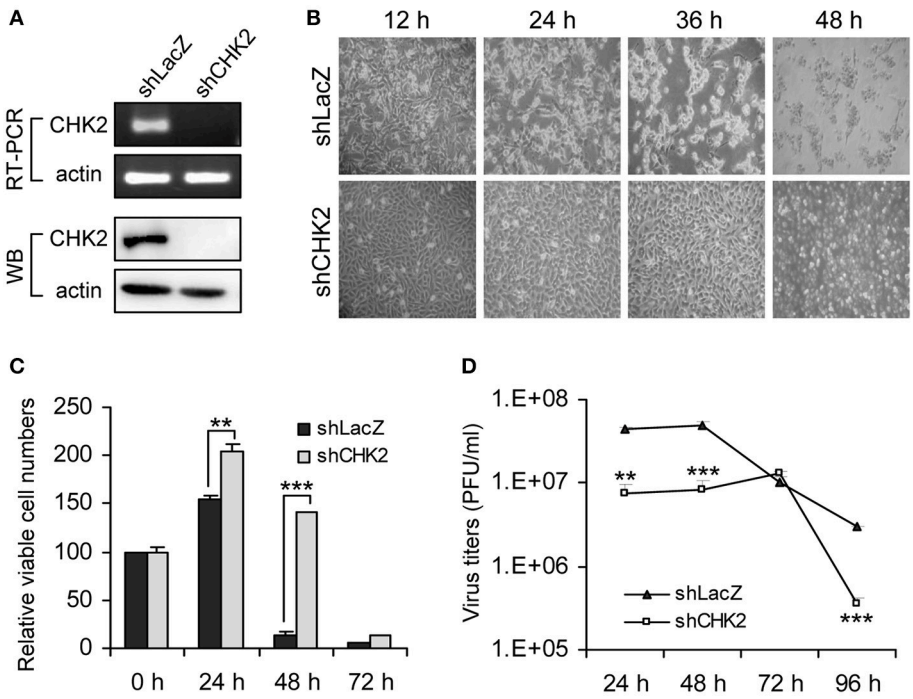


FIGURE 2 | Human kinase/phosphatase-wide RNAi screening identified CHK2 as a cellular factor involved in JEV infection. **(A)** Human U87 cells transduced with lentivirus expressing shRNA targeting CHK2 (shCHK2) or LacZ control (shLacZ) were verified for CHK2 knockdown by RT-PCR for mRNA level and immunoblotting analysis for protein expression. The cells were infected with JEV (MOI 5) for the indicated times. Cell morphology **(B)**, relative viable cell numbers ($n = 3$) were determined by trypan blue exclusion **(C)** and viral progeny production ($n = 3$) was determined by plaque-forming assays **(D)**. Data are mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.005$ by two-tailed Student *t*-tests.

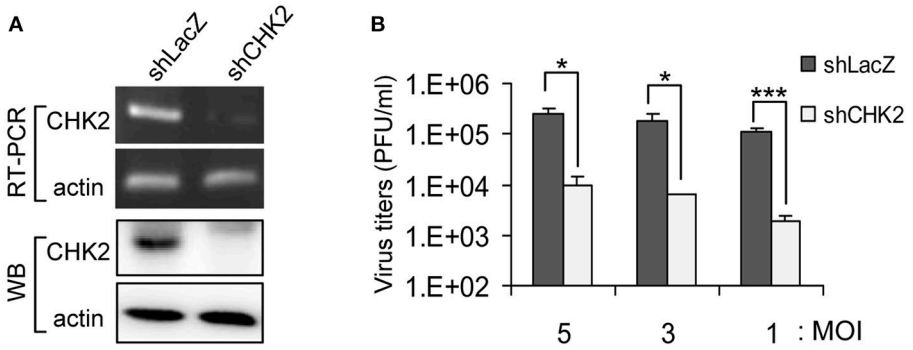


FIGURE 3 | Reduced CHK2 expression decreases JEV replication. Human A549 cells were transduced with shCHK2-expressing lentivirus and selected by puromycin. ShRNA-targeting LacZ was the non-targeting sequence control. **(A)** The RNA and protein levels of CHK2 were verified by RT-PCR with CHK2-specific primers and immunoblotting with anti-CHK2 antibody, respectively. Cells were infected with JEV at the indicated MOIs (5, 3, and 1); **(B)** at 24 h post infection, culture supernatants were collected for virus titration by plaque-forming assays. Data are mean ± SD virus titers ($n = 3$). $^{*}p < 0.05$; $^{***}p < 0.005$ by two-tailed Student *t*-test.

by shRNA were identified by sequencing and BLAST search.

Lentivirus Preparation

Lentiviral vectors (pLKO.1-puro) carrying the shRNAs targeting CHK2 (5′-GAACAGATAAATACCGAACAT-3′) and control LacZ (5′-TGTTCGCATTATCCGAACCAT-3′) were co-transfected into 293T cells with pMD.G and pCMVΔR8.91 (obtained from the National RNAi Core Facility, Taiwan) by using lipofectamine 2000 reagent. The culture supernatants were harvested and further concentrated by centrifugation at 20,000 × g for 3 h at 4°C.

Cell Survival

Cells were collected for survival determination by a trypan blue exclusion method. The virus-induced cytopathic effect (CPE) was analyzed by measuring the release of a cytoplasmic enzyme, lactate dehydrogenase (LDH), by using a commercial kit (Cytotoxicity Detection Kit; Roche).

Immunoblotting

Cells were lysed and subjected to western blot analysis as described (Chan et al., 2008). Primary antibodies used in this study included anti-CHK2 (sc5278, Santa Cruz Biotechnology; H00011200-M01, Abnova), anti-CHK2 (Thr68) (#2661, Cell Signaling Technology), and anti-β actin (Chemicon). The mouse monoclonal antibody specific to JEV NS3 was described previously (Wu et al., 2002).

RNA Preparation and RT-PCR Analysis

RNA was extracted by using an RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was reverse transcribed from 2 μg RNA with a random hexamer and a ThermoScript RT Kit (Invitrogen). PCR involved specific primer sets for CHK2 (5′-ATGTCTCGGGAGTCGGATGTT-3′ and 5′-ACTTTATTTCTGCTTAGTGACAGTGCA-3′) and β-actin (5′-TCCTGTGGCATCCACGAAACT-3′ and 5′-GAAGCATTTGCGGTGGACGAT-3′), respectively.

Cell Cycle Analysis by Flow Cytometry

The cell cycle status was analyzed by propidium iodide (PI) staining for nuclear DNA contents and by flow cytometry. At the indicated times after viral infection, cells were collected and fixed with 70% ethanol. Cells were stained with PI solution containing RNaseA at 4°C for 30 min, and then analyzed by flow cytometry (Attune NxT, Thermo Fisher).

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Two categorical data were compared by independent Student *t*-test. The statistical tests were two-tailed and significance was set at $P < 0.05$, < 0.01, and < 0.005. For immunoblotting, the band density was quantified by use of ImageJ (US National Institutes of Health).

RESULTS

Human Kinase/Phosphatase-Wide RNAi Screening Identified CHK2 as a Cellular Factor Involved in JEV Infection

We used a human kinase/phosphatase-wide RNAi screening strategy to search for potential kinases and phosphatases involved in JEV infection. U87, a human glioma cell line, was transduced by each of the seven VSV-G pseudotyped lentivirus pool (Human kinase and phosphatase set) provided by the National RNAi Core Facility. Each kinases/phosphatases pooled tube contains ∼180 kinase/phosphatase genes; each gene is targeted by 5 shRNAs that bind to distinct target sequences. The VSV-G pseudotyped lentivirus set that carries these shRNAs knocked down 1260 genes encoding kinase/phosphatases, which accounts for ∼90% of all kinase/phosphatase in accordance of the NCBI database. After selection with puromycin for lentivirus-transduced cells, cells were infected JEV at an MOI of 10 (**Figure 1A**). Surviving cell colonies were cultured to extract genomic DNA. DNAs encoding shRNA were amplified by PCR and sequenced to determine their targets by BLAST alignment with the NCBI database to further confirm the identities of these genes as kinase/phosphatase encoding genes. Seven host candidate genes (**Figure 1B**), were identified in cells survived from JEV challenge.

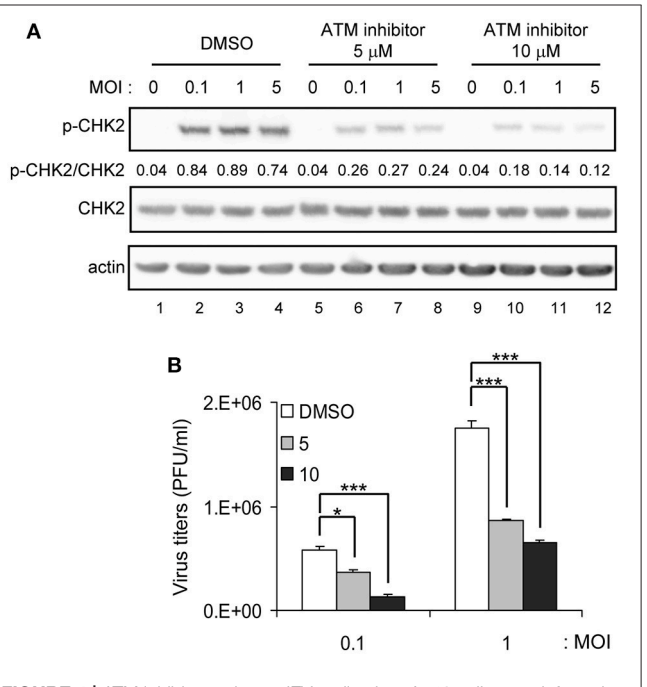


FIGURE 4 | ATM inhibitor reduces JEV replication. A549 cells were infected with JEV at MOI 0.1, 1 or 5 in the absence (DMSO solvent control) or presence of 5 or 10 μM ATM inhibitor (KU-55933) as indicated. **(A)** Immunoblotting analysis of cell lysates at 24 h with antibodies against T68-phosphorylated CHK2, CHK2 and actin. **(B)** A549 cells were treated with ATM inhibitor (KU-55933) at the indicated dose, followed by JEV infection at MOI 0.1 or 1 for 24 h. Culture supernatants were collected for virus titration by plaque-forming assays. Data are mean ± SD virus titers ($n = 3$). *$p < 0.05$; ***$p < 0.005$ by two-tailed Student *t*-test.

To verify whether knockdown of these candidate genes indeed rescued cells from JEV infection, we transduced U87 cells with the lentiviral vector targeting each candidate gene and infected the cells with JEV. Knockdown of one of these candidate genes, CHEK2, substantially rendered cell survival from JEV infection. U87 cells showed reduced expression of CHK2 by transduction with lentivirus expressing an shRNA targeting CHK2 (**Figure 2A**). Upon JEV infection, knockdown of CHK2 resulted in reduced CPE (**Figure 2B**), enhanced cell survival (**Figure 2C**) and reduced JEV progeny production (**Figure 2D**) as compared with control knockdown shLacZ cells. To ascertain the importance of CHK2 in JEV infection, we further checked the involvement of CHK2 in another human cell line, A549 cells. Similarly, JEV production was reduced in human A549 cells with knocked-down CHK2 expression (**Figure 3**).

JEV-Activated CHK2 Is Beneficial for JEV Replication

CHK2 is a protein kinase activated by ATM in response to DNA damage (Bakkenist and Kastan, 2004). To understand how CHK2 participates in JEV replication, we determined whether the ATM/CHK2 pathway was activated by JEV infection. CHK2 activation, as manifested by phosphorylation at Thr-68, was evident in JEV-infected cells in an MOI dependent manner, and an ATM inhibitor KU-55933 (Lau et al., 2005) reduced the CHK2 activation (**Figure 4A**). Furthermore, the ATM inhibitor significantly reduced JEV production (**Figure 4B**).

The involvement of CHK2 in JEV replication was further assessed by using a cell-permeable CHK2 inhibitor, CHK2 inhibitor II (Arienti et al., 2005). U87 cells were infected with JEV (MOI 0.1 and 5) in the absence or presence of non-cytotoxic CHK2 inhibitor II (25 and 50 μM). Immunoblotting analysis with anti-CHK2 (Thr68) antibody showed that CHK2 inhibitor II effectively reduced the JEV induced phosphorylation of CHK2 protein (**Figure 5A**). CHK2 inhibitor II also reduced virus production. With low MOI, 25 μM CHK2 inhibitor II readily decreased JEV production by ∼100-fold (**Figure 5B**). With high MOI, 25 μM CHK2 inhibitor II slightly suppressed JEV production, whereas 50 μM conferred 94% reduction in virus production. With the treatment of 50 μM CHK2 inhibitor II, virus progeny was inhibited in both high MOI (**Figure 5C**) and low MOI (**Figure 5D**) at various time points. The antiviral effect of the CHK2 inhibitor II was further verified in A549 cells and BE(2)C cells. CHK 2 inhibitor II reduced both the viral protein expression and CHK2 phosphorylation level (**Figure 6A**) Viral progeny production (**Figure 6B)** was suppressed by CHK2 inhibitor II at different time points (**Figures 6C, D**). Similar to U87, the CHK2 phosphorylation of BE(2)C neuroblastoma cells was induced by JEV and reduced by CHK2 inhibitor II **(Figure 7A)**. The virus progeny in both high MOI **(Figure 7B)** and low MOI **(Figure 7C)** was inhibited by 50 μM CHK2 inhibitor II in various time points. Collectively, blocking the

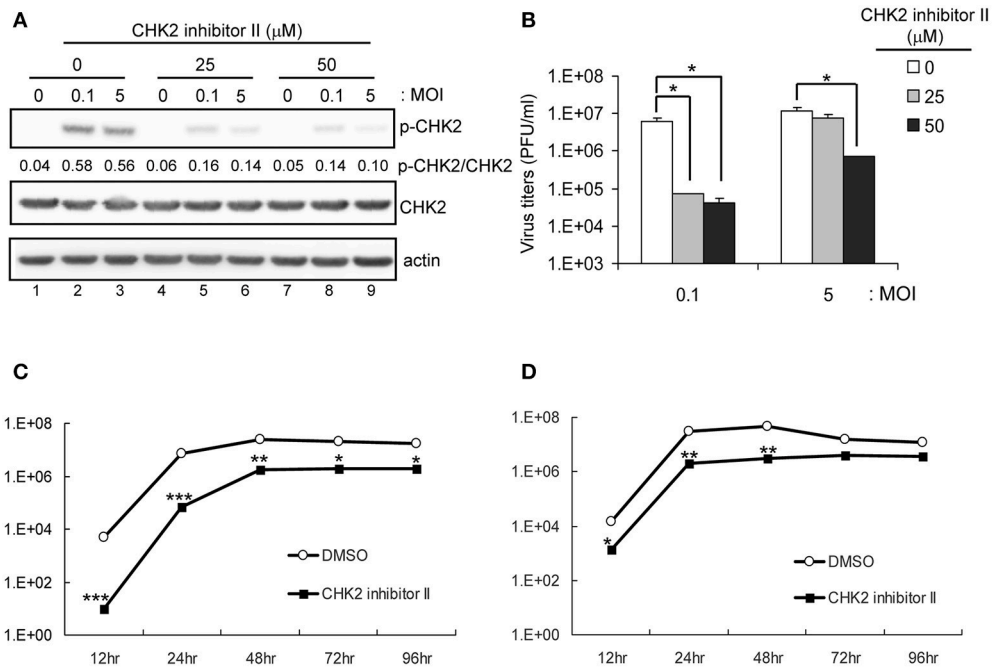


FIGURE 5 | Inhibition of CHK2 activity reduces JEV replication in human U87 glioma cells. U87 cells were infected with JEV (MOI 0, 0.1 and 5) with or without of CHK2 inhibitor II (0, 25 and 50 μM). **(A)** At 24 h post treatment, immunoblotting analysis of cell lysates at 24 h with antibodies against, T68-phosphorylated CHK2, CHK2, and actin was performed; virus titration **(B)** in culture supernatant was determined by plaque-forming assays. Cells were treated with DMSO or CHK2 inhibitor II (50 μM), followed by JEV infection at MOIs of 0.1 **(C)** or 5 **(D)** for the indicated times. Culture supernatants were collected for virus titration by plaque-forming assays. Data are mean ± SD virus titers ($n = 3$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ by two-tailed Student t-test.

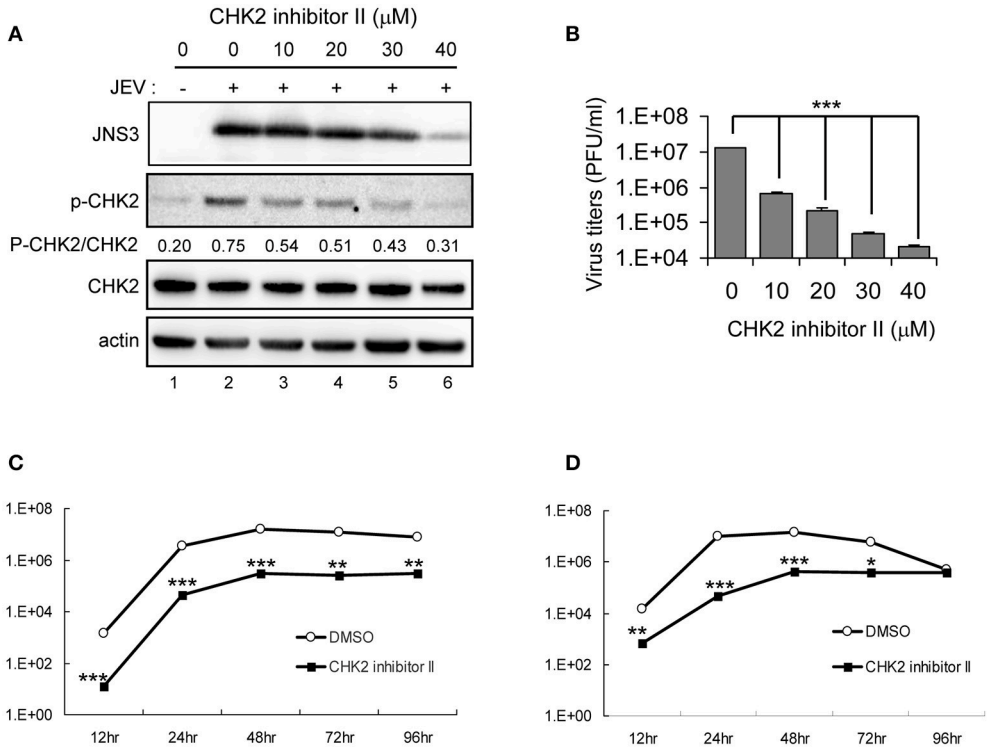


FIGURE 6 | Inhibition of CHK2 activity reduces JEV replication in human A549 lung carcinoma cells. A549 cells were mock-infected or infected with JEV (MOI 5) with various doses of CHK2 inhibitor II. **(A)** At 24 h post treatment, immunoblotting analysis of cell lysates at 24 h with antibodies against JEV NS3, T68-phosphorylated CHK2, CHK2, and actin and was performed; **(B)** virus titers determination in culture supernatants were determined by plaque-forming assays. Cells were treated with DMSO or CHK2 inhibitor II (50 μM), followed by JEV infection at MOIs of 0.1 **(C)** or 5 **(D)** for the indicated times. Culture supernatants were collected for virus titration by plaque-forming assays. Data are mean ± SD virus titers ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$ by two-tailed Student *t*-test.

ATM/CHK2 pathway by an ATM inhibitor or a CHK2 inhibitor reduced JEV replication in human glioblastoma, lung cancer carcinoma, and neuroblastoma cell lines tested.

JEV Infection Resulted in G1 Arrest in U87, A549, BE(2)C Cells

Given that the ATM/CHK2 pathway is activated upon DNA damage in order to modulate cell cycle progression, we assessed whether JEV infection results in cell cycle arrest. We analyzed the cell cycle by measuring DNA content with PI staining for mock- and JEV-infected cells at 24 h post-infection. JEV infection increased the proportion of cells in the G1 phase from 55.46% to 78.87% in U87 cells (**Figure 8A**). Similar G1 phase arrest was also noted in A549 cells (**Figure 8B**) and BE(2)C cells (**Figure 8C**). Therefore, JEV infection induced cell cycle arrest in the G1 phase.

DISCUSSION

With the recent development of screening strategies, RNAi-based technology has been extensively used to identify genes involved in cellular processes, such as signal transduction, cell cycle, cancer biology and host–pathogen interactions (Houzet and Jeang, 2011). Viruses are obligate intracellular parasites that may utilize host cell machineries during every step of their life cycles (Goff, 2007). In this study, we used a human kinase/phosphatase-wide RNAi strategy for large scale screening to identify a gene(s) involved in regulating JEV infection in U87 human glioma cells. Among the 1260 human kinases/phosphatases found, we identified a cell cycle-regulating molecule, CHK2, that facilitated JEV infection in the cells.

For infectivity, viruses have been found to interact with CHK2 and its related responses in host cells. Human papillomaviruses activate the ATM DNA response (Lai et al., 2008; Moody and Laimins, 2009). HSV-1 and SV40 recruit members of the ATM DNA damage pathway to specific sites in the nucleus during replication (Taylor and Knipe, 2004; Wilkinson and Weller, 2004; Zhao et al., 2008). Furthermore, the NS5B of HCV was found to interact with both ATM and CHK2, and the replication of subgenomic replicon RNA of HCV was suppressed in cells with CHK2 knockdown (Ariumi et al., 2008). In this study, we also found that downregulation of CHK2 and inhibition of CHK activity enhanced cell resistance to JEV infection. Likewise, progeny virus production and expression of viral proteins were reduced in cells with knockdown of functional CHK2. Because viral replication induces cellular stresses and triggers an ATR response, viruses may hijack CHK2 to elongate the cell cycle arrest, thus creating a time window for viral replication. Consequently, downregulation of CHK2 becomes a suitable way to inhibit virus propagation.

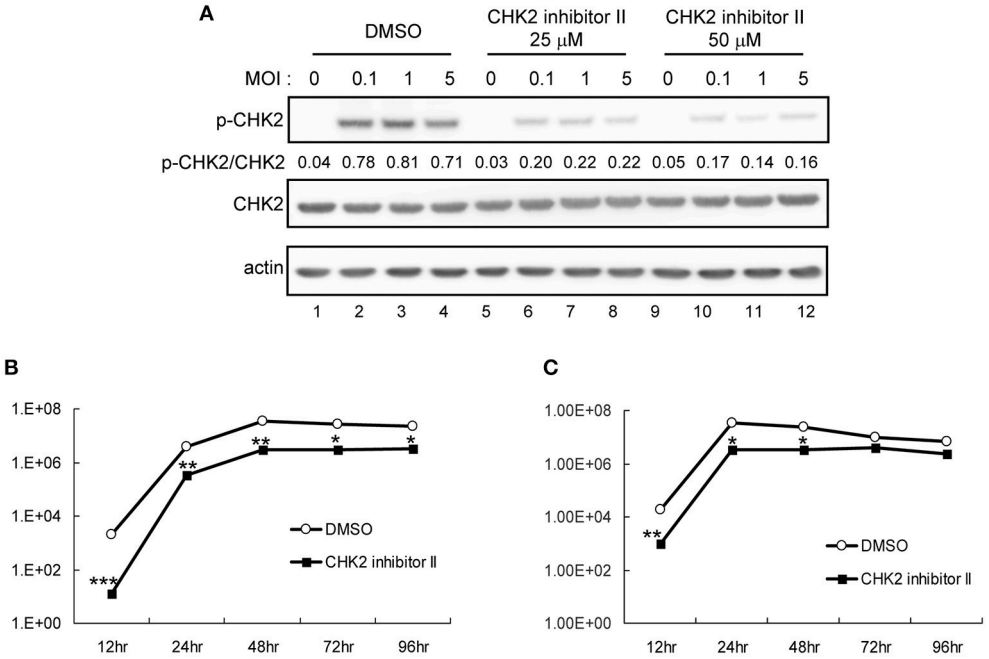


FIGURE 7 | Inhibition of CHK2 activity reduces JEV replication in human BE(2)C neuroblastoma cells. BE(2)C cells were infected with JEV at MOI 0.1, 1 or 5 in the absence (DMSO solvent control) or presence of 25 or 50 μM CHK2 inhibitor II as indicated. **(A)** Immunoblotting analysis of cell lysates at 24 h with antibodies against T68-phosphorylated CHK2, CHK2 and actin. BE(2)C cells were treated with DMSO or CHK2 inhibitor II (50 μM), followed by JEV infection at MOIs of 0.1 **(B)** or 5 **(C)** for the indicated times. Culture supernatants were collected for virus titration by plaque-forming assays. Data are mean ± SD virus titers ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$ $^{***}p < 0.005$ by two-tailed Student *t*-test.

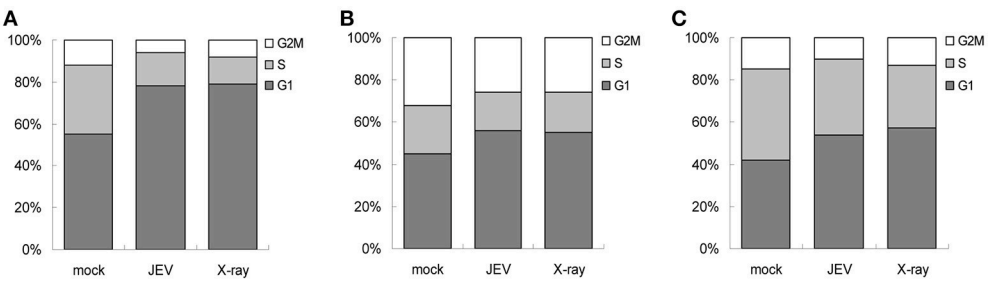


FIGURE 8 | JEV infection induces cell cycle arrest at G1 phase. U87 **(A),** A549 **(B)**, and BE(2)C **(C)** cells were mock-infected, JEV (MOI 5) infected, or X-ray (8 Gy) treated. At 24 h post-treatment, DNA content was determined by PI staining and analyzed by flow cytometry for proportion of cells in G1, S, and G2 phases.

Viruses are streamlined organisms featured in minimizing proteins required for genome replication and control of the host cell cycle. In analogy to some RNA viruses such as influenza A virus (He et al., 2010), mouse hepatitis virus (Chen and Makino, 2004), and severe acute respiratory syndrome coronavirus (Yuan et al., 2007), JEV infection is able to regulate cell-cycle arrest in the G1 phase, probably resulting from CHK2 activation (Lavin and Kozlov, 2007). Upon activation, CHK2 phosphorylates Cdc25a and Cdc25c (phosphatases), which results in G1/S or G2/M arrest via degradation or cytoplasmic sequestration, respectively (Antoni et al., 2007). Each phase of the cell cycle should represent a unique metabolic status. Indeed, the transcription activity of Pol II is much higher in the G1 phase than in the S or G2/M phase (Yonaha et al., 1995). As well, in non-dividing hepatocytes, the G0/G1 state has higher translation efficiency, which facilitates expression of the HCV genome (Fehr et al., 2012). Likewise, to proliferate the viral genome, JEV may arrest the cell cycle in the G1 phase to increase Pol II transcription activity and translation efficiency. Moreover, oncogenic viruses often possess mechanisms to induce G1/S cell cycle arrest via p53 inactivation, thus rescuing the infected cells from apoptosis (Yew and Berk, 1992; Scheffner et al., 1993; Härtl et al., 2008). JEV inducing G1 arrest may be advantageous to gain sufficient time and resources for viral replication and to avoid early apoptosis of infected cells.

Although the exact mechanism of how JEV induces the DNA damage response is not fully understood, our studies demonstrate that JEV executes its own replication by manipulating the host cell cycle via CHK2. The kinase/phosphatase-wide RNAi screening system can be an effective strategy to search for cellular factors involved in the regulation of JEV infection.

AUTHOR CONTRIBUTIONS

Y-LC and Y-LL designed research, analyzed data and wrote the paper. Y-LC performed research. C-LL provided critical reagents.

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Increased Relative Risk of Tick-Borne Encephalitis in Warmer Weather

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Tick-borne encephalitis (TBE) is a serious acute neuroinfection of humans caused by a tick-borne flavivirus. The disease is typically seasonal, linked to the host-seeking activity of *Ixodes ricinus* (predominantly nymphs), the principal European tick vector species. To address the need for accurate risk predictions of contracting TBE, data on 4,044 TBE cases reported in the Czech Republic during 2001–2006 were compared with questing activity of *I. ricinus* nymphs monitored weekly at a defined location for the same 6-year period. A time shift of 21 days between infected tick bite and recorded disease onset provided the optimal model for comparing the number of cases of TBE with numbers of questing nymphs. Mean annual distribution of TBE cases and tick counts showed a similar bimodal distribution. Significantly, the ratio of TBE cases to questing nymphs was highest in the summer-autumn period even though the number of questing nymphs peaked in the spring-summer period. However, this pattern changed during a period of extreme meteorological events of flooding and abnormally high temperatures, indicating that changes in climate affect the incidence of TBE. Previous studies failed to link human behavior with changes in incidence of TBE but showed extrinsic temperature impacts arbovirus replication. Hence, we hypothesize the apparent discrepancy between peak nymphal tick activity and greatest risk of contracting TBE is due to the effect of temperature on virus replication in the tick vector. Relative proportions of questing nymphs and the numbers of weeks in which they were found were greater in summer-autumn compared with spring-summer at near-ground temperatures >5°C and at standard day and weekly average temperatures of >15°C. Thus, during the summer-autumn period, the virus dose in infected tick bites is likely greater owing to increased virus replication at higher microclimatic temperatures, consequently increasing the relative risk of contracting TBE per summer-autumn tick bite. The data support the use of weather-based forecasts of tick attack risk (based on daytime ambient temperature) supplemented with weekly average temperature (as a proxy for virus replication) to provide much-needed real-time forecasts of TBE risk.

Keywords: tick-borne encephalitis, TBEV, *Ixodes ricinus*, arbovirus, seasonality, climate change

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INTRODUCTION

Tick-borne encephalitis (TBE) is a serious acute neuroinfection of humans caused by a member of the virus family, Flaviviridae. The principal vector of the European subtype of TBE virus (TBEV) is the most epidemiologically important European tick species, *Ixodes ricinus*. In 1948, across several areas of the Czech Republic, 56 cases of a viral neuroinfection were reported linked to tick-bite (Růžek, 2015); TBEV was isolated from the patients and from ticks collected in Central Bohemia and Moravia (Gallia et al., 1949; Krejčí, 1949; Rampas and Gallia, 1949). In subsequent years, TBE was recognized in other European countries and the virus isolated: Hungary (Fornosi and Molnár, 1952), Poland (Przesmycki et al., 1954), Bulgaria (Vaptsarov et al., 1954), Slovenia (Bedjanič et al., 1955), Austria (Pattyn and Wyler, 1955), Romania (Draganescu, 1959), Finland (Oker-Blom, 1956), Germany (Sinnecker, 1960), and Sweden (Kaäriainen et al., 1961). Presently in Europe, TBE is most prevalent in Southern Germany, Switzerland, Austria, the Czech Republic, Slovakia, Hungary, Slovenia, the Baltic countries, Poland, parts of Scandinavia, and Russia (Heinz et al., 2013). Longitudinal surveillance in Austria shows TBE emergence in previously unaffected regions consistent with similar findings in Norway, Sweden, and Denmark (Skarpaas et al., 2004; Johan et al., 2006; Fomsgaard et al., 2009; Heinz et al., 2013). During the period of our study (2001–2006), TBE mortality rate in the Czech Republic was 0.2% (EPIDAT, see below). Patients required hospitalization for 3–4 weeks followed by 6–8 weeks or more rehabilitation (Duniewiecz, 1999).

Since the first virus isolation, particular attention is paid to TBEV and its vector in the Czech Republic. As early as 1954, in the first European monograph on TBE, seasonal bimodal curves of weekly registered TBE cases and tick occurrence were recorded and the influence of meteorological factors was recognized (Raška and Bárdoš, 1954). Owing to the relatively high number of cases, TBE became a notifiable disease in the Czech Republic in 1970. Since 1971, records of laboratory confirmed cases are collated in an extensive database (EPIDAT, National Institute of Public Health, Prague), which includes clinical data on TBE cases as well as probable date and place of infection, and time of onset of symptoms. This unique database, comprising 21,847 TBE cases reported in 1970–2016, enables the evaluation of temporal changes in the epidemiology of TBE over several decades coinciding with major changes in climatic variables, and including regional analysis (Daniel et al., 2011; Kříž et al., 2012). Since the 1990s, increasing incidences of TBE have been reported in several Central and Western European countries. In 2012, TBE became an obligatory notifiable disease in all countries of the European Union (2012/506/EU: Commission Implementing Decision of 8 August 2012), with a unified case definition (Amato-Gauci and Zeller, 2012). As a consequence of the increasing incidence, a detailed study of the seasonal dynamics of *I. ricinus* tick activity was carried out in 2001–2006 within the framework of the WHO/EC project, Climate Change and Adaptation Strategies for Human Health (Menne and Ebi, 2006). Host-seeking activity of nymphs (epidemiologically the most important stage), and concurrent near-ground temperature and relative air humidity, were recorded 1 day each consecutive week for 6 years, and the data compared with standard weather data collected at a nearby meteorological station (Daniel et al., 2015). Temperature was shown to be the strongest predictor of the seasonality of tick host-seeking activity. These results are the basis of a computer model for predicting the risk of tick attack in the Czech Republic. Predictions are actualized according to routine weather forecasts modified by the 3–4 day synoptic situation, and publicized by the Czech Hydrometeorological Institute (CHMI) with risk categorized at 10 different levels (Daniel et al., 2010).

Given that only low numbers of ticks are infected with TBEV (see section Discussion), comparatively few people bitten by ticks develop clinical neurological disease (Duniewiecz, 1999). This also applies to people bitten by infected ticks. For example, in a highly active natural focus of TBEV transmission, the ratio of symptomatic to inapparent TBE cases was approximately 2:3, representing 6.1 and 9.6%, respectively of permanent residents (Luňáčková et al., 2003). Hence current predictions are of tick attack risk rather than of the risk of TBE clinical infections. Here we examine the relationship between abundance of questing nymphal ticks and incidence of TBE in order to test if weather-based forecasts of tick attack risk can be used to predict risk of contracting TBE.

MATERIALS AND METHODS

Incidence of TBEV Clinical Infections in Humans

Since 1970, laboratory-confirmed cases of TBE in the Czech Republic have been recorded in the EPIDAT database (http://www.szu.cz/publikace/data/infekce-v-cr), a national reporting system maintained by the National Public Health Institute (NIPH) in Prague, Czech Republic. Each case of TBE is reported by the diagnosing physician to the public health authorities, which then undertake epidemiological investigations. Through an interview with the patient, records are made of medical history, probable time and place of infection, and possible transmission route. These data are then collated on a weekly basis by NIPH. For our study, 4,044 cases of TBE registered in the Czech Republic during 2001–2006 were classified according to the date of the first recorded clinical symptoms.

Monitoring *Ixodes ricinus* Tick Activity

The tick collecting site and monitoring design to determine the seasonal variation in *I. ricinus* questing activity have been described previously (Daniel et al., 2015). Briefly, *I. ricinus* host-questing activities were investigated in 2001–2006 by the standard flagging method in a defined site in the southern outskirts of Prague, 4 km from the main observatory station of the CHMI. Ticks were counted at weekly intervals from March to November. Altogether 208 monitoring visits were conducted. Ticks were not removed from the plots but were immediately released back to the place of collection. Detailed numbers of ticks counted and concurrent records of microclimate temperature are published (Daniel et al., 2015). The 6-year study period allowed time for the development of three tick generations (the life cycle

of *I. ricinus* typically takes 2 years to complete in Central Europe) (Daniel and Dusbábek, 1994). Only data for nymphs were used as this stage poses the greatest risk to humans (Lindblom et al., 2013).

Meteorological Data

Three sets of temperature data were used for comparisons of meteorological conditions with *I. ricinus* host-questing activity:

1. Near-ground temperature measured using a mercury thermometer placed at 1 cm above the ground, in shadow and away from the sun (Daniel et al., 2015).
2. Standard day temperature recorded by the nearby CHMI observatory on the same day and at the same time as the near-ground temperature was recorded in the tick monitoring site.
3. Weekly average temperature for each week of the 2001–2006 monitoring period obtained from the CHMI database.

Near-ground temperature represents the real-time ecoclimatic temperature that ticks experience in the monitored site. Such measurements are not routinely available and therefore temperature measurements recorded daily by standard meteorological methods are used for tick questing activity forecasts. However, weekly average temperature better characterizes the temperature influence on virus replication in infected ticks (Danielová, 1975).

Statistical Analysis

To compare meteorological conditions and tick counts with the estimated times of human infection (i.e., infected tick bite), the predictor variables were lagged by performing a time shift (1–6 weeks) to take into account the incubation period between virus infection and clinical symptoms. The best model was selected using two different approaches, Akaike's and Bayesian information criteria, which measure relative quality of statistical models for given data. They take into account both the overall fit of the model (statistical goodness of fit measured via log-likelihood) and the number of parameters that have to be estimated to achieve the particular degree of fit, by imposing a penalty for increasing the number of parameters.

For statistical testing, square root transformation was applied to the data for tick activity and TBE cases to control for heteroskedasticity and obtain a near-normal distribution. Statistical analysis was based on the linear regression model to test the linear relationship between square root transformed counts of ticks/TBE cases. The model for each respective year is expressed as:

$$y_i \sim \beta_0 + \beta_1 x_i + \beta_2 P_i + \beta_3 x_i P_i, \qquad i = 1, 2, \ldots, n \qquad (1)$$

where

y_i is the square root of the number of TBE cases

x_i is the square root of the number of active nymphs

$P_i = \begin{cases} 0 \text{ if an observation belongs to spring} - \text{summer period} \\ 1 \text{ if an observation belongs to summer} - \text{autumn period} \end{cases}$

$\beta_0, \beta_1, \beta_2, \beta_3$ are regression coefficients

n is the number of analyzed weeks in the year.

Difference of the slopes for spring-summer (β_1) and summer-autumn ($\beta_1 + \beta_3$) period was tested via the regression coefficient β_3 for the interaction term between the period indicator P_i and nymphal tick counts. Chow's test was used to examine whether both parameters of one regression line are equal to those of another regression line. Temperature and the respective numbers of ticks were grouped into categories and the cumulative frequencies were calculated. The statistical significance level was set to 5%. Data were processed using R software, version 3.1.2 (R Core Team, 2014).

All data relevant to the conclusions of this manuscript are available upon request to the authors.

RESULTS

Seasonal Incidence of TBE

During 2001–2006, 4,044 cases of TBE were reported in the Czech Republic. Of these, 21 cases (0.52%) were attributed to alimentary infections from drinking/eating contaminated milk products (Kříž et al., 2009). The rest were assumed to have resulted from the bite of an infected *I. ricinus* tick. Determination of the seasonal incidence of infection was based on the distribution of cases by calendar week of the onset of symptoms with an accuracy of 1 week (**Figure 1**). Generally, the seasonal incidence of cases is characterized by a bimodal curve, as seen across much of Europe (European Centre for Disease Prevention and Control, 2016). In 2001, the late summer peak was almost as high as the spring peak while in 2006, the late summer peak was higher than the spring peak.

Comparison of the Seasonal Incidence of TBE and the Abundance of Host-Questing *I. ricinus* Nymphs

Mean annual distribution of TBE cases and tick counts show a similar bimodal profile (**Figure 2**). However, whereas the number of questing nymphs reached a maximum in the spring-summer period (∼week 21) and then declined but with a minor second peak in autumn (∼week 40), the peak incidence of TBE was at the beginning of the summer-autumn period (∼week 29), and showed a slower rate of decline compared to nymph activity at the end of the summer period and a relatively higher summer-autumn peak (∼week 40).

To investigate the apparent seasonal discrepancies, the linear regression relationship between the mean annual numbers of TBE cases and tick abundance was compared between the spring-summer and summer-autumn periods. Time shifts of 1–6 weeks were tested to account for the delay between time of infection and onset of TBE symptoms (**Figure 3**). There was a high degree of correlation in weekly count of TBE cases and weekly count of questing nymphs; the highest mean correlation was obtained with a 3-week shift ($r = 0.95$). However, even allowing for a 3-week delay between the time of an infected tick bite and the onset of clinically apparent disease, discrepancies were apparent in the seasonal dynamics of nymphal tick abundance and TBE incidence (**Figure 2**).

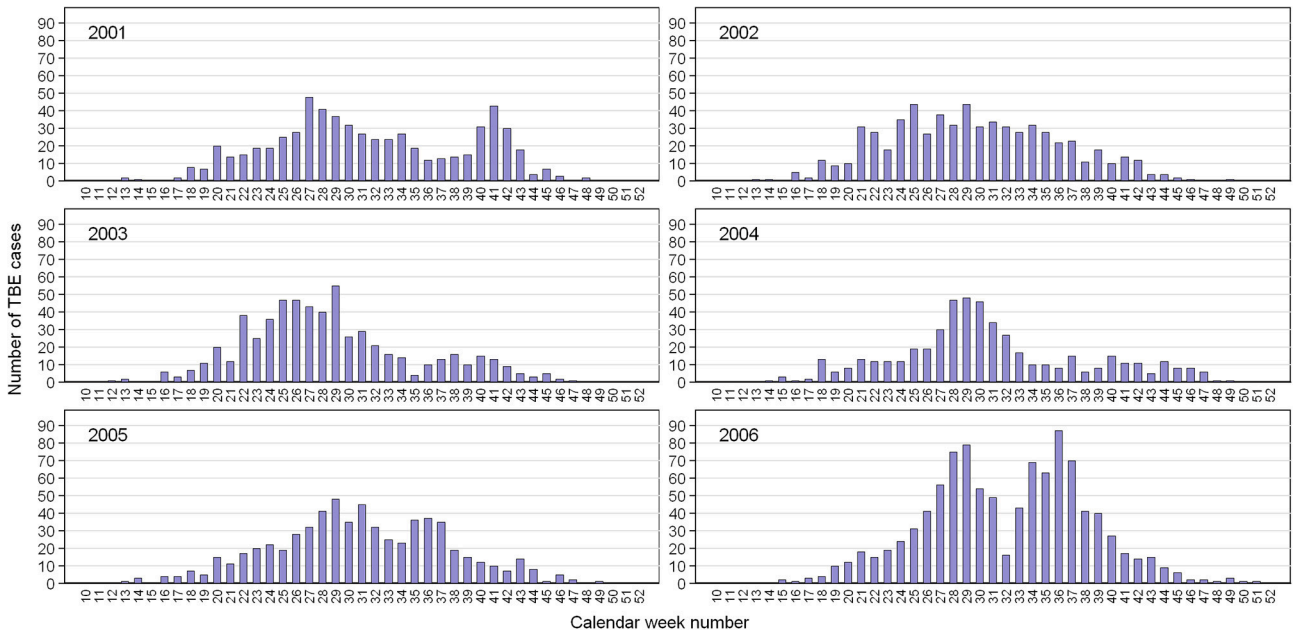

FIGURE 1 | Cases of TBE in the Czech Republic by calendar week of onset of clinical symptoms. Data from the Czech EPIDAT database for 2001–2006.

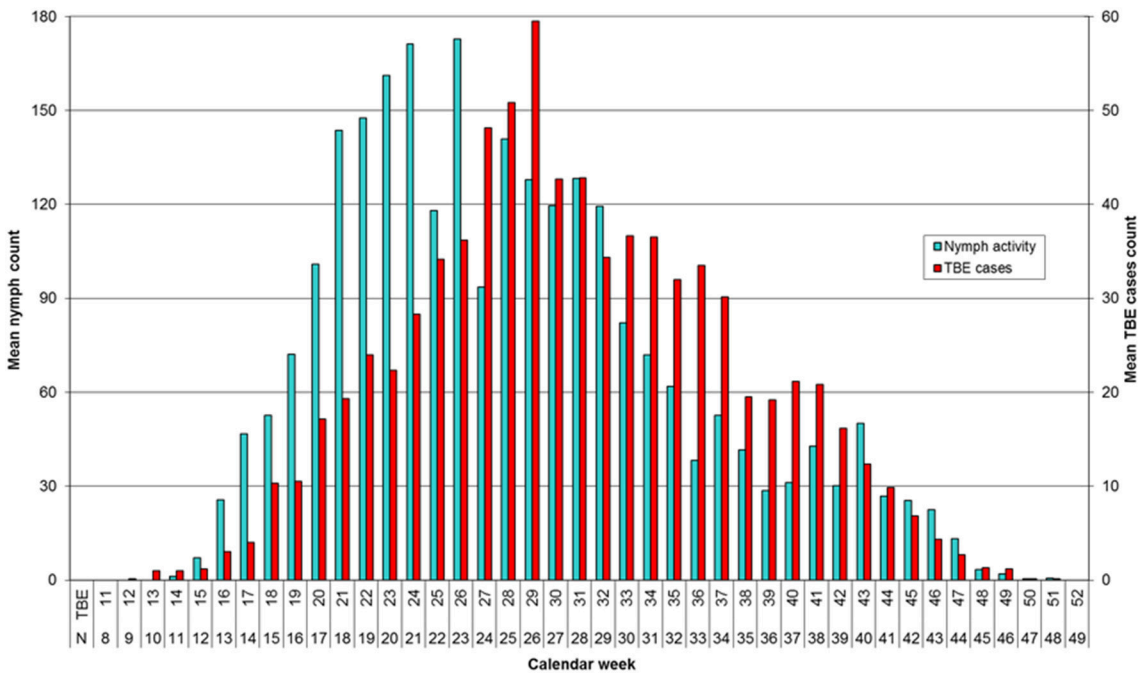

FIGURE 2 | Seasonal activity of *Ixodes ricinus* nymphs by week of collection and incidence of TBE by week of onset. Data for 2001–2006 are compared using a 3-week shift in alignment. Spring-summer and summer-autumn periods are distinguished by the sharp drop in number of questing nymphs around week 22 and fall in near ground temperature to 10–12°C (Daniel et al., 2015).

Comparison of annual regression lines and their slopes showed that summer-autumn was consistently steeper than spring-summer (**Figure 4**, Supplementary Table 1). Over the 6-year time span, the difference in the slope of the regression lines between the two seasonal periods was highly significant ($p < 0.001$) indicating that the incidence rate of TBE grew more rapidly in the summer-autumn period than in the spring-summer period in response to the unit increase in tick questing

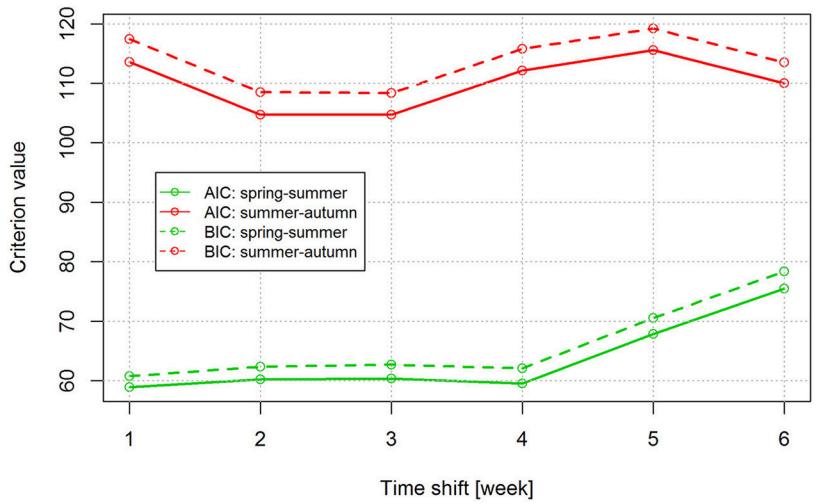

FIGURE 3 | Analysis of the effect of a 1–6 week time shift on the estimated time of infection using Akaike's (AIC) and Bayesian (BIC) information criteria. The two criteria were used to select the best model in the group of models (1–6 week shift) compared. Lower criterion values indicate the preferred model.

rate. The greatest difference was observed for 2006 ($p = 0.005$) (Supplementary Table 1), reflecting the remarkably high relative incidence of TBE in the summer-autumn period (**Figure 1**). Even when 2006 was removed from the model, the difference between regression slopes for spring-summer and summer-autumn remained significant ($p = 0.012$). Seasonal regression lines within 2002 and 2003 were similar ($p = 0.19$ and $p = 0.31$, respectively); however, in 2003 (the year following extensive flooding), they were reversed indicating there were relatively fewer cases of TBE per given number of questing ticks during the summer-autumn period compared with the spring-summer period. This anomaly was probably a consequence of floods and unusual meteorological conditions (see section Discussion).

Year-on-year variability in the relationship between *I. ricinus* abundance and TBE incidence was greater in spring-summer period compared with summer-autumn (**Table 1**). This inter-seasonal difference reflects greater variability in meteorological conditions during spring. All regression lines in the summer-autumn period were similar except 2006 (**Figure 5**).

Host-Questing Activity of *I. ricinus* Nymphs and Temperature: Comparison of Spring-Summer and Summer-Autumn Periods

Our previous study showed air temperature to be the best predictor of nymph questing activity compared with relative humidity or day length (Daniel et al., 2015), as was found in a study of the interaction between TBE incidence, ambient temperature and precipitation rate (Kříž et al., 2015). Hence the variation in relationship between the numbers of questing *I. ricinus* nymphs and the number of TBE cases for the spring-summer and summer-autumn periods was examined in relation to ambient air temperature to which host-seeking ticks were exposed. Questing activity was examined in ranges of 5°C, comparing near-ground temperature in the monitoring site with standard day and weekly average temperatures recorded at the nearby CHMI observatory. Standard day and weekly average temperatures were generally 4–5°C higher than near-ground temperatures with a maximum difference of 10°C (in 2006; **Figure 6**). During the total 66 weeks of the spring-summer period for the 6-year period examined, 6,903 questing nymphs were recorded, whereas 7,955 questing nymphs were counted during the total 102 weeks of the summer-autumn period (Supplementary Table 2).

Based on near-ground temperatures, the proportions of questing nymphs recorded in the two lowest temperature ranges (≤0°C and 0.1–5°C) differed substantially between the two seasonal periods (**Figure 7**). These low temperature ranges were comparatively rare in the summer-autumn period (Supplementary Table 2). In the categories 5.1–10 and 10.1–15°C, similar proportions of nymphs were questing in the two seasonal periods although there were more records for the summer-autumn period. However, there was a marked difference in the proportions and numbers of nymphs questing in the higher temperature ranges (15.1–20°C and >20°C) during summer-autumn compared with spring summer although, not surprisingly, there were more records in these categories (Supplementary Table 2). This difference was more striking when comparisons were made using standard day and weekly average temperatures (**Figure 7**). The relative proportions of questing nymphs and the numbers of weeks in which they were found were greater in summer-autumn compared with spring summer at near-ground temperatures >5°C, and at standard day and weekly average temperatures of >15°C (**Figure 8**).

DISCUSSION

Although most cases of TBE arise from an infected tick bite, the ratio of symptomatic human TBE infections to questing

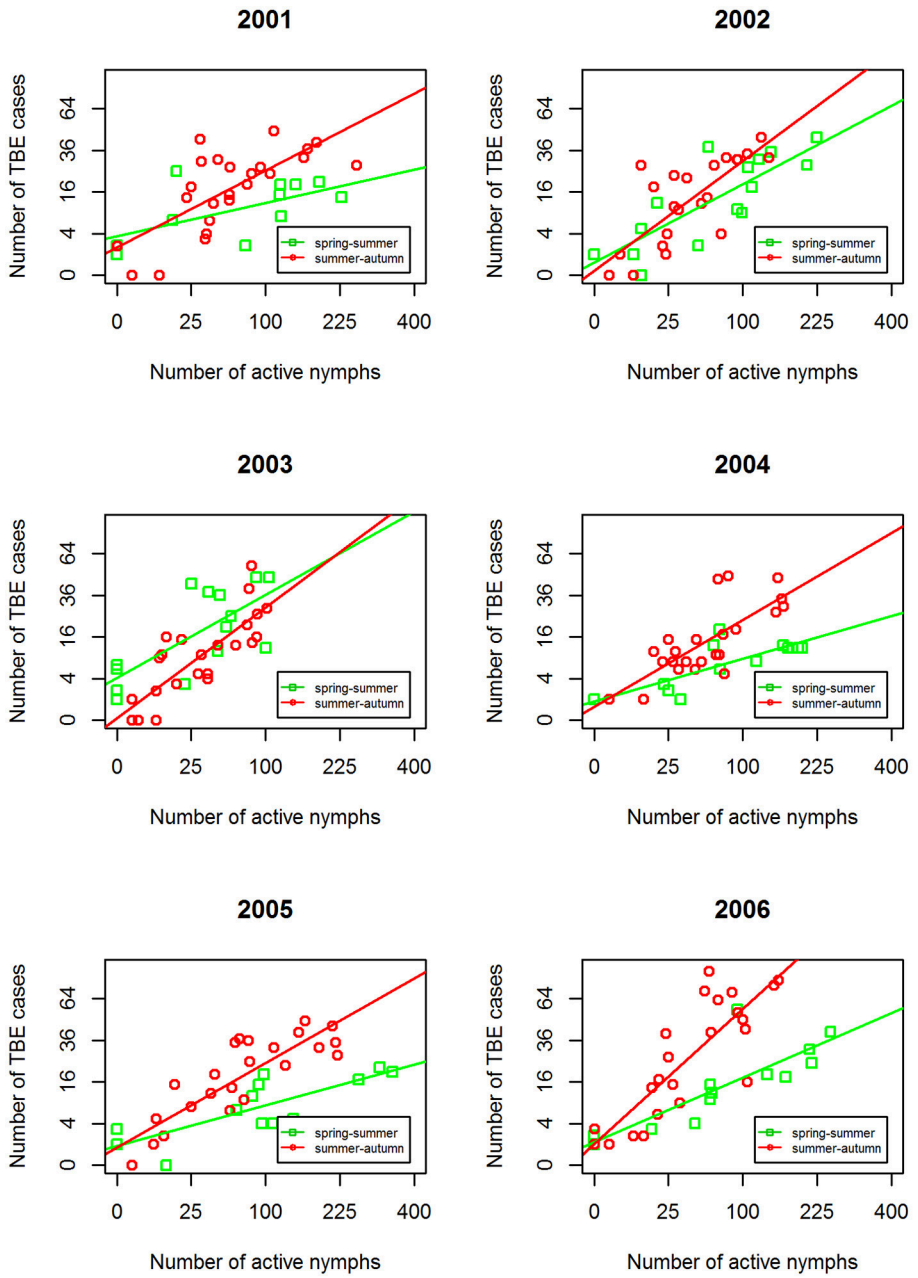

FIGURE 4 | Inter-seasonal comparison of the linear regression relationship between numbers of host-questing *Ixodes ricinus* nymphs and cases of TBE. Square root transformation was used for both axes together with a 3-week shift in alignment.

I. ricinus nymphs was found to differ significantly between spring-summer and summer-autumn periods. The seasonal difference is apparent even when allowance is made for the interval between infected tick bite and the first symptoms of TBE. This interval includes the incubation period for TBE, which is highly variable between individuals partly because of the typical bi-phasic clinical course. The first phase, characterized by non-specific influenza-like symptoms with an incubation period of 3–21 days, may be missed in up to one third of cases and disease recognized directly with neurological symptoms (Duniewiecz, 1999). Other factors influencing the incubation phase include age, overall state of physical and mental health, and the virus dose transmitted by tick bite (Duniewiecz, 1999; Penyevskaya, 2008). Modeling indicated that 21 days was the best estimate of the time between infected tick bite and onset of TBE.

Given the need for accurate risk predictions of contracting TBE, we investigated the apparent discrepancy between seasonal

TABLE 1 | Differences between numbers of ticks and TBE cases.

Year	2001	2002	2003	2004	2005	2006
SPRING-SUMMER PERIOD						
2001	–					
2002	0.075*	–				
2003	0.010	0.022	–			
2004	0.339	0.012	<0.001	–		
2005	0.332	0.005	<0.001	0.976	–	
2006	0.196	0.777	0.038	0.022	0.013	–
SUMMER-AUTUMN PERIOD						
2001	–					
2002	0.407	–				
2003	0.303	0.978	–			
2004	0.626	0.462	0.506	–		
2005	0.806	0.428	0.403	0.920	–	
2006	<0.001	0.002	0.001	<0.001	<0.001	–

**p-values from Chow's test of whether the coefficients are equal in pairwise regression line comparisons of abundance of host-questing nymphs and cases of TBE.*

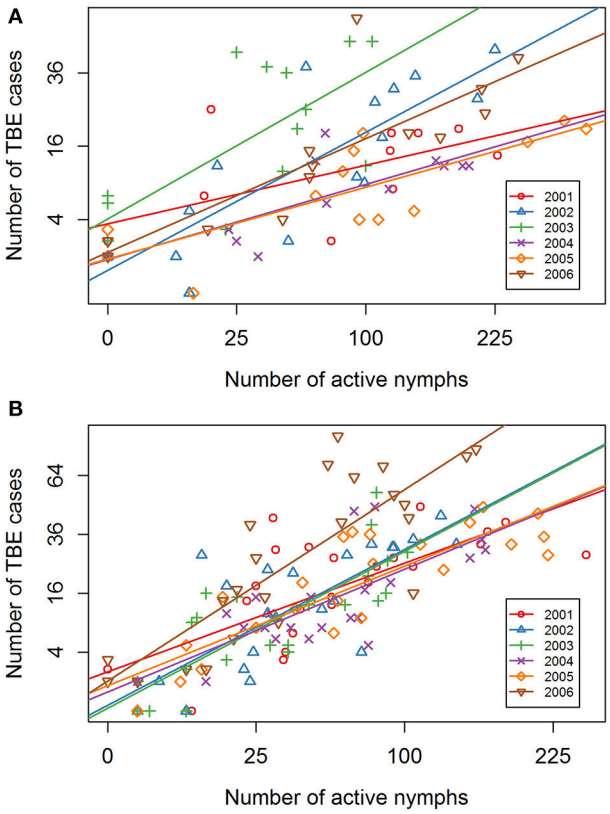

FIGURE 5 | Inter-annual comparison of the linear regression relationship between numbers of host-questing *Ixodes ricinus* nymphs and cases of TBE. Year-on-year comparison for 2001–2006 of the regression lines for **(A)** the spring-summer period, and **(B)** the summer- autumn period. Square root transformation was used for both axes with a 3-week shift in alignment.

incidence of TBE and abundance of host-questing nymphs. Our previous study showed near-ground temperature in the site of tick monitoring is the strongest predictor of tick activity, and showed a high correlation with local meteorological observations in Central Bohemia (Daniel et al., 2015). Similar observations apply to monitoring sites in northern Bohemia, northern Moravia, southern Bohemia, and in the Bohemian-Moravian Highlands (Daniel et al., 2016; Brabec et al., 2017). Hence seasonal discrepancies between numbers of active *I. ricinus* nymphs and TBE cases appear to be independent of locality. Likewise, the seasonal discrepancy in tick activity and TBE incidence is not explained by human activity (which has changed in the last 20 years), as exemplified by the rise in TBE cases at higher altitudes in the Czech Republic where land use and socio-economic conditions have remained unchanged (Daniel et al., 2011). Although promotion of healthy lifestyles encourages outdoor activity, greater environmental management reduces tick contact; furthermore, sustained national awareness campaigns have increased awareness of tick-borne diseases and preventative measures (Daniel et al., 2006, 2010). TBE vaccination in Austria of 85% of the population corresponds with a reduction in the number of TBE cases to ∼16% of pre-vaccination levels; however, in the Czech Republic TBE vaccination only reached 23% coverage in 2013 (Heinz et al., 2013; Prymula, 2015). Although it cannot be ruled out that human activity accounts for the greater relative risk of contracting TBE during summer-autumn, the lack of any clear evidence suggests an alternative explanation.

Seasonal variation in tick host-questing activity, represented by a bimodal curve, shows a significant relationship with microclimatic conditions (particularly temperature) in the *I. ricinus* habitat (Daniel et al., 2015). The first and always considerably higher activity peak corresponds with an average temperature range of 10.1–15.0°C while the second, substantially smaller peak corresponds with an average temperature range of 15.1–20.0°C. However, this pattern changed significantly in 2003 when the numbers of questing nymphs were significantly reduced. Interestingly, a similar anomaly was observed in the 2003 seasonal regression lines comparing the numbers of TBE cases: unlike other years, there were more cases per given number of questing ticks during spring-summer compared with summer–autumn (**Figure 4**). These coincident changes in the typical seasonal dynamics of questing ticks and TBE incidence followed record-breaking flooding in August 2002 in Central Europe, and coincided with an extremely dry period from March to the end of September in 2003 when temperatures exceeded the 30-year monthly averages by 2.9–4.1°C (Hladný et al., 2004; Menne and Ebi, 2006; Rebetez et al., 2009; Daniel et al., 2015). The correlation between extreme meteorological events and TBE incidence has not previously been reported.

Ambient temperature has a significant influence on the development and activity of arthropod vectors, and on the pathogens they transmit. Most studies of ambient temperature effects on arbovirus infection of vectors have involved mosquitoes rather than ticks (Danielová, 1975; Watts et al., 1987; Mellor and Leake, 2000; Dohm et al., 2002; Carpenter

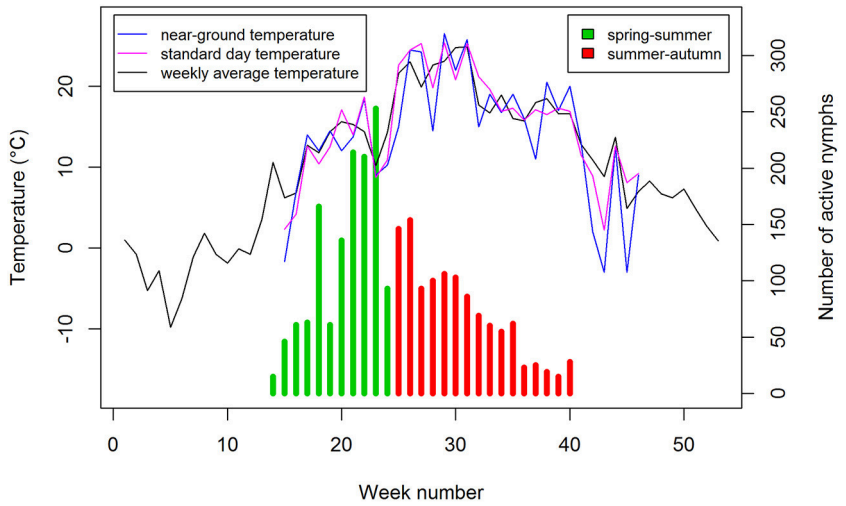

FIGURE 6 | Host-questing *Ixodes ricinus* nymphs in spring-summer and summer-autumn compared with near-ground, standard day, and weekly average temperatures. Data for 2006.

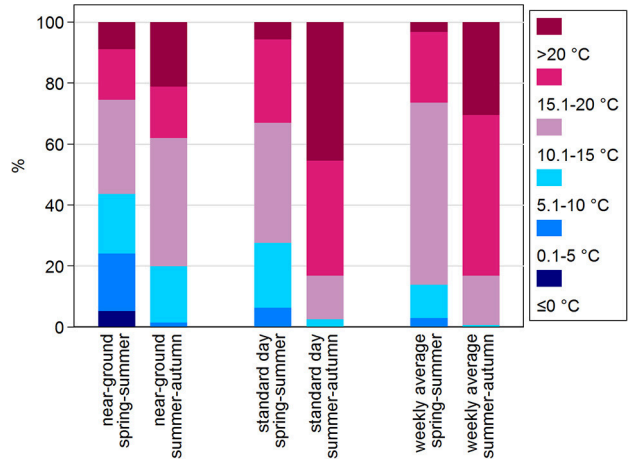

FIGURE 7 | Proportions of host-questing *Ixodes ricinus* nymphs active at different temperature categories in spring-summer compared with summer-autumn periods. Proportions are expressed as % questing nymphs evaluated against near-ground, standard day, and weekly average temperatures.

et al., 2011). The effects of temperature (15 and 24°C) and relative humidity (75 and 97% RH) on TBEV-infected *I. ricinus* ticks were examined in laboratory studies using 920 nymphs fed on viraemic laboratory mice (Danielová et al., 1983; Danielová, 1990). The highest infection rates (70 and 73%) were observed at 24°C + 75% RH and 24°C + 97% RH, respectively. Furthermore, nymphs that fed on viremic mice 1–2 months after molting showed 59% infection rates whereas older nymphs (3–4 months after molting from larvae) feeding on the same mice had infections rates of only 29% ($p < 0.001$), suggesting that physiological age affects infection levels. Given that spring host-questing nymphs are those metamorphosed in the preceding year and overwintered as unengorged nymphs while summer-autumn host-questing nymphs are predominantly nymphs metamorphosed in the same summer (Daniel and Dusbábek, 1994), a combination of physiological age and environmental temperature may influence TBEV replication in ticks and account for higher relative numbers of human TBE cases in the summer-autumn season. Studies on the Far Eastern subtype of TBEV in its principal vector, *Ixodes persulcatus*, provide similar indications that extrinsic climatic conditions affect virus infection of the tick vector (Korenberg and Kovalevskii, 1994; Korenberg, 2000). Russian authors also conclude that clinical disease occurs most often when humans are bitten by ticks carrying a relatively high virus dose (Korenberg and Kovalevskii, 1994, 1999; Penyevskaya, 2008). In a study of 1,496 adults and 345 children (all unvaccinated) bitten by adult *I. persulcatus* in a focus of TBEV transmission, the ticks were removed and classified according to the level of infectious virus they carried (Penyevskaya, 2008). Of those bitten by ticks with low virus titers (~1.0 $\log_{10}$ $TCID_{50}$), 4/1108 (0.4%) adults, and 2/240 (0.8%) children became symptomatically infected. Ticks with moderate infections (~2.17 $\log_{10}$ $TCID_{50}$) were associated with 6/147 (4.1%) adults and 5/44 (11.4%) children symptomatically infected while ticks with high infection levels (~3.08 $\log_{10}$ $TCID_{50}$) were associated with 34/241 (14.1%) adults and 20/61 (32.8%) children that developed TBE.

Given that higher temperatures are associated with higher levels of TBEV infection in ticks and with higher incidences of TBE in humans, environmental temperature explains the discrepancy reported here between tick activity and number of cases of TBE in spring-summer compared with summer-autumn periods. Although comparatively frequent in the spring period, the bites by *I. ricinus* ticks (particularly nymphs) during the sharp explosive onset of their host-questing activity (also the start of human outdoor leisure activities) cause

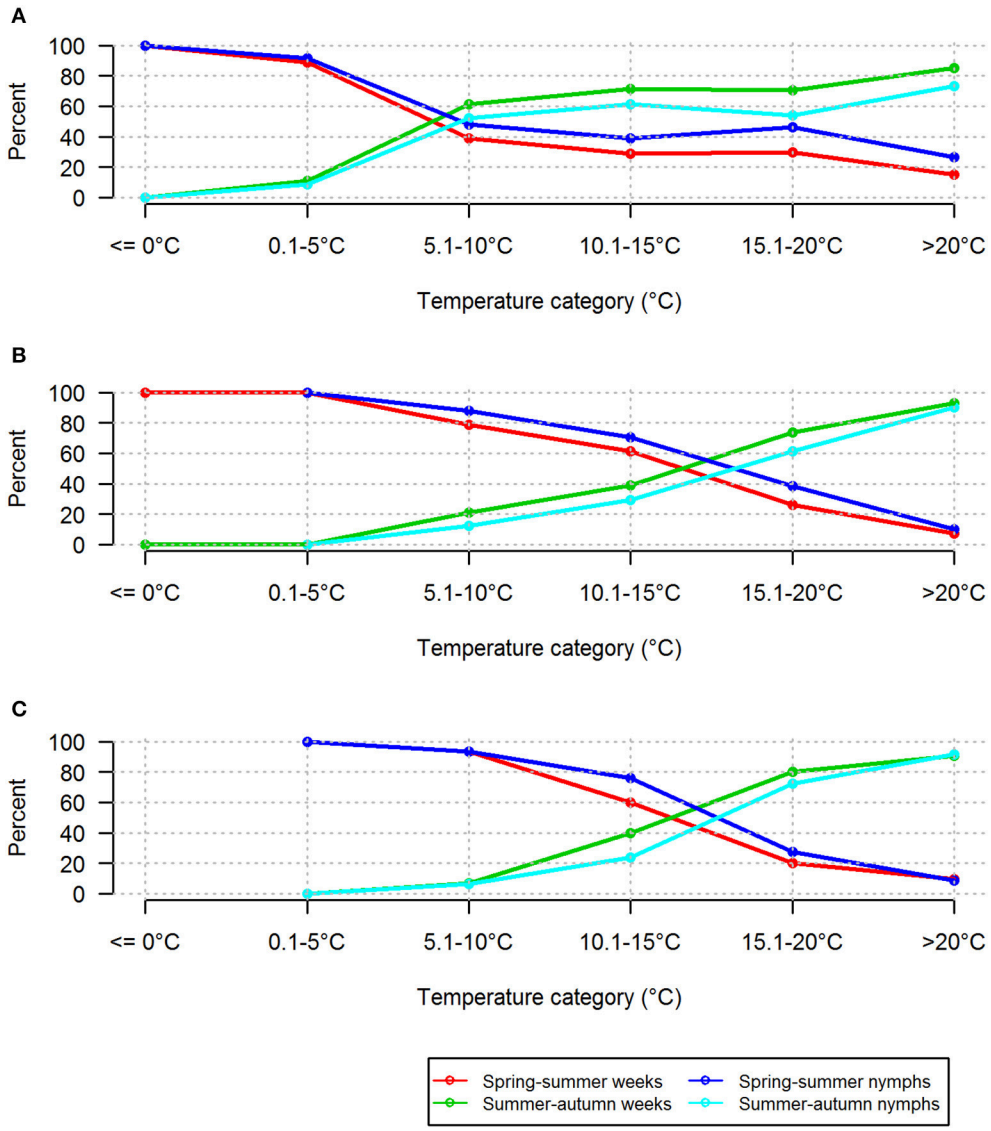

FIGURE 8 | Relative proportions of nymphs and numbers of weeks in which they were found at different temperature categories in spring-summer and summer-autumn. Based on **(A)** near-ground temperature measured in the tick habitat, **(B)** standard day temperature measured by the CHMI observatory, and **(C)** weekly average of temperature derived from the CHMI database.

relatively fewer cases of clinical disease in comparison with the summer period. Lower average temperatures in the tick habitat may constrain TBEV replication after overwintering such that the threshold dose needed for clinical infection in humans is not attained. In spring, humans bitten by infected ticks may receive only a sub-threshold dose of TBEV causing subclinical (asymptomatic) infection resulting in an immune response. This would explain, for example, the high prevalence of antibodies in individuals in South Bohemia without any previous clinical disease (Luňáčková et al., 2003). Higher average temperatures during the summer-autumn period may lead to higher levels of TBEV in ticks and consequently an increased risk that humans develop the disease following an infected tick bite. Our working hypothesis can be tested by extensive statistical comparison of virus infection levels in ticks collected in the same locality during the spring-summer and summer-autumn periods. Although the logistics of such an undertaking are formidable, given the very low TBEV infection rate in ticks in nature (estimated as <0.1%) modern sequencing technologies now make this challenge tractable (Daniel et al., 2008, 2016).

Daytime ambient temperature directly affects *I. ricinus* host-questing activity, the basis for prognoses of general tick attack risk. This daily warning, based on routine meteorological forecasts, has been implemented in the Czech Republic since 2007 as part of the awareness campaign for inhabitants in TBE

risk areas (Daniel et al., 2015). However, conditions affecting TBEV load in tick bites are better represented by weekly average temperatures. Thus the model of general tick attack risk (based on daytime temperature) supplemented with weekly average temperature provides the opportunity for real time forecasts of TBE clinical infection risk as part of an effective campaign to prevent TBE in humans.

ETHICS STATEMENT

The study uses national statistics on recorded cases of TBE that do not refer to individual case details and are freely available to academic researchers. Use of these data is exempt from requiring ethical consent.

AUTHOR CONTRIBUTIONS

MD, VD conceived and designed the study; AF, MM, and BK analyzed the data. PN, MD, and VD wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00090/full#supplementary-material

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MINI REVIEW
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Mapping Arbovirus-Vector Interactions Using Systems Biology Techniques

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Studying how arthropod-borne viruses interact with their arthropod vectors is critical to understanding how these viruses replicate and are transmitted. Until recently, these types of studies were limited in scale because of the lack of classical tools available to study virus-host interaction for non-model viruses and non-model organisms. Advances in systems biology "-omics"-based techniques such as next-generation sequencing (NGS) and mass spectrometry can rapidly provide an unbiased view of arbovirus-vector interaction landscapes. In this mini-review, we discuss how arbovirus-vector interaction studies have been advanced by systems biology. We review studies of arbovirus-vector interactions that occur at multiple time and length scales, including intracellular interactions, interactions at the level of the organism, viral and vector populations, and how new techniques can integrate systems-level data across these different scales.

Keywords: arbovirus, virus, arthropod, vector, interactions, systems biology

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INTRODUCTION

Arthropod-borne viruses (arboviruses), which are transmitted by arthropod vectors like mosquitoes, flies, and ticks, are a source of endemic, emerging, and re-emerging infectious diseases. Chikungunya virus (CHIKV), a mosquito-borne virus, causes severe arthritic disease with over one million estimated infections in a single epidemic (Powers and Logue, 2007). Mosquito-borne dengue virus (DENV) infects nearly 400 million people annually (Bhatt et al., 2013) and can cause severe illness such as dengue hemorrhagic fever and dengue shock syndrome (Gubler, 2002). Zika virus (ZIKV) is a recently emerged mosquito-borne virus that causes major developmental defects when fetuses are infected *in utero* (Mlakar et al., 2016; Delaney et al., 2018). Tick-borne viruses, including Powassan virus, can also be neuropathogenic and are increasing in prevalence. As habitats for arthropod vectors expand with global climate change, residents of densely-populated regions will be at risk of arbovirus infections.

Unraveling how arboviruses interact with their vectors is critical to understanding arbovirus replication and transmission and informing arbovirus mitigation strategies. While classical methods have provided significant insight into arbovirus-vector interactions, systems biology approaches, which serve to generate and integrate large unbiased datasets using "-omics"-based approaches, have several advantages. First, systems biology approaches offer an unbiased view of arbovirus-vector interactions, leading to discoveries that may not have been possible using hypothesis-driven approaches. Second, researchers can use technologies like next-generation sequencing (NGS) and mass spectrometry (MS) to directly sample vector nucleic acids and proteins to answer scientific questions that were previously intractable in non-model systems that lack many

classical genetic or biochemical tools. Finally, system approaches enable researchers to rapidly map arbovirus-vector interactions of newly emerging arboviruses. In this mini-review, we explore how systems biology approaches have been applied to study aspects of arbovirus-vector interactions at different scales (**Figure 1**). We review advances in identifying intracellular arbovirus-vector interactions that occur in response to infection. We also discuss how advances in NGS methods can provide insight into interactions occurring over larger time and length scales, and can be used to bridge arbovirus-vector interactions occurring over multiple scales. Finally, we consider how specific challenges in studying arbovirus-vector interactions may be addressed in the future.

IDENTIFYING INTRACELLULAR ARBOVIRUS-VECTOR INTERACTIONS

All viruses must hijack host machinery and resources to replicate. This can be done through changes in gene expression, or through direct physical contact with host machinery. With the advent of high-throughput techniques including transcriptomics, proteomics, yeast-two-hybrid (Y2H), affinity purification and MS (AP-MS), and lipidomics, there have been many advances in identifying these types of arbovirus-vector interactions.

Gene Expression Profiling

A classic systems biology analysis of virus-host interactions includes understanding how gene expression at the RNA and protein level changes in response to a virus infection. Several groups have used transcriptomic and proteomic profiling to understand how gene expression patterns in these vectors change in response to arbovirus infection (Bonizzoni et al., 2012; Paradkar et al., 2015; Dong et al., 2017; Etebari et al., 2017; Saucereau et al., 2017; Xin et al., 2017; Shrinet et al., 2018). These unbiased approaches can identify factors critical to arbovirus replication. For example, Paradkar et al. used RNA-seq data as a starting point to demonstrate the role of Cul4 in promoting West Nile virus (WNV) replication in *Culex quinquefasciatus* cells (Paradkar et al., 2015). More recently, Xin et al. used quantitative global proteomic profiling in *Aedes albopictus* C6/36 cells to identify several cellular pathways to be perturbed by ZIKV infection, including innate immunity and the unfolded protein response (Xin et al., 2017). They further identified the ubiquitin-proteasome system as conserved hub for virus replication in both mosquito and mammalian cells. The authors used Bortezomib, a FDA-approved inhibitor of the 20S proteasome to inhibit ZIKV replication in both *Aedes aegypti* cells and mice. A similar proteomic profiling study of *Ixodes scapularis* ISE6 cells revealed hundreds of changes in protein abundance following Langat virus (LGTV) infection (Grabowski et al., 2016). Here, Grabowski et al. identified changes pathways related to metabolism, protein biosynthesis, and mTOR signaling, which could be targeted using chemical inhibitors. While the limited annotation of the *I. scapularis* genome restricts the insight that can be gleaned from pathway level analysis, this is a first of its kind proteomic study of flavivirus-tick interactions, and led to the subsequent identification of several LGTV replication factors in the tick host (Grabowski et al., 2017).

Mosquitoes are notorious for having highly repetitive genome sequences, and this can make sequence alignment for transcriptomic studies challenging even if assembled genomes have been available for some time. Moreover, rapid expansion of gene families resulting from highly active transposons mean that assigning gene function can also be challenging as duplicate genes rapidly evolve to develop novel functions. Some groups used transcriptomics coupled with proteomics to improve mass spectra identification in vectors with poorly annotated or unsequenced genomes. In a recent study of *A. aegypti* Aag2 cells, Maringer et al. used this technique to improve identification of active transposons and identify genomic regions with incomplete annotation (Maringer et al., 2017).

Physical Interactions

Another type of arbovirus-vector interaction that can be probed using systems techniques is virus-host protein-protein interactions (PPIs). For example, two studies have identified PPIs between a DENV and *A. aegypti* proteins via Y2H screening (Mairiang et al., 2013; Tham et al., 2015). These studies have provided an initial landscape of flavivirus-vector PPIs, however flaviviruses proteins include many transmembrane domains. Alternative systems approaches, like the AP-MS approach used by Muñoz et al. to identify interactions between DENV membrane protein E and the *Aedes* vector (Muñoz et al., 2013), may be better suited to identifying such flavivirus-vector PPIs.

A key limitation for using AP-MS to identify arbovirus-vector PPIs is finding a system that allows for abundant viral protein expression and efficient vector protein identification. We found that codon optimization can help improve viral protein expression in cell lines derived from vectors with better proteome annotation. Using this strategy, we identified arbovirus-mosquito interactions for multiple DENV proteins directly in *A. aegypti* Aag2 cells using systematic AP-MS (Shah et al., 2018). In this study, we compared virus-host interactions between the human and mosquito hosts at the level of individual proteins, complexes and pathways. This multi-level analysis highlighted the Sec61 translocon as a conserved hub for flavivirus replication that could be pharmacologically modulated in both human and mosquito cells. In the future, systematic AP-MS will be a promising method for exploring the arbovirus-vector PPI landscape.

Lipidomics

While gene expression profiling is limited by reference genome availability and quality, lipidomics analysis is not subject to the same limitation and represents an avenue for studying arbovirus-vector interactions in non-model vectors. In fact, Perera et al. executed one of the first "-omics" studies of arboviruses directly in a mosquito system by performing lipidomic analysis of DENV-infected *A. albopictus* C6/36 cells (Perera et al., 2012). Here, the authors demonstrate that lipids are broadly redistributed in mosquito cells in a way that may support specific aspects of DENV replication following infection, such as maintaining fluidity, bending these membranes to form replication complexes, and providing the negative curvature

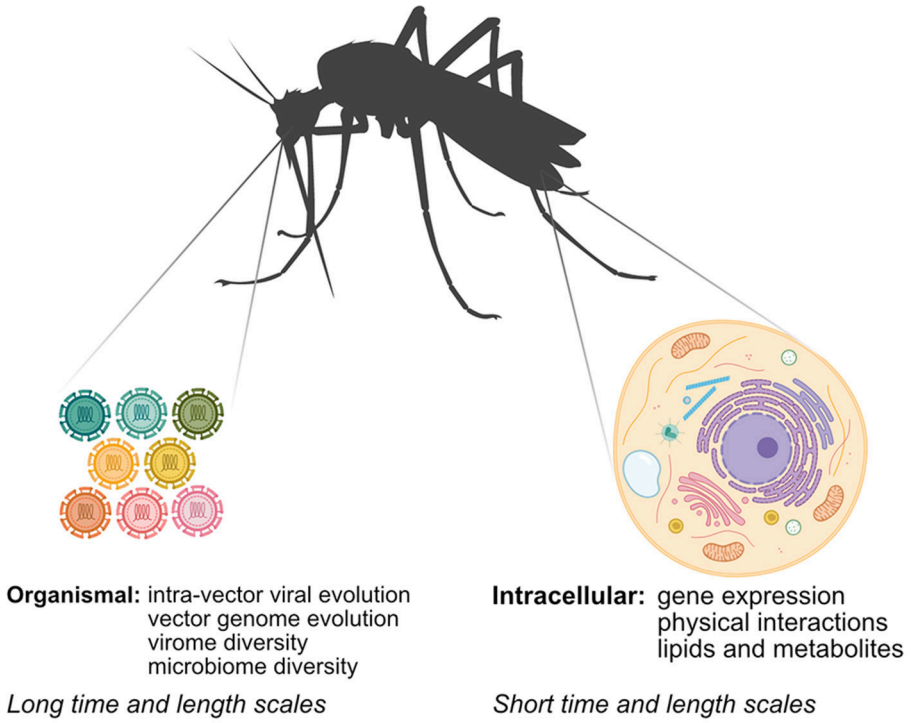

FIGURE 1 | Mapping arbovirus-vector interactions at different scales using systems biology. Arboviruses interact with their vectors at the intracellular and organismal scale. Multiple–omics techniques can be used at the intracellular scale, while interactions at the organismal scale rely primarily on NGS techniques.

required for DENV-induced double-membraned vesicles that have been observed by electron microscopy (Welsch et al., 2009). Additional experiments performed by Chotiwan et al. in *A. aegypti* midgut tissue offer a comprehensive temporal view of lipid regulation *in vivo* for over 10 days following DENV infection (Chotiwan et al., 2018). In a first of its kind study, the authors confirmed many of the observations made in cell culture experiments, such as overall increases in glycerophospholipid content and perturbation of the sphingolipid biosynthesis pathway. However, the changes observed *in vivo* also proved to be more complex than those observed in cell culture. More recently, *Wolbachia*-infected *A. albopictus* Aa23 cells were shown to alter sphingolipid content in a direction opposite of what is observed during DENV infection, suggesting that *Wolbachia* infection may inhibit DENV infection through perturbation of lipid homeostasis (Molloy et al., 2016). Taken together, these studies highlight the utility of lipidomic analysis as an avenue for systems-level interrogation of arbovirus-vector interactions and underline the importance of translating global techniques to *in vivo* models.

UNDERSTANDING VECTOR AND ARBOVIRUS EVOLUTION

Arboviruses replicate using error-prone polymerases and produce genetically diverse viral populations that facilitate their rapid evolution and adaptation to novel environments. Recent technological advances in sequencing can provide insight into how arbovirus-vector interactions impact arbovirus and vector evolution.

Intra-Vector Viral Evolution

Arboviruses must overcome multiple bottlenecks in the vector to travel from the midgut to the salivary gland and be transmitted to a host. While these bottlenecks in mosquito were first observed using classical virology techniques (Smith et al., 2008), understanding the effects of these bottlenecks on virus evolution and fitness is possible because of the development of NGS technology that allows researchers to assess the diversity of viral populations over time and through different tissues on a large scale. For example, Grubaugh et al. used NGS and single variant analysis to track WNV diversity through the multiple bottlenecks in different mosquito vectors (Grubaugh et al., 2016b). The authors found that enzootic *Culex* vectors generated more intra-vector WNV diversity to overcome genetic drift associated with the transmission bottleneck compared to the *A. aegypti* bridge vector. Despite this diversity, the virus transmitted to avian systems by *Culex* vectors had lower relative fitness due to weak purifying selection that allows the accumulation of deleterious mutations. Similar studies exploring tick- and mosquito-borne virus intra-vector diversity suggest that diversity is determined by both the virus and the vector, and results from differences in the strength of purifying selection, bottleneck effects, and positive selection from antiviral responses (Stapleford et al., 2014; Brackney et al., 2015; Sim et al., 2015; Grubaugh et al., 2016a;

Lequime et al., 2016; Patterson et al., 2018; Weger-Lucarelli et al., 2018). Further study will be needed to determine the impact of the different selective forces on viral genome evolution within their arthropod vector.

Long read sequencing, such as single-molecule real-time and nanopore sequencing, can provide additional information on viral diversity through the determination of recombination events and reconstruction of full-length genotypes. While long read technologies have a high error rate, multi-platform sequencing, which uses a combination of short and long reads, can compensate for this shortcoming. Jaworski and Routh used long and short read sequencing to detect recombination rates and determine wild type sequence frequency, which was essential to studying defective interfering RNA production in Flock House virus (Jaworski and Routh, 2017). Depledge et al. also used a multi-platform approach to analyze alternative splicing, transcription start sites and read through variants in viral transcripts (Depledge et al., 2018). While this approach was applied to herpes virus, it will be extremely valuable in the study of arbovirus-vector interactions, such as the impact of subgenomic flavivirus RNA on vector innate immunity and transmission (Göertz et al., 2016).

Another recent advance in NGS methodology, single cell RNA sequencing (scRNA-seq) (Tang et al., 2009), permits the study of how replication in specific cell types within various tissues contributes to viral diversity and transmission. A recent study by Severo et al. used the technique to characterize mosquito hemolymph cells (Severo et al., 2018). While this study was done in *Anopheles* mosquitoes, similar studies in arbovirus vectors will provide a foundational knowledge on vector immune cells. In a virus inclusive scRNA-seq analysis in human cells, Zanini et al. showed that DENV and ZIKV replication rates vary among cells and this variation correlates with differences in gene expression of host factors. The authors further used these correlations to identify host restriction and dependency factors (Zanini et al., 2018). Applying such scRNA-seq techniques to vector systems could provide insight into several open questions in the field of arbovirus-vector interactions, such as which vector pathways control viral replication and transmission in different tissues, and which cell types control viral persistence, vertical transmission, and the selection of viral variants *in vivo*. In this way, scRNA-seq provides a unique opportunity to bridge the study of intracellular arbovirus-vector interactions with viral evolution, immunity and transmission *in vivo*.

Long-Term Evolutionary Interactions

In addition to direct sequencing of viral populations, arthropod genome sequencing projects enabled by NGS technologies have improved the ability to understand long-term interactions between arboviruses and vector genomes. Endogenous viral elements (EVEs) are viral sequences that are inserted into the host genome. EVEs inserted into the germline can provide a record of past infections and may provide a source of antiviral immunity. Consequently, in contrast to arbovirus intra-vector evolution, arbovirus-derived EVE integration events reflect the long-term evolutionary relationship between arboviruses and their arthropod vectors.

The organization, evolution, and mode of action of EVEs in arthropod genomes are still poorly understood, but their characterization has benefited from progress in arthropod genomics. EVE integration occurs mainly in Piwi-interacting RNA (piRNA) clusters, genomic regions known to be composed of incomplete transposon sequences and a source of piRNAs production. piRNA are the main defense system against transposition in many species (Aravin et al., 2007) and described as a potential antiviral defense for mosquito (Miesen et al., 2016). Using small RNA NGS to profile piRNA production from EVEs, several studies have found the production of anti-sense piRNAs mostly restricted to EVEs in piRNA clusters (Palatini et al., 2017; Suzuki et al., 2017; Whitfield et al., 2017). This piRNA production suggests a role for EVEs in the antiviral response against new infection of arthropods, a hypothesis that is supported by a recent study that observed a difference in piRNA production during DENV infection of *Aedes* mosquitoes (Wang et al., 2018).

In addition to providing insights into EVE biogenesis, NGS techniques can also help identify more EVEs through the generation of new arthropod genomes to survey and improvements in genome assemblies. For example, while arthropod EVEs described in the literature belong to many different virus families, a comprehensive metagenomic study of 48 arthropod genomes identified over 4000 EVEs, and found that most belong to *Rhabdoviridae* and *Parvoviridae* families (ter Horst et al., 2018). Interestingly, no EVEs from *Togavirirdae* were found in this or other studies, even though family members like CHIKV are major arboviruses. Finally, Whitfield et al. used long read sequencing technology to increase the resolution of highly repetitive regions of *A. aegypti* Aag2 cell genome, allowing the discovery of unknown EVEs from the *Rhabdoviridae*, *Flaviviridae,* and *Chuviridae families* (Whitfield et al., 2017). This study suggests that the arthropod EVE population is underestimated, and also highlights the utility of new sequencing technologies for increasing our genomics resolution to aid in EVE discovery.

MAPPING ARTHROPOD-VIROME INTERACTIONS

The emergence of ZIKV has reignited concern about identifying arboviruses that pose a risk for emergence. Due to the declining cost and increasing portability of NGS technologies, researchers can now identify new arboviruses, determine their range, and understand vector competence, all of which can contribute to the arboviral emergence.

Arbovirus Discovery

There is great interest in identifying arboviruses in field-caught vectors, and several groups have now sequenced hundreds or even thousands of field-caught mosquitoes and ticks to survey the vector virome (Tokarz et al., 2014; Frey et al., 2016; Harvey et al., 2018; Sadeghi et al., 2018; Zakrzewski et al., 2018). Together, these studies have identified dozens of new arboviruses. In the future, virome sequencing could be especially useful for surveillance, tracking the spread of pathogenic viruses. The spread of low-cost

nanopore sequencing represents an interesting virus discovery tool, as the portable MinION was used to identify arboviruses in field-caught mosquito (Russell et al., 2018). In the future, these tools will advance virus discovery in understudied vectors like the sandfly.

Vector Competence

scRNA-seq can also be used inform on complex topics like vector competence. Several recent studies have shown that the presence of arthropod-specific viruses can inhibit or promote the replication of medically-relevant arboviruses (Goenaga et al., 2015; Nasar et al., 2015; Schultz et al., 2018) and could impact vector competence. Co-infection with bacteria like *Wolbachia* species can also reduce vector competence (Glaser and Meola, 2010). Virome and microbiome mapping of an individual vector is the first step in relating infection status to vector competence. Recently, Cross et al. commenced such an effort by coupling virome and microbiome analysis in individual *I. scapularis* ticks (Cross et al., 2018). The authors found a positive and negative correlations correlation between different arboviruses, and between levels of specific tick-borne virus RNA and co-infections with *Borrelia burgdorferi*, the bacterium that causes Lyme disease. These results support the current model that the virome and microbiome influence vector competence and highlight the need for more studies in this area.

CAVEATS AND POTENTIAL SOLUTIONS

Despite the advances made using systems biology approaches to study arbovirus-vector interaction, several challenges remain. First, the lack of reference genomes for many vectors limits the systems techniques that can be applied to them. However, the i5K initiative to sequence 5,000 arthropod genomes (Poelchau et al., 2018) will fill critical gaps in this area. Poor genome annotation also limits the utility of systems biology studies, and further development of methods to predict gene function will be important for progress. Finally, as with all systems biology studies, improvements in data collection enabled by systems biology approaches need to be accompanied by advances in techniques capable of transforming that information into mechanistic insight. Better genetic tools like the recent advances in heritable CRISPR genome editing of arthropods (Chaverra-Rodriguez et al., 2018) will enable rapid testing of specific arthropod genes that interact with arboviruses for their impact on virus replication *in vivo*. Ultimately, these tools could be used for arbovirus mitigation efforts. For example, CRISPR could be used to target non-essential arbovirus replication genes to engineer arthopod populations with reduced vector competence without compromising overall arthropod fitness.

CONCLUSIONS

Systems biology approaches offer global, unbiased views of arbovirus-vector interaction landscapes. By taking advantage of these approaches, researchers have the potential to transform our understanding of arbovirus replication, evolution, diversity, and vector competence. In the long-term, this will offer insight into the basic biology of arboviruses and their vectors, and lead to the development of arbovirus mitigation strategies.

AUTHOR CONTRIBUTIONS

MP and PS conceived of topics to be discussed and wrote the manuscript. MP wrote sections pertaining to virus and vector evolution. PS contributed to all sections of the manuscript.

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frontiers in Cellular and Infection Microbiology

REVIEW
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The Role of Host Cholesterol During Flavivirus Infection

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In recent years the emergence and resurgence of arboviruses have generated a global health alert. Among arboviruses, Dengue (DENV), Zika (ZIKV), Yellow Fever (YFV), and West Nile (WNV) virus, belong to the genus *Flavivirus*, cause high viremia and occasionally fatal clinical disease in humans. Given the genetic austerity of the virus, they depend on cellular factors and organelles to complete its replication. One of the cellular components required for flavivirus infection is cholesterol. Cholesterol is an abundant lipid in biomembranes of eukaryotes cells and is necessary to maintain the cellular homeostasis. Recently, it has been reported, that cholesterol is fundamental during flavivirus infection in both mammal and insect vector models. During infection with DENV, ZIKV, YFV, and WNV the modulation of levels of host-cholesterol facilitates viral entry, replicative complexes formation, assembly, egress, and control of the interferon type I response. This modulation involves changes in cholesterol uptake with the concomitant regulation of cholesterol receptors as well as changes in cholesterol synthesis related to important modifications in cellular metabolism pathways. In view of the flavivirus dependence of cholesterol and the lack of an effective anti-flaviviral treatment, this cellular lipid has been proposed as a therapeutic target to treat infection using FDA-approved cholesterol-lowering drugs. This review aims to address the dependence of cholesterol by flaviviruses as well as the basis for anti flaviviral therapy using drugs which target is cholesterol synthesis or uptake.

Keywords: flavivirus, arbovirus infection, host cholesterol, IFN response, lipid-lowering drugs

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INTRODUCTION

Viral infections transmitted by mosquitoes, such as those caused by the flaviviruses dengue (DENV), yellow fever virus (YFV), West Nile virus (WNV), and Zika virus (ZIKV) represent important health challenges. In the last years, the emergence or reemergence of different arboviruses have generated a global health alert. In this sense, DENV continues to increase in tropical and subtropical regions of the world, whereas outbreaks of YFV in humans have been reported in Angola and some countries in South America. Moreover, the emergence of ZIKV, which is strongly associated with microcephaly in newborns, Guillain-Barre syndrome in adults and the ability to be transmitted sexually and through trans placental route, as well as the neurotropic behavior of WNV; making these viruses a latent threat to global health (Barrett, 2017; Valderrama et al., 2017; Salles et al., 2018; Silva et al., 2018; Talero-Gutiérrez et al., 2018). Although, there is an approved and effective vaccine for YFV, an effective vaccine or treatment for DENV and ZIKV have not been achieved yet (Barrett, 2017; Silva et al., 2018). Therefore, it is urgent to develop

an effective therapy against these viruses. Flaviviruses use different strategies to replicate and to evade immune response; one of them is the inhibition of interferon (IFN) response mediated by at least, in the case of DENV, of the non-structural proteins NS2A, NS4A, NS4B, and NS5 which target the signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2) (Morrison et al., 2012). A second mechanism to inhibit immune response is common for many RNA viruses and is the use of the non-structural viral proteins to modify the endoplasmic reticulum (ER) membranes to generate partially isolated compartments known as replication complexes (RC) where the new viral particles are replicated and formed (Welsch et al., 2009; Neufeldt et al., 2018). The RC as replicative organelles are fundamental because they create a barrier that minimizes the detection of double-stranded RNA or 5′-phosphorylated RNA in the cytoplasm (Neufeldt et al., 2018). Interestingly, the formation of the RC requires fatty acids, cholesterol, glycerophospholipids, phospholipids, and sphingolipids (mainly ceramides) (Heaton et al., 2010; Neufeldt et al., 2018). To this regard, the NS3 protein sequesters the fatty acids synthase (FASN) to the RC, and viral infection increases the cholesterol uptake and cholesterol synthesis (Heaton et al., 2010; Soto-Acosta et al., 2013, 2017). A wide variety of lipids (fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterols, prenolic lipids, saccharolipids, and polychaetes) can be found in cell membranes (Fahy et al., 2005). Cholesterol is an abundant lipid in biomembranes of eukaryotes cells and is essential for adequate cellular functioning (Simons and Ikonen, 2000; Crane and Tamm, 2004; Fernández et al., 2004). Cholesterol levels in the cells are controlled by biosynthesis, efflux from cells, and uptake (Simons and Ikonen, 2000). Some cholesterol uptake receptors that participate during DENV infection are the low-density lipoprotein receptor (LDLr) and the scavenger receptor class B type I (SR-BI) (Betters and Yu, 2010; Li et al., 2013; Soto-Acosta et al., 2013), while the increase in cholesterol synthesis during DENV infection is mediated by the increase in the activity of the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR, limiting enzyme in the cholesterol synthesis pathway) being the activation of the HMGCR a consequence of the inhibition of the molecule considered as the cellular metabolism controller, the AMP-dependent kinase (AMPK) (Ikonen, 2008; Cerqueira et al., 2016).

On the other hand, there is a close correlation between cholesterol levels and type 1 IFN response. High levels of cholesterol induce a poor IFN response (Liu et al., 2013). Thus, the reduction in cholesterol levels during flavivirus infection grants an increase in IFN response. Despite how much is known about the role of cholesterol during DENV infection (Rothwell et al., 2009; Puerta-Guardo et al., 2010; Poh et al., 2012; Carro and Damonte, 2013; Soto-Acosta et al., 2013, 2017), its importance during YFV, WNV and ZIKV infection is understudied. Therefore, understanding the importance of host cholesterol during flavivirus infections allow us to design antiviral strategies with cholesterol-lowering drugs such as statins (Rothwell et al., 2009; Martínez-Gutierrez et al. 2011, 2014; Soto-Acosta et al., 2013; Bryan-Marrugo et al., 2016), or with AMPK activators such as metformin (Soto-Acosta et al., 2017; Htun et al., 2018), and the nordihydroguaiaretic acid (NDGA) compound (Soto-Acosta et al., 2014; Merino-Ramos et al., 2017); which reduce cholesterol synthesis as well as with drugs that inhibit cholesterol uptake such as ezetimibe. This review is mainly focused on the recent findings that demonstrate how the mosquito-borne flaviviruses take advantage of host-cholesterol to complete their life cycle (**Figure 1**) and host-directed antiviral (HDA) therapy strategies for flavivirus inhibition.

THE ROLE OF HOST CHOLESTEROL DURING THE FLAVIVIRUS LIFE CYCLE

Host Cholesterol in Flavivirus Entry

The arboviruses of the Flavivirus genus, Flaviviridae family members, as enveloped viruses, have a major and conserved E glycoprotein, which is composed of ninety dimers arranged with a quasi-icosahedral symmetry on the viral membrane (Nybakken et al., 2006; Li et al., 2008; Dai et al., 2016). This protein is highly conserved in the Flavivirus genus and is involved in viral attachment to the mosquito and mammalian host cells. The first step in viral infections is the binding and entry process. In this step, the viral particles have to bind to specific molecules on the cell surface such as receptors and coreceptors to enter into the host cell (**Figure 1A**). Since several of the attachment and receptor molecules described for viruses are present in lipid rafts (Takahashi and Suzuki, 2009), lipids and cholesterol-rich plasma membrane microdomains that are essential during entry of DENV (Lee et al., 2008; Puerta-Guardo et al., 2010; Soto-Acosta et al., 2013; García Cordero et al., 2014; Reyes-del Valle et al., 2014; Diwaker et al., 2015), and WNV (Medigeshi et al., 2008) (**Figure 1A**). The use of cholesterol-depleting drugs such as methyl-β-cyclodextrin (MβCD) or filipin, which binds to cellular cholesterol forming complexes and avoiding the formation of lipid rafts before viral entry. It has been demonstrated to induce an antiviral effect for DENV and WNV infection, which made it possible to confirm the importance of lipid rafts during entry for both viruses (Lee et al., 2008; Medigeshi et al., 2008; Rothwell et al., 2009). To the contrary, cholesterol and lipid rafts are not essential during the entry in the mosquito cell line C6/36 (*Aedes albopictus*) infected with DENV (Mosso et al., 2008) and WNV (Chu et al., 2006). Although insects cannot synthesize cholesterol *de novo*, they can take it to synthesize cell membranes and hormones (CLAYTON et al., 1962; CLAYTON, 1964; Krebs and Lan, 2003). Recently, cholesterol molecules were detected during the early stages of DENV replication in the middle intestine of *Aedes aegypti* mosquitoes (Chotiwan et al., 2018). However, the specific role of the cholesterol in this organ in mosquito-virus interaction remains unexplored.

The cholesterol dependence of DENV entry and post-entry steps have been observed in several mammalian cell lines (Lee et al., 2008; Soto-Acosta et al., 2013; Martínez-Gutierrez et al., 2014), it does not seem to be a general event, because it has been described that cholesterol is not required for Vero (green monkey epithelial kidney cell line) (Acosta et al., 2009; Carro and Damonte, 2013), HepG2 (Hepatocarcinoma cell line) and ECV304 cells entry (human endothelial cell line) (Rothwell et al.,

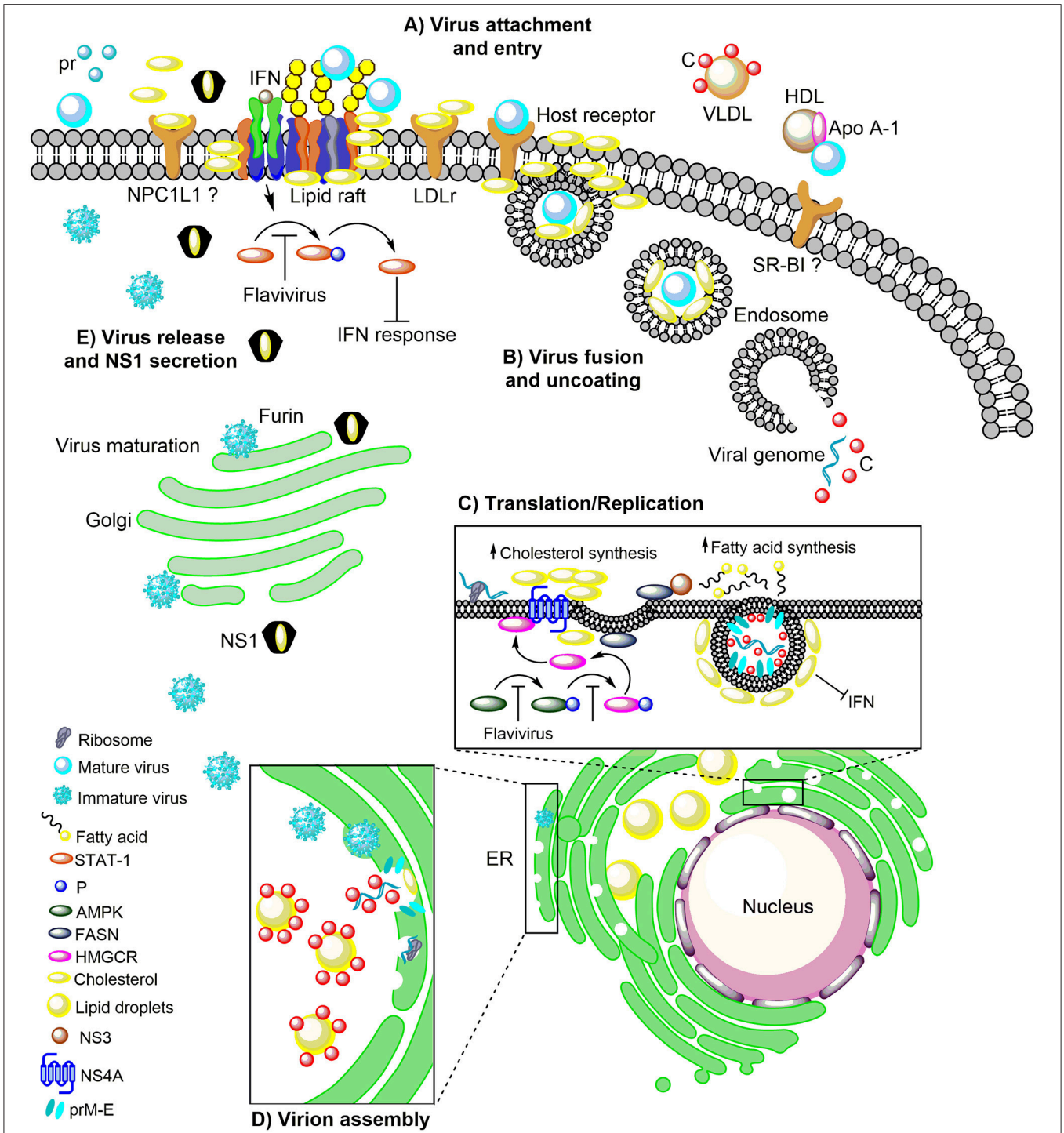

FIGURE 1 | The role of host cholesterol during the replicative cycle of the flaviviruses. **(A)** Upon attachment to the cell surface, virus particles bind to the distinct receptors found in lipid rafts, cholesterol-rich plasma membrane microdomains, to trigger clathrin-dependent endocytosis. Immediately after attachment and entry, there is an increase in cholesterol levels, which correlates with an augment of the LDLr, on the surface of the infected cell. The importance of NPC1L1 and SR-BI in flaviviral infection has been suggested. **(B)** Following, the low pH in the endocytic vesicle induces fusion between endosomal and viral membranes. **(C)** The viral RNA into the cytoplasm is transported to the ER where it is translated into the three structural proteins (C, prM, and E) and the seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). RNA replication occurs in NS4A protein-induced membrane invaginations called RC, where the fatty acids and cholesterol synthesis take place. In this event, NS3 recruit the FASN to the ER increasing its activity. Additionally, the flaviviral infection inhibits the AMPK activity inducing a reduction in the phosphorylation levels of HMGCR, which leads to an increase in its activity and cholesterol synthesis. This mechanism occurs in the ER where the FASN and HMGCR enzymes are found associated with the viral proteins NS3 and NS4A. **(D)** The flavivirus-induced membranes are rich in cholesterol and serve as a

(Continued)

FIGURE 1 | scaffold for the assembly of the viral progeny. In the ER the C protein is accumulated on the LDs for the packaging of the viral genome and the nucleocapsid formation. Later the nucleocapsids bud in the ER to complete the assembly of immature virions which are transported to the Golgi complex for its maturation. **(E)** The maturation of viral particles occurs along the secretory pathway where the low pH triggers rearrangements in the structural proteins (prM) to allow the proteolytic cleavage of the pr peptide by furin protease. Finally, mature (infectious) virions are released from the cell by exocytosis. The DENV NS1 protein is also secreted and has a peculiar three-dimensional fold as hexamer that forms a lipoprotein particle with an open-barrel protein shell and a central channel rich in lipids (cholesterol), reminiscent of the composition of high-density lipoprotein. Furthermore, NS1 play an essential role in the pathogenesis, viral replication, and immune evasion. In this sense, the flaviviruses block the IFN signal transduction pathway either by inhibiting of the phosphorylation of STAT-1 which is found in the cholesterol-rich microdomains (lipid rafts) or hijacking cell elements to form their cholesterol-rich RC that avoid the recognition of viral component for the PRR. LDLr, Low-density lipoprotein receptor; NPC1L1, Niemann Pick C1-like 1 receptor; VLDL, Very low-density lipoprotein; DENV, Dengue virus; Apo A-1, Apolipoprotein A-1; HDL, High-density lipoprotein; SR-BI, Scavenger receptor class B type I; ER, Endoplasmic reticulum; RC, Replicative complexes; FASN, Fatty acid synthase; AMPK, Adenosine Monophosphate-activated Protein Kinase; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; SREBP, Sterol regulatory element binding protein; LDs, Lipid droplets; IFN, Interferon; STAT-1, Signal transducer and activator of transcription 1; P, Phosphorylation; PRR, Pattern recognition receptors.

2009). In addition, it has been described that the molecular tweezer CLR01, a small molecule that previously has been shown to inactivate some viruses through a selective interaction with the host-membrane-derived lipid bilayer of the viral envelope, inhibit EBOV (ebola virus) and ZIKV infection (Röcker et al., 2018).

Immediately after attachment and entry of the viral particles by clathrin-mediated endocytosis (Chu et al., 2006; Mosso et al., 2008; Acosta et al., 2009), at one and 48 h post-infection, an increase in cholesterol levels is observed in infected cells in mammalian cells (Soto-Acosta et al., 2013). This increment correlates with an increase in the presence of the low-density lipoprotein receptor (LDLr) on the surface of infected cells and with an augment in the cholesterol uptake (Soto-Acosta et al., 2013), indicating that cholesterol is essential during the first few hours of infection. On the other side, has been described that the structural protein C of DENV can interact with very low-density lipoproteins (VLDL) (Faustino et al., 2014). Faustino et al., using atomic force microscopy-based force spectroscopy, dynamic light scattering, nuclear magnetic resonance, and computational approach; demonstrated that dengue viral capsid proteins (C protein) bind to very low density lipoprotein (VLDL) surfaces (Faustino et al., 2014) (**Figure 1A**). This observation suggests the formation of lipoviroparticles (LVPs) in DENV infection. However, the presence of LVPs has not been observed during *in vivo* DENV infection, and the direct function of LVPs in DENV attachment or entry steps has not been analyzed (Reyes-del Valle et al., 2014). Besides, there is a report where apolipoprotein A-1 (Apo A-1), the main component of high-density lipoprotein (HDL), interact with DENV particles and facilitates viral entry through the scavenger receptor class B type I (SR-BI), the cell receptor for Apo A-I (Li et al., 2013) (**Figure 1A**). These observations provide evidence on the functional importance of lipoproteins and cholesterol uptake through cholesterol receptors during DENV infection. Moreover, the importance of the intracellular trafficking of cholesterol during the DENV entry has been demonstrated when this traffic is inhibited by the drug U18666A which mimic Niemann-Pick type C disease (hereditary lysosomal storage disease), causing the accumulation of cholesterol and the entrapment of DENV particles in late endosomes and lysosomes, reducing levels of viral genome released into the cytoplasm of treated cells (Poh et al., 2012).

Host Cholesterol in Viral Fusion

As enveloped viruses, flaviviruses need to be uncoated to release the viral RNA into the cytoplasm (**Figure 1B**). Uncoating is induced by the low pH environment of the endosomes, where the viral proteins enter into a fusion-active state and initiate the merging of the viral envelope with the endosomal membrane, thereby releasing the viral RNA genome into the cytoplasm (Kaufmann and Rossmann, 2011; Smit et al., 2011). This process requires two steps, the fusion between the viral and endosomal membranes and the uncoating of the protective capsid shell (Rumlová and Ruml, 2018). To analyze the fusion event, the lipid composition of the viral membrane of different viruses has been characterized (Brügger et al., 2006; Kalvodova et al., 2009; Merz et al., 2011; Gerl et al., 2012; Reddy and Sansom, 2016). In the *Flaviviridae* family, an essential role of membrane virion-associated cholesterol has been demonstrated for all serotypes of DENV (Carro and Damonte, 2013). As it has been shown for HCV (Aizaki et al., 2008) (another member of the family *Flaviviridae*, but not an arbovirus), the presence of cholesterol in DENV virions was more crucial for infection (Carro and Damonte, 2013). In this regard, Carro and Damonte showed that after exposure of DENV particles to MβCD a loss of infectivity of the four serotypes, associated with a reduction in the cholesterol content of the virions was observed.

Moreover, the addition of exogenous water-soluble cholesterol or fetal bovine serum did not fully recover the infectivity of virions, except, when a simultaneous incubation with MβCD and serum cholesterol was performed (Carro and Damonte, 2013); this is consistent with others reports in which authors try to restore the cholesterol that has been depleted from viral particles (Sun and Whittaker, 2003; Lee et al., 2008; Desplanques et al., 2010).

Interestingly, when virions were incubated only with exogenous cholesterol, an inactivating effect on particle infectivity was observed. This effect is similar that the one observed by Lee et al., allowing to suggest that excess in cholesterol could induce an increase in envelope rigidity, which in turn would limit the ability of the viral membrane to fuse inhibiting viral entry (Lee et al., 2008). The MβCD removes not only cholesterol but also other components as phospholipids from the membranes. Therefore, the possibility that MβCD alters the composition of other phospholipids in the viral particle has to be considered (Zidovetzki and Levitan, 2007; Carro and Damonte, 2013). On the other hand, in an *in vitro*

study, using a liposomal model system, it has been revealed that flaviviruses such as WNV can fuse with these receptor-free artificial lipid membranes, consisting of phosphatidylcholine and phosphatidylethanolamine at low pH, although with low efficiency. However, the addition of cholesterol to the target membranes has a strong promoting effect on the fusion capacity of WNV (Moesker et al., 2010). Other studies of virus-liposome co-flotation have indicated that cholesterol stimulates the interaction of glycoprotein E with lipid membranes (Stiasny et al., 2003; Umashankar et al., 2008), confirming the importance of cholesterol and specifically of the 3-hydroxyl group of cholesterol for this function. In contrast, the glycoprotein E of alphaviruses does not appear to interact directly with cholesterol in the target membrane (Umashankar et al., 2008). These observations suggest that cholesterol could be required to induce changes in the fluidity of the viral membrane or changes in the physicochemical properties of the membrane required during viral and endosomal membranes fusion (Smit et al., 2011). In an attempt to analyze the molecular composition of the flavivirus envelope, the composition of the lipid envelope of WNV virions was studied. Authors describe that the viral membrane is enriched in sphingolipids (sphingomyelin) and contains reduced levels of phosphatidylcholine (Martín-Acebes et al., 2014). Interestingly, by constructing a computational model of the DENV envelope, in which the known structure of the DENV membrane proteins was combined with a lipidic bilayer model. The authors found that despite the absence of cholesterol in the envelope, the virion presented a biophysical robustness that coincided with the level of cholesterol in the membrane of the influenza A virus (Reddy and Sansom, 2016). Thus, more studies have to be performed to determine the contribution of cholesterol to the biophysical properties of the viral particle. It is evident that the viral membrane is acquired in the ER (Welsch et al., 2009). Thus, the properties of the envelope may depend on the host from which the virions are released.

Host Cholesterol in Viral Translation/Replication

After internalization and uncoating (Samsa et al., 2009), the viral RNA is translated into a polyprotein which is cleaved into structural and non-structural proteins. The viral proteins synthesized such as the NS4A protein induces the ER-membrane remodeling to form membrane curvatures (Roosendaal et al., 2006; Miller et al., 2007). Consequently, the extensive membrane curvature could reduce the effective surface area and functionality of the ER (Heaton et al., 2010). Moreover, these invaginations of the ER leads to the redistribution of the FASN (Heaton et al., 2010) and the cholesterol-synthesizing enzyme, the HMGCR (Soto-Acosta et al., 2017) required for the synthesis of mevalonate, a precursor of the cholesterol, which is upregulated in response to cholesterol depletion (Goldstein and Brown, 1990). It is suggested that this mechanism occurs when NS3 recruit to FASN in the ER where the N-terminal 180 amino acids of NS3 have interaction with FASN and this association increase the FASN activity and the fatty acid biosynthesis to augment the lipid biogenesis to form the replicative complexes (Heaton et al., 2010) (**Figure 1C**). Moreover, the HMGCR is redistributed from the outer membrane of the nuclear envelope, where it is synthesized and localized (Pathak et al., 1986) to sites of viral replication (Peña and Harris, 2012; Soto-Acosta et al., 2017). Interestingly, although the induction of HMGCR expression during DENV infection does not occur, an increase in the activity of the enzyme due to a reduction of its phosphorylation levels was observed (Soto-Acosta et al., 2013). However, the action of viral proteins NS3 and NS4A on the HMGCR activity is unknown and the mechanism by which NS3 stimulates FASN-specific activity needs to be investigated further (Heaton et al., 2010). In summary, the presence and the increased activity of the FASN and HMGCR enzymes in the ER and their association with viral proteins such as NS3 and NS4A increase the *de novo* synthesis of fatty acids and cholesterol (Heaton et al., 2010; Soto-Acosta et al., 2017) (**Figure 1C**). This event induces the formation of the RC that comprise membrane packets (Vp), double-membrane vesicles (Ve), tubular structures (T), and convoluted membranes (CM) (Welsch et al., 2009; Junjhon et al., 2014). Inside of the Ve where the NS1, NS3, and NS5 proteins and dsRNA are localized, the RNA replication occurs (Welsch et al., 2009; Junjhon et al., 2014).

The complex set of membranes required for the viral replication is host-dependent (Perera et al., 2012). Consequently, the structures of convoluted membranes (CM) are found in DENV-infected mammalian cells (Welsch et al., 2009), but they are not induced in DENV-infected C6/36 cells (Junjhon et al., 2014; Reyes-Ruiz et al., 2018). Since the cholesterol contributes to the stability of subcellular structures, it is possible that the absence of CM could be related with the fact that the mosquitoes are cholesterol auxotrophs (CLAYTON, 1964; Rawson, 2003) and the amount of this molecule is lower in mosquito cells compared to mammalian cells. Under experimental conditions, the bovine serum from the culture medium provides the cholesterol that mosquito cells required (CLAYTON, 1964; Krebs and Lan, 2003). Considering that ∼1% of unesterified cholesterol is found in the ER (Lange et al., 1999; Liscum and Munn, 1999), the cholesterol requirement during viral replication is high. Thus, viruses directly manipulate the host cell pathways involved in the uptake and biosynthesis of cholesterol to increase the levels. When the cholesterol is low in the ER, the cholesterol sensor SREBP (Sterol regulatory element binding protein)-SCAP (SREP cleavage activating protein) complex is transported to the Golgi apparatus where the cytoplasmic domain of SREBP is cleaved and the protein is translocated into the nucleus acting as a transcription factor to induce HMGCR and LDLr gene transcription (Vallett et al., 1996). It has been reported that upregulation of enzymes involved in the intermediate steps of cholesterol biosynthesis such as HMGCR and mevalonate diphospho decarboxylase (MVD) lead to higher cholesterol levels in the ER which favor the replication of WNV (Mackenzie et al., 2007) and DENV (Rothwell et al., 2009; Soto-Acosta et al., 2013, 2017). On the other hand, the modulation of exogenous cholesterol uptake by critical proteins such as the LDLr plays an essential role in flavivirus replication (Poh et al., 2012; Soto-Acosta et al., 2013). The use of drugs that inhibit cholesterol biosynthesis pathway can alter the RC formation inhibiting virus

infection (Mackenzie et al., 2007; Rothwell et al., 2009; Martínez-Gutierrez et al., 2014; Soto-Acosta et al., 2017); demonstrating the essential role of the *de novo* synthesis of cholesterol during viral replication.

In mosquito cells little is known about the role of cholesterol during replication. However, Perera et al. demonstrated that DENV induces changes in the lipids profile of infected mosquito cells (Perera et al., 2012). Unfortunately, their mass spectrometry analysis was unable to successfully separated cholesterol. On the other hand, in *Wolbachia*-infected mosquito cells, it has been reported the upregulation of the apolipoprotein D, the ATP-binding cassette protein A1 (ABCA1), and a homolog of cholesterol transporter NPC2, and the downregulation of the LDLr (Geoghegan et al., 2017). All these proteins are involved in the cholesterol homeostasis. These results are exciting because the mosquitoes are cholesterol auxotrophs (Rawson, 2003) as well as the *Wolbachia* bacteria (Wu et al., 2004). Thus, both depend on and compete for cholesterol (Caragata et al., 2013). This competence for cholesterol is one of the reason that justify the inhibition of viral replication by *Wolbachia* (Moreira et al., 2009; Bian et al., 2010; van den Hurk et al., 2012; Caragata et al., 2016; Dutra et al., 2016; Geoghegan et al., 2017). However, further studies have to be performed to completely elucidate the role of cholesterol in flaviviral replication in mosquito infected cells.

The Importance of Host Cholesterol in the Viral Assembly

The flavivirus-induced membrane rearrangements also serve as a scaffold for the assembly of the viral progeny. After uncoating, translation and genome replication, the assembly of the viral particles is carried out in the replication complexes induce in the ER. These assembly sites have a high activity of enzymes such as the cholesterol-synthesizing enzyme, HMGCR and MVD involved in the cholesterol biosynthesis pathway (Mackenzie et al., 2007; Rothwell et al., 2009; Soto-Acosta et al., 2013, 2017), which leads to the replication complexes being rich in cholesterol. The sites for viral assembly and budding have been reported as the membrane packets (Vp), which contain anchored the viral E and prM proteins to their membrane (Junjhon et al., 2014). Although the mosquito lacks the biosynthetic pathways to produce cholesterol, the Vp are present in both, mammalian and mosquito cells (Welsch et al., 2009; Junjhon et al., 2014; Reyes-Ruiz et al., 2018). The first step in the flavivirus assembly is the packaging of one copy of the viral RNA in multiple copies of the capsid (C) protein to form the nucleocapsid (Lindenbach and Rice, 2003). The viral C protein is located in a close proximity of the ER surrounding the structures called lipid droplets (LDs) (Samsa et al., 2009; Carvalho et al., 2012), which are structurally similar to circulating lipoproteins with a core of esterified lipids, where the cholesterol is an essential component (Ducharme and Bickel, 2008). The viral RNA recruitment process by the C protein to form the nucleocapsid is still unclear. However, it has been suggested that the C protein or the C protein-RNA complex is accumulated on the LDs during infection to be then mobilized to viral assembly sites (Samsa et al., 2009; Carvalho et al., 2012) (**Figure 1D**). Interestingly, the accumulation of C protein on LD occurs in both infected mammalian and mosquito cells, which suggest that the LD are organelles with a conserved function in the viral replication in different hosts (Samsa et al., 2009) and crucial for viral assembly. Once that the nucleocapsid is formed, this is enveloped by a lipid membrane (Kuhn et al., 2002), from the ER where are found the prM-E heterodimers to complete the assembly of immature virion and then the viral particle is transported to the Golgi complex for its maturation (Mukhopadhyay et al., 2005; Yu et al., 2008; Welsch et al., 2009).

Role of Cholesterol in Viral Release and NS1 Secretion

The last step of the flaviviruses replicative cycle is the release of viral particles by exocytosis (Barrows et al., 2018). The viral particles to become infectious have to maturate by the cleavage of prM protein by the host protease furin (Pierson and Diamond, 2012) (**Figure 1E**). In this step, the participation of cholesterol has not been described.

On the other hand, during flavivirus infection, the non-structural protein 1 (NS1) is secreted (Scaturro et al., 2015). The NS1 protein is one of the most enigmatic proteins of flaviviruses because it is the only protein that is secreted, playing essential roles in immune evasion, pathogenesis and viral replication (Scaturro et al., 2015). The reported crystal structure of the secreted DENV NS1 protein revealed its peculiar three-dimensional fold as hexamer that forms a lipoprotein particle with an open-barrel protein shell and a central channel rich in lipids, reminiscent of the composition of high-density lipoprotein (Gutsche et al., 2011). NS1-associated lipid species include triglycerides, as well as cholesterol and phospholipid esters, a composition that evokes plasma lipoproteins in humans involved in vascular homeostasis. Thus, the discovery that DENV NS1 carries lipids could have critical pathophysiological implications during the disease (Gutsche et al., 2011) and supports the idea that cholesterol is required for the secretion of NS1 (**Figure 1E**).

MODULATION OF THE HOST-IMMUNE RESPONSE MEDIATED BY CHOLESTEROL

All flaviviruses share similar replication strategies manipulating host cell functions for successful infection. One of these strategies is the evasion of the antiviral responses in both, the hematophagous invertebrate vectors and vertebrate hosts (Ciota and Kramer, 2010; Coffey et al., 2013). The type I interferon (IFN) response constitutes the first line of defense for the early control of viral infections (Muñoz-Jordán et al., 2003; Morrison et al., 2012; Castillo Ramirez and Urcuqui-Inchima, 2015). This response is started by the physical interaction of the viral molecules with the host-pathogen recognition receptors (PRR) (van Boxel-Dezaire et al., 2006; Basler and García-Sastre, 2007; Fernandez-Garcia et al., 2009). Then, the recognition triggers a signaling pathway that activates transcription factors which induce the expression of IFN-α/β, which bind to specific receptor on the cell surface triggering Jak-STAT antiviral signaling pathway (van Boxel-Dezaire et al., 2006; Basler and García-Sastre, 2007). However, several studies have demonstrated that

the flavivirus NS2A, NS2B-3, NS4A, NS4B, and NS5 proteins inhibit type I IFN response (Muñoz-Jordán et al., 2003, 2005; Fredericksen and Gale, 2006; Ashour et al., 2009; Mazzon et al., 2009; Laurent-Rolle et al., 2010; Rodriguez-Madoz et al., 2010). Moreover, the flaviviruses hijack cellular elements in the ER membranes to induce the formation of their cholesterol-rich RC (Mackenzie et al., 2007; Rothwell et al., 2009; Soto-Acosta et al., 2013) where the recognition of viral component for the PRR is avoided, inhibiting IFN response until the replication process is completed (Welsch et al., 2009; Gillespie et al., 2010; Uchida et al., 2014; Miorin et al., 2016).

On the other hand, the key components of the IFN signal transduction pathway are the STAT proteins (STAT1 and 2), which are found in the cholesterol-rich microdomains, lipid rafts (Sehgal et al., 2002; Shah et al., 2002; Marchetti et al., 2006). Interestingly, the flaviviral infection induces the biosynthesis and redistribution of the cellular cholesterol to the RC also leads to the disruption of the cholesterol-dependent surfaces domains founds in the plasma membrane at later times of infection, inactivating the IFN-regulated Jak-Stat signaling pathway (Mackenzie et al., 2007) (**Figure 1**). This pathway is also inactivated directly by the non-structural proteins as described before (Muñoz-Jordán et al., 2003, 2005; Fredericksen and Gale, 2006; Ashour et al., 2009; Mazzon et al., 2009; Laurent-Rolle et al., 2010; Rodriguez-Madoz et al., 2010). Additionally, it is important to note that there is a close correlation between cholesterol and the type I IFNs response during viral infections because high cholesterol levels induce a poor IFN response (Liu et al., 2013). Thus, the reduction of cholesterol levels during flavivirus infection could guarantee an increase in the response of the IFN.

CHOLESTEROL-LOWERING THERAPY AS POTENTIAL THERAPEUTICS IN FLAVIVIRAL INFECTIONS

Arbovirus infections, especially those caused by flaviviruses such as DENV, ZIKV, WNV, and YFV, represent an immense global health problem (Boldescu et al., 2017). Therefore, it is urgent to find broad-spectrum agents that provide an opportunity to treat these infections. Since there are no specific drugs to treat the flaviviral diseases and aspects such as high genetic variability (Lim et al., 2013), antibody-dependent enhancement (ADE), and cross-reactivity between flaviviruses, make difficult to obtain a fully effective vaccine against all flavivirus (Villar et al., 2015; Halstead and Russell, 2016). The traditional approach for antiviral design is to target critical viral factors. However, this strategy in RNA viruses which do not have a proofreading function leads to a high rate of mutation in the virions, which induce the development of resistance to the antiviral drugs (Acosta and Bartenschlager, 2016; Boldescu et al., 2017). The other possibility is to target host factors. The genus Flavivirus belonging to the Flaviviridae family, which includes DENV, ZIKV, and WNV differs in many aspects, such as the mode of transmission or the course of the infection. However, the fundamental replication strategy of the members of the family is similar, as it requires the formation of organelle-like structures defined as RC, where replication of the genome of positive-strand RNA [(+) RNA] viruses occur in close association with cellular endomembranes (Neufeldt et al., 2018). The RC formation requires among other components, cholesterol (Welsch et al., 2009; Acosta and Bartenschlager, 2016; Neufeldt et al., 2018). Changes in serum cholesterol have been correlated with clinical manifestations in DENV infected patients (van Gorp et al., 2002; Suvarna and Rane, 2009; Biswas et al., 2015; Durán et al., 2015; Osuna-Ramos et al., 2018). Therefore, the development of therapeutic strategies to restrict biosynthesis or cholesterol absorption with FDA-approved drugs, like statins, is an attractive option with broad-spectrum activity possibilities. Statins are molecules of fungal origin that inhibit a crucial step in the biosynthetic pathway of sterol (inhibitors of the HMGCR) (Sirtori, 2014). Statins are powerful cholesterol-lowering drugs used in the therapy of dyslipidemias and have contributed significantly to the prevention of cardiovascular disease (Sirtori, 2014). There are several studies on the use of statins in DENV infection, ranging from cellular to preclinical and clinical trials where opposite effects have been observed (Martínez-Gutierrez et al., 2011, 2014; Whitehorn et al., 2016). For example, *in vivo* models of infection and treatment found that the use of Lovastatin inhibits DENV RNA replication and viral secretion in primary cultures of human monocytes and other cell lines (Rothwell et al., 2009; Martínez-Gutierrez et al., 2011, 2014; Soto-Acosta et al., 2013; Bryan-Marrugo et al., 2016). In the AG129 mouse model, permissive for DENV infection, lovastatin treatment resulted in a 2-day delay in virus-induced mortality, which was independent of the time point at which treatment was initiated (Martínez-Gutierrez et al., 2014). The delay in mortality in the mice model did not correlate with a reduction in viral RNA levels. Considering that statins are widely used and well-tolerated, they could be candidates for a prophylactic emergency antiviral regimen in DENV infections (Boldescu et al., 2017). However, lovastatin treatment was unable to inhibit DENV infection in a clinical trial in humans (Whitehorn et al., 2016). Although it is well known that in humans statins modify systemic lipid levels, the real effect on cellular cholesterol content is unclear. Thus, it is possible that the inability of the statins to inhibit DENV infection could be related with only a partial reduction in cellular cholesterol content, which could be rapidly compensated.

On the other hand, there is an indirect way to induce inactivation of the HMGCR enzyme, using metformin, a drug commonly prescribed to treat patients with type 2 diabetes (Crofford, 1995). This drug increases the activity of the AMPK, the sensor of cellular energy, resulting in the inhibition of HMGCR activity and thus to the suppression of cholesterol biosynthesis (Kawaguchi et al., 2002; Foretz and Viollet, 2011; Koren-Gluzer et al., 2013). Interestingly, in recent work by Soto-Acosta et al. (2017) a significant inhibition in DENV replication was observed in cells treated with metformin, supporting its antiviral activity. Moreover, the use of other AMPK activators such as NDGA or 6-choloro-5-[4-(1-hydroxycyclobutyl) phenyl]-1*H*-indole-3-carboxylic acid (PF-06409577), also induces a strong inhibition in WNV, ZIKV, and DENV infection (Soto-Acosta et al., 2014; Merino-Ramos et al., 2017; Jiménez de

Oya et al., 2018). Interestingly, in a retrospective cohort study of adult diabetics patients with DENV infection treated with metformin had a ∼37% lower risk of developing severe dengue (Htun et al., 2018), suggesting that metformin has a potential protective effect against dengue disease.

Another compound related with the inhibition of cholesterol metabolism is that related with the blockage of enzyme 7-dehydrocholesterol-Δ7-reductase that performs the final conversion step of 7-dehydrocholesterol to cholesterol, by AY-9944 (Mackenzie et al., 2007), also inhibit flavivirus replication. Moreover, other compounds as the okadaic acid that modulate the of PP2A phosphatase activity (Soto-Acosta et al., 2017) and the hymeglusin and zaragozic acid A which inhibit the HMGCR synthase and squalene synthetase, respectively (Rothwell et al., 2009), display an anti-flaviviral effect. In the same line of evidence, the treatment with 25-hydroxycholesterol (25-HC) which decreases the amount of SREBP-1 (Wang et al., 1994), a factor that enhances transcription of the LDLr and HMGCR genes, inhibits the flaviviral infection (Mackenzie et al., 2007), and reduces the viremia providing protection against ZIKV in mice and rhesus macaques (Li et al., 2017). Additionally, the disruption of the lipid raft formation by MβCD that deplete cellular cholesterol; the cholesterol chelation with filipin (Lee et al., 2008); and the decrease of the fatty acid biogenesis by cerulenin and C75 (Heaton et al., 2010) induce viral inhibition at entry and replication levels. In the case of the C75 drug also decreases the number of lipid droplets affecting the production of the viral particles (Samsa et al., 2009) and the amphipathic steroid 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A) that reduces the cholesterol biosynthesis and also block the intracellular trafficking of cholesterol (Poh et al., 2012), decreases DENV infection.

Further analysis in animal models and clinical trials is essential to determine whether the use of drugs such as other statins, metformin or other AMPK activators in single-dose or in combination as a treatment against flavivirus infections is feasible.

Since the increase in cholesterol levels during flavivirus infection is the consequence of an increase in cholesterol synthesis and an increase in cholesterol uptake through the LDL receptors (Soto-Acosta et al., 2013), it may be possible to inhibit viral infection not only inhibiting synthesis but also decreasing uptake. Recent evidence suggests that pre-treatment with bovine lactoferrin inhibits infection of all four DENV serotypes in Vero cells blocking the binding of DENV to the cell membrane by interaction with heparan sulfate, DC-SIGN, and LDLr (Chen et al., 2017). Bovine lactoferrin might inhibit infection with DENV or other flaviviruses by binding to potential receptors, such as LDLr. Another receptor for cholesterol uptake that can be pharmacologically blocked by the FDA-approved drug ezetimibe is the Niemann-Pick C1 Like 1 NPC1L1 (Garcia-Calvo et al., 2005; Ge et al., 2008). Ezetimibe is a potent inhibitor of cholesterol absorption that has been approved for the treatment of hypercholesterolemia. Rodent livers express only a negligible amount of NPC1L1 (Altmann et al., 2004). However, humans have detectable levels of NPC1L1 in the liver cells (Betters and Yu, 2010). The liver is an important target organ in flavivirus infection (Thepparit et al., 2004). Thus, ezetimibe could be considered a suitable candidate against infections caused by flavivirus (DENV, ZIKV, YFV, or WNV), just as it already had against HCV (Sainz et al., 2012) and hepatitis B virus (HBV) (Lucifora et al., 2013). Since viral infection induces an increase in cholesterol uptake and synthesis to support flavivirus replication (Rothwell et al., 2009; Poh et al., 2012; Soto-Acosta et al., 2013, 2014), a combination of drugs, which inhibit uptake and synthesis, may induce a more efficient HDA therapy for flavivirus inhibition.

CONCLUDING REMARKS

Viral infections transmitted by mosquitoes, such as those caused by the flaviviruses DENV, YFV, WNV, and ZIKV represent essential health challenges. The presence of the four DENV serotypes in many parts of the world, the propagation of the mosquito vector and the climate conditions for mosquito proliferation increase the risk to contract severe dengue. On the other hand, the association between ZIKV infection with microcephaly in neonates and with Guillain-Barré syndrome in adults, in addition to its ability to be transmitted through sexual and transplacental routes, made it a threat to global health. Finally, although YFV was confined mainly to monkeys, outbreaks in humans in Angola and Brazil have generated an alarm. Although in Brazil part of the population is immunized and new vaccination programs are in progress, in the rest of America and the world, most of the human population is not immunized. These facts highlight the importance of the study of flaviviruses and the development of new control strategies. It is well-known that every step of the viral life cycle, from entry to viral release requires close interaction with cellular proteins. Therefore, viruses hijack cellular proteins, components and processes to be replicated in the host cell. One of these components is the cellular cholesterol, required for the formation of RC, present in the ER. The reduction in the cholesterol levels with FDA-approved drugs that inhibit cholesterol synthesis or uptake *in vitro* and *in vivo* models induce a reduction of infection with DENV and ZIKV among others. Additionally, the close interaction between cholesterol synthesis and IFN response pathway indicate that the reduction in cholesterol levels could also increase IFN response inhibiting viral infection. Thus, cholesterol can be considered an excellent target to inhibit flavivirus infection. The use of FDA-approved cholesterol-lowering drugs alone or in combination can be tested in new clinical trials.

AUTHOR CONTRIBUTIONS

JO-R, JR-R, and RdA preparing manuscript, writing, correction, and figures design. JR-R and JO-R performed infection and performed microscopy assays.

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REVIEW
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Cellular and Molecular Immune Response to Chikungunya Virus Infection

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Chikungunya virus (CHIKV) is a re-emergent arthropod-borne virus (arbovirus) that causes a disease characterized primarily by fever, rash and severe persistent polyarthralgia. In the last decade, CHIKV has become a serious public health problem causing several outbreaks around the world. Despite the fact that CHIKV has been around since 1952, our knowledge about immunopathology, innate and adaptive immune response involved in this infectious disease is incomplete. In this review, we provide an updated summary of the current knowledge about immune response to CHIKV and about soluble immunological markers associated with the morbidity, prognosis and chronicity of this arbovirus disease. In addition, we discuss the progress in the research of new vaccines for preventing CHIKV infection and the use of monoclonal antibodies as a promising therapeutic strategy.

Keywords: Chikungunya virus, immune response, immunovirology, innate immunity, adaptative immunity, immunological markers, vaccines

INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne virus that belongs to the *Togaviridae* family (genus *Alphavirus*), and was first isolated in 1952–53 from mosquitos and from human serum during an epidemic in Tanzania (Robinson, 1955). CHIKV causes a self-limiting disease known as Chikungunya fever (CHIKF) that is characterized by high fever, rash, myalgia, polyarthralgia and headaches (Burt et al., 2017). While many of the symptoms generally disappear within 1 week, joint pain can persist in some patients for up to a few years (Javelle et al., 2015; Rodriguez-Morales et al., 2016). Over the past decade, the disease caused by CHIKV re-emerged as a serious public health problem and resulted in several outbreaks around the world (Wahid et al., 2017). Although CHIKV has been studied for over 60 years, little is known about immunopathogenesis caused by this virus and about protective immune response against it. In this review, we briefly outline the characteristics of CHIKV, including its structure, transmission, epidemiology, and diagnosis. We then focus on the innate and adaptive immune responses and soluble immunological markers.

CHIKUNGUNYA VIRUS

CHIKV is an enveloped alphavirus of ∼60–70 nm in diameter. It has an 11.8 kb-long single-stranded positive-sense RNA genome that encodes six structural (C-E3-E2-6K/TF-E1; Metz and Pijlman, 2016) and four non-structural (nsP1, helicase nsP2, nsP3 and polymerase nsP4; Ahola and Merits, 2016) proteins. Genomic RNA associates with 240 copies of 261 amino acid-long structural capsid protein C that forms icosahedral nucleocapsid (Khan et al., 2002; Jose et al., 2009). E1 and E2 are surface glycoproteins, 439 and 423 amino acid-long, respectively (Khan et al., 2002). E1 and E2 carry the major viral epitopes and participate in the attachment and the entry of the virus into target cells, where E2 is responsible for receptor binding, and E1–for membrane fusion (Voss et al., 2010). E3 consists of 64 amino acids that are required for E3-E2-6K-E1 or E3-E2-TF polyprotein translocation into the endoplasmic reticulum for virus spike formation (Snyder and Mukhopadhyay, 2012). The 61 amino acid-long 6K protein is a cation-selective ion channel that is responsible for increased cell permeability to monovalent cations and virion budding during infection (Melton et al., 2002). Transframe protein TF is produced as a result of C-terminal extension of 6K protein in the −1 frame (Firth et al., 2008). It retains ion-channel activity similar to that of 6K and appears to be important for the virus particle assembly and release (Snyder et al., 2013). Although the non-structural proteins nsP1-nsP4 are primarily associated with the viral replication process (Solignat et al., 2009; Lum and Ng, 2015), they carry out additional functions during the viral infection, just like in other alphaviruses (Rupp et al., 2015). It is worth noting that non-structural proteins are not packaged into the final virions, and hence the structural proteins (mainly surface glycoproteins E2 and E1) are the key targets of the host humoral immune response and of most anti-CHIKV vaccines (Powers, 2018).

EPIDEMIOLOGY AND VECTORS

CHIKV is a zoonotic virus that uses several non-human primates (NHPs) and possibly other vertebrates as amplification hosts (Tsetsarkin et al., 2016), which could also serve as virus reservoirs (Althouse et al., 2018) during inter-epidemic periods.

The first reported case of CHIKV human infection happened in Tanzania in 1952–53. Since then, several outbreaks occurred throughout the African continent (Robinson, 1955; Powers et al., 2000). Between 1960 and 1980, the virus was identified in Central, Western and Southern Africa (Powers et al., 2000), and in following years—in India and other countries of Asia and Africa (Wahid et al., 2017). Phylogenetic reconstruction of CHIKV evolution identified Asian, East/Central/South African (ECSA) and West African lineages which until 2004 (Sam et al., 2015) were mostly confined to the geographic regions after which they were named (Schuffenecker et al., 2006; Sudeep and Parashar, 2008; Wahid et al., 2017).

The first case of autochthonous transmission of CHIKV in the Americas was reported on Saint Martin Island in 2013 (Leparc-Goffart et al., 2014), and it was shown that the risk exists that CHIKV will establish enzootic/sylvatic cycle in the tropical regions of the American continent (Lourenço-de-Oliveira and Failloux, 2017). Increased traveling in the recent years and the presence of appropriate vectors allowed for a further spread of CHIKV with reports from the United States (Kendrick et al., 2014), Brazil (Tanabe et al., 2018), Spain (Bocanegra et al., 2016), Italy (Zammarchi et al., 2016) and Australia (Huang et al., 2017), among others (Wahid et al., 2017). CHIKV virus spread and outbreaks around the world in the last years are shown on **Figure 1**.

CHIKV disease (CHIKVD) has enzootic/sylvatic and urban cycles of transmission (Weaver, 2013) and occurs through a bite of infected female mosquitoes of *Aedes* genus (Sudeep and Parashar, 2008). *Aedes aegypti* and *Aedes albopictus* are the two most significant and well-documented CHIKV vectors, associated with outbreaks worldwide (Mourya and Mishra, 2006). The urban cycle of transmission is possible because of the sufficiently high levels of viremia developed in the infected individuals (Go et al., 2014) and it can start with the spillover of enzootic/sylvatic CHIKV via bridge vectors, such as *Aedes furcifer* (Diallo et al., 2012). The spread of CHIKV in the United States and Europe was linked to the adaptation of the ECSA strains to *Aedes albopictus* mosquitoes that are abundant in these regions (Madariaga et al., 2016). This adaptation to a different vector was attainable due to a mutation in the envelope protein gene (E1-A226V; Tsetsarkin et al., 2007, 2011), which is sometimes regarded as giving rise to Indian Ocean lineage (Wahid et al., 2017). Several other mutations that further enhance fitness and adaptation of CHIKV to its hosts were identified in E1 and E2 proteins (Singh et al., 2012; Agarwal et al., 2016), and were shown to occur in the intrinsically disordered regions of these proteins (Singh et al., 2018).

Cases of maternal-fetal transmission were reported (Ramful et al., 2007; Gérardin et al., 2008; Economopoulou et al., 2009) and the virus was detected in human breast milk (Campos et al., 2017), although the data on the impact of the infection is somewhat controversial (Laoprasopwattana et al., 2015; Torres et al., 2016), and experimental data from Rhesus macaques (*Macaca mulatta*) speaks against possibility of trans-placental transmission (Chen et al., 2010).

DIAGNOSIS OF CHIKV INFECTION

To date, several different methods are used to diagnose the CHIKV infection. These methods are based on the detection of (i) viral RNA (Pfeffer et al., 2002; Pastorino et al., 2005), (ii) IgM and IgG antibodies against the virus (or viral antigens proper; Kashyap et al., 2010; Johnson et al., 2016a,b), or (iii) viral particles in the conditioned media of cell lines that were exposed to samples of patients' serum *in vitro* (Pan American Health Organization, 2011). It is important to keep in mind that the detection efficiency of these methods varies depending on both the presence of the viral particles in the bloodstream of a patient and on the time of sample collection (**Figure 2**).

PATHOLOGY OF CHIKV INFECTION

The incubation period of 2–10 days is usually followed by CHIKVD that can be divided into acute and chronic phases. The

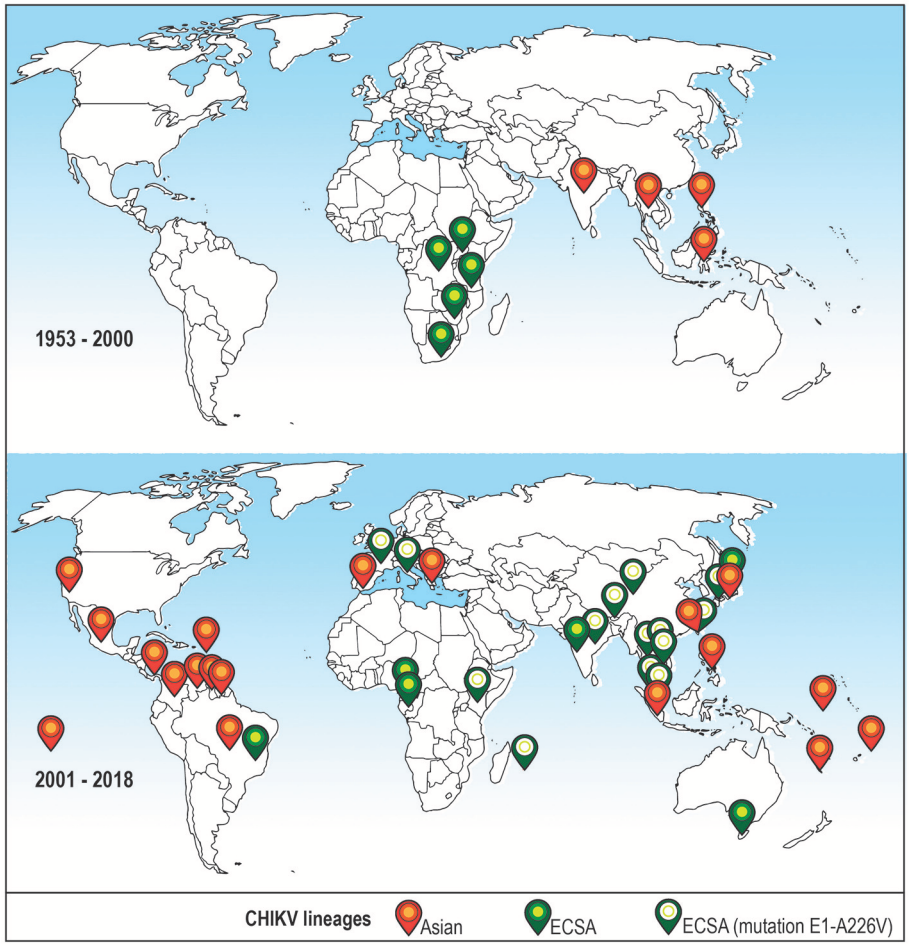

FIGURE 1 | Distribution of CHIKV lineages that are associated with recent outbreaks around the world before and after year 2000. Top: CHIKV cases reported in the XX century (1953–2000). Bottom: CHIKV cases reported recently (2001–2018). Only cases where the virus lineage was identified are shown. Orange icon–Asian lineage; green icon–East/Central/South African (ECSA) lineage; green icon with a white circle–ECSA strain with a mutation A226V in the E1 envelope glycoprotein, this strain is sometimes referred to as Indian Ocean lineage (Wahid et al., 2017).

acute phase occurs during the first 2 weeks after the onset of the disease and can be further subdivided into viral (before day 5 *p*ost-*i*llness *o*nset, *pio*) and convalescent (days 5–14 *pio*) stages (Thiberville et al., 2013a). Polyarthralgia, the most characteristic symptom of the acute phase, is reported in 87–98% of cases (Thiberville et al., 2013b). When and if the disease continues into the extended symptomatic-chronic phase, arthralgia that usually affects multiple joints can remain for several months or even years (Moro et al., 2012; Schilte et al., 2013).

Generally, acute clinical symptoms include high fever (>38.5°C) and shivers, severe joint and muscle pain, skin rash, weakness and headache (**Figure 3**). High viral load, lymphopenia and moderate thrombocytopenia are also observed in the acute phase (Thiberville et al., 2013b). In most cases, the symptoms remain for about 4–7 days as a self-limiting disease and are followed by a complete patient recovery (Schwartz and Albert, 2010). Nonetheless, clinical cases of symptomatic chronic disease for up to several years were reported (Brighton et al., 1983; Borgherini et al., 2008; Soumahoro et al., 2009; Gérardin et al., 2011; Moro et al., 2012; Schilte et al., 2013). Studies conducted after CHIKV outbreaks on Reunion Island in 2006 and in Italy in 2007 showed persistence of myalgia, asthenia and arthralgia in 60–67% of cases 36 and 12 months post-infection, respectively (Moro et al., 2012; Schilte et al., 2013).

Newborns and infants are predisposed to develop a more severe disease (Sebastian et al., 2009; Thiberville et al., 2013b). Fever is the main symptom in children (Simarmata et al., 2016). Both atypical and severe cases were frequently observed in this patient group, with the disease leading to hyperpigmentation, erythema (Nair, 2008; Rao et al., 2008), bullous skin lesions (Robin et al., 2010) and neurological symptoms, such as seizures and encephalitis (Robin et al., 2008), and a variety of other complications (Ramful et al., 2007).

Elderly individuals are another group with an increased risk of a more severe progression of the CHIKVD. Recently reported fatal cases of CHIKV infection in elderly people described liver

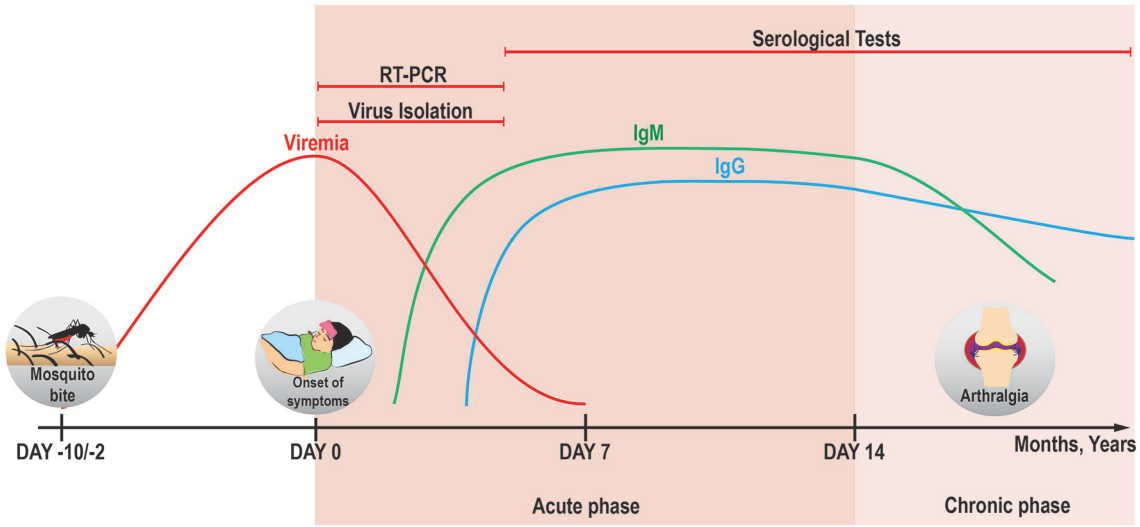

FIGURE 2 | Applicability of different diagnostic methods in the course of CHIKV infection. In the acute phase, viremia can persist until days 5–7 *pio* (Silva and Dermody, 2017) and CHIKV genomic RNA can be detected by RT-PCR reliably until day 7 *pio* (Edwards et al., 2017). It is therefore suggested that the detection of CHIKV RNA and virus isolation from serum samples for diagnostic purposes is done before day 5 *pio* (Johnson et al., 2016b), because the chance of false-negative results increases with the decrease in viral load. IgM and IgG antibodies against CHIKV begin to be produced at days 2 *pio* (Jain et al., 2018) and 4 *pio* (Prince et al., 2015), respectively. Stable titers of IgM can be seen in the serum from day 6 *pio* till around 4 months *pio* (Prince et al., 2015) [and can be detected mostly until 6 months *pio* (Chua et al., 2017)], whereas sustained levels of IgG can be present for more than 1 year (Chua et al., 2017). The antibodies against CHIKV can be detected by immunoassays after the development of humoral immune response (in case of IgG–long into the chronic phase, both–symptomatic or asymptomatic). A more detailed overview of the methods available for diagnostics of CHIKV is given in a review by Sam et al. (2015).

failure with subsequent cardiovascular collapse (Chua et al., 2010), and neurological and pulmonary deterioration followed by multiple organ failure (Hoz et al., 2015). Furthermore, 65 fatal cases after atypical CHIKV infection were reported during the 2006 outbreak on Reunion Island (Economopoulou et al., 2009). Mortality rate and overall severity of the disease increased there with age (Josseran et al., 2006), which seems to be a common theme for CHIKV infection in humans (Hoarau et al., 2010; Lang et al., 2017) and NHPs (Messaoudi et al., 2013). The incidence of atypical cases of CHIKF reported previously was <1%, taking into consideration the total number of infected people (Economopoulou et al., 2009). Some of the major atypical cases of CHIKV infection (as of 2010) are summarized in the review by Rajapakse et al. (2010).

After the mosquito bite, CHIKV replicates at the site of the inoculation and then spreads to peripheral organs and target cells via the circulatory system (Mourya and Mishra, 2006). CHIKV is able to infect a variety of adherent model cell lines and primary cells, but it fails to either infect or even bind to both–blood-derived cell lines (Jurkat, THP-1, U937, B-420) and primary blood cells (lymphocytes and monocyte-derived dendritic cells) (Sourisseau et al., 2007). Conflicting data is published by Sourisseau et al. (2007) and by Her et al. (2010) regarding susceptibility of primary human monocytes to CHIKV. Notably, monocyte-derived macrophages were found to be susceptible to CHIKV infection (Sourisseau et al., 2007; Solignat et al., 2009).

Similar to its behavior in humans, in a mouse model, CHIKV has a pronounced tropism to fibroblasts of the muscle, joint connective tissue and deep dermis (Couderc et al., 2008). From the infected skin fibroblasts and dermal macrophages, the virus spreads to lymph nodes, reaching spleen and liver in the acute phase, and muscles and joints through blood—later in the course of the disease (Roosenhoff et al., 2016). In severe cases—the spread of the virus into the central nervous system was shown in a mouse model (Couderc et al., 2008; Gardner et al., 2010) and in cynomolgus macaques (*Macaca fascicularis*) (Labadie et al., 2010).

IMMUNE RESPONSE TO CHIKV

Innate immune response against viruses consists of macrophages, dendritic cells (DCs) and natural killer cells (NKs) and is followed by the activation of B and T lymphocyte-mediated adaptive immune response. The subsequent generation of memory cells then leads to a specific response to the viral infection and protects from reinfection. In the following sections we review the current knowledge of cellular and molecular immune responses to CHIKV in humans and animal models.

Innate Immune Response

Natural Killer (NK) Cells

Acute phase of CHIKVD is marked by a significant increase in activation of components of the cell-mediated immunity led by an extensive activation of innate NK cells (Hoarau et al., 2010). The function of NK cells is regulated by a combination of signals from activating (e.g., CD94/NKG2C and NKG2D,

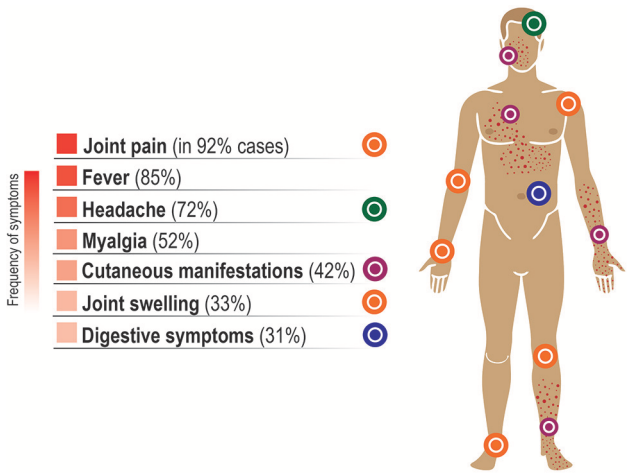

FIGURE 3 | Symptoms of the acute phase of CHIKV infection. Some minor variation exists in the frequency of symptoms reported in different studies. Typically, the clinical symptoms in the acute phase of the disease include high fever, pain and swelling in the joints, myalgia, and skin rash, often accompanied by headache, backache and fatigue. Here, the average percentage of symptomatic cases where a given symptom was reported is based on the data from Thiberville et al. (2013b), with the exception for the percentages for fever and headache that were taken from Huits et al. (2018). These symptoms usually remain for about 5–7 days as a self-limiting disease and are followed by a complete recovery within 2 weeks. However, severe joint pain can remain for months or even years in some individuals, often in distal joints (Roosenhoff et al., 2016) and in fluctuating manner (Hoarau et al., 2010). It has been estimated that ∼16% of cases are asymptomatic (Thiberville et al., 2013b). The symptoms in CHIKV-infected children differ from those in adults and are listed in Table 1 from the study by Simarmata et al. (2016).

activating killer cell immunoglobulin-like receptors KIRs–KIR2DS, KIR3DS) and inhibitory receptors (e.g., CD94/NKG2A, inhibitory KIRs–KIR2DL, KIR3DL) on their cell membranes (Pegram et al., 2011).

Recent data associate the expression of KIR and NKG2 receptors with susceptibility (Petitdemange et al., 2014) to CHIKV infection and viral clearance (Petitdemange et al., 2011). KIRs are involved in recognition of human leukocyte antigen (HLA) class I molecules (HLA-A, -B and -C) on nucleated cells, and specific KIR ligand/receptor combinations were implicated in HIV and hepatitis C (Jamil and Khakoo, 2011). In a similar fashion, a significant increase in the frequency of HLA-C subtype 2 allele (HLA-C2) in combination with the expression of *KIR2DL1* gene (encodes the receptor that can interact with HLA-C2) was found in CHIKV-infected patients during the CHIKV outbreak in Gabon in 2010 (Petitdemange et al., 2014).

At the same time, high viral load during the acute phase of infection and subsequent clearance of the infected cells were both associated with the expansion of the subpopulation of $CD3^{-}CD56^{+}$ NK cells that co-expressed the activating NKG2C receptor and KIR2DL2/KIR2DL3 inhibitory receptors for HLA-C subtype 1. This $NKG2C^{+}$ subpopulation of NK cells rapidly increased in the acute phase (at the expense of $NKG2A^{+}$ population) and demonstrated strong cytolytic response and reduction in IFN-γ production. This argues for a dichotomy between cytolytic and immunoregulatory functions of NK cells in the acute phase of infection (Petitdemange et al., 2011). In contrast, compared to controls, NK and NKT-like ($CD3^{+}CD56^{+}$) cells had lower cytotoxicity and higher expression of IFN-γ in the chronic phase. In addition, more of these cells expressed the inhibitory NKG2A receptor, while fewer were positive for the activating NKG2D (Thanapati et al., 2017).

Strong cytolytic response and decreased responsiveness to cytokine stimulation are typical for terminally differentiated NK cells that mature in progression from $CD56^{bright}CD57^{-}$ to $CD56^{dim}CD57^{-}$ and then—to $CD56^{dim}CD57^{+}$ phenotype (Nielsen et al., 2013). In agreement with that, the shift from $CD56^{bright}$ to cytolytic and mostly unresponsive to cytokines $CD56^{dim}$ cells was observed among $CD3^{-}CD56^{+}$ NK cells in CHIKV-infected patients (Petitdemange et al., 2011). The number of terminally differentiated $CD57^{+}$ NK cells peaked at the early (up to day 3 *pio*) acute phase. Persistence of these NK cells correlated with the viral load, and extended past the day 30 *pio* in some patients, all of which later developed chronic arthralgia (Petitdemange et al., 2016). Interestingly, acute joint pathology in CHIKV-infected mice was associated with NK cell activity that also becomes detectable in the early acute phase of the disease (Teo et al., 2015).

Monocytes, Macrophages And Dendritic Cells (DCs)

Monocytes and monocyte-derived macrophages appear to play a central role in the CHIKV-associated joint pathology. During CHIKV outbreaks, patients generally develop polyarthritis as an arthritis-like syndrome (Amdekar et al., 2017) in synovial joints (Phuklia et al., 2013). The inner lining of synovial joints is formed by macrophage-like synovial cells and fibroblast-like synoviocytes, and the latter are known to be important for the pathogenesis of rheumatoid arthritis (Bartok and Firestein, 2010). Similar to primary human osteoblasts (Noret et al., 2012), *in vitro* cultures of primary human fibroblast-like synoviocytes are susceptible to CHIKV infection, which results in the secretion of IL-6, IL-8, CCL2/MCP-1 and RANKL by the infected cells. The supernatants from these CHIKV-infected synoviocytes induce migration of monocytes as well as differentiation of monocytes/macrophages into osteoclast-like cells that produce high levels of arthritis mediators, such as IL-6 and TNF-α (Phuklia et al., 2013). In the joint, osteoclast-like cells can damage joint structure and contribute to the arthritic-like syndrome, as it was shown for rheumatoid arthritis (Schett, 2007).

In the chronic phase, macrophages were proposed to act as cellular reservoirs of persistent CHIKV (Labadie et al., 2010) and as regulators of the local Th1/Th2 balance (Dupuis-Maguiraga et al., 2012) in the damaged tissues that they infiltrate together with other mononuclear inflammatory cells. Such infiltrates were observed in the muscles of CHIKV-infected mice (Ziegler et al., 2008), in the muscles, joints, lymphoid tissues and liver of the infected macaques (Labadie et al., 2010), and in human biopsy samples. For example, in one chronic patient, both CHIKV RNA and proteins in the perivascular synovial macrophages were detected 18 months post-infection. While the synovial fluid contained activated $CD56^{+}$ NK and $CD4^{+}$ T cells, the majority (∼50%) of infiltrating cells were $CD14^{+}$ monocytes (Hoarau

et al., 2010). Virus persistence and active monocyte trafficking into the synovial tissue and fluid were associated with the robust expression of IFN-α (Hoarau et al., 2010), a potent inhibitor of CHIKV replication (Sourisseau et al., 2007). In agreement with that, high levels of IFN-α were produced by monocytes and whole blood cultures that were infected with CHIKV *in vitro* (Her et al., 2010). The importance of monocytes for limiting CHIKV infection is further illustrated in a mouse model, where depletion of $Ly6C^{hi}$ $CCR2^{+}$ (receptor for CCL2/MCP-1) monocytes *in vivo* promoted a more severe disease (Haist et al., 2017).

In a mouse model, macrophages were shown to be important for both–viral clearance and development of arthritic symptoms (Gardner et al., 2010). Similar to human primary macrophages (Sourisseau et al., 2007), primary mouse macrophages (Gardner et al., 2010) and transformed RAW264.7 mouse macrophage cells (Kumar et al., 2012b; Nayak et al., 2017) are also susceptible to CHIKV *in vitro*. Nonetheless, in the two recent studies conflicting results were obtained regarding CHIKV ability to cause apoptosis in the infected cells. No apoptosis was observed by Kumar et al. (2012b), while the study by Nayak et al. reported apoptosis induction through both–intrinsic and extrinsic pathways (Nayak et al., 2017). Both studies recorded an upregulation of IL-6 and TNF-α levels (Kumar et al., 2012b; Nayak et al., 2017). We note here, that two protective mechanisms of the host—apoptosis and autophagy—can play both pro- and antiviral roles in CHIKV infection, as discussed in the review by Long and Heise (2015).

DCs, another monocyte-derived cell type, participate in antigen presentation and therefore connect the innate and adaptive immune responses. Although shown to be susceptible to CHIKV in cynomolgus macaques (Labadie et al., 2010), cultures of primary human DCs (unlike monocyte-derived macrophages) appear to be resistant to CHIKV (Sourisseau et al., 2007). There are only a few studies related to the interaction of DCs with CHIKV. One of those assessed the role of dendritic cell immunoreceptor (DCIR) in CHIKV infection in mice. In this work, CHIKV decreased the number of $DCIR^{+}$ cells at the site of infection. It also altered cytokine expression in cultures of bone marrow-derived dendritic cells from $DCIR^{-/-}$ mice. In addition, infected $DCIR^{-/-}$ mice developed more severe disease symptoms, such as edema, increased inflammation and weight loss, suggesting a role for this receptor in limiting CHIKV-induced inflammatory response (Long et al., 2013). In another study, intracerebroventricular injection of CHIKV in neonate mice promoted the infection of astrocytes that was accompanied by a robust mobilization of DCs restricted to the site of infection (Das et al., 2015).

It is important to mention, that the current understanding of the cell-mediated immune responses to CHIKV is often based on the research done in animal models [reviewed comprehensively elsewhere (Broeckel et al., 2015; Fox and Diamond, 2016; Haese et al., 2016)], and none of those models completely recapitulates the course of CHIKVD in humans (Roosenhoff et al., 2016). In line with that, the roles of the less studied immune cell types, such as γδ T cells (Long et al., 2016), neutrophils and eosinophils (Poo et al., 2014) to our knowledge are only described in the context of CHIKVD in mouse models. Therefore, further research is required to fully understand the role of those cell types in the protection from or development of CHIKV-associated pathology in humans.

Humoral and Cellular Adaptive Immune Response to CHIKV

Pathogen-specific, humoral and cell-mediated immune responses that together constitute the adaptive immunity are carried out by B and T lymphocytes, respectively. An induction of anti-CHIKV antibodies that subsequently led to rapid clearance of the virus was demonstrated in a mouse model. In accordance with that, B cell (μMT) knockout mice showed a more severe disease and persistent viremia (for over a year) highlighting the importance of these antibody-producing cells for CHIKV clearance (Lum et al., 2013). In $Rag1^{-/-}$ mice that lack both B and T cells, prophylactic administration of anti-CHIKV monoclonal antibodies was sufficient to prevent virus persistence (Hawman et al., 2013). In addition, therapeutic administration of a human neutralizing monoclonal antibody in rhesus monkeys at days 1 and 3 after CHIKV infection blocked virus spread and inflammation in several tissues including joints and muscles (Broeckel et al., 2017).

In humans, anti-CHIKV IgG is first detected in the early convalescent stage, when naturally-acquired IgG response is dominated by the antibodies of IgG3 subtype. Early appearance of these antibodies correlates with protection against complications of the chronic CHIKVD (Kam et al., 2012b).

Both in humans and mice the antibody-mediated immune response seems to primarily target the envelope glycoprotein E2 of CHIKV (Kam et al., 2012a; Smith et al., 2015; Weber et al., 2015; Weger-Lucarelli et al., 2015). Moreover, the majority (70–80%) of the antibodies are estimated to be directed against the single linear epitope (E2EP3) in the N-terminus of the viral E2 protein. Accordingly, CHIKV infection in mice vaccinated with E2EP3 peptides was characterized with reduced infectivity of the virus and better clinical outcomes with decreases in viremia and joint inflammation. In plasma samples from patients in convalescent and recovery stages, anti-E2 antibodies were also shown to be the most persistent—they were detectable 21 months *pio*, unlike anti-E3, anti-capsid and anti-nsP3 antibodies that had been present only earlier in the course of the disease (Kam et al., 2012a). According to epitope mapping, monoclonal antibodies produced only against the epitopes on the outer surfaces (and not facing the interior of the E2/E1 trimer structure) were neutralizing (Fong et al., 2014).

The role of specific anti-CHIKV antibodies in the disease immunopathology has also been studied. Recently, two peptides of CHIKV E1 glycoprotein were identified by *in silico* bioinformatic analysis and showed similarity to human proteins. These E1 peptides were recognized by the serum from CHIKV-infected patients and were able to induce muscle inflammation in mice, thus showing that molecular mimicry between virus and host proteins

contributes to CHIKV pathology (Reddy et al., 2017). In another study, sub-neutralizing levels of CHIKV-specific antibodies aggravated the disease in mice, showing thereby that antibody-mediated enhancement of CHIKVD severity is also possible and requires consideration (Lum et al., 2018).

Cytotoxic $CD8^+$ T cells represent one of the major resources of antiviral immunity and are responsible for destruction of the infected cells. Analysis of circulating T lymphocytes showed that in acutely infected patients the early stage of the CHIKVD is accompanied by activation and proliferation of $CD8^+$ T lymphocytes with a peak at day 1 *pio* (Wauquier et al., 2011). Higher percentages of activated $CD8^+$ cells remained in the blood 7–10 weeks post-infection in the patients with CHIKV-associated arthritis symptoms. Elevated numbers of $CD8^+$ cells, as compared to healthy controls, were also observed in patients with untreated rheumatoid arthritis (Miner et al., 2015).

While $CD8^+$ T cell response marks the early stage of CHIKV infection, $CD4^+$ T cell lymphocyte-mediated immune response increases toward the end of the acute phase, peaking at day 4 *pio* (Wauquier et al., 2011).

The main function of $CD4^+$ T helper cells is to support and modulate the activity of other immune cells. This is achieved via production of cytokines that stimulate cell-mediated immunity and antibody responses. The role of these cells in CHIKV infection was studied in $CD4^{-/-}$ and $CD8^{-/-}$ KO mice. CHIKV-specific $CD4^+$ and not $CD8^+$ T cells were directly linked to the IFN-γ-independent inflammation in the joints, without evident role in replication and dissemination of the virus in the body (Teo et al., 2013). Moreover, a transfer of splenic $CD4^+$ T cells from CHIKV-infected wild-type mice into T cell receptor-deficient CHIKV-infected mice promoted a severe joint disease in the latter, further illustrating the essential role of $CD4^+$ T cells in the CHIKV-associated joint inflammation (Teo et al., 2017).

Therapeutic strategies based on the inhibition of $CD4^+$ T cells were developed and proved to be promising. For example, a treatment with FTY720 (fingolimod), an agonist of a phosphorylated sphingosine 1-phosphate receptor 1 (S1PR1), successfully abrogated joint pathology in CHIKV-infected mice by blocking the S1PR1-mediated emigration of $CD4^+$ T cells from the lymph nodes into the joints (Teo et al., 2017). In a parallel study, CHIKV-infected mice were treated with a fusion protein CTLA4-Ig (abatacept) that blocks costimulatory receptors on the surface of T cells and prevents activation of the latter. The treatment resulted in decreased inflammation and lower numbers of $CD11b^+/Ly6C^+$ monocytes, NK and T cells in the joints of the infected animals. Although unsuccessful at completely clearing the viral RNA, a combined therapy with CTLA4-Ig and anti-CHIKV monoclonal antibodies quickly eliminated the infectious virus and further improved disease pathology (Miner et al., 2017). Both works focused on acute joint pathology and called for testing of abovementioned approaches for the treatment of the chronic CHIKV-induced arthritis (Miner et al., 2017; Teo et al., 2017).

CYTOKINES AS IMMUNOLOGICAL MARKERS IN CHIKV-ASSOCIATED DISEASE

A vast number of samples were collected during the recent outbreaks around the world. In combination with increasing availability of high-throughput screening platforms, this allowed researchers to link various aspects of CHIKVD to expression profiles of cytokines, chemokines and growth factors in humans. We outline these expression profiles on **Figure 4** and summarize the principal findings below.

Ng et al., Wauquier et al., and Reddy et al. used plasma samples from acutely infected patients and uninfected individuals to compare levels of cytokines, chemokines and growth factors. The study by Ng et al. analyzed the levels of 30 such molecules, of which 12 were upregulated, and 4–downregulated (Ng et al., 2009). A similar comparison by Wauquier et al. included 50 soluble proteins, of which 25 were upregulated (including ICAM-1, VCAM-1, and RANTES that were undetectable in controls) and 10–downregulated. Notably, the exclusion of older individuals from the analysis did not affect these results. Many of the upregulated proteins (e.g., IFN-γ, IL-6, CXCL10/IP-10, CCL2/MCP-1, and others) showed dynamic expression pattern with levels changing across the sampling timeline (day 0–7 *pio*). The levels of many cytokines varied sufficiently not only at different time points, but also between individuals. IFN-α2, whose inter-individual levels were somewhat homogenous, represented one of the few exceptions (Wauquier et al., 2011). Out of 15 cytokines and chemokines tested by Reddy et al. (2014), seven were upregulated, one was downregulated, and only the upregulation of IL-6 and CXCL10/IP-10 was in agreement with both of the abovementioned studies (Ng et al., 2009; Wauquier et al., 2011).

Dynamic expression of cytokines and chemokines is not exclusive to the early acute phase. Venugopalan et al. showed that Th1 cytokines (e.g., IFNs -α, -β, -γ, IL-1β, CXCL10/IP-10, CCL2/MCP-1) reach maximum levels between days 0 and 5 *pio*, and Th2 cytokines (e.g., IL-4, IL-13)—between days 15 and 30 *pio* (Venugopalan et al., 2014). Chow et al. also reported the bias toward Th2 cytokines in the early convalescent stage (around 10 days *pio*). In the same study, the levels of RANTES and EGF peaked in the late convalescent stage (4–6 weeks *pio*) and of IL-17—in the chronic phase (2–3 months *pio*) (Chow et al., 2011). Later in the chronic phase Kelvin et al. found that high IgG levels were accompanied by increased levels of IL-6, CXCL9/MIG, and CXCL10/IP-10 in the 6-months follow-up of CHIKVD patients. In the 12-months follow-up, high IgG levels coincided with higher levels of CXCL9/MIG and lower—of IL-10 (Kelvin et al., 2011).

The relationship between the expression profile of cytokines and chemokines in the early phases of CHIKVD and the severity of the disease was studied in various contexts and yielded different results. The severity of symptoms in the 2007 outbreak in Singapore was associated with high levels of IL-1β and IL-6 and a decrease in the level of RANTES (Ng et al., 2009). At the same time, in the 2007 outbreak in Italy the severity of

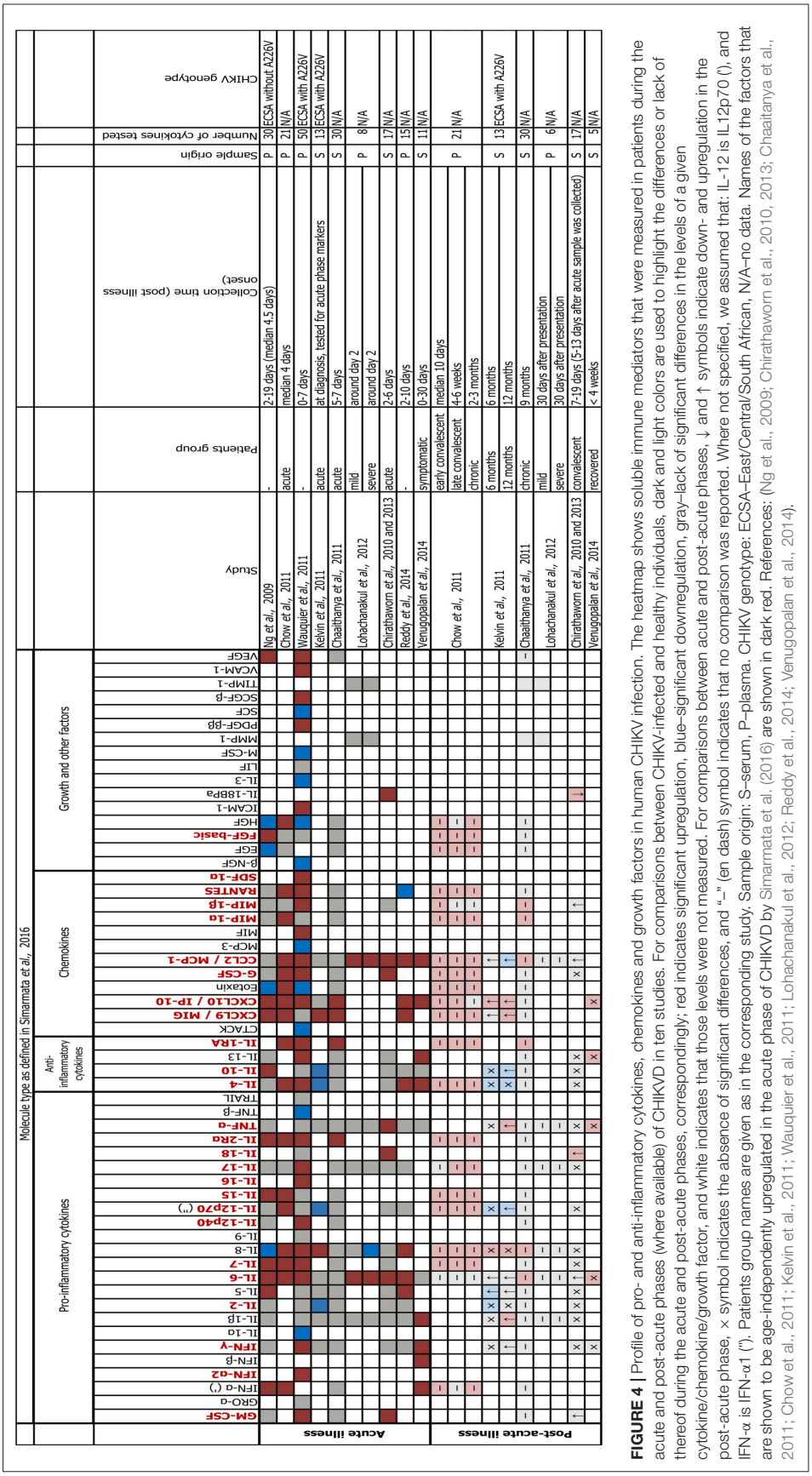

FIGURE 4 | Profile of pro- and anti-inflammatory cytokines, chemokines and growth factors in human CHIKV infection. The heatmap shows soluble immune mediators that were measured in patients during the acute and post-acute phases (where available) of CHIKVD in ten studies. For comparisons between CHIKV-infected and healthy individuals, dark and light colors are used to highlight the differences or lack of thereof during the acute and post-acute phases, correspondingly; red indicates significant upregulation, blue–significant downregulation, gray–lack of significant differences in the levels of a given cytokine/chemokine/growth factor, and white indicates that those levels were not measured. For comparisons between acute and post-acute phases, ↓ and ↑ symbols indicate down- and upregulation in the post-acute phase, × symbol indicates the absence of significant differences, and "–" (en dash) symbol indicates that no comparison was reported. Where not specified, we assumed that: IL-12 is IL12p70 (), and IFN-α is IFN-α1 (') Patients group names are given as in the corresponding study. Sample origin: S–serum, P–plasma. CHIKV genotype: ECSA–East/Central/South African, N/A–no data. Names of the factors that are shown to be age-independently upregulated in the acute phase of CHIKVD by Simarmata et al. (2016) are shown in dark red. References: (Ng et al., 2009; Chirathaworn et al., 2010, 2013; Chaaitanya et al., 2011; Chow et al., 2011; Kelvin et al., 2011; Wauquier et al., 2011; Lohachanakul et al., 2012; Reddy et al., 2014; Venugopalan et al., 2014).

CHIKVD was associated with increased levels of CXCL9/MIG, CXCL10/IP-10, and IgG (Kelvin et al., 2011). During the 2009–2010 outbreak in Thailand, the severity of CHIKVD was linked to elevated levels of IL-6 and CCL2/MCP-1 and decreased levels of IL-8 (Lohachanakul et al., 2012). In CHIKV-infected mice, the expression levels of monocyte chemoattractant proteins MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7 were increased in joints and the treatment with MCP inhibitor reduced the virus-induced bone loss in these animals (Chen et al., 2015).

Other relationships of cytokine and chemokine levels in the course of CHIKVD were established as well. For example, strong Th2 cytokine response was associated with prolonged presence of musculoskeletal symptoms of CHIKVD (Venugopalan et al., 2014), and increased levels of IL-6 (Chaaitanya et al., 2011; Chow et al., 2011) and of GM-CSF (Chow et al., 2011)—specifically with persistent arthralgia.

Another interesting observation regards the correlation of cytokine and chemokine levels with viral loads. High viral load positively correlates with the levels of IFN-α, IL-6, IL-12, IL-1RA, CXCL10/IP-10, and CCL2/MCP-1 (Chow et al., 2011), which seems to be in agreement with the recent data from Teng et al. (2015). Similarly, CCL2/MCP-1 correlated strongly, and IL-6—moderately with high viral load in the patients in the acute phase that were positive for CHIKV RNA (Reddy et al., 2014).

A comprehensive catalog of genes that are differentially expressed upon CHIKV infection was obtained by using RNAseq in a mouse model. Gene expression changes were in agreement with previously published mouse, monkey and human studies, and allowed for identification of an emerging role for granzyme A in CHIKV-associated arthritis (Wilson et al., 2017). Nonetheless, we would like to emphasize that extreme caution should be taken when extrapolating conclusions from studies in animal models to humans. For example, anti-CHIKV IgG3 antibodies, abundant in humans (Kam et al., 2012b), were not detected in mice at all (Patil et al., 2012; Teo et al., 2015). Opposite to humans (Venugopalan et al., 2014), the cytokine response in mice is shifted from Th2 early in the acute phase (Patil et al., 2012; Teo et al., 2015) toward Th1 later in the course of the disease (Patil et al., 2012). This early Th2 response was further enhanced when mice were infected via mosquito bite (Thangamani et al., 2010; Saraswat et al., 2016). This finding highlights the importance of both–the choice of the animal model and of the virus transmission route in the experimental system.

The authors of the abovementioned works further discussed the roles of Th1 and Th2 cytokine responses (Venugopalan et al., 2014), type I and II IFN signaling (Chirathaworn et al., 2010; Wauquier et al., 2011; Long and Heise, 2015), and the involvement of individual cytokines and chemokines: IL-6 (Chow et al., 2011; Kelvin et al., 2011), IL-13 (Venugopalan et al., 2014), IL-7, IL-15, RANTES (Ng et al., 2009), IL-18 (Chirathaworn et al., 2010), IL-1β, TNF-α, CXCL9/MIG, CXCL10/IP-10, and CCL2/MCP-1 (Kelvin et al., 2011). Simarmata et al. elaborated on the association of lower levels of pro-inflammatory cytokine GM-CSF with joint pain in CHIKV-infected children (Simarmata et al., 2016). Recent work by Chen et al. also connected the activation of NLRP3 inflammasome by CHIKV with upregulation of IL-1β and IL-18, as well as with the inflammation and osteoclastogenic bone loss in the CHIKVD (Chen et al., 2017).

Several explanations were suggested for discrepancies between cytokine, chemokine and growth factor expression profiles observed in patients with CHIKVD. One variable that needs to be considered is the genotype of the virus, which can affect the degree of joint pathology, the extent of inflammatory cells infiltration, and the intensity of cytokine response (Teo et al., 2015). Other important factors were pointed out by the authors of the studies that are shown here on **Figure 4**. In particular, attention was drawn to the differences in experimental approaches (Wauquier et al., 2011; Venugopalan et al., 2014), cohort sizes (Chow et al., 2011; Wauquier et al., 2011; Venugopalan et al., 2014), genetic backgrounds (Kelvin et al., 2011; Reddy et al., 2014), disease stages included into analyses (Chirathaworn et al., 2013), disease severity (Reddy et al., 2014), sources (e.g., plasma or serum) of the samples (Teng et al., 2015) and their collection times (Chirathaworn et al., 2013; Venugopalan et al., 2014).

CHIKV VACCINES AND ANTI-CHIKV MONOCLONAL ANTIBODIES

As of September of 2018, after over 50 years of development, there are no licensed vaccines or antiviral therapeutic strategies for prevention or treatment of CHIKV infection (Ljungberg et al., 2016; Powers, 2018).

The first formalin-inactivated CHIKV vaccine was produced in the culture of green monkey kidney tissue in the 1970s and was shown to be tolerated and immunogenic in 16 healthy human adults (Harrison et al., 1971). Recently, a formalin-inactivated CHIKV vaccine produced in Vero cells neutralized the virus infectivity by stimulation of both humoral and cellular immune response in the immunized mice (Tiwari et al., 2009). Additionally, both recombinant E2 protein and whole-inactivated virus vaccines protected mice from CHIKV infection, and no virus was detected in the tissues of immunized animals (Kumar et al., 2012a).

Over the years, several vaccine candidates were evaluated as possible preventive approaches. Among those are inactivated (Rudd et al., 2015; DeZure et al., 2016) and live attenuated (Edelman et al., 2000; Plante et al., 2011; Chu et al., 2013; Hallengärd et al., 2014; Roy et al., 2014; Roques et al., 2017) viruses, DNA (Mallilankaraman et al., 2011; Bao et al., 2013; Hallengärd et al., 2014; Tretyakova et al., 2014; Muthumani et al., 2016; Roques et al., 2017) and subunit (Metz et al., 2011, 2013; Khan et al., 2012) vaccines, as well as vaccines that are based on virus-like particles (VLPs) obtained from yeast (Saraswat et al., 2016), insect (Metz et al., 2013) and mammalian cells (Akahata et al., 2010; Chang et al., 2014). A large group of promising vaccine candidates takes advantage of chimeric avirulent backbones of measles (Brandler et al., 2013; Ramsauer et al., 2015) and vaccinia viruses (García-Arriaza et al., 2014; Weger-Lucarelli et al., 2014), adenovirus (Wang et al., 2011a), vesiculovirus (Chattopadhyay et al., 2013) and alternative alphaviruses (Wang et al., 2011b; Erasmus et al., 2017). Candidate

vaccines are tested in mouse and NHP models, and some of them have completed—NCT02861586 (Edelman et al., 2000) or are currently in the phase 2 clinical trials NCT02562482 (Chang et al., 2014).

Each of these approaches has its own advantages and limitations, and their immunogenicity should be carefully balanced with tolerability, lack of adverse effects and overall safety. For example, one of the early candidate vaccines was based on the live attenuated CHIKV strain 181/clone 25. In phase 2 clinical trials, it elicited neutralizing antibodies in 98% of recipients by day 28, and 85% of vaccinated individuals remained seropositive 1 year after immunization. Nonetheless, 8% of vaccinees developed transient arthralgia, although without arthritic signs or flu-like syndromes (Edelman et al., 2000).

Recently, vaccine candidates were developed that employ a picornavirus internal ribosome entry site (IRES) to render them incapable of infecting mosquitos and to reduce the expression of CHIKV structural protein genes (Plante et al., 2011; Roy et al., 2014). A single dose of such live attenuated virus was shown to be highly immunogenic and prevented the development of the hyperthermia and acute viremia in cynomolgus macaques (Roy et al., 2014). Interestingly, this Indian Ocean lineage-based vaccine can provide both–protection against other CHIKV lineages (Langsjoen et al., 2018) and cross-species protection against O'nyong'nyong virus (Partidos et al., 2012).

Current antiviral development strategies rely on exploitation of known antiviral agents and chemicals against other pathogens and synthesis of novel compounds (Ravichandran and Manian, 2008; Abdelnabi et al., 2015; Powers, 2018; Subudhi et al., 2018), nucleic acid-based therapies (Dash et al., 2008; Parashar et al., 2013; Lam et al., 2015) and anti-CHIKV monoclonal antibodies (Pal et al., 2013, 2014; Selvarajah et al., 2013; Clayton, 2016; Broeckel et al., 2017). In the last years, the use of monoclonal antibodies (mAbs) as therapeutic agents against CHIKV infection was evaluated by several groups, as reviewed by April M. Clayton (Clayton, 2016). Human anti-CHIKV mAbs were shown to have both–prophylactic and therapeutic effects in an adult wild-type mouse model of CHIKVD (administered 8 or 18 h post-virus challenge; Selvarajah et al., 2013), and to protect immunocompromised *Ifnar1*$^{-/-}$ mice from lethal virus challenge (Smith et al., 2015). Similarly, in the screening of 230 mouse anti-CHIKV mAbs, 36 were found to be neutralizing, of which a combination of two was the most potent in protecting *Ifnar1*$^{-/-}$ mice against CHIKV-induced death (Pal et al., 2013). This combination was used in the follow-up study, where it neutralized infectious CHIKV in blood and reduced viral burden in the joints and muscles of the legs of infected rhesus macaques (Pal et al., 2014). Recently, it was also shown that the treatment of rhesus macaques with an anti-CHIKV mAb (SVIR0001) administered after virus infection reduced viremia, joint disease, cellular inflammatory infiltration and the levels of pro-inflammatory cytokines and chemokines (Broeckel et al., 2017).

CONCLUSIONS

In the light of recent outbreaks, interest in CHIKV from the scientific community has grown significantly. Despite an outstanding progress in the CHIKV research, several questions regarding its immunopathology and associated arthritic syndrome remain to be answered. It is clear that further research is necessary to establish better *in vitro* and *in vivo* systems to study CHIKV infection and a consistent and reproducible picture of molecular immune response elicited against it, which in turn would pave the way to the discovery of markers that may be associated with disease morbidity and prognosis. Lastly, the treatment of CHIKVD is mostly symptomatic and no approved vaccine or antiviral drug currently exists. We believe that the development of safe and robust prevention and treatment approaches for CHIKV infection needs to be given top priority among researchers.

AUTHOR CONTRIBUTIONS

All authors performed the literature search and wrote the initial draft of the manuscript. IT, ET, LA, and DY prepared the manuscript figures. LA, DY, and ÊB reviewed the initial draft and wrote the final version manuscript. NC revised the manuscript. All authors revised and agree with the final manuscript version.

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REVIEW
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The Role of Mammalian Reservoir Hosts in Tick-Borne Flavivirus Biology

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Small-to-medium sized mammals and large animals are lucrative sources of blood meals for ixodid ticks that transmit life-threatening tick-borne flaviviruses (TBFVs). TBFVs have been isolated from various organs obtained from wild-caught *Myodes* and *Apodemus* species in Europe and Asia. Thus, these rodents are well-established reservoirs of TBFVs. Wild-caught *Peromyscus* species have demonstrated seropositivity against Powassan virus, the only TBFV known to circulate in North America, suggesting that they may play an important role in the biology of the virus in this geographic region. However, virus isolation from *Peromyscus* species is yet to be demonstrated. Wild-caught medium-sized mammals, such as woodchucks (*Marmota monax*) and skunks (*Mephitis mephitis*) have also demonstrated seropositivity against POWV, and virus was isolated from apparently healthy animals. Despite the well-established knowledge that small-to-medium sized animals are TBFV reservoirs, specific molecular biology addressing host-pathogen interactions remains poorly understood. Elucidating these interactions will be critical for gaining insight into the mechanism(s) of viral pathogenesis and/or resistance.

Keywords: tick-borne flavivirus, tick-borne encephalitis virus, ixodid ticks, mammalian reservoirs, small-to-medium sized mammals

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INTRODUCTION

The tick-borne flaviviruses (TBFVs) cause up to 15,000 cases each year in Europe despite the availability of several licensed vaccines (Gritsun et al., 2003; Dobler, 2010; LaSala and Holbrook, 2010; Heinz et al., 2013, 2014). This group of closely related agents is comprised of the tick-borne encephalitis virus sero-complex group (TBEV), Kyasanur Forest disease virus (KFDV), Omsk hemorrhagic fever virus (OHFV), Powassan virus/deer tick virus (POWV/DTV) and the naturally attenuated Langat virus. Humans are accidental hosts and suffer from infection mainly following a tick bite from infected ticks. The pathognomonic features of acute TBFV infection are severe neurological syndromes, which include meningitis and encephalitis, but OHFV and KFDV infections are typically associated with a hemorrhagic fever syndrome and may show encephalitis and/or meningoencephalitis as well. The case fatality rate associated with TBFV infections is varied, but can be up to 40%, depending on the virus (Mandl, 2005).

TBFV particles enclose an 11 kb (+)RNA genome and measure ~60 nm in diameter (Füzik et al., 2018). The genome has a single uninterrupted open reading frame (ORF), which serves as both a template for (–)RNA synthesis as well as the mRNA. Translation of the ORF results in a single polyprotein, which is cleaved by host and viral proteases into 3 structural proteins (C, prM/M, and E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). An

in-depth review of the functions of the TBFV proteins is available elsewhere (Mlera et al., 2014). Untranslated 5′- and 3′-regions (UTRs) that flank the ORF carry signals for replication, translation, cellular localization, and virion packaging (Bidet and Garcia-Blanco, 2014).

In nature, the TBFVs are maintained in a cycle involving infected hard-bodied (ixodid) ticks and small-to-medium sized mammals from which they obtain blood meals (Deardorff et al., 2013; Mlera et al., 2014). This cycling is particularly noteworthy in that the TBFVs must be well-adapted to replication in both arachnid as well as mammalian host systems. The precise ixodid tick species that are responsible for TBFV transmission differ by geographic region. For example, Powassan virus (POWV) is mainly transmitted by *Ixodes scapularis* and *Ixodes cookei* in the USA, but *Hemaphysalis longicornis* is a vector for the same virus in Asia (Ebel, 2010; Fatmi et al., 2017). The European TBEV subtype is transmitted by *Ixodes ricinus,* whereas *I. persulcatus* transmits the Siberian and Far Eastern TBEV subtypes (Rieille et al., 1920, 2014; Dzhivanyan et al., 1974; Lundkvist et al., 2001; LaSala and Holbrook, 2010).

The small-to-medium sized mammals that function as unwitting blood banks for ticks may also play a role as TBFV reservoir hosts. Here we define TBFV reservoir hosts as the ecological systems in which the viruses can be indefinitely harbored and from which they may be transmitted to other organisms (Ashford, 2003). This definition would include the ticks, but our focus is on mammalian reservoir hosts. When viremic, these animals can transmit TBFVs to feeding ticks (Khasnatinov et al., 2016). Interestingly, through a process called "co-feeding," ticks can transmit virus to other ticks when they feed on the same host in close proximity, even if the host is non-viremic (Labuda et al., 1993b,c, 1997; Nuttall and Labuda, 2003; Havlikova et al., 2013). This may be the most important route of transmission from tick-to-tick (Labuda et al., 1993a, 1997). The small-to-medium sized mammals include different rodent species, such as woodchucks (*Marmot monax*), skunks (*Mephitis mephitis*), and squirrels (*Scuiridae*) among others (**Figure 1**). The role as natural reservoir host is well defined for some of these animals but remains uncertain for others. In this review, we discuss the role played by wild mammalian reservoir hosts of TBFVs in the biology of these increasingly important human pathogens. We hope the review reinvigorates interest and research aimed at understanding this complex host-pathogen relationship.

TBFV RESERVOIR HOSTS

Several feral species have been implicated as potential natural hosts of TBFVs. Definitive reservoirs are those from which infectious virus can be isolated, and/or those with high positivity in surveillance studies. Potential reservoir species may have evidence of seropositivity and/or the presence of viral RNA. However, virus may be isolated from spill-over animals not normally considered reservoir hosts.

A general ecological consideration for the rodent reservoir is that they are typically at their peak densities 1 year after a heavy seed crop, usually in the next autumn, with rapid decline over winter (Stenseth et al., 2002). As seeds of various tree species, such as oak and beech are known to provide excellent food for the rodents, it is likely that the rodents' survival is improved for some time following a mast crop (Hansson, 1998). Throughout the next year, they most often remain much below the long-term average and return to "normal levels" as late as on the third year after a heavy seed crop (Hansson, 1971, 1998; Jensen, 1982; Pucek et al., 1993). During each year, the rodent population densities are highest in early winter, followed by a marked decline in spring and a gradual increase in autumn (Gurnell, 1978). These cyclic variations in mammalian reservoir densities may directly impact the tick populations and subsequently the pathogen burden in nature.

In the following sections, we review the animal species that play a major role in the biology of TBFVs and discuss the interactions between host and pathogen.

TBFVs in *Myodes* Species

The *Myodes* genus comprises several species that have a global distribution. The *Myodes glareolus* (bank vole) is a common rodent in Europe and North Asia and its distribution overlaps with regions in which TBEV cases are high (Torre and Arrizabalaga, 2008; Knap et al., 2012). In North America, *M. gapperi* (southern red-backed vole) and *M. rutilus* (northern red-backed vole) are the more common species. The red-backed vole is a major component of the rodent population in deciduous forests of North America (Boonstra and Krebs, 2012). The *M. glareolus* breeding season extends from April to the end of September and the females produce 3–4 litters each (Stenseth et al., 2002). In general, the vole populations are characterized by a marked increase to high populations, followed by a "crash" every 3–4 years (Krebs and Myers, 1974; Ecke et al., 2017).

There is conclusive evidence that the *Myodes* rodents are natural reservoirs for various TBFVs. For example, the TBEV strain CGI223 was isolated from the brain of a *M. glareolus* rodent in 1990 in Záhorská Ves, Slovakia and could be propagated in mouse brains as well as in Vero E6 cells (Frey et al., 2014). TBEV was also isolated from spleen, lung and kidney tissues collected from wild-caught *M. glareolus* which were incidentally coinfected with hantaviruses (Weidmann et al., 2006). The Oshima-C1 TBEV strain is another example of a TBEV isolated from the spleen of wild *M. rufocanus* in Hokkaido, Japan (Takeda et al., 1999). Thus, the *Myodes* species can harbor the TBFVs in different organs.

The percentage of wild-caught *Myodes* rodents with viral RNA in their organs varies from region to region, but rodents from Siberia may be highly infected. The viral loads associated with reservoir host infection may be determined by quantifying genome copy numbers using quantitative PCR. A report from a study done in Siberia showed that 46.2% (18/39) *M. rufocanus* (gray, red-backed vole) were positive for TBEV RNA, whereas 78.1% (25/32) *M. rutilus* had viral RNA in the brain or spleen (Bakhvalova et al., 2016). In some samples, the RNA suggested a mixture of the Siberian (TBEV-Sib) and Far Eastern (TBEV-FE) subtypes (Bakhvalova et al., 2016); this could potentially lead to the generation of recombinant viruses with altered virulence

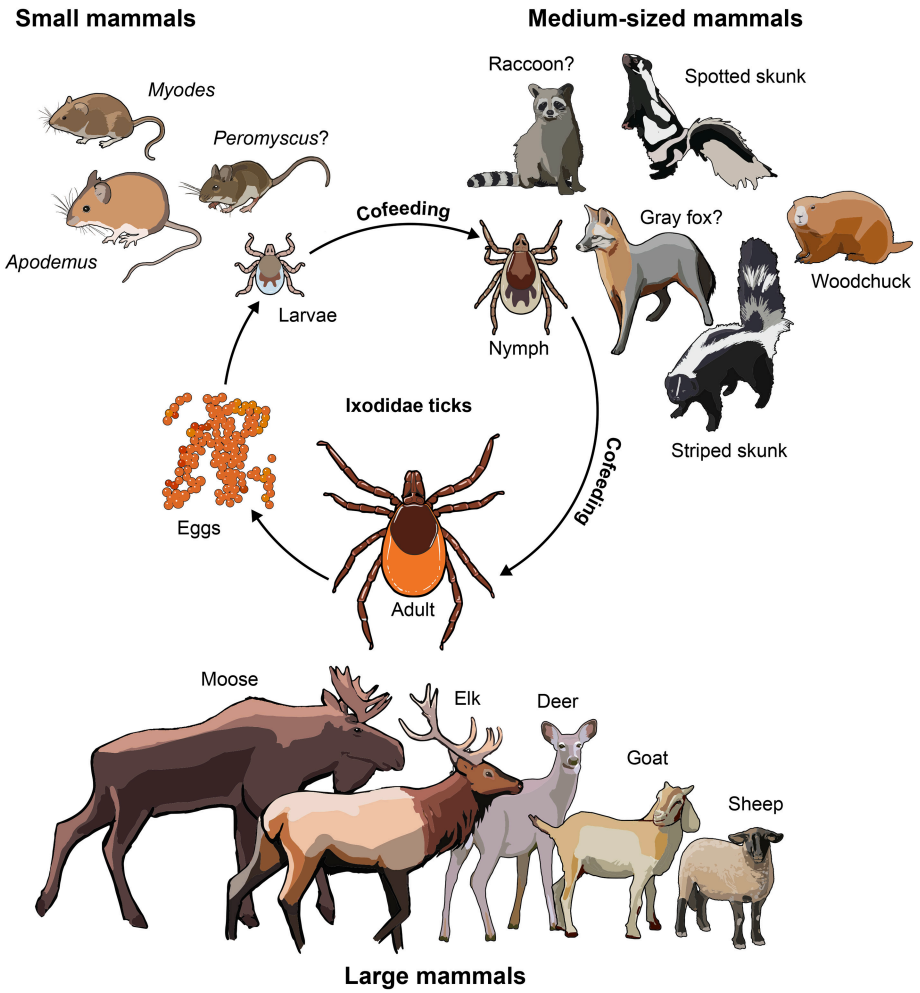

FIGURE 1 | Animals involved in the biology of TBFVs**.** Small-to-medium sized mammals are known reservoirs of TBFV. Animals with a question mark have been shown to have seroconverted to TBFV infection, but no virus isolation has been demonstrated yet. Large animals play the role of supporting adult tick populations by providing blood meals. Nymphal ticks prefer to feed on medium-sized mammals, whereas larvae favor small rodents. TBFV transmission to naïve ticks is believed to be mostly via co-feeding, and from viremic hosts to a lesser extent (Labuda et al., 1993a, 1997).

(Bertrand et al., 2012; Fajs et al., 2012; Norberg et al., 2013). A very high TBEV viral load average of 2.5×10^9 RNA copies/mL was reported per organ in *M. glareolus* (Knap et al., 2012), although the levels varied. In this specific case, viral RNA was also detectable in various *M. agrarius* tissues, such as spleen, kidney, lung, liver, heart, blood clots, and brains (Knap et al., 2012). In a separate study, Tonteri and colleagues captured wild rodents in Finland from 2 sites and analyzed the tissue samples for TBEV RNA by PCR. Over a 2-year period, they collected 202 *M. glareolus*, 23 (11.4%) of which were positive for TBEV and in which the RNA was primarily detected in the brain (Tonteri et al., 2011). Almost all the rodents with viral RNA were seropositive for TBEV antibodies (Tonteri et al., 2011). There was no attempt to isolate virus in these studies, but the fact that viral RNA was present in the brain in the absence of clinical signs of disease is a very interesting feature. Viral RNA was also identified in an ecological study in which 4% (6/150) of wild-captured *M. glareolus* rodents were positive for TBEV RNA in the liver (Pintér et al., 2014). The presence of viral RNA in multiple tissues indicates that TBFV infection in the *Myodes* species is not confined to a single organ, and, furthermore, viremia levels suggests that these animals may transmit virus to naïve ticks that feed on them. The high level of viremia, quantified by PCR, is thought to last only for a few days to enable transmission to ticks (Heigl and von Zeipel, 1966; Randolph et al., 1996; Nuttall and Labuda, 2003; Achazi et al., 2011). However, it is noteworthy that the *Myodes* rodents are apparently able to remain asymptomatic while coping with high viral loads in their organs, and this could lead to a sustained virus "leak" into the circulation, hence perpetuating viremia and enabling transmission.

Seroprevalence studies provide additional surrogate evidence for exposure of reservoir hosts to TBFVs, and seropositivity has

been demonstrated for the *Myodes* species in several countries. In one study conducted in Switzerland, 3.6% (12/333) wild-captured rodents were seropositive for anti-TBEV antibodies, and 8 of the 12 mice were *M. glareolus* (Burri et al., 2012). In another study conducted in Slovenia, a high prevalence of anti-TBEV antibodies was also observed in *M. agrarius* at 12.5% (39/272) and this seropositivity rate was higher than in other rodent species captured at the same time (Knap et al., 2012). A study in Hungary involving 541 rodents captured over a 4-year period from 2010 to 2014 found 20.5% *M. agrarius* were seropositive against TBEV (Zöldi et al., 2015). Interestingly, in this study, a rate of 0% (0/10) for *M. glareolus* was recorded in 2010, but the incidence increased to 44.4% (8/18) in 2013. However, these incidence rates may be confounded by the small sample sizes. Another interesting observation in this study was the high tick infestation on *M. glareolus*, but not on other rodent species. These differences could be a result of different animal behavior because *Myodes* species move slowly and are more likely to be infested (Zöldi et al., 2015). Higher seropositivity was also associated with older rodents (Zöldi et al., 2015), suggesting repeated exposure and/or a more robust immune response in the adults compared to juveniles.

In North America, *M. rutilus* rodents captured in Central Alaska were 5.8% (14/243) seropositive for POWV/DTV antibodies (Deardorff et al., 2013). *M. gapperi* rodents were captured in Southern Alaska and 6.7% (6/89) of these were positive for antibodies against POWV/DTV (Deardorff et al., 2013). However, this study was limited to serological testing of blood samples only, without virus isolation. Based on the findings that TBEV was readily detectable in *Myodes* species in Europe, attempts to isolate POWV from the same species in the USA should be pursued.

Experimental TBFV Infection and Molecular Studies in *Myodes* Species

A limited number of reports have described *in vivo* molecular interactions between *Myodes* rodents and TBFVs, or host-pathogen interactions using *Myodes*-derived *in vitro* cell culture models. Such investigations are critical for understanding the basis of TBFV persistence in these reservoir species. Experimental subcutaneous infection of pathogen-free *M. glareolus* with 3 TBEV strains each representing the three subtypes led to viremia by 4 days post infection (dpi) (Tonteri et al., 2013). In these studies, all rodents infected with both the TBEV-Eur and TBEV-FE produced specific IgG antibodies against the virus and had viral RNA in their organs, but only 8/13 rodents that were inoculated with TBEV-Sib tested positive by either method (Tonteri et al., 2013). This showed that there were strain-dependent outcomes in the *Myode*s rodents in the acute phase of the study. A very interesting outcome for the voles was the observation of clinical illness in 2 mice that were inoculated with TBEV-FE. The clinical illness was associated with non-suppurative encephalitis and viral RNA was detected in the brain, spleen and kidney and lung with 1 animal also being viremic. The *Myodes* rodents used in this study were colonized inbred animals and it is not clear if they had acquired any genetic changes that could sponsor susceptibility to severe disease. It is also not known if some rodents that get infected in the wild develop clinical and severe illness, which could lead to their death.

Tonteri and colleagues extended the experimental *Myodes* rodent infection to study viral persistence over a 168-day period. After 109 dpi, viral RNA could only be detected in the brain, an observation which was different from wild-caught *Myodes* which were positive for virus in other organs apart from the brain (Knap et al., 2012; Tonteri et al., 2013). Although viral RNA was detected in the organs of experimentally infected *Myodes* rodents, it is not certain if the RNA was from infectious virus particles because the authors did not attempt to isolate infectious virus. However, virus could be isolated from some, but not all, experimentally infected *Microtus arvalis* rodents, a different vole species, at 100 dpi (Achazi et al., 2011). These differences could be attributed to differences in the way the virus is introduced into the host i.e., tick infection with all the components of tick-saliva vs. needle-inoculation (Hermance and Thangamani, 2015).

One study to examine the response of *Myodes* rodents to TBFVs is that by Stoltz et al. (2011). In this work, the authors experimentally inoculated primary cells obtained from *M. glareolus* fetuses with a human clinical TBEV isolate 1993-783 (Haglund et al., 2003; Stoltz et al., 2011). Inoculation of the *M. glareolus* cells with this TBEV isolate resulted in an infection, which was demonstrated by immunofluorescent staining of viral proteins. The TBEV titer, determined by immunofocus assay, at 12 h post infection (hpi) was surprisingly high at just over 10^6 ffu/mL, and remained fairly constant as long as 96 hpi (Stoltz et al., 2011). Using qPCR, Stoltz and colleagues further analyzed the expression of *IFN-β* and *MX2*, and reported that *IFN-β* mRNA expression was induced ∼100-fold at 12 hpi and remained constant out to 96 hpi. *MX2* mRNA expression rose from a little over 1-fold at 6 and 12 hpi to 100-fold at 24 hpi and peaking at 10,000-fold by 48 hpi. The cells used in this study were a heterogenous population derived from whole-fetus tissues, excluding the head and liver. Thus, the IFN response observed following infection cannot be attributed to a specific cell type. Despite this, it is interesting to note that the cells mounted a strong but ineffective antiviral response, indicating that the IFN response does not necessarily restrict virus infection. Perhaps, the factors used to engage the virus *in vitro* are not as complete as those used *in vivo*.

Powassan Virus in *Peromyscus* Species

The *Peromyscus* genus represents the most abundant mixed forest rodent in North America (Bedford and Hoekstra, 2015). The *P. maniculatus* and their congeneric *Peromyscus leucopus* species are mainly distributed in the eastern regions of the USA, coinciding with the geographic regions from which human Powassan virus (POWV) infections have been mostly reported. The optimal habitat for *Peromyscus* mice is the mature woodland with shrubby underwood (Krohne, 1989; Mosheh and O, 2002). Like other rodents, the densities of *Peromyscus* mice are also influenced by seed-crop production as well as weather and habitat changes. For example, the severe ice storm in January 1994 resulted in a decline in *Peromyscus* numbers in northern Illinois from 16.7 individuals per plot to 0.79 individuals (Yunger,

2002). In addition, increased male agonistic behavior is thought to contribute toward poor survival of juvenile *P. maniculatus* during spring and summer (Watts, 1969).

POWV (lineage I) and its close relative DTV (Lineage II) are the only TBFVs known to circulate in North America (Ebel et al., 1999, 2000; Ebel and Kramer, 2004; Ebel, 2010). Some wild-caught mice, such as *P. maniculatus* and *P. truei* were seropositive against POWV/DTV at 6% (2/33) and 22.2% (9/22), respectively (Deardorff et al., 2013). *Peromyscus* mice are also established reservoirs for other pathogens such as *Borrelia* species responsible for Lyme disease and hantaviruses, which cause hantavirus cardio-pulmonary syndrome (Schmaljohn and Hjelle, 1997; Morzunov et al., 1998; Monroe et al., 1999; Barbour, 2016). However, infectious POWV/DTV has not been isolated from any wild-caught *Peromyscus* species to date. This may be because very few studies have attempted to isolate POWV from *Peromyscus* but have rather focused on serological surveys. Isolation of infectious virus from wild-caught *Peromyscus* would be indisputable evidence that these mice are a natural reservoir, but this has yet to be accomplished.

Experimental Infection of *Peromyscus* Mice

Based on the studies of (Deardorff et al., 2013), we developed an experimental model of POWV infection in *P. leucopus* (Mlera et al., 2017). Inoculation (by injection) of 4-week old *P. leucopus* mice with 10^3 PFU of POWV (lineage I/LB strain) did not result in overt clinical signs of disease (Mlera et al., 2017). This observation was similar to results of a study in which POWV lineage II was used to subcutaneously infect adult *P. leucopus* mice (Telford et al., 1997). In our study, the lack of an apparent clinical disease was observed even when the mice were intracranially (ic) inoculated. However, mild signs of inflammation, such as perivascular cuffing and microgliosis were evident when brain sections were examined (Mlera et al., 2017). *In situ* hybridization also revealed that POWV was restricted to the olfactory bulb and ventricle in ic-inoculated *P. leucopus* mice. Analysis of the *P. leucopus* brain transcriptome following ic inoculation with POWV revealed that the mice responded by activation of the IFN-signaling system. *In vitro* experiments with *P. leucopus*-derived fibroblasts supported our observations that interferon is secreted in response to POWV (Izuogu et al., 2017). It remains undetermined, however, whether the IFN signaling pathway is the sole or most important system restricting POWV infection. We are in the process of data mining the genome of *P. leucopus* to gain further insight into the restriction of POWV in *P. leucopus*. Although the IFN secretion in *Myodes* cells does not eliminate virus replication (Stoltz et al., 2011), POWV is restricted in *P. leucopus* fibroblasts.

TBFVs in *Apodemus* Species

The *Apodemus* genus comprises more than 20 species (Bugarski-Stanojević et al., 2008). The center of origin of *A. agrarius* rodents is believed to be Eastern Russia, and this has dramatically expanded westward without human assistance (Aguilar et al., 2008; Hildebrand et al., 2013). The expansion is exemplified by the identification of *A. agrarius* in 59 new localities in south western Slovakia (Tulis et al., 2016). *A. flavicolis* breeding season begins in March and ends in October and females produce 2–3 litters each (Stenseth et al., 2002). Except for winters following heavy mast years, winter reproduction does not occur in *Apodemus* species (Adamczewska, 1961; Pucek et al., 1993).

The *A. agrarius*, species is the most abundant in Europe and Asia (Bugarski-Stanojević et al., 2008). A study investigating tick infestation of small mammals in an English woodland showed that *A. flavicolis* (giant yellow-necked mouse) was the most abundant (52.5% of 217), followed by *Apodemus sylvaticus* (wood mouse) at 35.5% (Cull et al., 2017). Unfortunately, this study did not look for evidence of virus infection either in the animals or in the ticks collected from the infested animals.

Several TBEV strains have been isolated from the *Apodemus* mouse species, indicating that the genus is a reservoir host for TBFVs. Examples include the TBEV strains KrM 93 and KrM 213, which were isolated from lung and spleen tissue harvested from *A. agrarius* (striped field mouse) caught in South Korea (Kim et al., 2008; Yun et al., 2011). In Hokkaido, Japan, the TBEV strains Oshima 08-As and Oshima-A-1 were isolated from spleens of wild-caught *A. speciosus* (large Japanese filed mouse). The studies reporting isolation of TBEV from *Apodemus* mice seem to suggest that organ tropism of TBEV in *Apodemus* mice is different from that in *Myodes* species. In other studies, TBEV RNA was also detected predominantly in the spleen and infrequently other organs, such as the brain, lung and blood clots (Knap et al., 2012). There was no TBEV RNA detected in the kidneys and liver (Knap et al., 2012). In addition, the viral loads in *Apodemus* mouse organs were generally lower (range 6.48–3.7 $\times$ 10^5 copies/ml) when compared to those observed in *Myodes* species (Knap et al., 2012). Thus, it is apparent that TBFV organ tropism and extent of restriction varies between reservoir hosts.

Although the *Apodemus* mice seroconvert following exposure to TBFVs, the antibody titers reported in one study were several orders of magnitudes lower than in *Myodes* rodents. The titers determined by indirect immunofluorescence ranged from 0 to 80 in *A. flavivcolis* mice, compared to 0–1,280 in *Myodes* rodents (Knap et al., 2012). Furthermore, the study by Knap and colleagues showed lower seropositivity/infection rates in *Apodemus* than in *Myodes* rodents (Knap et al., 2012). They found that wild-caught *A. flavivcolis, A. sylvaticus,* and *A. agrarius* were seropositive for TBEV at 3.9, 9.6, and 2.4%, respectively (Knap et al., 2012). In a Hungarian study, 3.7% (3/327) *A. flavicolis* mice were positive for antibodies against TBEV whereas 4.6% (8/174) *A. agrarius* mice were seropositive for TBEV antibodies (Zöldi et al., 2015), further indication that the infection rate in *Apodemus* is relatively low.

The seropositivity and viral load differences between *Apodemus* and *Myodes* could be attributed to ecological factors. For example, a study looking at the effect of weather on the activity of the 2 rodent species showed that activity of *M. glareolus* rodents was positively influenced by moon phase regardless of cloud cover (Wróbel and Bogdziewicz, 2015). In contrast, rainfall positively impacted *A. flavicolis* but decreased activity of *M. glareolus* (Wróbel and Bogdziewicz, 2015). Thus, decreased rodent activity due to ecological factors could enhance infestation by ticks hence affecting seropositivity rates between species.

Experimental Infection of *Apodemus* Mice

Peroral, intraperitoneal or intramuscular inoculation of *Apodemus* mice results in no overt clinical signs of disease (Kopecky et al., 1991; Egyed et al., 2015). Wild-caught *A. sylvaticus* mice experimentally inoculated intraperitoneally with the virulent Central European (TBEV-Eur) strain were viremic for only 3 dpi (Kopecky et al., 1991). In *Apodemus* mice, TBEV was not detectable in the brains from 1 to 7 dpi (Kopecky et al., 1991), but, a recent study using only 2 wild-caught *A. agrarius* showed neuroinvasion and subclinical encephalitis following peroral inoculation (Egyed et al., 2015). Rodents that were intramuscularly inoculated showed no histological alterations in the brains, but mice that were orally inoculated with 1.5×10^3 PFUs presented with viral antigens in the brain and this was accompanied by mild lympho-histiocytic vasculitis, which was restricted to the anterior olfactory nucleus (Egyed et al., 2015). The animal numbers used in this study ($n = 2$ per group) limit the conclusions that can be drawn from the study. The lack of neuroinvasion by TBEV in *Apodemus* mice (Kopecky et al., 1991) is similar to our observations when we inoculated *P. leucopus* mice via the peripheral route (Mlera et al., 2017).

In the studies by Kopecký and colleagues, the authors compared the response of *A. sylvaticus* mice to that of outbred ICR laboratory mice and reported that the macrophages from ICR mice had higher virus titers that were sustained for 7 dpi. In contrast, viral titers in *A. sylvaticus* macrophages rapidly declined from just above 10^4 PFUs at day 0 to below 10 PFUs by 7 dpi (Kopecky et al., 1991). Macrophages are important cells in viral pathogenesis and they may function as virus reservoirs when infected by flaviviruses (Mogensen, 1979; Kreil and Eibl, 1995). Subversion of the anti-viral activities of macrophages by flaviviruses can facilitate viral replication and spread, enhancing the intensity of immune responses, leading to severe immune-mediated disease which may be further exacerbated during the subsequent infection with some flaviviruses (Ashhurst et al., 2013). Thus, the ICR mouse macrophages may be incapable of curbing TBEV infection, or the TBFVs may have evolved mechanisms to antagonize ICR mouse macrophage responses, but not those of *the Apodemus* species. This is in addition to the possibility that the *Apodemus* mice have evolved broader TBFV restriction mechanisms than ICR mice. Differences in the mechanistic responses of macrophages from various rodent species may be useful in understanding TBFV pathogenesis and could assist in the development of antiviral therapies.

IFN responses were mounted in both ICR and *A. sylvaticus* mice, but the serum levels peaked 1 dpi in ICR mice, the peak in *A. sylvaticus* mice was reached at 2 dpi. Notably, the highest IFN titers in *A. sylvaticus* mice were lower at 1,280, but the titer was extremely high at 10,240 in ICR mice. Thus, the overtly high IFN induction in ICR mice could lead to a runaway cytokine storm, perhaps leading to more aggravated pathogenesis in these mice.

Seroconversion appeared to be faster in the *A. sylvaticus* mice, detectable at 3 dpi and reaching titers of 256 at 7 dpi. In ICR mice, the titer reached 32 at 7 dpi. Although *A. sylvaticus* mice respond by an IFN and antibody response, it seems they are not able to clear TBFV infection completely, hence the detection of TBFV in wild-caught mice persists albeit at low level. Only mice inoculated with TBEV doses starting from 100 PFU seroconverted in the study reported by Egyed et al. (2015).

The Role of Medium-Sized Mammals

The role played by medium-sized mammals (**Figure 1**) in the biology of TBFV infections remains poorly studied. Woodchucks (*Marmota monax*) are one such medium sized mammal that might play a role in TBFV ecology. The *I. cookei* ticks are believed to transmit mostly POWV Lineage I and these ticks preferentially infest woodchucks for blood meals (Ebel, 2010). Seroprevalence of POWV antibodies in adult wild-caught woodchucks in Ontario, Canada during the mid-summers of 1964 and 1966 was quite high in the range 43–60% (McLean et al., 1964, 1967). Juvenile woodchucks tested in 1964 had relatively lower seroprevalence rates in May (33%), but the rate increased dramatically to 47% by July (McLean et al., 1964). POWV was isolated from 3/60 pools of *I. cookei* ticks removed from these woodchucks caught in 1966, as well as from the blood of 2 animals sampled in 1964 (McLean et al., 1964, 1967). In a different study, a pool of 56 *I. cookei* nymphs collected in 1981 from a feral yearling woodchuck in Guelph, Ontario (Canada) was also positive for POWV (Artsob et al., 1984). Virus was also isolated from the blood obtained from the same woodchuck (Artsob et al., 1984), indicating that the animal was viremic and a possible source of virus for naïve ticks. However, it was not clear whether the viremia was due to an active recent infection or rather a persistent infection that had been acquired at an earlier time. Viremia was sustained for 8–11 days following experimental subcutaneous inoculation of woodchucks with POWV lineage I (Kokernot et al., 1969). Nevertheless, these reports demonstrate that woodchucks play an important role in the ecology of POWV in nature and might be useful as experimental models of infection to delineate specific host-pathogen interactions.

Studies conducted in Connecticut and Massachusetts showed that 16% (12/75) and 83% (10/12), respectively, of the striped skunk (*M. mephitis*) had hemagglutinin inhibiting antibodies against POWV (Main et al., 1979). Additional POWV seropositivity in skunks was reported for 1/5 of spotted skunks (*Spilogale putorius*) caught in Alameda County, California (Johnson, 1987), however, none of the 4 striped skunks caught in the same study tested positive. There is a single isolation of POWV from the kidneys of an apparently healthy male spotted skunk in California (Johnson, 1987). However, efforts to isolate virus from the brain, trachea, lungs or throat swabs from the same animal failed (Johnson, 1987). These results suggest that POWV may persist in the kidneys of spotted skunks, which is in contrast to the brain as in the *Apodemus* or *Myodes* species. Considered together, these results suggest that the skunk is a true reservoir of POWV in nature, but additional studies are required to evaluate the current seropositivity status and/or isolation of virus from tissues obtained from these animals.

The raccoon (*Procyon lotor*) is another medium sized mammal that can be infested by TBFV-transmitting *Ixodes* and *Hemaphysalis* ticks (Jinnai et al., 2009; Inoue et al., 2011). Thirty three raccoons captured in New York State harbored

POWV/DTV-infected ticks, but none of the animals were seropositive for POWV (Dupuis et al., 2013). Inoculation of captured raccoons with POWV lineage I did not result in viremia or clinical signs of disease (Kokernot et al., 1969). Apart from these reports, no other study that we are aware of describes POWV biology in the raccoon. Thus, additional studies are required to elucidate the relationship between this ubiquitous animal and TBFVs.

Apart from the isolation of the WV77 DTV in West Virginia (USA) from a fatal case of encephalitis in the gray fox, *Urocyon cinereoargenteus* (Kuno et al., 2001), no field reports have described seroprevalence. However, experimental inoculation of this member of the Canidae family with a very large dose of 540,000 LD_{50} POWV (lineage I) resulted in no obvious clinical signs of disease although viremia was observed for 3 dpi (Kokernot et al., 1969). In addition, no signs of clinical disease were observed following subcutaneous challenge of the red fox (*Vulpes vulpes*) (Kokernot et al., 1969). This is interesting considering that the WV77 caused a fatality, and it begs the question as to what extent experimental inoculation can mimic natural infection via tick bite. Perhaps, the animal might have had other underlying conditions or a mixed infection, which aggravated the DTV infection.

Companion animals in the Canidae family, such as dogs, are also prone to tick infestations that could lead to transmission of TBFVs (Bajer et al., 2013; Chen et al., 2014). Clinical TBEV infection in dogs is rarely diagnosed, and is likely to be fatal in most cases (Pfeffer and Dobler, 2011). Fatality is preceded by fever, aggressiveness, optic neuritis, and encephalitis (Stadtbaumer et al., 2004; Pfeffer and Dobler, 2011; Bajer et al., 2013). Surveillance studies done in Austria, Belgium, the Czech Republic, Denmark, Germany, Japan, Norway and Sweden showed seropositivity in dogs ranging from 0.1 to 24.1% (reviewed in Pfeffer and Dobler, 2011). The studies from Germany and the Czech Republic indicated that there were some neurological symptoms observed in the sero-surveillance, but the clinical outcomes were not specified (Pfeffer and Dobler, 2011). Thus, TBFV infection of canines requires further study.

Cervids and Other Large Mammals

Large mammals, such as deer and elk (**Figure 1**) are inadvertent and transient TBFV hosts, which play an important role predominantly in maintenance of tick populations by providing blood meals (Carpi et al., 2008). Non-viremic transmission from infected ticks to naïve ticks cofeeding on the same host in proximity is well documented and the large animals also contribute to this process. From 1979 to 2010, 32% (84/266) of white-tail deer serum samples collected from Connecticut were positive for antibodies against POWV/DTV (Nofchissey et al., 2013). A recent survey also found evidence of antibodies against POWV in 4.2% of Eastern US white tail deer, indicating that virus-infected ticks continue to feed on this large mammal (Pedersen et al., 2017). Seropositivity in these large animals is a useful sentinel marker of TBFV prevalence in geographic regions in which the animals are found (Tonteri et al., 2016). It is evident from seropositivity that the cervids use the adaptive immune response to ward off TBFV infection, but specific innate and molecular responses need to be elucidated further especially since there is no evidence of neuroinvasion/neuropathology.

In some rural parts of Europe, goats and sheep are reared for milk. The milk may be consumed raw or as processed milk products, such as cheese. Recent reports have shown that sheep may develop encephalitis after natural TBEV infection (Böhm et al., 2017) Infected milk goats infected with TBEV can shed the virus in the milk and the virus can be transmitted to humans who consume it unpasteurized (Ernek et al., 1968; Cisak et al., 2010; Balogh et al., 2012; Hudopisk et al., 2013; Offerdahl et al., 2016). Goats have also been proposed as sentinels for TBEV in endemic areas (Klaus et al., 2012; Rieille et al., 2017; Salat et al., 2017). Experimental infection of goats with TBEV leads to no clinical signs of disease even when virus is being shed in the milk (Balogh et al., 2012). This is interesting, and it would be interesting to know which cells of the animals' mammary apparatus harbor the virus.

FUTURE PROSPECTS

In this paper, we reviewed the role of small-to-medium-sized mammals in TBFV biology. Rodents, particularly the vole (*Myodes*) and yellow-necked mouse (*Apodemus*), are true reservoirs of the viruses because viruses have been isolated from animals without clinical signs of disease. These are probably the most important rodent reservoirs of TBFVs. Although the role of the rodents is indisputable, very little research has been done to evaluate the specific host-pathogen interactions in these animals. The dearth of knowledge extends to medium-sized mammals, although some early reports indicate that woodchucks and skunks are critical players in the ecology and biology of TBFVs.

Studies designed to understand the role of reservoirs species will be important to develop the complete natural history of TBFV. Our lab is actively pursuing *in vivo* experiments as well as with cell cultures derived from some of these animals as surrogates for understanding permissiveness to infection as well as elucidating host cell factors which are critical for either susceptibility or resistance to TBFV infection.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Cellular and Infection Microbiology

MINI REVIEW
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The Immune Responses of the Animal Hosts of West Nile Virus: A Comparison of Insects, Birds, and Mammals

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Vector-borne diseases, including arboviruses, pose a serious threat to public health worldwide. Arboviruses of the flavivirus genus, such as Zika virus (ZIKV), dengue virus, yellow fever virus (YFV), and West Nile virus (WNV), are transmitted to humans from insect vectors and can cause serious disease. In 2017, over 2,000 reported cases of WNV virus infection occurred in the United States, with two-thirds of cases classified as neuroinvasive. WNV transmission cycles through two different animal populations: birds and mosquitoes. Mammals, particularly humans and horses, can become infected through mosquito bites and represent dead-end hosts of WNV infection. Because WNV can infect diverse species, research on this arbovirus has investigated the host response in mosquitoes, birds, humans, and horses. With the growing geographical range of the WNV mosquito vector and increased human exposure, improved surveillance and treatment of the infection will enhance public health in areas where WNV is endemic. In this review, we survey the bionomics of mosquito species involved in Nearctic WNV transmission. Subsequently, we describe the known immune response pathways that counter WNV infection in insects, birds, and mammals, as well as the mechanisms known to curb viral infection. Moreover, we discuss the bacterium *Wolbachia* and its involvement in reducing flavivirus titer in insects. Finally, we highlight the similarities of the known immune pathways and identify potential targets for future studies aimed at improving antiviral therapeutic and vaccination design.

Keywords: innate immunity, West Nile virus, *Culex*, host response, vector-borne disease

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INTRODUCTION

West Nile virus (WNV) belongs to the flavivirus genus, which also includes dengue virus (DENV), yellow fever virus (YFV), and Zika virus (ZIKV). WNV is endemic to the United States (U.S.) and Canada, Africa, Europe, the Middle East, and West Asia (WHO, 2011).WNV has a single-stranded positive-sense RNA genome encoding approximately 11,000 nucleotides. It is translated as a polyprotein and processed into 3 structural and 7 nonstructural viral proteins (reviewed in Brinton, 2013). The virus amplifies, or replicates to high titer (**Figures 1A,B**), within the bird population, making them likely to transmit the infection to mosquitoes (**Figure 1C**), primarily of the *Culex* genus. Mosquitoes can then reinfect the bird population, further perpetuating enzootic

infection (**Figure 1E**), or can bridge the infection to mammals, most commonly humans and horses (**Figure 1F**). It is at this interface that public health becomes a concern. Human symptoms can be mild, presenting with headache, weakness, or fever, or more severe, presenting with meningitis or encephalitis (Petersen et al., 2013). In this review, we discuss mosquito populations in North America with particular attention to species that bridge WNV infection to humans, and then survey the innate immune response pathways of the animals commonly infected with WNV: mosquitoes, birds, horses, and humans. While the adaptive immune response is important for mammalian survival to WNV, this review focuses on innate pathways and rapid immune activation during WNV infection. We review possible avenues for therapeutic design, including antibodies for passive immunity and the endosymbiont *Wolbachia* to reduce infection in insects. Lastly, we identify new areas for investigation, especially those focused on vaccine development and disease therapeutics.

CULEX MOSQUITOES AS VECTORS OF INFECTION

Mosquitoes in the culicine family carry WNV, and each species has a preferred geographical tropism, blood meal host, and daily and seasonal feeding pattern. *Culex quinquefasciatus* is located between the latitudes 36°N and 36°S (Barr, 1957), and in the U.S. from coast to coast (Darsie and Ward, 2005). *Cx. quinquefasciatus* has been observed as far north as 39°N, giving it some geographical overlap with *Cx. pipiens*, which lives above 36°N (Barr, 1957). *Cx. pipiens* ranges north into British Columbia and through Maine. *Cx. tarsalis* can be found in most of the U.S., but is not usually found in the easternmost states, likely due to competition with *Cx. salinarius*, which prefers warmer, coastal temperatures (Darsie and Ward, 2005). Finally, *Cx. restuans* is found in more urban areas, as the larvae are better able to tolerate pollution than other mosquito species (Johnson et al., 2015). *Culex* population genetics, mating patterns, and host selection, with emphasis on data collected in California, has been reviewed by Reisen (2012), which concludes that urbanization will favor *Cx. pipiens* and hinder *Cx. tarsalis*. While not discussed in Reisen (2012), *Cx. restuans* is also likely to thrive in metropolitan areas, perhaps making *Cx. pipiens* and *Cx. restuans* of greatest importance for the study of vector-borne disease.

Although research on *Culex* mosquitoes in North America has been ongoing for over 70 years, the arrival of WNV to the U.S. spurred deeper research into the species-specific and region-specific differences between *Culex* mosquitoes that allow them to be enzootic vectors (**Figure 1E**) and bridges to human infection (**Figure 1F**). *Culex* species feed on avian hosts, either as a primary source of blood meal or more opportunistically (Molaei et al., 2006; Reisen, 2012). A variety of studies have investigated the competence of *Culex* mosquito populations in specific geographic locations to transmit WNV to mammals. In a study completed in Connecticut, researchers found that *Cx. restuans* and *Cx. pipiens* tend to feed on birds, determined by the presence of bird blood meal, making these important species for enzootic infection. Moreover, *Cx. salinarius* is most likely to feed on both vertebrates and birds, making it an important bridge to humans in this region (Molaei et al., 2006). In another study in the northeastern U.S., researchers determined that vector competence can vary over time and is dependent on environmental factors, such as temperature, and genetic factors, such as ancestry. Furthermore, the authors conclude that *Cx. restuans* are more likely to transmit WNV than *Cx. pipiens* (Kilpatrick et al., 2010).

The *Cx. quinquefasciatus* mosquitoes are the primary species in the southern U.S. and Mexico. *Cx. quinquefasciatus* in Cancún and Chetumal often feed on humans, but rarely on birds, so this species is not a likely bridge between bird and human WNV infection (Janssen et al., 2015). However, in East Baton Rouge Parish, Louisiana, *Cx. quinquefasciatus* frequently feed on avian hosts, as well as human and other mammalian sources of blood meal, suggesting that *Cx. quinquefasicatus* is a vector for human transmission of WNV in southern Louisiana (Mackay et al., 2010). In Bernalillo County, New Mexico, researchers determined that *Cx. quinquefasciatus* mosquitoes are likely the primary vectors for enzootic infection in birds, but *Cx. tarsalis* are more likely the bridge to infect humans, as these mosquitoes feed on both mammals and birds throughout the feeding season (Lujan et al., 2014).

Laboratory studies have further investigated the species-specific differences between *Culex* mosquitoes when infected with WNV. *Cx. tarsalis* infected with WNV have decreased fecundity and increased feeding rates, but no change in survival (Styer et al., 2007). Conversely, *Cx. pipiens* show no difference in survival, fecundity, or feeding rates when infected with WNV (Ciota et al., 2011). These findings complicate the ability to predict WNV infection rates in specific mosquitoes. Ciota et al. predicts that susceptible *Cx. pipiens* mosquitoes will be maintained in a community, as there is no cost for infection, but there is a cost for resistance. The results in Styer et al. suggest that in *Cx. tarsalis* the cost for infection, namely decreased fecundity, may be overcome by an increase in feeding rate. This behavioral compensation is supported by the finding that *Cx. tarsalis* have a higher estimated rate of transmission than *Cx. pipiens* (Turell et al., 2001, 2002). The same research group determined that *Cx. restuans* and *Cx. salinarius* are both efficient vectors of WNV infection, while *Cx. quinquefasciatus* is moderately efficient (Sardelis et al., 2001). Additionally of note, *Cx. pipiens* can perpetuate WNV infection by vertical transmission (Dohm et al., 2002); consequently multiple generations of mosquitoes are infective (**Figure 1D**).

Alarmingly, the geographic range of *Culex* mosquitoes is expanding. Models of *Cx. pipiens* and *Cx. tarsalis* reveal that climate change is likely to contribute to the expansion of the mosquito population in Canada and extend the WNV transmission season by the year 2050 (Hongoh et al., 2012; Chen et al., 2013). Additionally, *Cx. quinquefasciatus* season is predicted to increase in length by a few weeks at both the beginning and end of the summer in the U.S. (Morin and Comrie, 2013). Taken together, these models conclude that the geographic range of mosquitoes, and consequently WNV infections, will

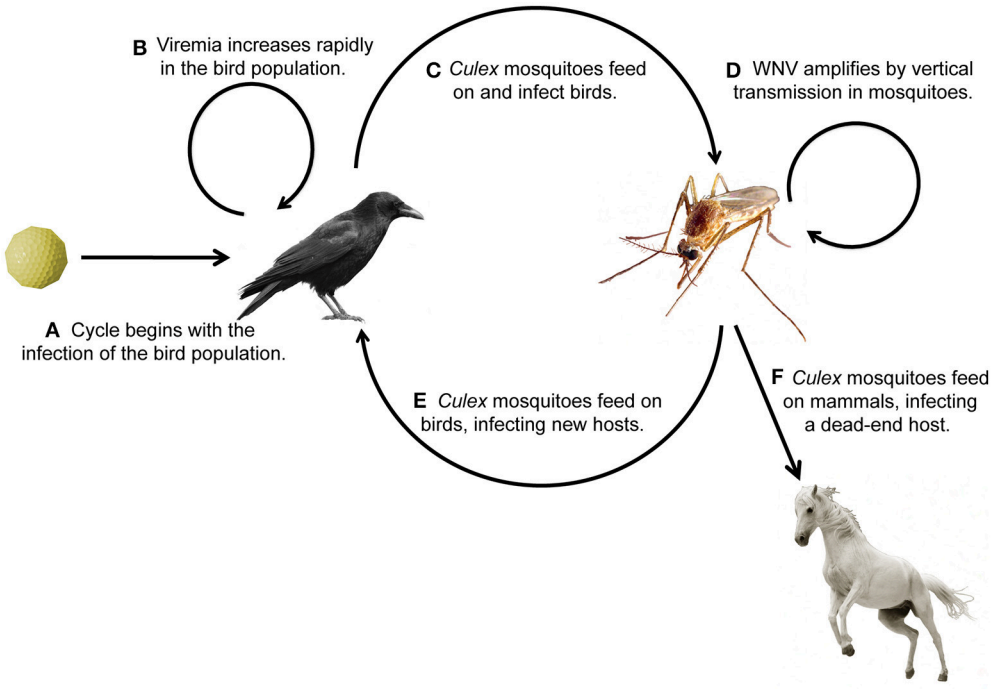

FIGURE 1 | Transmission cycle of West Nile virus through its animal hosts. **(A,B)** Birds become infected with WNV and viral titer increases, **(C,D)** birds transmit the infection to mosquitoes, **(E)** which transmit the infection to birds, causing enzootic infection, or **(F)** bridge the infection to humans and horses, the common dead-end hosts.

increase. A summary of the effects of climate change on several insect-borne infections is provided in Andersen and Davis (2017).

THE MOSQUITO IMMUNE RESPONSE TO WNV

Mosquitoes utilize an innate immune response to WNV to prevent mortality from infection. The RNA interference (RNAi) pathway is conserved across diverse phyla and provides host protection against virus infection, including arboviruses (Olson and Blair, 2015). Dicer-2, the viral nucleic acid sensor of the RNAi pathway, is utilized in the response to WNV infection in *Cx. quinquefasciatus* cells, and orally-infected *Cx. quinquefasciatus* mosquitoes respond to WNV (Kunjin strain) challenge via the RNAi pathway (Paradkar et al., 2014). In fact, WNV (Kunjin strain) has been shown to antagonize the host RNAi response in *Cx. quinquefasciatus* by generating viral noncoding sfRNA (subgenomic flavivirus RNA) that interacts with Dicer and Argonaute 2 (Moon et al., 2015). sfRNA is viral genomic RNA that resists degradation by the host cell by forming pseudoknot structures (Jones et al., 2012; Chapman et al., 2014).The RNAi pathway even drives WNV population diversity in both mosquitoes and *Drosophila melanogaster*, as the RNAi pathway selects for the more diverse virus variant (Brackney et al., 2009, 2015). Like mosquitoes, *D. melanogaster* utilize the RNAi pathway for resistance to WNV infection, determined by the detection of siRNA (small interfering RNA) (Chotkowski et al., 2008), validating the fruit fly as a possible model organism to study mosquito immunity.

Mosquitoes also utilize the JAK/STAT pathway in the immune response to WNV. Transcriptional profiling reveals that *Aedes aegypti* mosquitoes utilize this pathway in response to WNV, DENV, and YFV (Colpitts et al., 2011). Mechanistically, in *Culex* cells the immune response to WNV utilizes a secreted molecule called Vago that, like interferon in mammals, is hypothesized to act as a second messenger to activate the JAK/STAT pathway (Paradkar et al., 2012). Finally, apoptosis, a conserved immediate immune response, occurs in the salivary glands and midgut of *Cx. quinquefasciatus* mosquitoes to control viral load (Vaidyanathan and Scott, 2006; Girard et al., 2007).

Lastly, of note, the endosymbiont *Wolbachia* affects the mosquito host response to WNV. *Wolbachia* is a bacterium originally identified in *Cx. pipiens* (Hertig and Wolbach, 1924), reviewed in Johnson (2015). It is estimated that 40% of all arthropod species (Zug and Hammerstein, 2012) and 7% of *Cx. pipiens* mosquitoes in California (Rasgon and Scott, 2004) are infected with *Wolbachia*. There are a few strains of *Wolbachia* used in laboratory experiments, discussed in Woolfit et al. (2013): *w*Mel was identified in *D. melanogaster* and is benign (Teixeira et al., 2008), *w*MelPop was identified in *D. melanogaster* and has a pathogenic effect (Min and Benzer, 1997), and *w*MelPop-CLA is a strain of *w*MelPop adapted for *Ae. aegypti* (McMeniman et al., 2008). Inaugural experiments in *D. melanogaster* determined that

Wolbachia infection increases host resistance to the Drosophila C virus, Nora virus, Flock House virus, and WNV (Teixeira et al., 2008; Glaser and Meola, 2010). Subsequently, others determined that the same effect occurs in mosquitoes: The presence of *Wolbachia* (*w*Mel and *w*MelPop-CLA strains) in *Ae. aegypti* mosquitoes is correlated with a reduction in DENV titer (Walker et al., 2011) and WNV titer (Hussain et al., 2013). One study determined that the amount of secreted WNV decreases significantly in *Aedes* cells that are also infected with *Wolbachia*, indicating restriction of the virus. Furthermore, it determined that the *Wolbachia* strain *w*MelPop, but not *w*Mel, has an inhibitory effect on WNV infection *in vivo* (Hussain et al., 2013). Taken together, the strain of *Wolbachia* is important for inhibition of WNV in *Aedes* mosquitoes.

Perhaps of greater biological importance for WNV is the effect of *Wolbachia* on *Culex* mosquitoes. In a study using *Cx. quinquefasciatus*, researchers concluded that *Wolbachia* increases host resistance to WNV infection (Glaser and Meola, 2010). However, this is in contrast to another study that suggests that the presence of *Wolbachia* (*w*AlbB) can increase WNV titer in *Cx. tarsalis* (Dodson et al., 2014). *w*AlbB is a strain originally isolated from *Ae. albopictus* (Zhou et al., 1998).

Because of the controversial results, one study specifically compared the effects of *Wolbachia* strain *w*AlbB in *Ae. aegypti* on DENV and WNV (Kunjin strain). The study concluded that both somatic infection and stable transinfection of *Wolbachia* lead to inhibition of DENV and WNV replication and transmission (Joubert and O'Neill, 2017). Some researchers even suggest that introducing *Wolbachia* into the wild mosquito population will reduce DENV infection in humans (Schmidt et al., 2017), and modeling predicts that WNV could be eradicated subsequent to the introduction of *Wolbachia* in the ecosystem (Farkas et al., 2017).

Because *Wolbachia* is a bacterium, it would follow that it is priming an immune response in mosquitoes. However, this does not seem to be the case during DENV infection (Rancès et al., 2012). Rainey et al. describe hypotheses for the mechanism by which *Wolbachia* reduces viral titer (Rainey et al., 2014). One possible mechanism of antiviral action could be competition between *Wolbachia* and a virus for cellular resources. This is supported by Moreira et al. (2009) which determined that *Wolbachia* and DENV are not found in the same cells. Another putative mechanism is modulation of the autophagy pathway. DENV utilizes the autophagy pathway for replication (Lee et al., 2008), however, *Wolbachia* (*w*AlbB) has been shown to manipulate this pathway for its own survival (Voronin et al., 2012). This mechanism may not be relevant for all flaviviruses, as WNV does not utilize autophagy for replication in mammalian cells (Vandergaast and Fredericksen, 2012). More work will need to be completed in the insect model to determine the role of autophagy in WNV pathogenesis.

THE BIRD IMMUNE RESPONSE TO WNV

Birds are an important reservoir of WNV, as the virus replicates to high titers in several bird species (**Figure 1B**; Komar et al., 2003). Additionally, the migration of bird populations aids in the distribution of WNV beyond the range of mosquitoes (Reed et al., 2003; Owen et al., 2006). Similarly to human and horse immunity, birds utilize the 2′-5′-oligoadenylate synthase (OAS) pathway in the immune response to WNV. Briefly, OAS proteins detect double-stranded RNA from viruses and undergo a conformational change to synthesize 2′-5′-oligoadenylates. These second messengers bind inactive RNase L, which then dimerizes to become active and cleave viral RNA. The OAS response is often utilized during flavivirus and alphavirus infections, likely because these positive-sense RNA viruses develop double-stranded RNA as replication intermediates in higher concentrations, as compared to a negative-sense RNA virus (Silverman, 2007). This response pathway ultimately inhibits the virus and induces apoptosis (Castelli et al., 1998; Tag-El-Din-Hassan et al., 2012).

While antibodies are a hallmark of adaptive immunity, passive immunity is a form of rapid immune activation, similar to innate immunity. Several bird species develop neutralizing antibodies to WNV, with long-lasting protection over multiple WNV seasons, including the house sparrow (*Passer domesticus*) (Nemeth et al., 2009) and raptor species (Nemeth et al., 2008). Importantly, young chicks can receive maternally-inherited passive immunity for rapid protection from virus infection. Maternally-inherited antibodies to WNV have been measured in flamingo chicks (*Phoenicopterus chilensis* and *Phoenicopterus ruber ruber*) (Baitchman et al., 2007), Eastern screech owls (*Megascops asio*) (Hahn et al., 2006), and rock pigeons (*Columba livia*; Gibbs et al., 2005), indicating that this is an effective strategy for protecting chicks.

Additionally, there is some cross-protection in birds to multiple flavivirus types. House finches that are first challenged with St. Louis encephalitis virus (SLEV) first and then WNV have an antibody response to WNV. Interestingly, finches first challenged with WNV and then SLEV have an elevation in WNV antibody titers, but no increase in SLEV antibody titers during the second infection (Fang and Reisen, 2006). This information could be useful in vaccine design to protect birds against flavivirus infection. Perhaps antibodies to WNV could confer resistance to multiple flaviviruses, theorizing a universal flavivirus vaccine. Some researchers did vaccinate birds with the goal of saving rare species. One study used a DNA vaccine to protect captive California condors (*Gymnogyps californianus*) during the initial spread of WNV in the U.S. The study determined that the vaccine is safe for California condors, stimulates protective antibodies, and protects against naturally circulating WNV (Chang et al., 2007).

THE HORSE IMMUNE RESPONSE TO WNV

Horses are also susceptible to WNV infection (**Figure 1F**), and since its entry to the U.S. in 1999, WNV has caused 27,726 confirmed equine cases (data through 2016; USDA APHIS, 2017). In a WNV outbreak in 2002, a survey determined that 22% of infected horses died from infection (Schuler et al., 2004). Like humans, horses have a robust immune

response to WNV that utilizes both the innate and adaptive responses.

In the early immune response, horses utilize an interferon-mediated (IFN) response. In one study that used WNV (Kunjin subtype) authors found increased levels of type I and type II interferon in blood leukocytes, lymph nodes, and spleen. They also noted increases in IFN-α, CXCL10, TLR3, ISG15, and IRF-7 in the brain, but no neuroinvasion of the virus (Bielefeldt-Ohmann et al., 2017). In a project that investigated global gene expression of the central nervous system (CNS) of horses by sequencing the transcriptome of the brain and spinal cord, researchers identified gene ontology groups utilized in the WNV immune response. These include IL-15, IL-22, MAPK, and JAK/STAT signaling, as well as apoptosis pathways and B cell and T cell receptor expression (Bourgeois et al., 2011). These pathways also exist in humans, indicating similarities between the human and horse immune responses to WNV.

Horses also have an OAS1 response to WNV that is inducible by interferon, and variation in the horse *OAS1* gene has been associated with changes in WNV susceptibility (Rios et al., 2007, 2010). Furthermore, like birds, horses also mount an immune response to WNV using antibodies (Bielefeldt-Ohmann et al., 2017). Pony foals have been shown to receive maternally-inherited antibodies as a means of passive immunity (Wilkins et al., 2006). This strategy utilizes antibodies to rapidly activate the immune response to protect foals from infection.

THE HUMAN IMMUNE RESPONSE TO WNV

Because of the negative impact of WNV on the human population throughout the U.S., many researchers have characterized the human immune response to WNV, reviewed by Suthar et al. (2013). Briefly, the viral RNA sensors RIG-I and MDA5 detect WNV intracellularly, activating the adaptor protein MAVS, leading to IRF-3 activation for interferon induction and downstream induction of interferon-stimulated genes (ISGs) (Fredericksen et al., 2004, 2008; Fredericksen and Gale, 2006). The cytokines IFN-α and IFN-β are important for controlling WNV tropism by inducing an antiviral state (Samuel and Diamond, 2005). Priming an IFN response with the unrelated virus Invertebrate Iridescent virus 6 actually reduces WNV (Kunjin strain) titer *in vitro* (Ahlers et al., 2016). The downstream ISGs include the IFIT (interferon-induced protein with tetratricopeptide repeats) genes and viperin, which inhibit viral infection and replication (Jiang et al., 2010; Szretter et al., 2011; Gorman et al., 2016). Notably, the nonstructural protein NS5 of WNV inhibits the interferon response by preventing the expression of IFN-α receptor 1 on the surface of host cells (Lubick et al., 2015). The virus can also evade host restriction by IFIT proteins via 2'-O methylation of WNV (Daffis et al., 2010; Szretter et al., 2012). This strategy of antagonizing the IFN response is common to flaviviruses. ZIKV and DENV NS5 target human STAT2 for degradation (Morrison et al., 2013; Grant et al., 2016) by different mechanisms. YFV binds to STAT2 after host cells are stimulated with IFN to prevent it from binding to promoter elements (Laurent-Rolle et al., 2014).

Apoptosis is another innate immune response in mammals that restricts WNV replication, and the mechanisms of apoptosis induction have been studied in murine models. In one, mouse embryonic fibroblasts utilize CHOP (cyclic AMP response element-binding transcription factor homologous protein) to induce apoptosis and reduce WNV titer (Medigeshi et al., 2007). However, while apoptosis can be an effective method for eliminating virus from a host, it has a damaging effect on neurons. Although caspase 3 is activated during WNV infection, possibly in an attempt at an immune response, caspase 3 knockout mice have higher survival during WNV infection and less neuronal death than their wild-type counterparts (Samuel et al., 2007). Moreover, inhibition of caspase 8 during WNV infection reduces CNS tissue injury (Clarke et al., 2014). These findings suggest that the net beneficial or detrimental outcome of apoptosis as an immune response could be dependent on the type of tissue and the specific pro-apoptotic pathway activated.

While the interferon response is critical for restricting WNV, the human immune response to WNV also utilizes the OAS and RNase L pathway (Hornung et al., 2014). Indeed, a single nucleotide polymorphism in the *OAS1b* gene, namely rs34137742, that contains a C to T substitution in the second intron of the gene, is a risk factor for human West Nile encephalitis and paralysis from WNV infection (Bigham et al., 2011). *OAS1* has been demonstrated to undergo positive selection in Old World primates (Fish and Boissinot, 2016), indicating a historic interaction between flaviviruses like WNV and host immunity (Daugherty and Malik, 2012). This pathway is conserved in birds and horses, as discussed in earlier sections.

Passive immunity is also useful for rapid host protection to WNV. B cell and antibody-deficient (μMT) mice and B cell activating factor receptor (BAFFR)-deficient mice are susceptible to infection, but, if treated with immune sera from a wild-type mouse with antibodies to WNV, can be protected from infection (Diamond et al., 2003; Giordano et al., 2017). Strikingly, the BAFFR-deficient mice can develop sustained protective immunity after treatment with immune sera (Giordano et al., 2017). Together, this indicates that passive immunity could be utilized as a therapeutic option for human infection to induce a robust immune response. To the best of our knowledge, no studies have determined if antibodies to WNV are maternally-inherited in humans.

In summary, the animal hosts of WNV have both shared and divergent immune response pathways (**Table 1**). While mammals do possess an RNAi pathway like insects, the IFN immune response takes precedence as the primary innate immune response (Benitez et al., 2015). Both insects and mammals utilize apoptosis as a rapid response to virus infection. Birds, horses, and humans all utilize an OAS response and passive immunity, which are both activated rapidly during infection and are effective at restricting WNV.

THE FUTURE OF WNV RESEARCH

Presently, no approved vaccine or therapeutic exists for human use to prevent or treat WNV infection. There are, however,

TABLE 1 | Summary of the host responses of the animal hosts of West Nile virus.

	Mosquito	Bird	Horse	Human
RNAi response	Yes	Unknown	Unknown	Not utilized
Interferon-mediated response	Possibly, using Vago	Unknown	Yes	Yes
Apoptosis	Yes	Unknown	Yes	Yes
OAS response	Absent	Yes	Yes	Yes
Passive immunity	Absent	Yes	Yes	Yes

Various classes of conserved host responses are noted if they are utilized in the response to WNV (yes), present in the host, but not utilized in the response to WNV (not utilized), or not present/undiscovered in the host (absent).

four approved horse vaccines in use in the U.S., which have greatly aided in the reduction of equine cases. Veterinary options include two inactivated whole virus vaccines, a non-replicating live recombinant canary pox vector vaccine, and an inactivated flavivirus chimera vaccine (Ishikawa et al., 2014; Balasuriya et al., 2015). A number of human vaccines have been proposed, with some in clinical trials.

One promising vaccine is ChimeriVax-WN02, which is a live, attenuated vaccine created by inserting the genes for the pre-membrane (prM) and envelope (E) proteins from WNV into the yellow fever 17D clone (Arroyo et al., 2004). The vaccine completed a successful phase I clinical trial and two phase II clinical trials (Monath et al., 2006; Biedenbender et al., 2011; Dayan et al., 2012). Another chimeric vaccine that passed a phase I trial, rWN/DEN4Δ30, also utilizes prM and E from WNV but uses the live attenuated vaccine candidate rDEN4 Δ30 as a vector (Durbin et al., 2013). Another strategy utilizes a DNA vaccine with the prM and E proteins of WNV, either with the CMV promoter (Martin et al., 2007) or a modified CMV promoter (CMV/R) (Ledgerwood et al., 2011). Both versions of this vaccine completed successful phase I clinical trials.

Despite these successful early clinical trials, no WNV vaccine has moved into phase III trials. Some challenges for a phase III trial for a WNV vaccine are discussed in Ishikawa et al. (2014). One notable impediment is the low and sporadic incidence of WNV activity, which would make it difficult to establish vaccine efficacy. Because of the logistical challenges of developing and licensing a vaccine for WNV, perhaps a more feasible avenue for prevention is the introduction of *Wolbachia* into the mosquito population. As discussed in an earlier section, *Wolbachia* reduces flavivirus titer in mosquitoes, and models predict that WNV eradication is possible with the introduction of *Wolbachia* (Farkas et al., 2017). Certainly, great caution should be taken to determine if the introduction of *Wolbachia* into the *Culex* population would have any detrimental effects on the greater ecosystem.

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LA wrote the manuscript in consultation with AG.

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Antibody-Dependent Enhancement and Zika: Real Threat or Phantom Menace?

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Keywords: Zika virus, dengue virus, West Nile virus, antibody-dependent enhancement (ADE), vaccines

INTRODUCTION

Host-pathogen interaction between Zika virus (ZIKV) and other medically relevant flaviviruses is a hot topic, in part due to the potential risk of disease exacerbation by antibody-dependent enhancement (ADE). Current data are controversial, thus care should be taken when facing this aspect of ZIKV infection, particularly during vaccine development.

ZIKA VIRUS EPIDEMIC

Mosquito-borne flaviviruses are (re-)emerging pathogens responsible for several human diseases that lately have been raising alarms both socially and in healthcare. Colonization of new geographical areas by vectors and spread of ZIKV to regions with competent vectors allows its increased settling, exemplified by the recent epidemic across new areas where other important flaviviruses, such as dengue virus (DENV), Yellow Fever virus (YFV) and West Nile virus (WNV), co-circulate. The alarm was mainly due to the rapid spread of the virus across the American continent (Ali et al., 2017; Zhang et al., 2017). Previously, ZIKV infection was associated with mild, flu-like symptoms, but currently several serious neurological complications including Guillain-Barré syndrome and fetal/neonatal microcephaly have been directly linked to it. In this scenario, the viability of a vaccine against ZIKV as a preventive strategy is gaining force (Saiz et al., 2017), supported by previous experiences with flavivirus vaccines such as that against YFV. However, development of flaviviral immunity may also carry disadvantages that must be taken into account before undertaking massive vaccination campaigns.

ANTIBODY-DEPENDENT ENHANCEMENT OF INFECTION

Flaviviruses often show antigenic cross-reactivity, with shared immunogenic epitopes for stimulation of both humoral and cell-mediated immune responses. This cross-reactivity can be beneficial and result in cross-protection; however, humoral cross-reactivity can also exacerbate disease by the phenomenon of antibody-dependent enhancement (ADE) (Halstead, 2014), of which DENV is the prototypic model. Cells with phagocytic activity and bearing Fc receptors (FcR) are able to help to the clearance of pathogens coated with antibodies. The process implies the recognition of the constant portion of the antibody by the receptor in a class-dependent manner and greatly improves phagocytic activity of cells but also can become a type of immunopathology when is "exploited" by pathogens as DENV. Primary DENV infection results in a mild, acute disease with production of efficient neutralizing antibodies, in which virus-antibody complexes are recognized by FcR, internalized and destroyed. Problems may arise when a second DENV infection of a different serotype occurs, as the antibodies produced during the first infection can recognize and bind the second infecting strain, but with sub-neutralizing capability (Halstead, 2014). Thus, cells bearing FcR uptake and internalize antibody-coated viruses that are able to replicate within (**Figure 1A**). This phenomenon can also enable the virus to infect non-permissive cells, and has

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been related to lower antiviral responses. Severe disease outcome is therefore related not only to early high viremia, but also to lower levels of innate immune mediators such as nitric oxide (NO) or interferon (IFN) transcripts, and to a higher production of interleukines such as IL-10 (Halstead, 2014). Although recent studies also support an increased risk of developing severe dengue disease in humans with pre-existing anti-DENV antibodies (Katzelnick et al., 2017), the controversy persists, because cases of dengue hemorrhagic fever have been reported in primary infections by dengue (Khurram et al., 2014; Halstead and Cohen, 2015; Soo et al., 2016). Dengue ADE has been linked to the production of cross-reactive antibodies against the precursor-membrane protein (prM) of the DENV viral surface, which might increase the infectivity of immature virions carrying high amounts of uncleaved prM (Dejnirattisai et al., 2010). Apart from data obtained *in vitro*, ADE activity has not been described for other flaviviruses. However, due to the potential disastrous consequences of ADE between ZIKV and other flaviviruses, this possibility must be kept in mind.

POTENTIAL FOR ADE BETWEEN ZIKA AND OTHER RELATED VIRUSES

A prerequisite for ADE to occur is the existence of antigenic cross-reactivity. Antibodies from DENV- or ZIKV-infected humans (Dejnirattisai et al., 2016; Paul et al., 2016; Sapparapu et al., 2016; Stettler et al., 2016; Kam et al., 2017) and non-human primates (Keasey et al., 2017; Pantoja et al., 2017), and from WNV-infected patients (Bardina et al., 2017) have shown a wide variety of *in vitro* cross-reactivity, with a primary tendency toward high cross-reactivity, especially between ZIKV and DENV. In other cases, highly specific or no cross-reactive antibodies against ZIKV or WNV have been observed (Stettler et al., 2016; Keasey et al., 2017; Vázquez-Calvo et al., 2017). Even cross-reactive antibodies can be protective if neutralization of heterotypic virus still occurs; however, *in vitro* seroneutralization assays have shown an absent or poor neutralizing capability for the majority of ZIKV and DENV cross-reactive antibodies tested, with few inducing *in vitro* cross-neutralization (Dejnirattisai et al., 2016; Kawiecki and Christofferson, 2016; Paul et al., 2016; Stettler et al., 2016; Bardina et al., 2017; Kam et al., 2017). Seroneutralization between WNV- and ZIKV-specific antibodies was also lacking (Bardina et al., 2017; Vázquez-Calvo et al., 2017). In contrast, the vast majority of DENV- and ZIKV-specific antibodies tested were able to increase heterotypic viral replication in cell culture (Dejnirattisai et al., 2016; Kawiecki and Christofferson, 2016; Paul et al., 2016; Bardina et al., 2017; Kam et al., 2017; Londono-Renteria et al., 2017). This has been postulated to be an ADE-mediated phenomenon, as it was prevented by abolishment of Fc-Fc$_\gamma$R interaction by antibody mutation (Stettler et al., 2016) or pre-treatment with α-FcR antibodies (Paul et al., 2016). Similarly, WNV-specific antibodies also enhanced ZIKV infection (Bardina et al., 2017). Although these results agree with previous experimentally-described viral enhancement upon heterotypic flavivirus infection in cultured cells (Fagbami et al., 1987), they are difficult to extrapolate *in vivo*; in fact, animal studies have shown disparate results. A significant increase in mortality was described in ZIKV-infected Stat2$^{-/-}$ mice, with altered IFN responses and permissivity to ZIKV and DENV infections when pretreated with DENV-immune plasma (Bardina et al., 2017). In addition, ZIKV-specific antibodies delivery in AG129 immunocompromised mice resulted in an enhancement of DENV infection (Stettler et al., 2016). Similar results were observed in non-human primates (Rhesus macaques) commonly used as models of dengue disease (George et al., 2017). In contrast, IFNAR$^{-/-}$ mice pre-treated with anti-DENV antibodies were protected against a lethal ZIKV challenge (Kam et al., 2017), nor was ADE observed in 129Sv/ev immunocompetent mice pre-treated with anti-DENV antibodies and challenged with ZIKV (Stettler et al., 2016), or in natural models of ZIKV infection (Rhesus macaques) previously exposed to DENV (McCracken et al., 2017; Pantoja et al., 2017) or YFV (McCracken et al., 2017). Likewise, ZIKV infection protect immunocompetent mice against WNV challenge (Vázquez-Calvo et al., 2017). The difference between ADE and cross-protection could be a product of the varied type and dose of the antibody/immunization route and scheme used. Indeed, in Stat2$^{-/-}$ mice, high concentrations of DENV-immune plasma protected against ZIKV infection, as did pretreatment with WNV-specific antibodies (Bardina et al., 2017). It is worth noting that ZIKV and DENV share immunodominant epitopes that elicited cross-reactive T cells (Grifoni et al., 2017; Wen et al., 2017) with *in vivo* protective roles (Wen et al., 2017), which could also explain the differences observed between experiments using plasma administration or virus immunization.

CURRENT EPIDEMIOLOGICAL SCENARIO

Regarding the current data on the clinical relevance of ADE for ZIKV infection, ZIKV has co-circulated with DENV and other flaviviruses in several countries with high rates of seroprevalence, and to-date, no change in clinical outcome has been correlated with the presence of other flaviviruses. During an outbreak in French Polynesia (2008), ZIKV infection was linked to Guillain-Barré development independent of previous DENV infection (Cao-Lormeau et al., 2016). Other neurological complications, such as microcephaly, have also been related to ZIKV infection (Martines et al., 2016). The hypothesis of ADE behind ZIKV-related microcephaly has been raised by expertise in the field, but remains to be explored (Miner and Diamond, 2017). However, a recent work, has shown that changes in the virus, specifically a mutation in the prM protein, will be more likely responsible for the development of microcephaly (Yuan et al., 2017) which could explain the increased rate during the last epidemic in the Americas, since evolutionary analysis indicated that this substitution was not present in previous ancestors. Additionally, the ZIKV epidemic in the Americas, where DENV is a very important concern, has not altered the downward trend in severity and lethality of dengue (**Figures 1B,C**). However, these data should be taken with caution, because they are based on clinical reported cases and not all of them have been assayed for laboratory confirmation. Nevertheless, they point to a lack of DENV-infection enhancement due to ZIKV circulation. Accordingly, recent epidemiological surveys have showed that patients with prior DENV infection exhibited no signs correlated

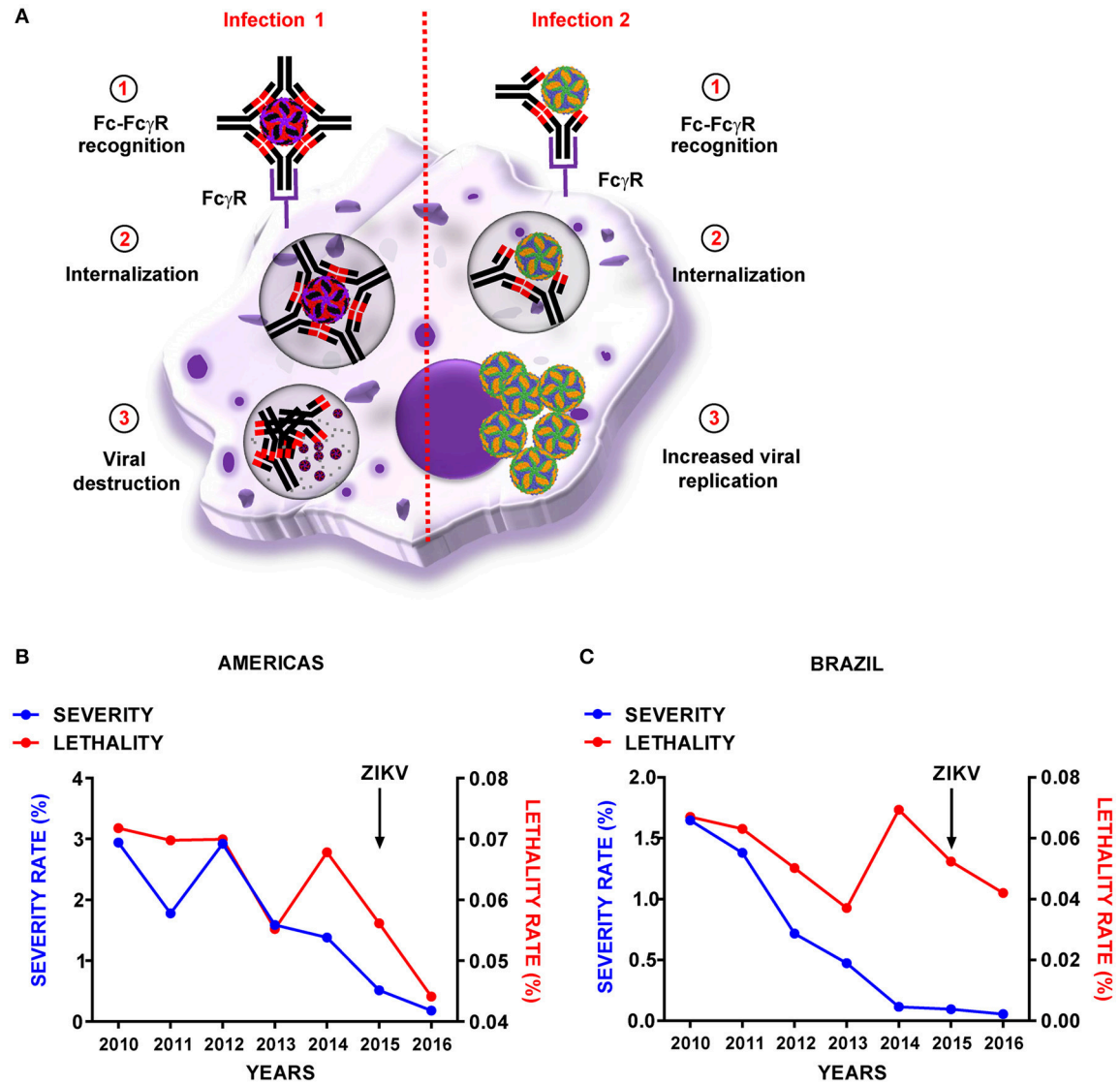

FIGURE 1 | Representation of dengue virus antibody-dependent enhancement and reported cases in the Americas. **(A)** Schematic representation of dengue virus antibody-dependent enhancement phenomenon. Graphical representation of DENV-severity and-lethality rates across the years in the Americas **(B)** and Brazil **(C)** Zika virus (ZIKV) epidemic is indicated with an arrow. Dengue severity was calculated as the number of cases including severe dengue and hemorrhagic dengue fever/total dengue cases. Lethality rate was calculated as the number of dengue-associated deaths/total dengue cases. Data were obtained from the Pan American Health Organization/World Health Organization (PAHO/WHO) website: (http://www.paho.org/hq/index.php?option=com_topics&view=readall&cid=3273&Itemid=40734&lang=en).

with ADE (as increased viral loads or pro-inflammatory cytokine profiles) in subsequent ZIKV infections (Bernardes-Terzian et al., 2017). Additionally, epidemiologic data suggests that vaccination against YFV could be related with protection against ZIKV (De Góes Cavalcanti et al., 2016), which further supports the lack of ADE among ZIKV and other flaviviruses. Nonetheless, more efforts, ideally with adequate immunocompetent animal models, must be performed to answer the questions about ZIKV and ADE. It is expected that, together with seroepidemiological surveillance data, these studies will contribute to a better understanding of the interplay between flaviviruses and the risk-to-reward ratio of ZIKV vaccines.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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