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Aedes Aegypti saliva enhances chikungunya virus replication in human skin fibroblasts via inhibition of the type I interferon signaling pathway

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ABSTRACT

Chikungunya virus (CHIKV) transmission occurs through the bite of an infected *Aedes* mosquito which injects virus-containing saliva into the skin of the human host during blood feeding. In the present study, we have determined the effect of *Aedes aegypti* saliva on CHIKV replication in human skin fibroblasts, a major cell type for viral entry, which mimics the events that occur during natural transmission. A significant increase in the expression of viral transcripts and infectious viral particles was observed in fibroblasts infected with CHIKV in the presence of saliva, as compared with those infected with virus alone. CHIKV-infected human fibroblasts were found to express significantly increased levels of various type I IFN-responsive genes, as demonstrated by specific PCR array analysis. In contrast, the expression of these genes was markedly decreased in cells infected with CHIKV in the presence of mosquito saliva. Moreover, Western blotting analysis revealed that STAT2 and its phosphorylated form were down-regulated in the presence of mosquito saliva. Our data demonstrate for the first time the significance of *Aedes aegypti* saliva in promoting CHIKV infection via down-regulation of several type I IFN-responsive genes in infected human skin fibroblasts via the JAK-STAT signaling pathway.

Chikungunya virus (CHIKV), a mosquito-borne alphavirus, has caused large outbreaks of disease throughout Asia, Africa and several islands in the Indian Ocean with recent epidemics in the Americas (Gallian et al., 2017). CHIKV is the agent of an acute febrile illness characterized by myalgia, rash, severe joint pain and, often debilitating, complications that can persist for years (Oviedo-Pastrana et al., 2017). Transmission of CHIKV occurs through the bite of an infected *Aedes* (*Ae.*) mosquito when the virus is injected, together with mosquito saliva, into the skin of the human host. The mosquito salivary glands secrete various pharmacologically active molecules that contribute to successful blood feeding by inhibiting host hemostasis, inflammation and immune responses (Wichit et al., 2016). In addition, the presence of saliva during CHIKV infection has been reported to significantly suppress the expression of various inflammatory genes and the production of chemokines (Agarwal et al., 2016).

Human dermal fibroblasts have been reported to be susceptible to CHIKV replication in vitro (Sourisseau et al., 2007) and we have recently demonstrated that these cells contained the highest concentration of CHIKV antigens following viral infection via the skin (Ekchariyawat et al., 2015). In the present study, we have determined the effect of *Ae. aegypti* saliva on CHIKV replication in human skin fibroblast cell line which better mimics the events that occur during natural transmission. To this end, the latter cells were infected with CHIKV at a multiplicity of infection of 1 in the absence or presence of *Ae. aegypti* saliva from 3 mosquitoes (1 μg/ml) which was maintained throughout the infection. *Ae. aegypti* saliva had no effect on the viability of the cells (Supplementary Fig. 1). At 48 h post infection a significant increase of > 2 logs in the expression of viral transcripts, measured by real-time quantitative PCR, was observed in fibroblasts infected with CHIKV in the presence of saliva, as compared with that in cells infected with virus alone (Fig. 1A). Moreover, results from a plaque assay confirmed the strong induction in the release of infectious CHIKV particles which increased in a time-dependent manner (Fig. 1B). These data support previous observations showing that *Ae. aegypti* saliva has an important role in the inhibition of an antiviral immune response by the host by creating an environment that favors the replication of CHIKV (Agarwal et al., 2016).

Type I interferons (IFNs) are known to combat viruses during viral infections and it has been well-established that CHIKV infection elicits a type I IFN response alongside the production of other pro-inflammatory

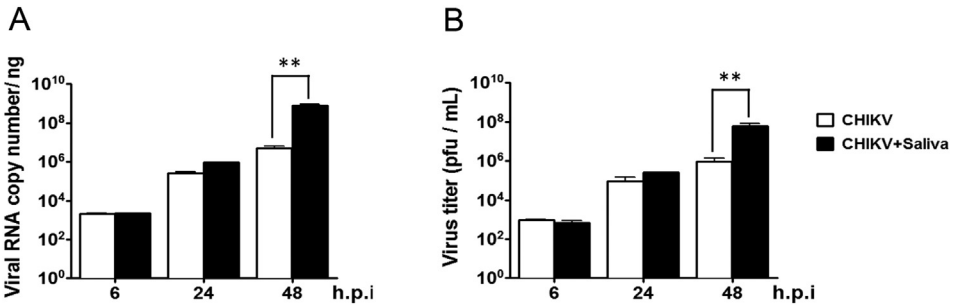
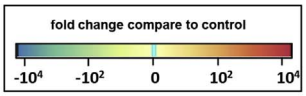
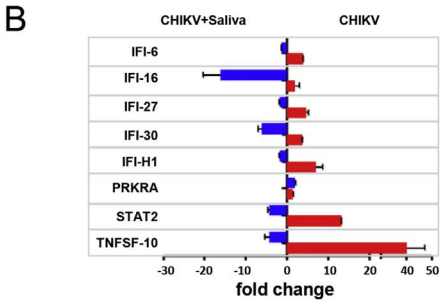


Fig. 1. *Ae. aegypti* saliva enhances CHIKV replication in human skin fibroblasts. Cells were infected with CHIKV LR2006_OPY1 strain at a multiplicity of infection of 1 in the presence or absence of *Ae. aegypti* saliva. After 6, 24 and 48 h, intracellular viral RNA and infectious virus production were quantified by (A) quantitative real-time PCR and (B) plaque assay, respectively. Data are representative of three independent experiments, each performed in duplicate ± standard deviation of the mean. The Student's *t*-test was used to analyze the differences between sets of data. A value of $p < 0.01$ was considered significant. ** *p*-values < 0.01.

A

Control	CHIKV	CHIKV+Saliva	Gene Name	Description	Functional Gene Grouping
1	115.0	-1.4	*BAG3*	BCL2-associated athanogene 3	Apoptosis
1	8.8	-21395.2	*IFI16*	Interferon, gamma-inducible protein 16	
1	2990.1	1.0	*MAL*	Mal, T-cell differentiation protein	
1	25110.4	-19.0	*MET*	Met proto-oncogene (hepatocyte growth factor receptor)	
1	73.8	1.0	*PSME2*	Proteasome activator subunit 2 (PA28 beta)	
1	5.9	-283.1	*SHB*	Src homology 2 domain containing adaptor protein B	
1	5503.0	-267.8	*TNFSF10*	Tumor necrosis factor superfamily, member 10	Apoptosis/Cytokine
1	435.3	-8.5	*ADAR*	Adenosine deaminase, RNA-specific	Apoptosis / Innate Immunity
1	6.4	-894.5	*IFI27*	Interferon, alpha-inducible protein 27	
1	204.5	-10.4	*IFI6*	Interferon, alpha-inducible protein 6	
1	570.5	-21.0	*IFIH1*	Interferon induced with helicase C domain 1	
1	20537.9	-1172.2	*TRAF3*	TNF receptor-associated factor 3	
1	2969.5	-3.5	*HLA-C*	Major histocompatibility complex, class I, C	Immunity
1	3864.3	-3.8	*HLA-DOA*	Major histocompatibility complex, class II, DO alpha	
1	305.7	1.1	*HLA-DQA1*	Major histocompatibility complex, class II, DQ alpha 1	
1	417.6	1.4	*HLA-F*	Major histocompatibility complex, class I, F	
1	2.4	-17.7	*BST2*	Bone marrow stromal cell antigen 2	Innate Immunity
1	129.4	-2.2	*GBP1*	Guanylate binding protein 1, interferon-inducible	
1	11237.2	-100.1	*HLA-B*	Major histocompatibility complex, class I, B	
1	1140.9	1.5	*HLA-E*	Major histocompatibility complex, class I, E	
1	73.8	3.7	*HLA-G*	Major histocompatibility complex, class I, G	
1	6.6	-1222.0	*IFI30*	Interferon, gamma-inducible protein 30	
1	70.8	-2.9	*IRF7*	Interferon regulatory factor 7	
1	1395.0	-29.3	*IRF9*	Interferon regulatory factor 9	
1	336.9	-8.8	*ISG20*	Interferon stimulated exonuclease gene 20kDa	
1	39130.3	-11.7	*NMI*	N-myc (and STAT) interactor	
1	773.9	-11.1	*OAS1*	2'-5'-oligoadenylate synthetase 1, 40/46kDa	
1	62.9	-2.5	*SH2D1A*	SH2 domain containing 1A	
1	3182.6	-106.5	*STAT2*	Signal transducer and activator of transcription 2, 113kDa	
1	-5.1	-11.9	*MYD88*	Myeloid differentiation primary response gene (88)	Innate/Adaptive Immunity/Apoptosis
1	6.2	-459.8	*TAP1*	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	Innate/Adaptive Immunity
1	22.4	1.3	*VEGFA*	Vascular endothelial growth factor A	Adaptive Immunity/Apoptosis/Cytokine
1	253.5	-3.5	*IFNA4*	Interferon, alpha 4	Interferons & Receptors/Innate Immunity
1	160.5	-6.1	*IFNAR1*	Interferon (alpha, beta and omega) receptor 1	
1	48.7	-1.9	*IFNAR2*	Interferon (alpha, beta and omega) receptor 2	
1	624.3	-22.1	*ANXA11*	Annexin A11	Cell cycle/Division
1	2070.8	-303.4	*CD70*	CD70 molecule	Cytokine
1	3.7	-23.8	*ARL5A*	ADP-ribosylation factor-like 5A	GTP binding
1	747.5	-30.8	*SAMSN1*	SAM domain, SH3 domain and nuclear localization signals 1	Negative regulation of adaptive immune response
1	435.3	-6.9	*HSPA1L*	Heat shock 70kDa protein 1-like	Protein folding
1	25.4	6.8	*PRKRA*	Protein kinase, IFN-inducible ss RNA dependent activator	RNA-mediated gene silencing
1	20.3	2.4	*GCH1*	GTP cyclohydrolase 1	Tetrahydrobiopterin biosynthesis
1	201.7	-6.1	*HOXB2*	Homeobox B2	Transcription regulation
1	3227.0	-29.8	*MNT*	MAX binding protein	





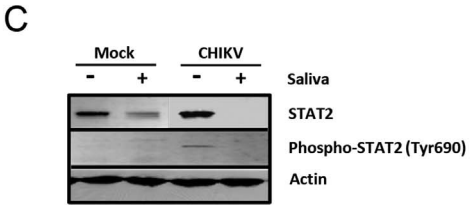


Fig. 2. Expression of genes involved in the regulation of the human type I IFN response by CHIKV in human skin fibroblasts. Cells were exposed to CHIKV in the presence or absence of saliva. (A) The expression of significantly up- or down-regulated genes involved in the human type I IFN response was measured by PCR array (PAHS-016Z; SABiosciences, Frederick, MD). The fold changes of gene expression were color-coded from blue to red (showing down-regulation to up-regulation) (B) mRNA levels of the indicated gene were validated by qRT-PCR. Results are expressed as fold change of transcripts in CHIKV-infected cells in the presence or absence of saliva, relative to those in mock-infected cells (Control). (C) Mock and infected cells were lysed and analyzed by immunoblotting against STAT2, phospho-STAT2 (Tyr690) and β-actin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cytokines (Lum and Ng, 2015). In the present study, CHIKV-infected human fibroblasts were found to express significantly increased levels of many type I IFN-responsive genes, as demonstrated by specific PCR array analysis. In contrast however, these genes were markedly decreased in cells infected with CHIKV in the presence of mosquito saliva (Fig. 2A). The expression of the interferon-inducible (IFI) genes *IFI6*, *IFI16*, *IFI27*, *IFI30* and *IFIH1* (*MDA5*), as well as *STAT2*, *TNFSF10* (*TRAIL*) and *PRKRA*, were further validated by real-time quantitative PCR (Fig. 2B). In contrast, and used as a negative control, expression levels of the *PRKRA* did not change under either experimental condition in accordance with the microarray data. Moreover, immunoblotting analysis revealed that *STAT2* and its phosphorylation form were down-regulated in the presence of mosquito saliva (Fig. 2C). Since, mosquito saliva can down-regulate these genes expression (Supplementary Fig. 2), it is therefore reasonable to hypothesize that mosquito saliva directly affects the host immune response, at least in part, via JAK-STAT signaling pathway.

Following CHIKV infection, viral RNA is recognized by cytosolic RNA sensors that trigger the expression of various pro-inflammatory genes, resulting in an anti-viral type I IFN response. In particular, *MDA5* efficiently detects dsRNAs which leads to activation of the IRF3-dependent signaling pathways and, ultimately, type I IFN production. Consequently, triggering of the canonical pathway of IFN type I signaling through JAK-STAT activation results in the assembly of the ISGF3 complex that translocates to the nucleus to activate gene transcription (Schwartz and Albert, 2010). These concerted activation pathways converge to restrict CHIKV replication. Support for this notion is provided by the recent observation that alphavirus-infected cells were unable to induce a type I IFN response in the absence of *MDA5* (Akhrymuk et al., 2016). Moreover, the enhanced *TRAIL* expression in CHIKV-infected fibroblasts is reminiscent of published results showing that triggering of the *STAT1* signaling cascade by HIV-1 Vpr also enhanced expression of this molecule, which would presumably help to eliminate HIV-1-infected cells through *TRAIL*-mediated cell death (Zahoor et al., 2014). Therefore, the decrease in the expression of these molecules by mosquito saliva is aimed to impair the host immune responses which, as a consequence, favors viral replication.

As a typical adaptive immune response does not develop immediately following infection, the innate immune system seems unable to control the early phase of CHIKV infection. Concordantly, in this study, most of down-regulated genes in skin cells challenged with saliva belong to those of innate immune response. In particular, *IFI6*, *IFI16*, *IFI27* and *IFI30* expression was significantly decreased in CHIKV-infected skin cells in the presence of saliva. As shown previously, increased *IFI6* expression levels are associated with a strong decrease in yellow fever, Dengue type 2, as well as West Nile, virus infection and *IFI6* was found to also limit HCV entry and replication in human hepatoma cells (Meyer et al., 2015). It is of note that *IFI6* and *IFI27* are two related proteins belonging to the FAM14 family on the basis of sequence similarity and that are commonly and concomitantly induced by IFNs. However, unlike *IFI6*, the latter molecule is not known to be involved in the modulation of virus infection. Another IFI gene induced in CHIKV infected skin cells, *IFI16*, reportedly acts as a restriction factor for several other DNA viruses (Orzalli et al., 2015), whereas it also induces anti-viral inflammasome activity against KSHV and EBV (Roy et al., 2016).

To our knowledge, this is the first study showing the importance of *Ae. aegypti* saliva in promoting CHIKV infection via downregulation of the expression of several type I IFN-responsive genes in infected human skin fibroblasts involving at least in part the JAK-STAT signaling pathway.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2017.08.032.

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