

High Mobility Group Box 1: a Novel Host Restriction Factor in Zika Virus Replication

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Abstract

Neonatal microcephaly and adult Guillain-Barré syndrome are severe complications of Zika virus (ZIKV) infection. The robustly induced inflammatory cytokine expressions in ZIKV-infected patients may constitute a hallmark for severe disease. In the present study, the potential role of high mobility group box 1 protein (HMGB1) in ZIKV infection was investigated. HMGB1 protein expression was determined by the enzyme-linked immunosorbent assay (ELISA) and immunoblot assay. HMGB1's role in ZIKV infection was also explored using treatment with dexamethasone, an immunomodulatory drug. Antiviral effects of dexamethasone treatment on both wild-typed (WT) and HMGB1-knockdown (shHMGB1) Huh7 cells were determined by the focus-forming assay. Results showed that the Huh7 cells were highly susceptible to ZIKV infection. The infection was found to induce HMGB1 nuclear-to-cytoplasmic translocation, resulting in a >99% increase in the cytosolic HMGB1 expression at 72h.p.i. The extracellular HMGB1 level was elevated in a time- and multiplicity of infection (MOI)-dependent manner. Dexamethasone 150 µM treatment of the ZIKV-infected cells reduced HMGB1 extracellular release in a dose-dependent manner, with a maximum reduction of $71 \pm 5.84\%$ ($p < 0.01$). The treatment also reduced virus titers by over $83 \pm 0.50\%$ ($p < 0.01$). The antiviral effects, however, was not observed in the dexamethasone-treated HMGB1-knockdown cells, suggesting the importance of the intracellular HMGB1 in ZIKV infection. Overall, these results suggest that translocation of HMGB1 occurred during ZIKV infection and inhibition of the translocation reduced ZIKV replication. These findings highlight the potential of developing therapeutics against ZIKV infection by affecting the translocation of HMGB1 from the nucleus to the cytoplasm.

Introduction

Outbreaks involving Zika virus (ZIKV), an arthropod-borne virus (arbovirus) that belongs to the genus *Flavivirus*, family *Flaviviridae*, has been declared as Public Health Emergency of International Concern (PHEIC) in February 2016¹ following the epidemics in the Pacific and Americas². ZIKV infection becomes a major concern as it has been associated with severe neurological disorders, including microcephaly in newborns and Guillain-Barré syndrome in adults^{3,4}. To date, there is no specific antiviral or vaccine available to treat or prevent ZIKV infection.

HMGB1 is a highly conserved DNA-binding protein and mainly resides in the nucleus, regulating the DNA transcription and nucleosome stability⁵. During infection and cell injury, acetylated HMGB1 is translocated from the nucleus to the extracellular milieu to act as a proinflammatory cytokine, causing inflammatory responses⁶ as seen in various virus-infected cells such as hepatitis C virus, dengue virus, West Nile virus, herpes simplex virus, human immunodeficiency virus and severe acute respiratory syndrome coronavirus-2^{7–13}. HMGB1 levels in dengue and hepatitis B infected patients' sera were found to be positively correlated to viral load and disease symptoms^{14,15}. Although the HMGB1 canonical pathway was found to be activated in both *in vitro*¹⁶ and ZIKV presymptomatic or asymptomatic patient plasma¹⁷, the role of HMGB1 in ZIKV infection remains largely unknown.

Dexamethasone is a glucocorticoid that has been broadly used due to its rapid anti-inflammatory and immunosuppressive impacts. The protective role of dexamethasone against various diseases, including sepsis

¹⁸, severe acute pancreatitis ¹⁹, acute lung injury ²⁰ through inhibiting HMGB1-mediated inflammation, has been proposed. Dexamethasone has been shown to inhibit HMGB1 nuclear-to-cytoplasmic translocation via acetyltransferase attenuation, hence reducing the HMGB1-TLR4 inflammatory pathway ²¹. On the other hand, recent studies showed that dexamethasone possesses the antiviral potential against many viruses such as human rhinovirus ²², foot-and-mouth disease virus ²³ and dengue virus ²⁴. The following study aimed to examine the dynamic of HMGB1 expression following ZIKV infection *in vitro* and its role in the pathogenesis of Zika.

Results

ZIKV caused productive infection in Huh7 cells.

The Huh7 cells were infected with the Asian ZIKV strain P6-740 at multiplicity of infection (MOI) of 1 in a 96-well plate and virus replication was monitored at selected time points for 4 days post-infection. At 24, 48, 72 and 96 h.p.i, two hundred microliters (200 µl) of supernatant of the infected cell cultures was harvested for virus titration using the focus-forming assay as previously described ²⁵. A gradual increase in the production of infectious virus was observed over the infection period (Fig. 1). From a titer of $3 \pm 0 \log_{10}$ FFU/mL at 0 h.p.i, the ZIKV replicated to $3.511 \pm 0.033 \log_{10}$ FFU/mL at 24 h.p.i. ($p > 1.0$), $4.734 \pm 0.010 \log_{10}$ FFU/mL (1-log increase, $p > 1.0$) at 48 h.p.i, $5.744 \pm 0.012 \log_{10}$ FFU/mL (2-log increase, $p > 0.1$) at 72 h.p.i, and a maximum titer of $6.362 \pm 0.061 \log_{10}$ FFU/mL (3-log increase, $p < 0.001$) at 96 h.p.i (Fig. 1). These findings suggested that Huh7 cells supported ZIKV replication well.

ZIKV infection induced translocation of nuclear HMGB1 into the cytosol.

The nuclear and cytosolic HMGB1 protein expressions of the mock-infected or ZIKV-infected Huh7 cells were determined using immunoblot assay. The nuclear and cytosolic HMGB1 expressions were normalized to the PARP-1 and GAPDH levels, respectively (Fig. 2). The HMGB1 was detected mainly in the nucleus of the mock-infected Huh7 cells after 72 h of incubation period (Fig. 2). During ZIKV infection, the accumulation of nuclear HMGB1 was reduced to 51% at 24 h.p.i ($p < 0.01$), to 7.2% at 48 h.p.i ($p < 0.001$) and 0% at 72 h.p.i. ($p < 0.001$), in comparison to that of the mock-infected Huh7 cells (Fig. 2a). On the other hand, the ZIKV infection resulted in increased level of HMGB1 in the cytoplasm by 11% at 24 h.p.i ($p < 0.001$), 39% at 48 h.p.i ($p < 0.001$) and 99% at 72 h.p.i. ($p < 0.001$) as compared to the mock-infected cells (Fig. 2b). These results suggested that the ZIKV infection induced the translocation of HMGB1 from the nucleus to the cytoplasm in Huh7 cells.

ZIKV infection induced HMGB1 release from Huh7 cells in a time- and MOI-dependent manner.

The extracellular HMGB1 levels in the culture supernatant of mock-infected Huh7 cells remained constant over the 72 h incubation period, with an average value of 346.83 ± 20.19 pg/ml. In contrast, the ZIKV infection at MOI of 1 significantly increased the extracellular HMGB1 levels at 48 h.p.i (1074.75 ± 91.25 pg/ml, $p < 0.001$) and 72 h.p.i (1397.25 ± 13.75 pg/ml, $p < 0.001$), in comparison to those of the mock-infected cells (Fig. 3a). Our results also showed that the HMGB1 release from the infected cells was MOI-dependent. At 72 h.p.i, the levels of extracellular HMGB1 increased from 1671.25 ± 162.5 pg/ml ($p < 0.05$) at MOI of 1, to 2115 ± 81.25 pg/ml ($p < 0.05$) at MOI of 3 and 8165 ± 531.25 pg/ml ($p < 0.001$) at MOI of 5 (Fig. 3b).

Dexamethasone inhibited the release of extracellular HMGB1 and ZIKV replication in a dose-dependent manner.

Dexamethasone showed low cytotoxicity for Huh7 cells; almost 100% of the Huh7 were still viable following treatments with 50–200 µM dexamethasone (Fig. 4). After ZIKV infection at MOI of 1, the Huh7 cells were treated with 0, 5, 80 and 150 µM of dexamethasone and the HMGB1 release of infected cell and virus titers were assessed at 72 h.p.i (Figs. 5 and 6). The extracellular HMGB1 levels of ZIKV-infected cells were significantly reduced from 1326 ± 54 pg/ml for the mock treatment, to 829 ± 69 pg/ml ($p < 0.05$), 564 ± 68 pg/ml ($p < 0.01$) and 385 ± 93 pg/ml ($p < 0.01$) by treatment with dexamethasone at 5, 80 and 150 µM, respectively (Fig. 5). Furthermore, the treatment of ZIKV-infected Huh7 cells with 5 µM, 80 µM and 150 µM of dexamethasone decreased the infectious virus titers by 25% ($p > 0.05$), 67% ($P < 0.05$) and 83% (1 log, $P < 0.01$), respectively (Fig. 6), in comparison to the mock-treated control.

Anti-ZIKV effect of dexamethasone was dependent on the presence of HMGB1.

The involvement of HMGB1 in the dexamethasone's anti-ZIKV effect was further investigated using the HMGB1-knockdown cells, shHMGB1 cells (Supplementary Fig. S1). Both shHMGB1 cells and wild-type (WT) Huh7 cells were infected with ZIKV at MOI of 1, followed by treatment with 150 µM of dexamethasone. In general, the ZIKV replicated to higher titer in the shHMGB1 Huh 7 cells than in the WT Huh7 cells (Fig. 7). Dexamethasone treatment of the infected shHMGB1 cells and WT Huh7 cells showed a reduction of 36% ($p > 0.05$) and 75% of ZIKV titer ($P < 0.05$), respectively, in comparison to the virus titers in the mock-treated infected cells (Fig. 7). This suggested that the HMGB1 may play a role in the antiviral actions of dexamethasone.

Discussion

Although the HMGB1 canonical pathway was found to be activated in both *in vitro*¹⁶ and ZIKV presymptomatic or asymptomatic patient plasma¹⁷, there is still no experimental evidence to support these suggestions. The findings of the present study demonstrated that the ZIKV infection induced translocation of HMGB1 from the nucleus to the cytoplasm and release from the infected Huh7 cells. Treatment with dexamethasone, a well-known anti-inflammatory drug, significantly reduced ZIKV infection and the extracellular HMGB1 levels. In the HMGB1 knockdown Huh7 cells, however, the treatment with dexamethasone did not cause significant inhibition of ZIKV replication, suggesting the involvement of HMGB1 in regulating the antiviral mechanisms of dexamethasone (Fig. 8).

HMGB1 is a nuclear transcription factor which acts as a pro-inflammatory cytokine when released from the cells in response to infection, cell injury and inflammation^{6,26}. In normal circumstances, the HMGB1 accumulates in the nucleus, giving a nuclear-to-cytoplasmic HMGB1 ratio of approximately 30:1²⁷. During infection or cell injury, HMGB1 undergoes post-translational modifications, including acetylation, methylation and phosphorylation, causing its translocation from the nucleus to cytoplasm before being released into the extracellular environment^{28,29}. For instance, the host cell p300/CBP-associated factor (PCAF) acetylase complex, triggered by dengue virus capsid protein, has been demonstrated to enhance the HMGB1 release³⁰. Moreover, excessive production of reactive oxygen species (ROS) induced by respiratory syncytial virus and porcine circovirus-2 have been found to promote the release of HMGB1^{31,32}. Similarly, our study showed that

ZIKV infection could induce HMGB1 nuclear-to-cytoplasmic translocation and release in a time-dependent manner, most likely through the similar mechanisms described.

In the previous study, augmented serum levels of HMGB1 was observed in secondary dengue virus infection¹⁵, which has been associated with higher viral load and prolonged viremia, most probably due to the delayed virus clearance as reported earlier³³. Meanwhile, our study also revealed that the secreted HMGB1 was elevated in MOI-dependent manner. Because virus-induced cell death is mainly infectious dose-dependent, ZIKV infection may result in necrosis- or apoptosis-dependent HMGB1 passive release, which requires further confirmation in the future.

Extracellular HMGB1 induces innate immune response via interaction with the receptor for advanced glycation end products (RAGE) or toll-like receptor (TLR-2 and -4), activating MyD88 and NF-κB signaling pathways, which then leads to the production of pro-inflammatory cytokines for virus clearance³⁴. However, excessive extracellular HMGB1 during virus infection has been associated with the pathogenesis of diseases³⁵⁻³⁷. For instance, extracellular HMGB1 was related to the pathogenesis of dengue hemorrhagic fever-dengue shock syndrome (DHF/DSS), presumably through the vascular barrier disruption³⁰. Besides, extracellular HMGB1 has been correlated to neuroinvasion of ZIKV and Japanese encephalitis virus (JEV), possibly via disruption of the blood-brain barrier^{38,39}. Therefore, preventing the overproduction of extracellular HMGB1 could be a therapeutic approach against the pathogenesis of viral infection.

Many studies have demonstrated that intracellular and extracellular HMGB1 play different roles in virus replication. For example, intracellular HMGB1 facilitates influenza virus and hepatitis C virus replication by directly interacting with viral RNA or nucleoprotein^{40,41}. Vice versa, intracellular HMGB1 limits HIV replication by downregulating the long terminal repeat (LTR)-mediated transcription⁴². Extracellular HMGB1 has also been reported to promote HIV replication in monocytic cells but decreases viral replication in primary macrophages⁴³. Moreover, the antiviral effect of extracellular HMGB1 against hepatitis C virus has been established by the activation of the interferon signaling in virus-infected human hepatoma cell line⁴⁴. Although HMGB1 has been found to have significant effects on various virus replication, the role of HMGB1 in ZIKV infection remains largely unknown.

This study revealed that dexamethasone treatment decreased both extracellular HMGB1 release and ZIKV replication. Dexamethasone has been shown to inhibit HMGB1 nuclear-to-cytoplasmic translocation via acetyltransferase attenuation, hence reducing the HMGB1-TLR4 inflammatory pathway²¹. Moreover, dexamethasone's anti-inflammatory action, which reduces TNF-α, IFN-α, and IL-10 production, has also been demonstrated to reduce dengue virus replication²⁴. Therefore, the inhibition of ZIKV replication by dexamethasone treatment was likely through the prevention of HMGB1 extracellular release.

To further investigate the role of intracellular HMGB1 in the antiviral mechanism of dexamethasone against ZIKV infection, HMGB1-depleted cells were used. This study showed no antiviral effects in dexamethasone-treated HMGB1-knockdown cells, signifying that the presence of intracellular HMGB1 is critical for the dexamethasone's antiviral activity. This finding is consistent with several earlier studies which demonstrated that HMGB1 retained in the nucleus plays a role in the antiviral response against dengue virus⁴⁵, duck reovirus,

duck Tembusu, duck plague virus⁴⁶ and human immunodeficiency virus⁴². In addition, the role of nuclear HMBG1 retention in upregulating interferon-stimulated genes (ISGs) expression to antagonise virus replication has been reported^{45,47}. Thus, the anti-ZIKV mechanism of dexamethasone is most likely through preventing HMGB1 translocation, which results in HMGB1 retention in the cells. In conclusion, current findings suggest that ZIKV infection triggers HMGB1 translocation from the nucleus to cytoplasm and extracellular release in a time- and MOI-dependent manner. The findings of this study provide a better insight into the underlying anti-ZIKV mechanisms by dexamethasone, particularly through the regulation of HMGB1. Further *in vivo* investigation could provide better understanding of HMGB1's role in ZIKV infection.

Methods

Cell culture and virus. Human hepatocellular carcinoma cells (Huh7, JCRB 0403) were cultured in Roswell Park Memorial Institute 1640 medium (RPMI; RBRC-RCB1942), enriched with 10% of heat-inactivated fetal bovine serum (FBS; Bovogen, Australia) and L-glutamine (Gibco, NY, USA). Huh7 cells were incubated at 37°C in a humidified incubator with 5% CO₂. Asian Zika virus (ZIKV) strain P6-740 (from Malaysia) was used in this study. ZIKV was archived in Tropical Infectious Diseases Research & Education Centre (TIDREC) at University of Malaya, Kuala Lumpur, Malaysia. African Green monkey kidney cells (Vero, ATCC- CCL81) in Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA) were used for virus propagation and titration.

Virus infection and virus titer determination. Huh7 cells were infected with ZIKV at various MOIs, with and without dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) added to cells at different concentrations. The cell culture supernatants were then collected at different time points (e.g., 24 h, 48 h and 72 h) for ZIKV titer determination using the focus-forming assay and expressed as Focus-Forming-Unit per ml (FFU/ml). The assay was carried out as previously reported²⁵.

Enzyme-linked immunosorbent assay (ELISA). The amount of extracellular HMGB1 in cell culture supernatant was quantified using a commercially available human HMGB1 ELISA kit (Cloud-Clone Corp, USA; cat. SEA399Hu 96 tests) according to the manufacturer's instructions.

Immunoblot assay. Huh7 cells were harvested at 24, 48 and 72h after ZIKV infection. Proteins were extracted from Huh7 cells as directed by the manufacturer using the NE-PER cytoplasmic and nuclear protein extraction kit (Thermo Scientific Pierce, Rockford, IL, USA). Micro BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA) was used to quantify the protein concentration of each sample. Equal amounts of proteins were denatured with sodium dodecyl sulphate–sample loading buffer, separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane for 14 minutes by the Bio-Rad semi-dry transfer system (Hercules, CA). Afterward, the membranes were blocked with 3% skimmed milk in PBS for 1 h at room temperature before being incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study were as follows: rabbit anti-HMGB1 antibody (ab79823; 1:10000–1:50000 dilution; Abcam, Cambridge, UK), rabbit anti-GADPH antibody (ab9485, 1:2500; Abcam, Cambridge, UK) and rabbit anti-PARP1 antibody (PLA0184, 1:2000-1:10000 dilution, Sigma-Aldrich, St. Louis, MO, USA). Blots were then incubated for 1 h at room temperature with a secondary goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (65-6120, 1:3,000–1:10,000; Invitrogen, Carlsbad, CA,

USA). The chemiluminescence Western blotting kit (BioRad, Hercules, CA) was used to detect the presence of proteins.

HMGB1 gene knockdown in Huh7 cells. The shRNA approach was used to achieve stable knockdown of HMGB1 gene expression (shHMGB1)⁴⁸. The recombinant lentiviruses carrying shRNA plasmid targeting human HMGB1 gene were purchased from OriGene Technologies (TL316576V, Rockville, MD, USA). A 50 percent confluent Huh7 cell monolayer was infected with the recombinant lentiviruses at a MOI of 20 in the presence of 8 µg/ml of polybrene, which was then incubated at 37°C overnight. After 24 hours, the culture medium was changed to a puromycin-containing selective medium with a final concentration of 1 µg/ml (Sigma-Aldrich, St. Louis, MO).

Statistical analysis. The statistically significant differences between the cell groups were determined by employing a one-way analysis of variance (ANOVA) and Bonferroni's multiple-comparison test with a 95 percent confidence interval (C.I.) to determine the significance of the variance. Graph-Pad Prism 8 was used to perform all statistical tests. The statistical significances of the groups were as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

Declarations

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Author Contributions

K.L.C., N.Z., S.A.B. and S.S.S. involved in the study and experimental design. K.L.C and N.Z. conducted the experiments and analysed the results. K.L.C., N.Z., S.A.B., S.S.S., P.H. and R.L. wrote, reviewed and edited the manuscript. All the authors have reviewed the manuscript.

Additional Information

Competing interests: The authors declare no competing interests.

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Figures

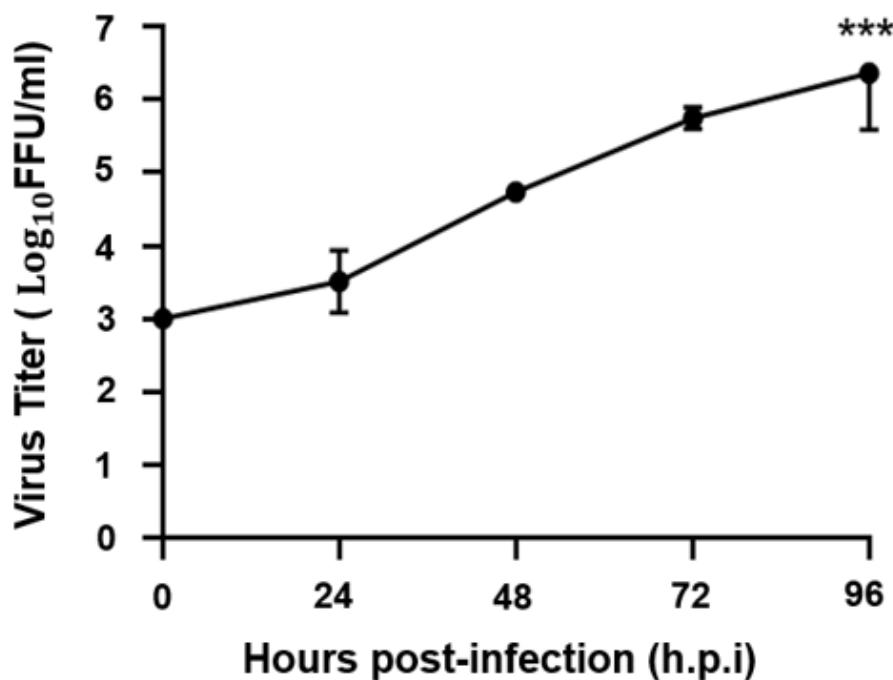
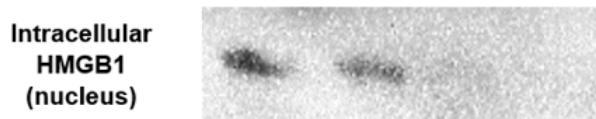
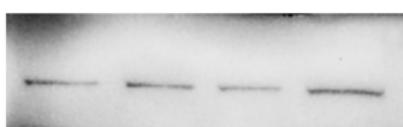


Figure 1

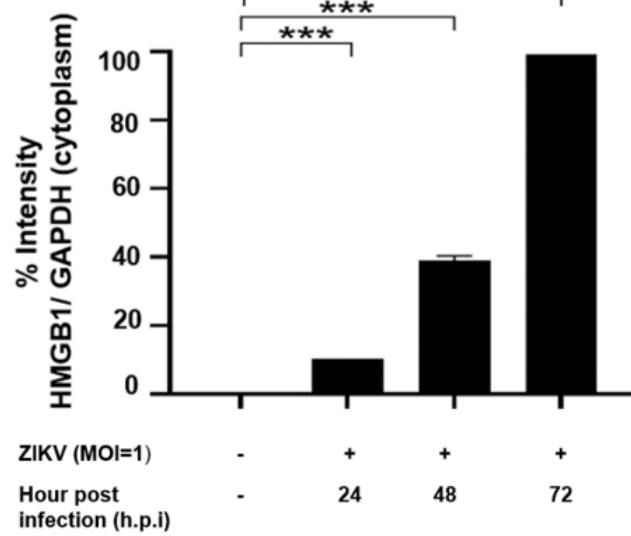
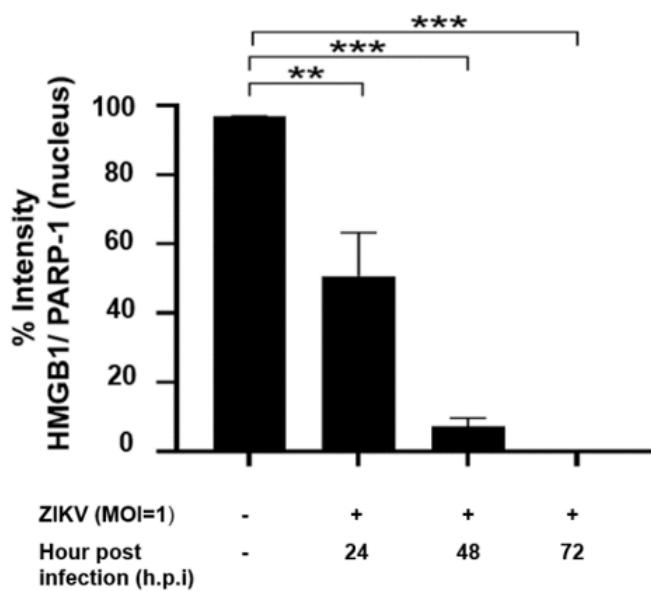
Replication kinetics of ZIKV in Huh7 cells. Huh7 cells were infected at a multiplicity of infection (MOI) of 1 in 96-well plate and the replication of virus was studied after 1-4 days post-infection (p.i.). Focus-forming assay on Vero cells was performed to determine virus titers of infected cell culture supernatants. Asterisks (***) indicate significant difference ($P<0.001$).

a**b**

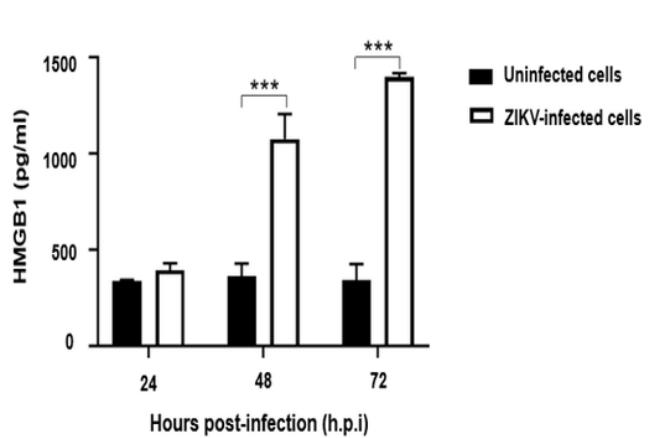
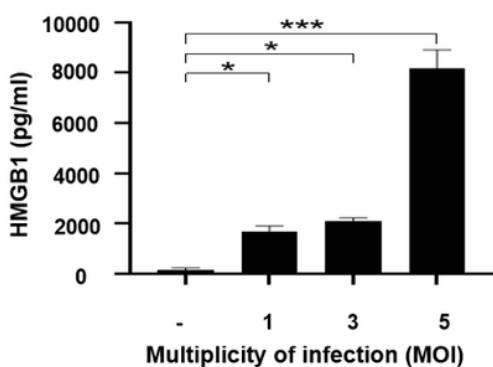
PARP-1



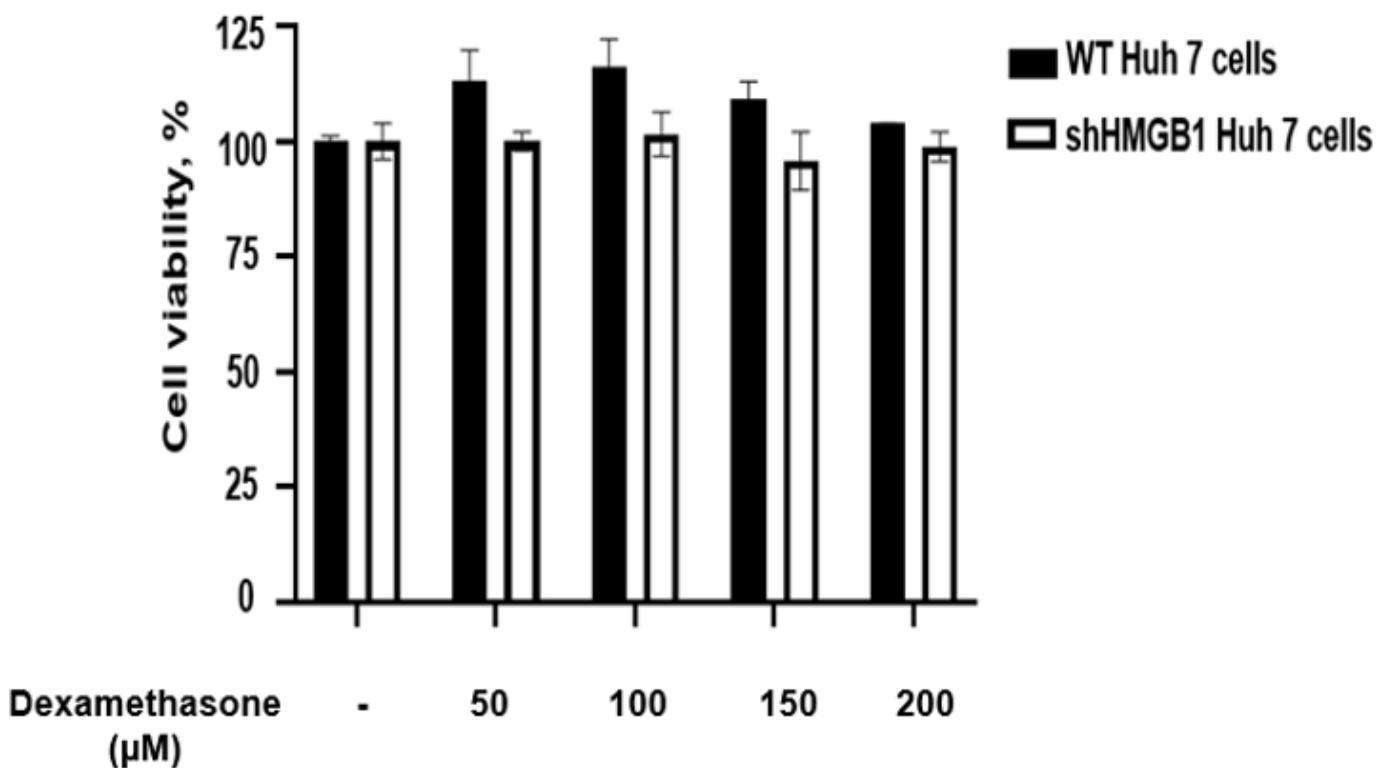
GAPDH

**Figure 2**

HMGB1 translocation during ZIKV infection. Huh7 cells were mock-infected or infected with ZIKV at an MOI of 1. Cell lysates were harvested at 24, 48 and 72 h post infection (p.i.). The nuclear (A) and cytosolic (B) proteins were determined by immunoblot assay for HMGB1 detection. Statistical significances between the groups were indicated: *P<0.05, **P<0.01, ***P<0.001.

a**b****Figure 3**

Extracellular HMGB1 levels during ZIKV infection: (A) Huh7 cells were mock-infected or infected with ZIKV at an MOI of 1. Supernatants of cell culture were harvested at 24, 48 and 72 h post-infection (p.i) and the level of HMGB1 was measured by ELISA. Asterisks (**) indicate significant difference ($P<0.01$). (B) Huh7 cells were mock-infected or infected with ZIKV at an MOI of 1, 3 and 5. Supernatants were then harvested at 72 h post infection (p.i) for extracellular HMGB1 detection by ELISA. Statistical differences between the groups were indicated: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

**Figure 4**

Cytotoxic effects of dexamethasone on wild-type (WT) and HMGB1-knockdown (shHMGB1) Huh7 cells. The cytotoxicity of dexamethasone was determined using the MTS assay. Both cells were treated with dexamethasone at concentrations of 0 μ M, 50 μ M, 100 μ M, 150 μ M and 200 μ M for 72 h. The experiments were performed in triplicates and the data obtained were analyzed using Graph Pad Prism 8.

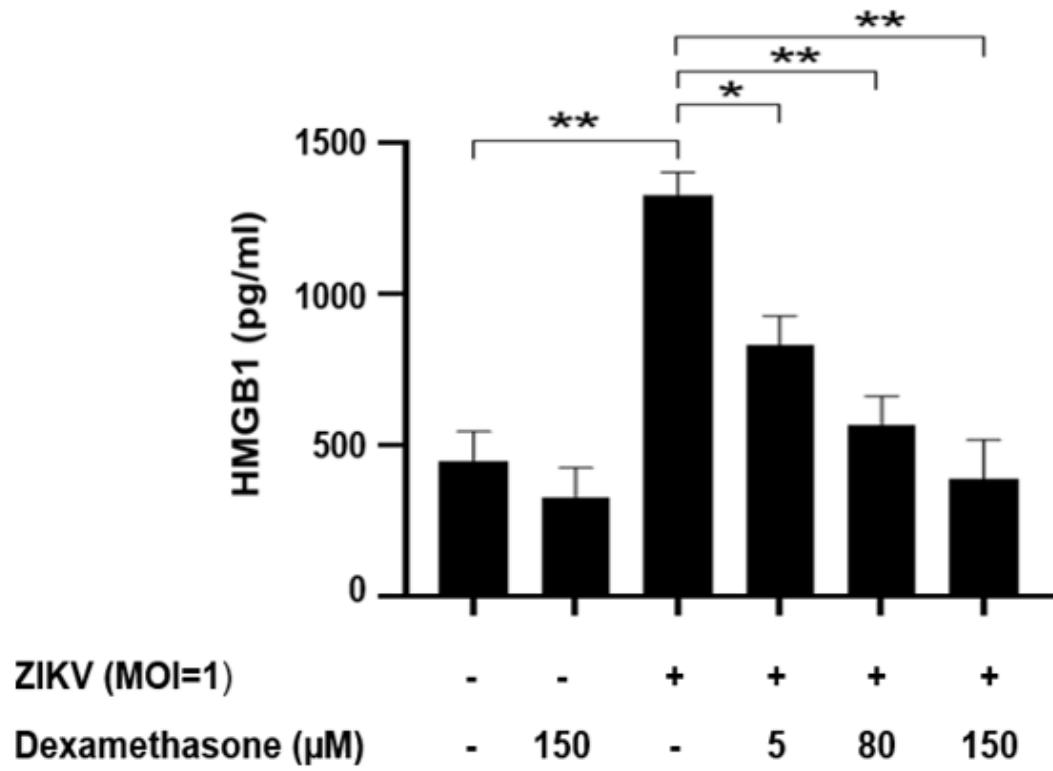


Figure 5

Effects of dexamethasone treatment on extracellular HMGB1 level during ZIKV infection. Huh7 cells were treated with various concentrations of dexamethasone (0, 5, 80, 150 μ M) after ZIKV infection of ZIKV at MOI of 1. After 72 h, supernatants of cell culture were harvested and the level of HMGB1 was analyzed by ELISA. Significant differences between the groups were indicated: * $P<0.05$ and ** $P<0.01$.

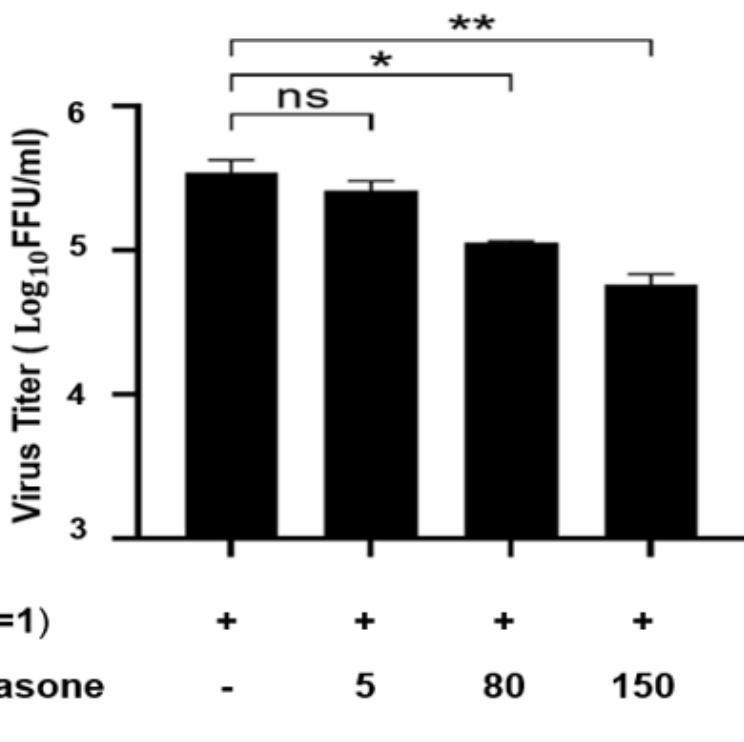


Figure 6

Inhibitory effects of dexamethasone against ZIKV replication. Wild-type (WT) Huh7 cells were treated with various concentrations of RESV (0, 5, 80, 150 μ M) after ZIKV infection at MOI of 1. The cell supernatants were harvested at 72 h.p.i and virus titers were determined using the focus-forming assay. Statistical significances between the groups were indicated: *P<0.05 and **P<0.01.

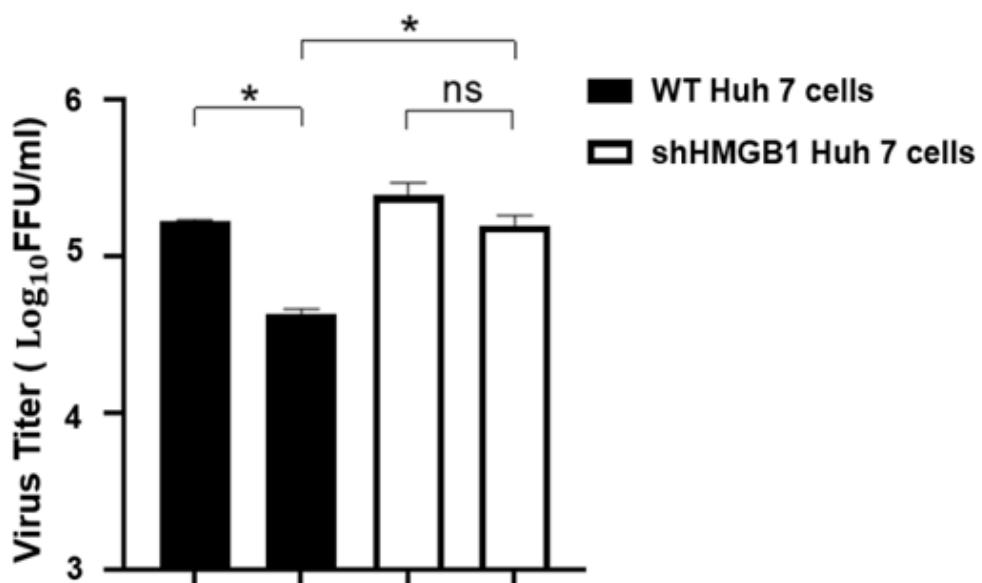


Figure 7

Role of HMGB1 in dexamethasone's antiviral response against ZIKV. Wild-type (WT) and HMGB1-knockdown (shHMGB1) Huh7 cells were treated with 0 and 150 μ M of RESV after ZIKV infection at MOI of 1. The cell supernatants were harvested at 72 h.p.i and virus titers were determined using the focus-forming assay. Asterisks (*) indicate significant difference ($P<0.05$).

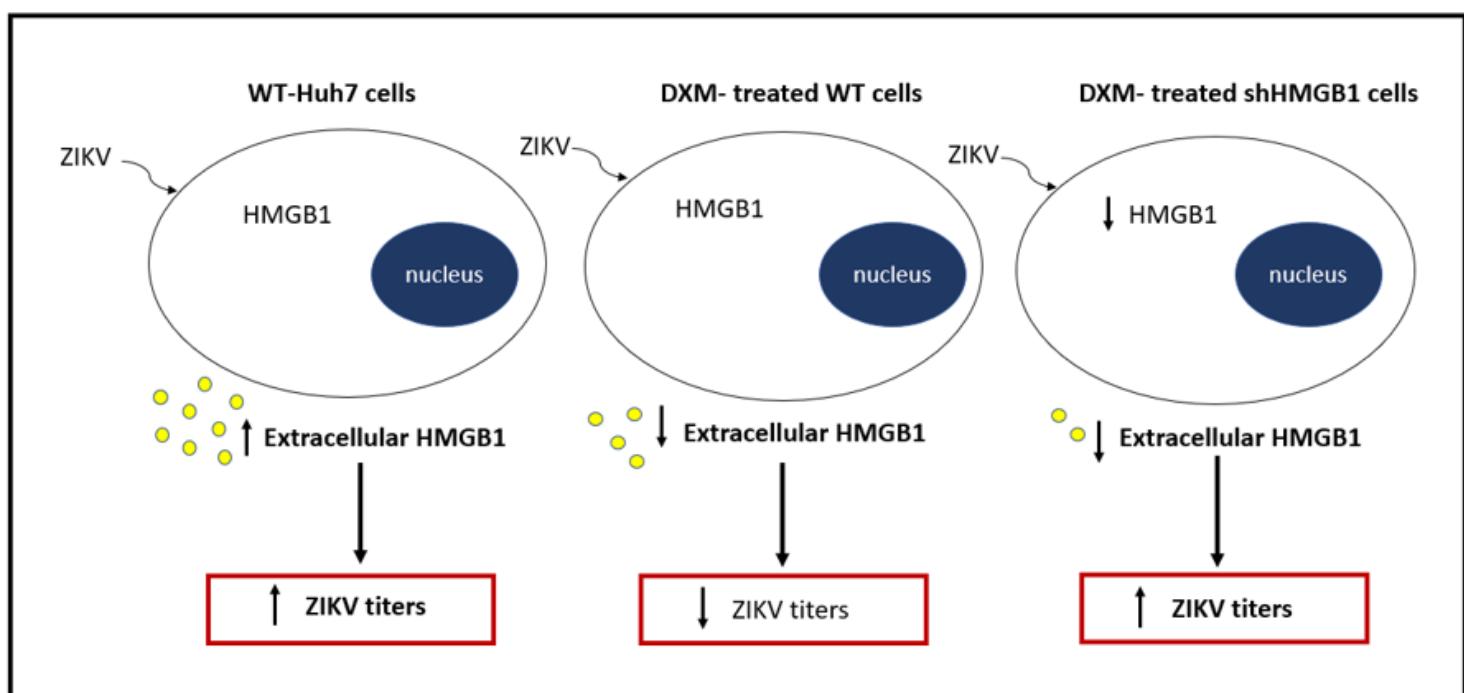


Figure 8

Model of HMGB1 and its role in ZIKV infection. ZIKV infection induces the translocation and release of HMGB1 in WT Huh7 cells. Treatment of dexamethasone in the ZIKV-infected WT cells inhibits the release of extracellular HMGB1 and ZIKV replication. However, the treatment in shHMGB1 cells does not cause the reduction of ZIKV titers, indicating the importance of the intracellular HMGB1 in ZIKV infection.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryfigureS1HMGB1anovelhostrestrictionfactorincontrolofZikavirusreplicationCKL220621.docx](#)
- [SupplementaryFig.2fulllengthblotsofFig.2aandb.docx](#)
- [SupplementaryFig.3fulllengthblotsofS1.docx](#)