Zika virus infection impairs NLRP3-dependent inflammasome activation

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Directed by Professor Je-Wook Yu

The Master's Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Eunji Gim

June 2019

This certifies that the Master's Thesis
of Eunji Gim is approved.

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The Graduate School
Yonsei University

June 2019

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ABSTRACT

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Zika virus (ZIKV), single-stranded positive-sense RNA virus within the genus of Flavivirus, is neurotropic virus. ZIKV infection causes neurological impairments such as Guillain-Barre syndrome in adults and microcephaly in fetus through inflammation and neural cell death. Host immune response against virus infection is important for the clearance and prevention of infection. One of the initial innate immune responses of virus infection is the expression of antiviral cytokines such as type I interferon (IFN). It is crucial for anti-viral response through suppresses viral replication. Adaptive

immunity against ZIKV infection is mediated by antibodies, which suppress virus binding and entry into host cells and by cytotoxic T lymphocytes, which eliminate the infection by killing ZIKV infected cells. Viruses have developed to evade host immune responses. Recently, it is reported that ZIKV inhibits type I IFN-mediated signaling pathway. However, effect of ZIKV infection on inflammasome activation has not been studied in detail yet. Here, I examined whether ZIKV infection induced pro-inflammatory cytokine production in bone marrow-derived macrophages (BMDMs) and mixed glial cells. Pro-inflammatory cytokine mRNA levels were induced by ZIKV infection. But, ZIKV infection did not increase LPS-mediated toll-like receptor4 (TLR4) signaling pathway in BMDMs. Interestingly, ZIKV infection inhibited NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome activation but did not inhibit absent in melanoma 2 (AIM2) inflammasome activation in BMDMs and mixed glial cells. Taken together, ZIKV infection counteracts not only type I IFN signaling but also NLRP3 inflammasome activation. These findings offer new insights into ZIKV-mediated evasion strategy.

Key words : zika virus, inflammasome, NLRP3, evasion

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I. INTRODUCTION

Zika virus (ZIKV) belongs to the flavivirus genus that is transmitted to human by mosquitoes. Flavivirus includes dengue virus, Japanese encephalitis virus, yellow-fever virus and West Nile virus.[1] ZIKV is structurally similar to other flavivirus. It is spherical and lipid-enveloped virus of 40~65 nm that contain a positive-single strand RNA.[2] The ZIKV genome translates into one large polyprotein. Then, it is divided into three structural (Capsid (C), pre-membrane (prM), and Envelope (E)) proteins and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins. The prM protein constitutes a virus membrane, the C protein encapsulates the virus genome, and the E protein binds to membrane of host cell.[3] NS proteins

are associated with virus replication. NS3, serine protease, has proteolytic enzyme activity. Cofactor of NS2B enhances the enzymatic activity and substrate specificity. NS5 plays a role in RNA-dependent RNA polymerase and methyltransferase. NS1, NS3 and NS5 are highly conserved.[4,5]

ZIKV is characterized by neurotropism and neurovirulence. ZIKV infection causes neuropathic diseases through inflammation and neuroapoptosis.[6-8] Pro-inflammatory cytokines are increased in ZIKV-infected patients.[9,10] Symptoms happen within 6 days following ZIKV infection. Mild symptoms of ZIKV infection accompany fever with rash and joint pain.[11,12] In a more severe case, ZIKV infection causes Guillain-Barre syndrome[13-15] and encephalitis[16,17] in adults and congenital malformation such as microcephaly in fetuses.[18-21]

Infection by viruses is associated with production of pro-inflammatory cytokines such as type I interferon (IFN), tumor necrosis factor (TNF) –α and interleukin (IL)-1β. Pro-inflammatory cytokines were produced by pattern-recognition receptors (PRRs), in myeloid lineage cells and activate and recruit immune cells.[22] PRRs, which are dominantly expressed in myeloid cells, play a crucial role in induction of innate immune against pathogen infections and tissue damages. There are various kinds of PRRs, including Toll like receptors (TLRs), retinoic acid inducible gene-I (RIG-I) like receptors (RLRs), Nod-like receptors (NLRs), C-type lectin receptors.[23,24] Several PRRs, such as recognize the virus single strand RNA or double strand DNA, produce type I IFN, which is the important anti-viral cytokine.[25-27] Virus causes not only anti-viral response, but also inflammation.[28-30] Secretion of pro-inflammatory cytokines such as IL-1β and IL-18, related to inflammasome activation, is an important component of inflammatory response against pathogens.[31,32]

Inflammasome is cytosolic multiprotein complex that activate inflammatory cascade in response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Inflammasome is characterized by a particular sensor protein, such as absent in melanoma 2 (AIM2), IFN-γ-inducible protein (IFI16) and various NOD-like receptor (NLR) subsets.[31,33,34] Activation of inflammasome induces caspase-1 activation, which subsequently cleaves pro-IL-1β and pro-IL-18 to their active form.[31,35] Gasdermin D (GSDMD) is also cleaved by activated caspase-1. Then cleaved N-terminal of GSDMD generates plasma membrane pores. Pore formation by N-terminal of GSDMD triggers pyroptosis, which is inflammatory caspase-dependent lytic cell death.[35-37] Secreted IL-1β and IL-18 act as chemokines, recruit other immune cells to infected region and pyroptosis eliminates virus infected cells. It makes immune response go further.

Antigen recognition by T cells induces cytokines production in CD4+ T cells, clonal expansion as a result of differentiation of the T cells into effector cells or memory cells. Most virus specific cytotoxic T cell is CD8+ T cells that sense cytosolic and viral peptides presented by major histocompatibility complex (MHC) class I. The antiviral effects of cytotoxic T lymphocytes (CTLs) are mainly due to killing of infected cells, but other mechanisms include activation of nucleases within infected cells that degrade viral genomes. Effector CD4+ T cells responds to antigen by producing cytokines that have several functions, such as activation of macrophages and B cells.[38] In ZIKV infected patients, CD8+ T cells preferentially focus on structural protein, whereas CD4+ T cells target structural and NS proteins.[39]

Antiviral antibodies bind to viral envelop or capsid proteins and function mainly as neutralizing antibodies to prevent virus attachment and entry into host cells. The E protein, prM protein and NS1 are the main targets of the humoral immune response in case of flavivirus.[39] Thus, antibodies prevent both initial infection and infectious

burden. Antibodies opsonize viral particle and promote their clearance by phagocytes.[40] Adaptive immune responses are mediated by CTLs and antibodies, which remove infected cells and block infection, respectively.

Viruses have evolved various strategies to evade the host immune system.[41-44] ZIKV infection impairs host immune system such as type I IFN signaling pathway.[45-47] ZIKV infection inhibits both RIG-I and TLR3-mediated IFN-β production. NS5 protein of ZIKV suppresses the type I IFN receptor signaling pathway through induction of STAT2 degradation.[48-50] Further, NS4A protein of ZIKV directly binds to mitochondrial antiviral signaling protein (MAVS) then inhibits IFN-β production through inhibition of RIG-I and MAVS interaction.[51,52]

Virus also counteracts both inflammasome activation and resulting effector functions through suppression of inflammasome assembly, inhibition of caspase-1 function and neutralization of IL-1β and IL-18.[53] The Measles virus (MV) V protein inhibits NLRP3 oligomerization.[54] The influenza A virus (IFAV) NS1 protein inhibits ASC recruitment, thereby preventing caspase-1-dependent IL-1β production.[55,56] Downstream of inflammasome activation is prevented by inhibition of caspase-1. Cowpoxvirus (CPV) CrmA protein suppresses caspase-1 by binding and acts as a pseudosubstrate for caspase-1. [57] Viral evasion strategies of host immune response suppress early anti-viral immune response and are used for increase the viral replication.

Recent study has suggested that ZIKV infection activates NACHT, LRR and PYD domains containing protein 3 (NLRP3) inflammasome. NS5 protein of ZIKV enhances the self-oligomerization of NLRP3 through direct interaction with NLRP3.[10,58,59] Further, ZIKV infection induces expression of NLRP3 and IL-1β in fetal brain and ZIKV infected mice show induction of IL-1β and IL-18 levels in serum and brain.[10,60-62] However effect of ZIKV infection on host inflammatory

response has not been studied in detail yet. And the association of ZIKV infection with congenital defects has led to questions of how ZIKV is capable of crossing the placental barrier to reach the fetal brain. ZIKV is to use macrophage and monocyte as the carrier. And then, ZIKV infects the fetal brain and causes diseases.[63] There have been no reports of inflammasome activation after ZIKV infection in glial cells. Here, I used the BMDMs and mixed glial cells related to pathogenesis. I examined whether ZIKV infection could activate the inflammasome in mouse bone marrow-derived macrophages (BMDMs) and mixed glial cells.

II. MATERIAL & METHODS

1. Reagents and antibodies

Lipopolysaccharide (LPS), ATP, nigericin sodium salt, poly (dA;dT) and puromycin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Mouse monoclonal anti-caspase-1 (p20) antibody and mouse monoclonal anti-NLRP3/NALP3 antibody were purchased from Adipogen (San Diego, CA, USA). Goat polyclonal anti-IL-1β/IL-1F2 antibody was purchased from R&D systems (Minneapolis, MN, USA). Rabbit polyclonal anti-caspase-1 (p20) antibody was purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Mouse monoclonal anti-β-actin antibody was purchased from Santa Cruz (Santa Cruz, CA, USA).

2. Mice

C57BL/6 mice purchased from Orient Bio (Gyeonggi-do, Korea). All mice care was administered in compliance with the Guide for Care and Use of Laboratory Animals and approved by the Animal Care Committee of Yonsei University College of Medicine. Entire mice were maintained in exact pathogen-free conditions.

3. Cell cultures

Primary BMDMs were isolated from femurs of C57BL/6 mice. Bone marrow progenitor cells were differentiated into BMDM with 8% L929 conditional media contained Dulbecco's modified Eagle's medium (DMEM, Corning, NY, USA), that

is supplemented with 10% fetal bovine serum (FBS, Corning), 100 U/mL penicillin and streptomycin (Gibco, Gran Island, NY, USA). After 4 days, culture medium replaced fresh complete DMEM containing 5% L929-conditional media. On the second day of the replace of media, adherent cells were detached by 0.25% trypsin-EDTA and plated in 6 or 12 well plate with 5% L929 conditional media contained complete DMEM. Primary mouse mixed glial cells were prepared from postnatal P1-3 day mouse brain. Mouse brain makes an incision to removing all the meninges and washed with Hank's buffered salt solution. Brain tissues were dissociated by pipetting with Pasteur pipettes in DMEM/F12 (Gibco, Gran Island, NY, USA) medium. Then filter out cell suspension by cell strainer. Isolated mixed glial cells were cultured in DMEM/F12 supplemented with 10% FBS (Gibco) and antibiotics. After 7 days, culture medium substituted fresh completed medium. Since then one third of the medium volume was replaced every 4 days. All cells were maintained in a 37℃ incubator filled with 5% CO_2.

4. Zika virus infection

ZIKV (African strain MR766) was kindly provided by Ph.D. Ok Sarah Shin (Korea University College of medicine, Seoul, Korea). Cells were infected with ZIKV at a multiplicity of infection (M.O.I) of 10. At 2 hours (hrs) post-infection, cells were washed by phosphate-buffered saline (PBS, Corning) and replaced with regular growth medium for 20-24 hrs before being harvest.

5. Stimulations for pattern recognition receptor

To stimulate the TLR4 in macrophages and mixed glial cells, 0.25 μg/ml LPS was treated to macrophage for 3 hrs. BMDMs and mixed glial cells were primed with

0.25 μg/mL LPS for 3 hrs followed by 1.5 mM or 2.5 mM ATP and 1 μM nigericin for 30 minutes (mins) in order to activate NLRP3 inflammasome activation. For the purpose of AIM2 inflammasome activation, Poly (dA:dT) (1 μg/ml) were transfected in BMDMs and mixed glial cells through Lipofectamine 2000 (Invitrogen).

6. Measurement of cytokine production

Secreted IL-1β, IL-6 and IFN-β were measured in cell-free supernatants using ELISA. Mouse IL-1β, IL-6 enzyme-linked immunoassay (ELISA) kits were obtained from Biolegend (San Diego, California, USA) and R&D (Minneapolis, MN, USA). Mouse IFN-β ELISA kits were purchased from R&D (Minneapolis, MN, USA).

Total RNAs were extracted with TRIzol reagent (Invitrogen). Reverse transcription used PrimeScript™RT Master Mix (TaKaRa Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The cDNA were amplified by reverse transcription PCR using AccPower Hotstart PCR premix (Bioneer, Daejeon, Korea). Real-time PCR was performed using SYBR Premix Ex Taq™II (TakaRa) and detected by a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The transcription of target mRNA was normalized to Rn18s mRNA transcription using the $2^{-\triangle\triangle ct}$ method. All primers were purchased from Genotech (Daejeon, Korea). Specific primer pairs (Table 1).

7. Immunoblot analysis

Cell lysates and supernatants were harvested after treatment. Proteins of

supernatants were precipitated by methanol-chloroform extraction. Soluble cell lysates were prepared by 20 mM HEPES (pH 7.5) buffer containing 1 mM EGTA, 50 mM KCl, 150 mM NaCl, 0.5% NP-40, 1.5 mM $MgCl_2$, phosphatase inhibitors and protease inhibitors. Using SDS-PAGE, the proteins in supernatants and cell lysates were separated by size. And then, proteins were transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 3% skim milk in PBS with 0.1% Tween 20 for 30 mins at room temperature. The proper primary antibodies were incubated with membranes at 4℃. The membranes were washed by PBST. After incubation with HRP-conjugated secondary antibody, proteins were detected by Pierce ECL Western Blotting Substrate (32106, Thermo Fisher Scientific, Waltham, MA, USA)

8. Statistical analysis

DATA was expressed as the mean ± standard error of the mean (SEM). Groups were compared using t-test. P-value of < 0.05 demonstrates statistically significant.

Table 1. Primer sequence for PCR

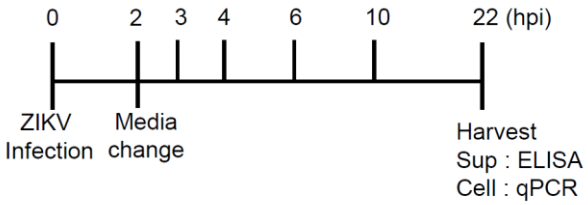
Primer sequence	
Target	Sequence
Mouse IL-6	Forward: 5'- AGTTGCCTTCTTGGGACTGA -3'
	Reverse: 5'- TCCACGATTTCCCAGGAGAC -3'
Mouse IL-1β	Forward: 5'- GCCCATCCTCTGTGACTCAT -3'
	Reverse: 5'- AGGCCACAGGTATTTTGTCG -3'
Mouse IFN-β	Forward: 5'-TTCCTGCTGTGCTTCTTCAC -3'
	Reverse: 5'- CTTTCCATTCAGCTGCTCCA -3'
Mouse Rn18s	Forward: 5'- CGCGGTTCTATTTTGTTGGT -3'
	Reverse: 5'- AGTCGGCATCGTTTATGGTC -3'
ZIKV	Forward: 5'- TTGGTCATGATACTGCTGATTGC -3'
	Reverse: 5'- CCTCCATGTTCCAAGACAACATC -3'

III. RESULT

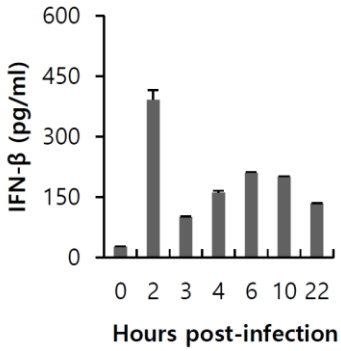
1. Zika virus infection induces interferon-β secretion in bone marrow-derived macrophages

Initial innate immune signaling causes the production of type I IFN and antiviral effector molecules that prevent viral replication during virus infection. The cytosolic RLR recognizes viral dsRNA intermediates and induces type I IFNs to control viral replication.[64] Previous studies suggested that ZIKV NS proteins inhibit type I IFN production. ZIKV NS4A protein interacts with MAVS thus inhibits RIG-I and MAVS interaction[51] and ZIKV NS1 and NS4B protein suppress TBK1 phosphorylation.[52] To investigate the production of type I IFN in ZIKV infected BMDMs, BMDMs were infected with ZIKV. Change the media after ZIKV infection in order that ZIKV attaches the cells. Culture supernatants and cells were harvested at 2, 3, 4, 6, 10 and 22 hrs post-infection (Fig 1A). The IFN-β was induced by ZIKV infection immediately. The reason why IFN-β is the highest at 2 hrs post-infection was because there was less volume when I first gave the virus injection. Therefore there was a relatively large in quantity (Fig 1B). Type I IFN was an early reaction that results in the largest amount of secretion in 4~6 hrs post-infection after virus infection. The data demonstrated on induction with ZIKV-infected BMDMs accumulate IFN-β mRNA for 2~4 hrs, after which time the mRNA was rapidly degraded. However, there was not distinguished from protein level compared to the decrease in mRNA because of the accumulation of IFN-β secretion during that time. The reason ZIKV mRNA was decreasing because IFN has cleared ZIKV (Fig 1C).

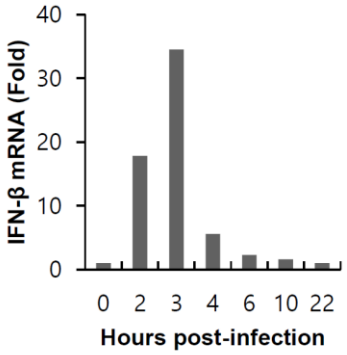
(A)



(B)



(C)



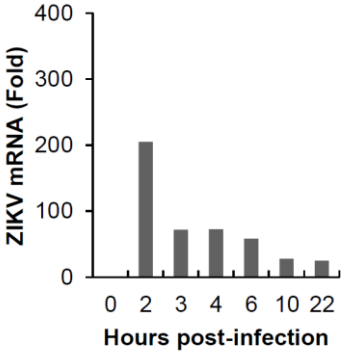


Figure 1. IFN-β production is induced by ZIKV infection in BMDMs. (A) Experimental scheme. After infection ZIKV (MOI = 10), I changed the media 2 hrs later. The culture supernatants and cells were harvested in various times (0, 2, 3, 4, 10 and 22 hrs post infection). (B) IFN-β protein levels were measured using enzyme-linked immunosorbent assay (ELISA) in the supernatants of ZIKV (MOI = 10) infected BMDMs collected at indicated post infection times (n = 2). (C) IFN-β and ZIKV genome mRNA levels were measured using quantitative polymerase chain reaction (qPCR) in ZIKV (MOI = 10) infected BMDMs collected at indicated post infection times. Changes in the levels of each mRNA were first normalized to the Rn18s and then the fold-change in ZIKV-infected cells was calculated in comparison to corresponding uninfected cells. Data was expressed as the mean ± SEM. MOI : multiplicity of infection, sup : culture supernatants

2. Zika virus infection induces pro-inflammatory cytokine production, but does not induce inflammasome activation in bone marrow-derived macrophages

It is reported that pro-inflammatory cytokines such as IL-6, IL-1β and IFN-β are increased in ZIKV infected patients.[10] In order to examine whether pro-inflammatory cytokines such as IL-1β and IL-6 are triggered by ZIKV infection from mouse BMDMs, ZIKV was used to infect BMDMs. The level of secreted IL-1β did not induce when compared to mock and ZIKV infection. In contrast, the IL-6 secretion was induced (Fig 2A). The mRNA transcription levels of IL-1β and IL-6 were detected in ZIKV infected BMDMs. IL-1β secretion is considered to be a two-step process. First, inactive pro-IL-1β is synthesized through the upregulation of transcription level. And then, processed into mature form, active IL-1β by activation of caspase-1, and subsequently released.[31] Data was not shown. Pro-IL-1β expression was detected in cell lysates of ZIKV infected BMDMs. In case IL-6, peak IL-6 transcription in the ZIKV-infected BMDMs occurs at 4~6 hrs post infection, and IL-6 mRNA level gradually decreased. But secreted IL-6 proteins were accumulated in culture supernatants. And therefore there caused a difference between mRNA and protein level of IL-6. ZIKV infection alone failed to induce IL-1β secretion. It was confirmed that ZIKV infection occurred by checking the increase of the ZIKV genome (Fig 2B).

In order to check whether the amount of pro-IL-1β to cleave in the active form to be too little to produce pro-IL-1β, the TLR4-mediated pro-IL-1β was induced and tested. Priming with TLR4 ligand LPS increases in pro-IL-1β in macrophages.[65] I try to examine whether the ZIKV infection act as a second signal to trigger IL-1β maturation, ZIKV was infected in LPS-primed BMDMs. However there did not increase in IL-1β and IL-6 secretion in ZIKV infection compared to mock infection in LPS-primed BMDMs (Fig 3A). While the secretion of the equal IL-6 was

detected for ZIKV infection in untreated- or LPS primed-BMDMs, mRNA level was different (Fig 3B). This was because the LPS caused IL-6 to become stronger than virus infection, which caused IL-6 to fail to degrade.[66]

The pro-IL-1β was increased in ZIKV infected BMDMs. Therefore I examined whether ZIKV infection derived IL-1β activation in presence of ATP in BMDMs as determined by presence of mature form of IL-1β in culture supernatants. Normally, extracellular ATP is induced K+ efflux. It is considered a common second signal for NLRP3 inflammasome activation, which requires two independent signals, a priming signal such as LPS and an activation signal such as ATP.[67] Therefore, I checked the secretion of IL-1β in BMDMs infected with ZIKV followed by ATP treatment. Despite pro-IL-1β was inducted in ZIKV infected cell lysates, co-stimulation with ATP after ZIKV infection did not lead to activation of IL-1β and caspase-1. Equal levels of pro-caspase-1 were detected in cell lysates (Fig 4A, B).

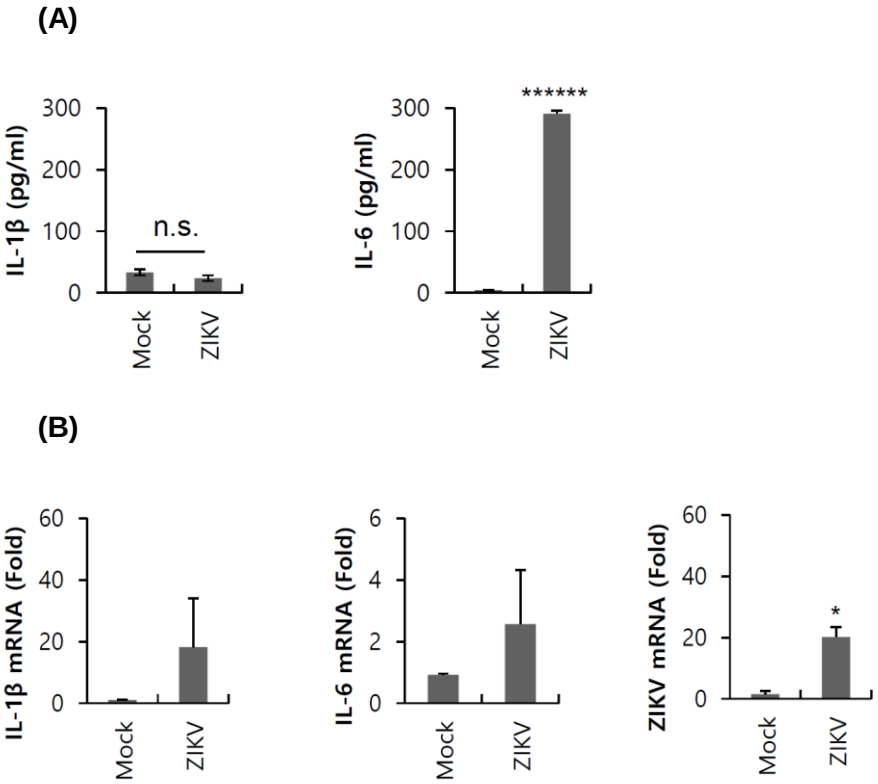


Figure 2. ZIKV infection triggers IL-1β mRNA transcription but does not induce IL-1β secretion in BMDMs. (A) IL-1β and IL-6 protein levels were measured using ELISA in the culture supernatants of ZIKV (MOI = 10) infected BMDMs collected at 26 hrs post-infection (n = 3). (B) IL-1β, IL-6 and ZIKV genome mRNA levels were measured by qPCR in ZIKV (MOI = 10) infected BMDMs collected at 26 hrs post-infection. Changes in the levels of each mRNA were first normalized to the Rn18s and then the fold-change in ZIKV infected cells was calculated in comparison to corresponding mock infected cells (n = 3). Data was expressed as the mean ±SEM. n.s. : not significant, *$p<0.05$, ******$p<0.0000001$, MOI : multiplicity of infection.

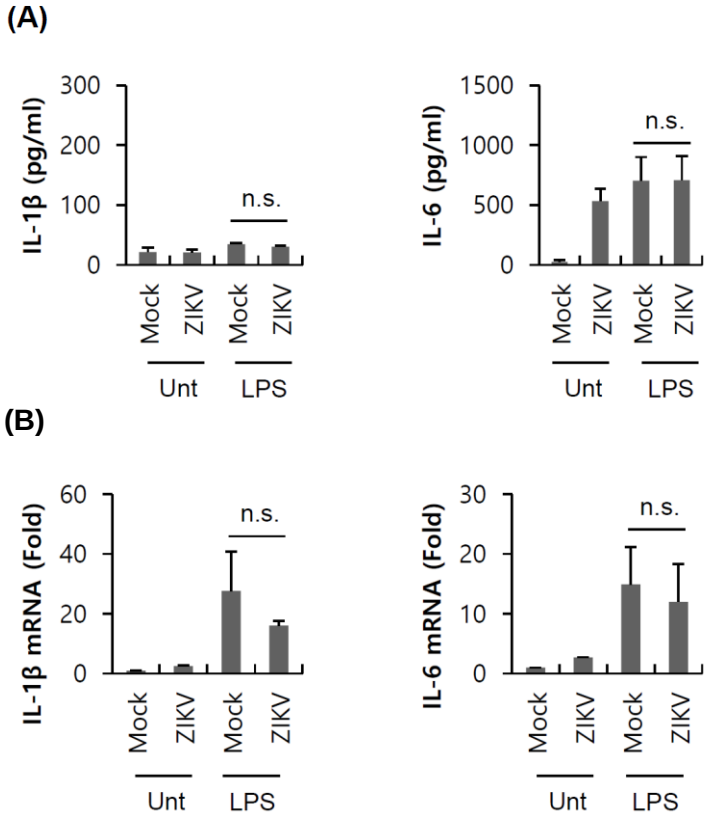


Figure 3. ZIKV infection fails to induce secretion of IL-1β in LPS-primed BMDMs. (A) ELISA of supernatants IL-1β and IL-6 protein levels for unprimed BMDMs (Unt) or LPS-primed (0.25 μg/ml, 3 hrs) BMDMs infected with ZIKV (MOI = 10) for 22 hrs. Uninfected cells serve as mock control (n = 2). (B) qPCR of IL-1β and IL-6 mRNA levels for unprimed BMDMs (Unt) or LPS-primed (0.25 μg/ml, 3 hrs) BMDMs infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. Changes in the levels of each mRNA were first normalized to the Rn18s and then the fold-change in ZIKV-infected cells was calculated in comparison to corresponding mock infected cells (n = 2). Data was expressed as the mean ±SEM. P>0.05, n.s.: not significant, MOI : multiplicity of infection, Unt : untreated, LPS : lipopolysaccharide (TLR4 agonist)

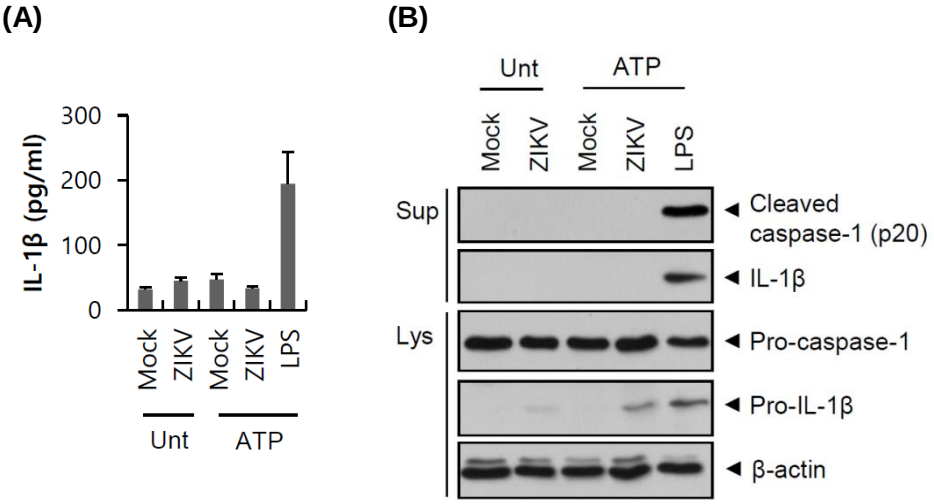


Figure 4. ZIKV infection does not promote NLRP3-dependent caspase-1 activation in the presence of ATP. (A) ELISA of IL-1β in culture supernatants of BMDMs pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. The cells were then treated with ATP (2.5 mM) for 30 minutes (mins). Quantification of IL-1β in culture supernatants of BMDMs primed with LPS (0.25 μg/ml, 3 hrs), followed by treatment with ATP (2.5 mM) for 30 mins were used as a positive control (n = 2). (B) Immunoblots of supernatant (Sup) and cell extracts (Lys) of BMDMs pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. Then the cells were treated with ATP (2.5 mM) for 30 mins. ATP (2.5 mM) was treated for 30 mins in LPS-primed (0.25 μg/ml, 3 hrs) BMDMs. The cells were used as a positive control. MOI : multiplicity of infection, Unt : untreated, LPS : lipopolysaccharide (TLR4 agonist)

3. Zika virus infection fails to increase pro-inflammatory cytokine productions in LPS treated bone marrow-derived macrophages

After infecting the ZIKV for 22 hrs, the media was changed and LPS was treated. LPS is the ligand of typical TLR4 and subsequently activates TBK1 and NF-Kb, increasing the expression of pro-inflammatory cytokines, such as IL-1β, IL-18 and IL-6.[68] To examine whether ZIKV infection enhanced LPS-mediated TLR4 signaling pathway, LPS was treated following ZIKV infection in BMDMs. Unlike Fig 2A, IL-6 did not appear the secretion because IL-6 induced by ZIKV infection changed the media before LPS treatment. Treatment of LPS following ZIKV infection induced mRNA transcription levels of pro-IL-1β and IL-6. IL-6 was expressed and secreted, but pro-IL-1β was not cleaved into activated form of IL-1β, thus the secretion of IL-1β had not been detected. When LPS was treated in ZIKV or mock infected BMDMs, there was no difference between the secretion and transcription level of IL-6. It suggested that the ZIKV infection did not affect the LPS-mediated TLR4 signaling (Fig 5A, B).

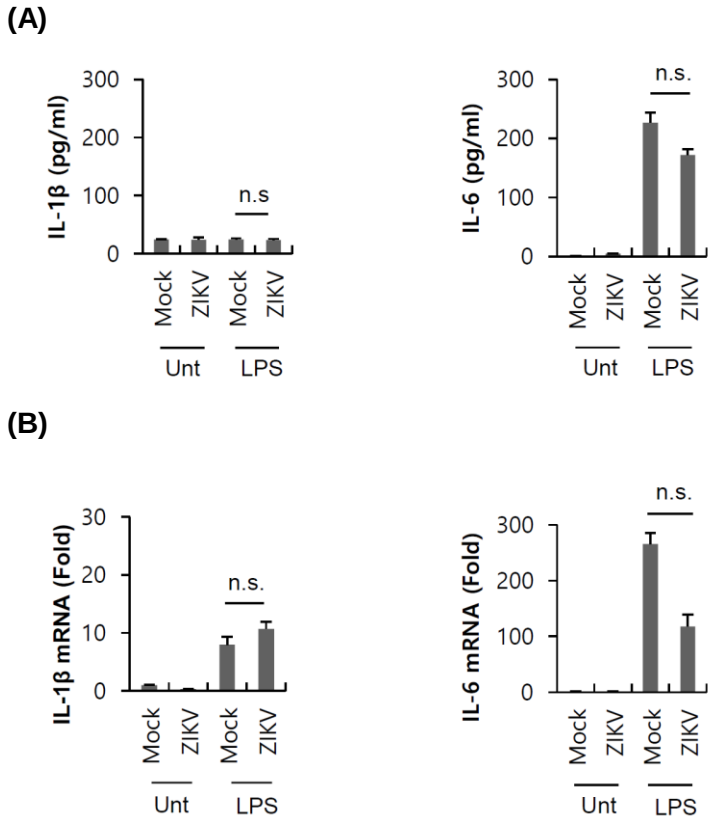


Figure 5. ZIKV infection does not increase IL-6 and IL-1β secretion in LPS treated BMDMs. (A) Quantification of IL-1β and IL-6 in culture supernatants from BMDM infected with ZIKV (MOI = 10) for 20 hrs post-infection following LPS treatment as determined by ELISA (n = 2). (B) Quantification of IL-1β and IL-6 mRNA levels of BMDM infected with ZIKV (MOI = 10) for 20 hrs post-infection following LPS treatment (n = 2). DATA was expressed as the mean ±SEM. $p>0.05$, n.s. not significant. MOI : multiplicity of infection, Unt : untreated, LPS : lipopolysaccharide (TLR4 agonist)

4. Zika virus infection specifically inhibits NLRP3 inflammasome activation in bone marrow-derived macrophages

Inflammasome has crucial role in maturation and secretion of IL-1β by promoting pro-caspase-1 cleavage. Activated caspase-1 is essential factor of IL-1β secretion.[31] Secretion of IL-1β did not detect in ZIKV infected BMDMs (Fig 2-4). To examine whether ZIKV infection inhibited the IL-1β secretion, I measured IL-1β secretion from untreated or NLRP3 agonists-treated (LPS plus ATP or nigericin) BMDMs following infection with mock or ZIKV. I observed that ZIKV infection attenuated the activation of inflammasomes, since less cleaved IL-1β was detected in BMDMs infected with ZIKV followed by LPS and ATP or nigericin treatment to activate NLRP3 inflammasome (Fig 6A, C). Immunoblots were also suggested that ZIKV infection inhibited the caspase-1 cleavage and IL-1β maturation in BMDM treated with LPS plus ATP or nigericin (Fig 6B, D).

To confirm that caspase-1 inhibitory effect is NLRP3-dependent manner, I conducted pre-infected with mock or ZIKV followed by poly (dA; dT) transfection. ZIKV infection did not inhibit activation of caspase-1 in presence of AIM2 inflammmasome activator (Fig 7).

Summarized, AIM2 is activated by DNA virus and NLRP3 is activated by RNA virus.[69] ZIKV is a positive single stranded RNA virus that is recognized by NLRP3. ZIKV infection inhibited the activation of NLRP3 inflammasome particularly. The IL-1β secretion and pyroptosis are decreased. It leads to increase of viral replication.

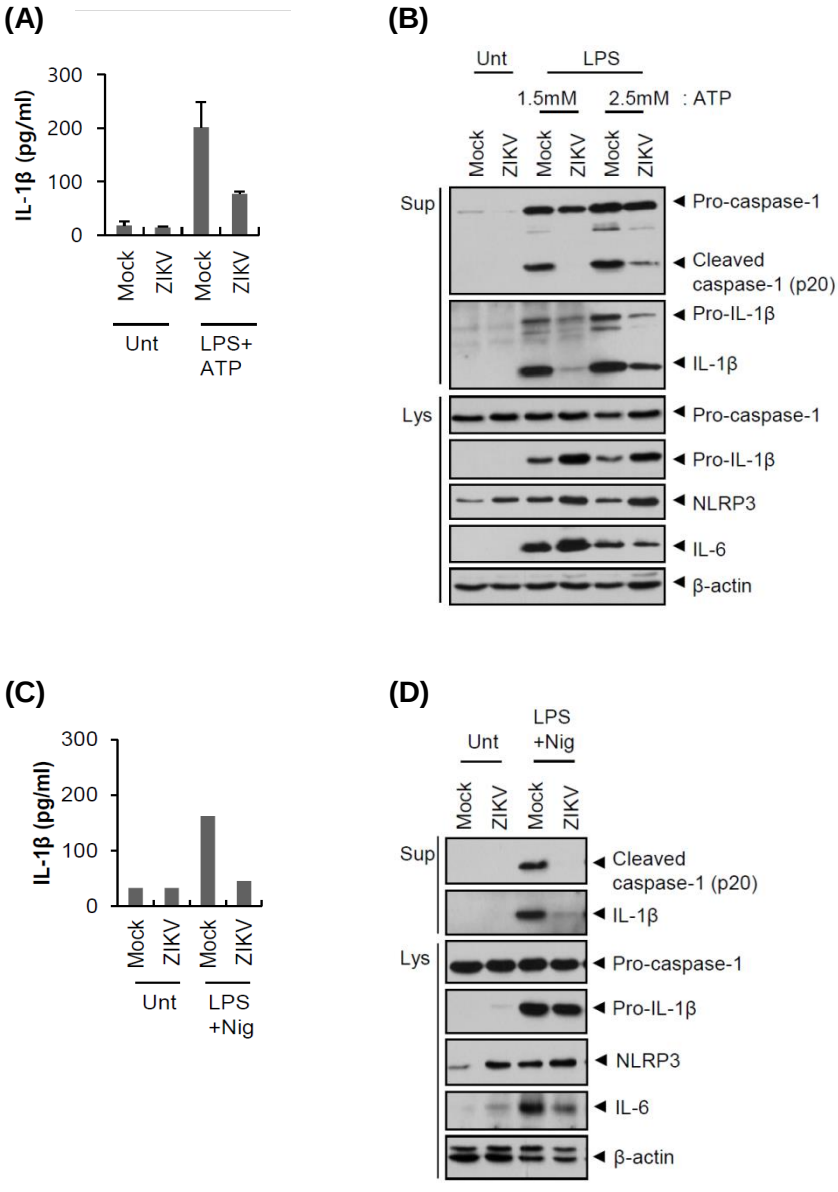


Figure 6. ZIKV infection inhibits NLRP3-dependent caspase-1 activation in BMDMs (A) ELISA of IL-1β protein levels in culture supernatants of BMDMs were pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection followed by LPS (0.25 μg/ml) for 3 hrs and ATP (2.5 mM) stimulation for 30 mins (n = 4). (B) Immunoblots of BMDMs pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. Then the cells were treated with LPS (0.25 μg/ml) for 3 hrs followed by ATP (1.5 mM or 2.5 mM) stimulation for 30 mins. (C) ELISA of IL-1β protein levels in culture supernatants of BMDMs were pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection followed by LPS (0.25 μg/ml) for 3 hrs and nigericin (1 μM) for 30 mins. (D) Immunoblots of BMDMs pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. Then the cells were treated with LPS (0.25 μg/ml) for 3 hrs followed by nigericin (1 μM) stimulation for 30 mins. MOI : multiplicity of infection, Unt : untreated, LPS : Lipopolysaccharide (TLR4 agonist), Nig : Nigericin (NLRP3 inflammasome agonist), sup : supernatant, Lys: lysate

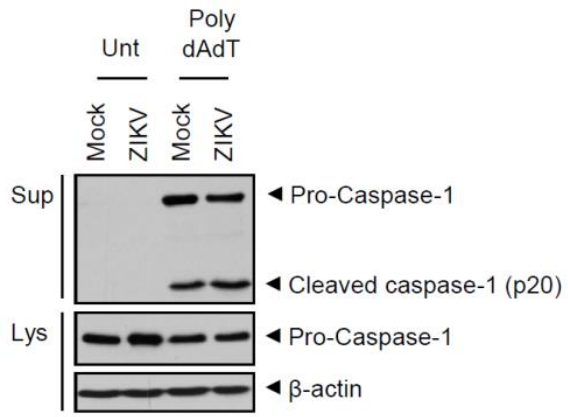


Figure 7. ZIKV infection does not inhibit AIM2-dependent caspase-1 activation in BMDMs. Immunoblots of BMDMs pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. Then the cells were transfected with poly (dA; dT) (1 μg/ml) for 5 hrs. MOI : multiplicity of infection, Unt : untreated, poly (dA; dT) : double strand DNA (AIM2 inflammasome agonist), sup : supernatant, Lys : lysate

5. Zika virus infection promotes pro-inflammatory cytokine production, but fails to trigger inflammasome activation in mixed glial cells

Neuron cells and glial cells exist in brain, and these cells have PRRs are known to sense pathogens and tissue damages in central nervous system (CNS). When microglia and astrocyte including glial cells are excessively activated and released in cytokine, the surrounding neuron cells have not only damages but also cell death, causing disease.[70] ZIKV has neuro-tropism and neuro-virulence. Therefore ZIKV infection causes neuropathic diseases.[71]

In this study used mixed glial cells, which consist of astrocytes, microglial cells and oligodendrocytes. To examine whether the increase of pro-inflammatory cytokines is occurred during ZIKV infection in mixed glial cells, I measured the secretion of IL-1β and IL-6 into the supernatants by infected mixed glial cells at 26 hrs post-infection. ZIKV infection did not induce IL-1β secretion but IL-6 secretion was induced (Fig 8A). Despite of undetectable protein secretion of IL-1β, IL-1β transcription level was triggered in ZIKV infected mixed glial cells (Fig 8B).

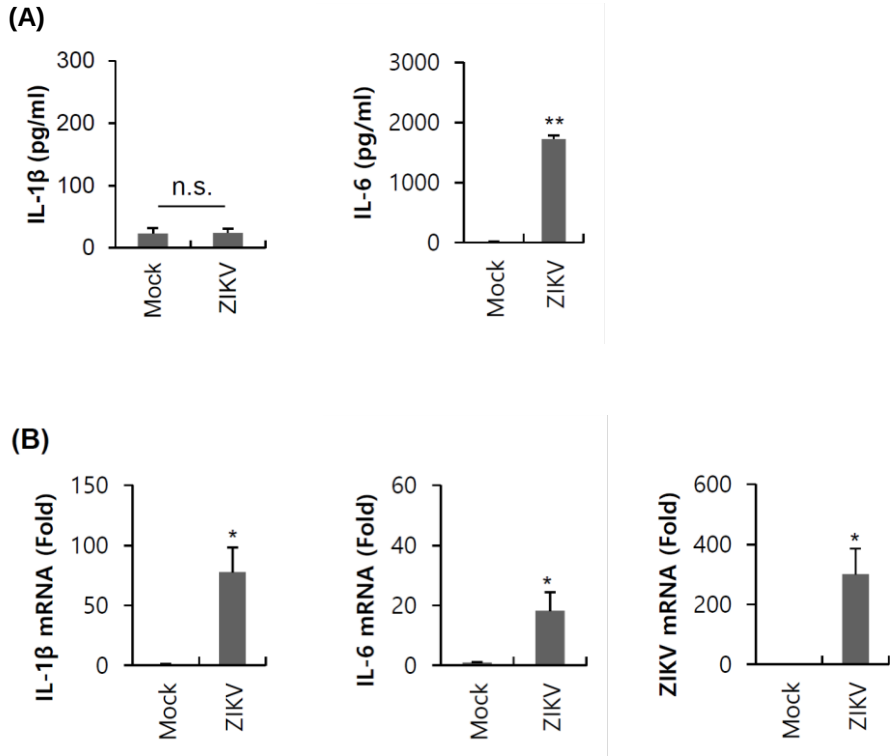


Figure 8. ZIKV infection induces IL-1β mRNA transcription but fails to trigger IL-1β secretion in mixed glial cells. (A) IL-1β and IL-6 protein levels were measured using ELISA in the culture supernatants of ZIKV (MOI = 10) infected mixed glial cells collected at 26 hrs post-infection (n = 3). (B) IL-1β, IL-6 and ZIKV genome mRNA levels were measured by qPCR in ZIKV (MOI = 10) infected mixed glial cells collected at 26 hrs post-infection. Changes in the levels of each mRNA were first normalized to the Rn18s and then the fold-change in ZIKV infected cells was calculated in comparison to corresponding mock infected cells (n = 3). Data was expressed as the mean ±SEM. n.s. : not significant, *p<0.05, **p<0.01, MOI : multiplicity of infection.

6. Zika virus infection suppresses NLRP3 inflammasome activation in mixed glial cells

Mixed glial cells were also confirmed for the same response as in BMDMs during ZIKV infection alone. In order to examine whether ZIKV infection inhibited the NLRP3 inflammasome, I measured IL-1β secretion from untreated or NLRP3 activators-treated (LPS plus ATP or nigericin) mixed glial cells infected with mock or ZIKV. Immunoblots and ELISA analyses showed that ZIKV infection inhibited the activation of caspase-1 and IL-1β in mixed glial cells with NLRP3 activators (Fig 9A-D).

In order to confirm that caspase-1 inhibitory effect was NLRP3-dependent manner, I conducted pre-infected with mock or ZIKV followed by poly (dA; dT) transfection in mixed glial cells. ZIKV infection did not inhibit activation of caspase-1 in presence of AIM2 inflammmasome activator (Fig 10).

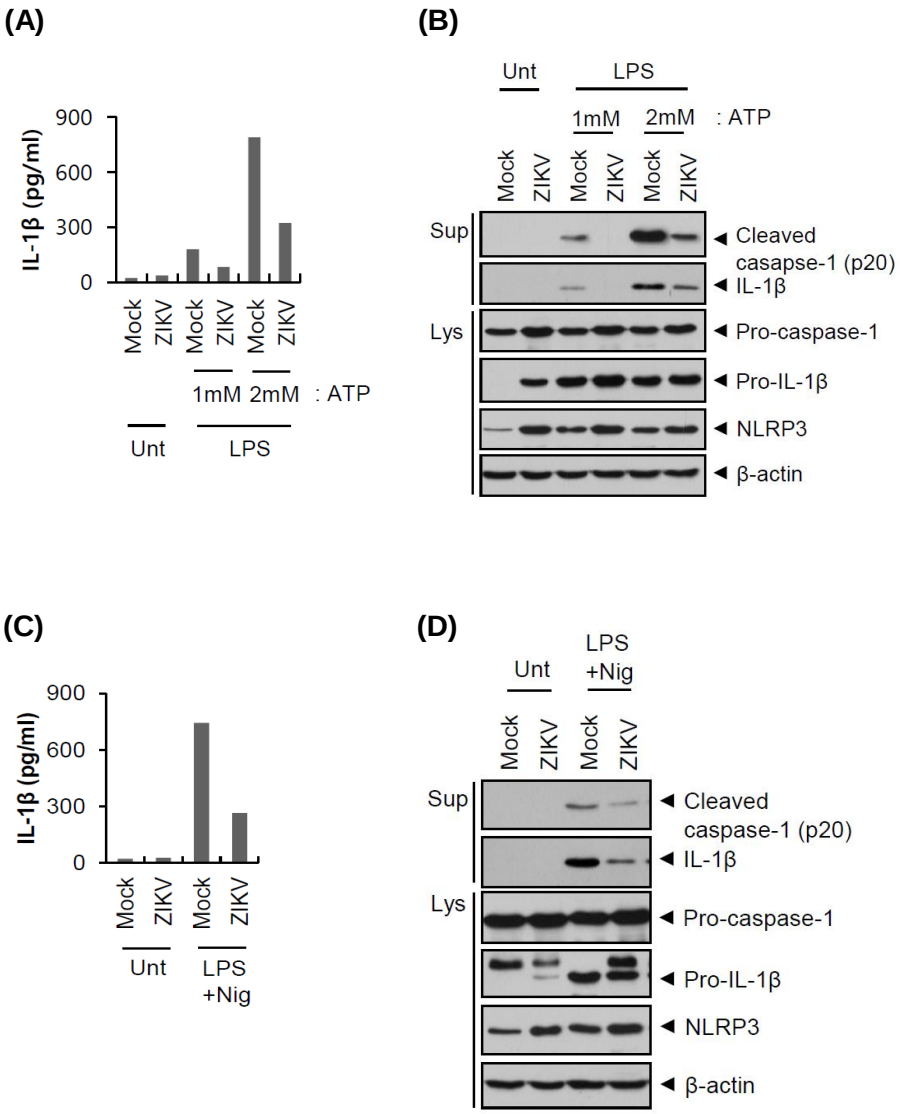


Figure 9. ZIKV infection inhibits NLRP3 inflammasome activation in mixed glial cells. (A) ELISA of IL-1β protein levels in culture supernatants of mixed glial cells were pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection followed by LPS (0.25 μg/ml) for 3 hrs and ATP (2.5 mM) stimulation for 30 mins. (B) Immunoblots of mixed glial cells pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. Then the cells were treated with LPS (0.25 μg/ml) for 3 hrs followed by ATP (1 mM or 2 mM) stimulation for 30 mins. (C) ELISA of IL-1β protein levels in culture supernatants of mixed glial cells were pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection followed by LPS (0.25 μg/ml) for 3 hrs and nigericin (1 μM) for 30 mins. (D) Immunoblots of mixed glial cells pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. Then the cells were treated with LPS (0.25 μg/ml) for 3 hrs followed by nigericin (1 μM) stimulation for 30 mins. MOI : multiplicity of infection, Unt : untreated, LPS : Lipopolysaccharide (TLR4 agonist), Nig : Nigericin (NLRP3 inflmmasome agonist), sup : supernatant, Lys: lysate

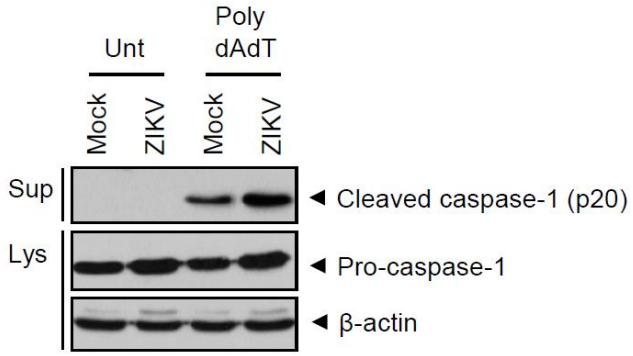


Figure 10. ZIKV infection does not inhibit AIM2 inflammasome activation in mixed glial cells. Immunoblots of mixed glial cells pre-infected with mock or ZIKV (MOI = 10) for 22 hrs post-infection. Then the cells were transfected with poly (dA; dT) (1 μg/ml) for 5 hrs. MOI: multiplicity of infection, Unt : untreated, poly (dA; dT) : double strand DNA (AIM2 inflammasome agonist), sup : supernatant, Lys : lysate

IV. DISCUSSION

Recent studies suggested that ZIKV infection activates NLRP3 inflammasome activation in THP-1 and BMDMs. Mechanistically, ZIKV NS5 promotes NLRP3 oligomerization and it facilitates subsequent signaling pathway.[9,10] However, I confirmed that the ZIKV infection does not induce the activation of inflammasome.

These opposing responses probably arise from characteristic of each ZIKV strain and cell type. In this study, I used African strain of ZIKV (MR766), and in previous reports used Asian strains of ZIKV (z16006, KU820898 and KU866423)[10,58,59]. Two strains, African and Asian lineage, have suggested differential pathogenesis and molecular response.[72] A phylogenic analysis particularly reveals 50 amino acid lineage-specific differences. All variations occur in prM protein, NS1 protein and NS5 protein.[2] ZIKV NS5 protein including methyltransferase and RNA-dependent RNA polymerase contains all the enzymatic activities required to synthesize the viral RNA genome. Difference between African and Asian strain in ZIKV NS5 protein may affect the replication rate. It is reported that the African strain more rapidly replicates itself. Difference between African and Asian strain in ZIKV NS5 protein may affect the replication rate. Moreover, ZIKV NS1 protein also has changes in amino acid. It is reported that ZIKV NS1 plays a role in evasion of the innate immune response such as IFN-β signaling pathway. It was Asian that ZIKV was reported to have been used, but African strains were frequently used.[72-74]

And second potential is different cell type. Previous studies used THP-1, human monocytes cell lines, but I used murine primary macrophages and mixed glial cells. The amount of IL-1β that is secreted by the activation of inflammasome in ZIKV-infected mouse BMDMs is small.[59] ZIKV causes the diseases in primates such as human. It may be the cause of the disease because the immune response caused by the ZIKV is more active in humans. Therefore, the strain and cell type-dependent effect of ZIKV on caspase-1 activation requires further experiments.

In contrast with the previous reports, ZIKV infection inhibits NLRP3 inflammasome activation upon NLRP3 activators (LPS plus ATP or nigericin). RNA virus promotes NLRP3 inflammasome activation.[69,75,76] AIM2 is a PYHIN protein that localizes to the cytosol and performs inflammasome in response to dsDNA virus.[77] In this study, I was confirmed that ZIKV, positive single stranded RNA virus, does not inhibit AIM2 inflammasome activation and suppresses NLRP3 inflammasome activation. It was possible to confirm that NLRP3 inflammasome played a role with the host immune response that worked when ZIKV infection. And I have examined that it is mechanism that ZIKV is evading it. Both NLRP3 and AIM2 form inflammasome as ASC and pro-caspase-1 oligomerization. The reason ZIKV infection specifically inhibits NLRP3 inflammasoem is expected to block subsequent reactions through interaction with ZIKV protein with NLRP3. This need more confirm later on.

IL-1β is crucial role in acute inflammatory response against pathogen infection. Virus triggers inflammasome formation and subsequent caspase-1 activation. When active caspase-1 cleaves the pro-IL-1β, a mature form of IL-1β is released out of the cells.[26] The secreted IL-1β induces vasodilation and attracts granulocytes to the inflamed tissue. In addition, if excessive IL-1β is generated in the central nervous system (CNS), it will cause a pain and cause a symptom during viral infection.[78] However, protection by IL-1β is suppressed by ZIKV infection. The immune response to the virus cannot be properly performed.

This finding has allowed us to offer a few explanations. First, ZIKV infection does not activate inflammasome. But, ZIKV infection induced NF-kB signaling, causing upregulation of IL-6 and pro-IL-1β mRNA level. It also led to induce expression of NLRP3 or other inflammasome components. This state is easier to trigger inflammation. Second, ZIKV infection impaired the NLRP3 inflammasome activation. Collectively, this study indicates that ZIKV infection induces

inflammation states but inhibits NLRP3-dependent inflammasome activation. It has not been determined which proteins of ZIKV can inhibit NLRP3 inflammasome activation yet. Further study will be need and focus on how to ZIKV protein affects NLRP3 inflammasome activation in molecular mechanism. A novel antagonistic mechanism employed by ZIKV protein to inhibit NLRP3 inflammasome activation, might guide the design of therapeutic targets in the future.

V. CONCLUSION

Here, I examined that ZIKV infection did not activate inflammasome. ZIKV infection alone induced IL-6 secretion but not IL-1β secretion. Even if other NLRP3 activators were treated with ZIKV, the secretion of IL-1β was not detected. ZIKV infection specially inhibited NLRP3 inflammasome activation in mouse BMDMs and mixed glial cells. Which viral protein could play a role in inhibition of NLRP3 inflammasome activation is not confirmed. Therefore further studies will be needed. This finding provides a new evasion strategy of ZIKV on NLRP3 inflammasome activation and pro-inflammatory cytokine production. If further studies are conducted to confirm which protein can be regulated in inhibiting, then it could be the therapeutic target.

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ABSTRACT (in Korean)

지카 바이러스 감염에 의한 NLRP3 의존적 인플라마좀 활성화의 저해

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김은지

지카 바이러스는 단일 가닥의 RNA 를 게놈으로 가지며 신경조직에 감염될 수 있는 바이러스이다. 지카 바이러스 감염 시 염증반응과 뇌세포의 사멸에 의해서 성인의 경우 길랑-바레 증후군이 태아의 경우 소두증이 발병된다고 보고 된다. 바이러스 감염에 대한 선천면역반응은 제 1 형 인터페론과 같은 염증성 사이토카인을 발현해 다른 면역세포들의 활성을 유도하거나 바이러스의 전사를 억제하는 반응이다. 후천면역반응은

바이러스가 숙주세포에 감염되지 못하도록 바이러스의 막 단백질을 인식할 수 있는 항체를 형성하거나 바이러스 감염세포를 제거하는 세포독성 T 세포를 통해서 이루어진다. 바이러스들은 면역반응을 회피하도록 진화되어왔다. 지카 바이러스의 경우, 제 1 형 인터페론 신호경로와 관련된 면역반응과 관련된 회피 기작에 대해서는 보고되었다. 하지만 지카 바이러스와 인플라마좀 활성화 사이의 관계에 대해서는 아직 보고되지 않았다. 본 연구에서는 마우스 골수 대식세포와 신경교세포에 지카 바이러스가 감염에 의해서 염증성 사이토카인의 분비가 유도되는지 여부를 확인해보았다. 지카 바이러스 감염 시 염증성 사이토카인의 전사는 증가됨을 확인하였다. 그리고 지카 바이러스 감염이 지질다당류 (LPS) 를 통한 toll-like receptor 4 (TLR4) 신호경로는 촉진시키지 못하는 것을 확인하였다. 흥미롭게도, 지카 바이러스가 감염 시 NLRP3 인플라마좀의 활성이 유도되는 조건에서 인플라마좀의 활성이 저해됨을 확인할 수 있었다. 종합해보자면, 대식세포와 신경교세포는 지카 바이러스 감염에 의해 염증성 사이토카인을 분비하지만, NLRP3 인플라좀 활성은 저해시킬 수 있다는 것을 확인하였다. 이는 새로운 지카 바이러스의 면역 회피기작을 제시하는 바이다.

핵심 되는 말: 지카 바이러스, 인플라마좀, NLRP3, 회피

PUBLICATION LIST

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