MECHANISM OF PROTEIN INVOLVEMENT IN CHIKUNGUNYA VIRUS INFECTION IN MACROPHAGES

Nattaya KAMCHOMPOO[1], Nathamon KOSOLTANAPIWAT[1], Onrapak REAMTONG[1] and Kobporn BOONNAK[2]

1 Faculty of Tropical Medicine, Mahidol University, Thailand; nattaya.kam@student.mahidol.edu (N. K.); onrapak.rea@mahidol.edu (O. R.)

2 Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand; kobporn.boo@mahidol.edu

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ABSTRACT

Chikungunya virus (CHIKV) is a mosquito-borne virus transmitted to humans by the bite of an infected *Aedes* mosquito. CHIKV is the causative agent for chikungunya fever which may cause chronic arthritis in some patients. CHIKV can replicate in various cell types, including epithelial cells, endothelial cells, fibroblasts, and immune cells such as monocytes and macrophages. Among immune cells, macrophages are susceptible to CHIKV infection and play a critical role in immune defense against invading viruses. Thus, this study aims to investigate the protein expression in mouse macrophage cell line (RAW264.7) following CHIKV infection using mass spectrometry (LC-MS/MS) technique. The results showed a total of 1,104 altered phosphoproteins in CHIKV-infected RAW264.7 cells compared to the mock control. One hundred thirty-nine phosphoproteins were significantly up-regulated phosphoproteins, whereas forty-four down-regulated phosphoproteins were observed following CHIKV infection. A STRING network analysis determined the protein interaction in both up-regulated and down-regulated phosphoproteins that are essential in biological processes. The analysis of protein-protein interactions revealed the presence of three major networks among the up-regulated phosphoproteins including the RNA metabolic process, immune system process, and response to the virus. The major network observed among the down-regulated phosphoproteins was macromolecule metabolic processes. Our findings indicate that CHIKV is capable of infecting and replicating in RAW264.7 mouse macrophage cell line and has a significant impact on the protein expression of these cells which alters several phosphoproteins in the host biological processes.

Keywords: Chikungunya Virus, Macrophages, Phosphoproteomics, Protein-Protein Interaction

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INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-borne virus transmitted to humans by the two main vectors which are *Aedes aegypti* and *Aedes albopictus* (Gasque et al., 2015). CHIKV is a positive-strand RNA virus which belongs to the *Togaviridae* family and *Alphavirus* genus (Her et al., 2010). In Thailand, the first report of a CHIKV outbreak was in 1958 in Bangkok (Khongwichit et al., 2021). CHIKV is the causative agent for chikungunya fever which has a broad range of symptoms such as high fever, headache, rash, and joint/muscle pain. In some cases, joint pain may persist for a long period, which can lead to chronic arthritis (Her et al., 2010). Interestingly, CHIKV can infect and replicate in various human cell types including epithelial cells, endothelial cells, fibroblasts, and immune cells, including monocytes and macrophages (Lombardi Pereira et al., 2019). Although these immune cells are important for eliminating the virus, they are also susceptible to CHIKV infection. Macrophages are susceptible cells and the main reservoirs for CHIKV infection, which may facilitate viral dissemination in the host (Her et al., 2010; Labadie et al., 2010; Sourisseau et al., 2007). Previous studies have shown that CHIKV can infect macrophages both in mouse (Gardner et al., 2010; Kumar et al., 2012) and in non-human primates (Labadie et al., 2010). The study on RAW264.7 mouse macrophages showed the ability of CHIKV to infect these cells that seem to be polarized to enhance viral survival through the interferon (IFN) signaling and regulation of the apoptosis (Kumar et al., 2012). Phosphorylation of proteins is one of the most common and significant post-translational modifications (PTMs). Protein phosphorylation plays an important role in the regulation of most cellular processes, such as signal transduction pathways, protein synthesis, cell division, and cell growth (Ardito et al., 2017). Recently, many studies have used proteomics to analyze the levels of host protein expression upon viral infection. To enhance the understanding of how viral infection regulates host proteins, some studies have used phosphoproteomics to investigate the response of host proteins which are involved in signaling pathway following viral infection (Miao et al., 2019; Pang et al., 2021; Rawarak et al., 2019; Wongtrakul et al., 2020). Prior phosphoproteomics studies have shown that CHIKV infection induced phosphorylation at p38, JNK, and c-jun in RAW264.7 macrophages, which increases the pro-inflammatory cytokine production (Nayak et al., 2019). Dengue virus (DENV) infection has shown that DENV can alter the expression of host proteins involved in numerous processes. The studies in the U937 cell line have indicated that Protein Disulfide Isomerase (PDI) is important for protein translation and virion assembly had up-regulated following Antibody-Dependent Enhancement (ADE) of DENV infection (Rawarak et al., 2019). Another study has shown that pyruvate kinase M2 (PKM2) has increased phosphorylation (Wongtrakul et al., 2020) in response to DENV 2-infected U937 cells under an ADE protocol infection. Although the protein alteration upon DENV have been well studied, the studies of phosphoprotein expression response to CHIKV infection are still limited.

In this study, phosphoproteomic analysis following CHIKV infection in RAW264.7 mouse macrophages has been performed. Following infection, the phosphoproteins of macrophages were analyzed by mass spectrometry (LC-MS/MS). The protein-protein interaction in both up-regulated and down-regulated phosphoproteins were indicated by the STRING network. Our findings showed that CHIKV could replicate in RAW264.7 macrophages and affects the protein expression of RAW264.7 macrophages. Several host proteins were up- and down-regulated in many biological processes during the CHIKV infection. The knowledge obtained from this study provides insight into the involvement of host protein response to CHIKV infection in macrophages which may be involved in the pathogenesis of CHIKV diseases.

RESEARCH METHODOLOGY

Cell culture

RAW264.7 mouse macrophage cell line was kindly provided by the Siriraj Center of Research Excellence in Dengue and Emerging Pathogens, Bangkok, Thailand. The macrophages were sub-cultured to a new passage in a T75 flask. Briefly, the macrophages were seeded into a flask in 15 ml of RPMI 1640 complete medium (supplemented with 2 g/L sodium hydrogen carbonate, 2 mM L-glutamine, 10% heat-inactivated FBS, penicillin (10,000 units/mL), streptomycin (10,000 µg/mL), and amphotericin B (25 µg/mL)) and then incubated at 37ºC in a 5% CO_2 incubator for 2 days. After that, the macrophages were kept in liquid nitrogen as the cell stock.

Infection of RAW264.7 cells with CHIKV for replication kinetics

RAW264.7 macrophages were infected with CHIKV viral stock (CHIKV strain TM009-1A2 was isolated from CHIKV infected patient from Maesod Hospital in 2019). The macrophages at a cell density of 1×10^6 cells/ml were infected with CHIKV at MOI (multiplicity of infection) 1, 5 and 10 and incubated at 37°C in 5% CO_2 for 2 hrs. After incubation, virus inoculum was discarded, and the cells were washed with 2 ml/well of 1X PBS 5 times and Plain RPMI 1640 (no FBS) 10 times. After washing, Plain RPMI 1640 was added into the 6-well plate (3 ml/well) and incubated at 37ºC in a 5% CO_2 incubator. The cell supernatant and cell pellets were collected at 0, 2, 4, 6, 8, 12, 24, 48 and 72 hpi (hours post-infection). The cell supernatant was used for viral titer determination ($TCID_{50}$ assay), whereas cell pellets were used to detect cell viability (trypan blue staining). The mock was performed in parallel but without CHIKV infection. MOI was calculated by the following equation:

$$\text{MOI} = \frac{\text{Viral titer (pfu/ml)} \times \text{Virus volume (ml)}}{\text{Number of cells}}$$

CHIKV-infected RAW264.7 macrophages preparation for phosphoproteomic analysis

The optimal condition for replication kinetics of CHIKV-infected macrophages was selected to infect RAW264.7 macrophages for phosphoproteomic analysis. The macrophages at a cell density of 1×10^7 cells/ml were infected with CHIKV at optimal condition (MOI 1 and 24 hours). The flask was then incubated at 37°C in 5% CO_2 for 2 hours and rocked gently every 15 mins. After incubation, virus inoculum was discarded, and the cells were washed with 10 ml/well of 1X PBS 5 times and Plain RPMI 1640 (no FBS) 10 times. After washing, Plain RPMI 1640 was added into a flask (15 ml/flask) and incubated at 37ºC in a 5% CO_2 incubator, for 24 hours. After incubation, the culture supernatant and cell pellets were harvested using the cell scraper and centrifuged at 1500 rpm, 4°C, for 5 mins. After centrifugation, the culture supernatant was kept at -80°C, whereas cell pellets were washed with 1X PBS 3 times and kept at -80°C for protein extraction.

$TCID_{50}$ (50% Tissue Culture Infectious Dose) assay

Vero cells were prepared in 96-well plate and incubated at 37ºC in a 5% CO_2 incubator for 2 days or until the cells reached a confluency of 90-100%. The medium was then discarded and the plate was washed with 180 µl/well of 1X MEM supplemented with 2.2 g/L sodium hydrogen carbonate, twice. Then, 180 µl/well of virus culture medium (1x MEM no FBS) was added to every well. Next, 20 µl of samples (supernatant from RAW264.7 infected with CHIKV at MOI 1, 5 and 10) were added into first well of row A-D (use 4 wells for each dilution). Making the 10-fold dilution at 10^{-1}-10^{-11} in well 1-11, and using well 12 as control. Twenty microliter of dilution was transferred from well 1 to well 2, until well 11 and 20 µl discarded after mixing in well 11. The 96-well plate was then incubated at 37ºC in a 5% CO2 incubator for 4 days. After incubation, the CPE (Cytopathic effect) was observed and recorded at the dilution in which the CPE appears and using that to calculate the titer as $TCID_{50}$/ml.

Detection of cell viability

Cell viability of CHIKV-infected cells was determined at 0, 2, 4, 6, 8, 12, 24, 48 and 72 hpi by trypan blue exclusion assay. The pellets were resuspended with RPMI 1640 medium. Twenty microliter of cell suspension was mixed with 20 µl of 0.4% trypan blue (Thermo Fisher Scientific,Waltham, MA, USA) and 20 µl of stained cells was placed in a hemocytometer for counting the number of cells under a microscope. The percentage of viable cells was calculated using the following equation:

$$\% \text{ Viability} = \frac{\text{Total live cells count}}{\text{Total live cells count} + \text{Total dead cells count}} \times 100\%$$

Phosphoprotein enrichment

Phosphoproteins were purified using a TALON PMAC Magnetic Phospho Enrichment Kit (Clontech, Mountain View, CA, USA). Briefly, cells were collected by centrifugation at 1500x g for 5 mins, washed with PBS 3 times, followed by addition of 200 µl of Extraction/Loading Buffer (Buffer A). The cells were incubated at 4°C for 10 mins and then the cell lysate was transferred to a microcentrifuge tube. The cell lysate was desalted by centrifuge at 10000x g for 20 mins at 4°C. The cell lysate was added into the beads and mixed on a rotary shaker at room temperature for 30 mins. Then, 500 µl of wash buffer was added into the beads and discarded; first and second washes. Finally, 50 µl of elution buffer was added into the beads and the eluate fraction was collected. The concentration of phosphoproteins was determined using the Bradford method. For mass spectrometric analysis (LC-MS/MS), phosphoproteins were sent to Central Instrument Facility (CIF), Faculty of Science, Mahidol University.

Data analysis

A total of phosphoproteins was identified using cut-off values based on a 95% level of confidence. Perseus software version 2.0.7.0 was used to identify the up-regulated and down-regulated phosphoproteins with fold-change of $\log_2(2)$ and p-value < 0.05. The volcano plot was generated by GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Protein-protein interaction was predicted using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://www.string-db.org/). Classification of altered proteins was performed by PANTHER version 17.0 (http://www.pantherdb.org/). Data were represented as mean ± standard error of mean (SEM). Comparison of MOI and time points were performed using Two-Way ANOVA with Bonferroni multiple comparisons tests. Statistically significant results were defined to have $p < 0.05$.

RESEARCH RESULTS

Replication kinetics of CHIKV infection in RAW264.7 macrophages

CHIKV replication kinetics in RAW264.7 was measured to investigate the optimal CHIKV infection time and MOI for further phosphoproteomics analysis. RAW264.7 cell line was infected at MOIs of 1, 5, and 10, and then the virus titer was determined by $TCID_{50}$ assay using the supernatants collected at 0, 2, 4, 6, 8, 12, 24, 48, and 72 hpi. We found that the replication patterns for all three MOIs were similar. For MOI 1, the viral titer gradually increased and highest viral replication was observed between 12 and 24 hpi. with the titer at $10^{5.85}$ and $10^{5.25}$ $TCID_{50}$/ml, respectively (Figure 1A). The cell viability was determined following CHIKV infection in RAW264.7. The cell viability remains approximately 93-88% within 0-12 hpi at all MOIs compared to mock control condition (Figure 1B). For MOI 1 at 24 hpi, the percentage of cell viability was 85.82% which showed a significant difference compared with MOI 5 (71.50%) and 10 (71.81%). A lot of cell death leads to the necrosis of the apoptosis process and may affect to increase or decrease of specific proteins, not related to viral replication. These results indicated that the optimal condition for phosphoproteomics should have high viral replication at low cell death. Therefore, MOI 1 and time at 24 hpi. were chosen as the optimal condition for phosphoproteomics analysis.

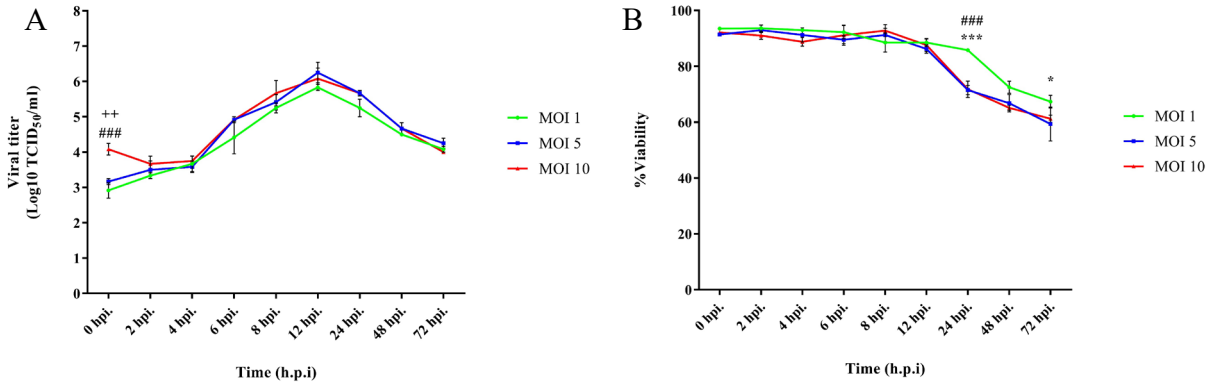

Figure 1 CHIKV replication kinetics in RAW264.7 macrophages. (A) Viral titer after RAW264.7 were infected with CHIKV at MOI 1, 5, and 10 measured by $TCID_{50}$ assay. (B) Cell viability of RAW264.7 after CHIKV infection at various MOI and time points. Two-Way ANOVA with Bonferroni multiple comparisons tests were performed. Significant differences between MOI 1 and MOI 5 are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0002$, **** $p < 0.0001$; significant differences between MOI 1 and MOI 10 are indicated as # $p < 0.05$, ## $p < 0.01$, ### $p < 0.0002$, #### $p < 0.0001$; significant differences between MOI 5 and MOI 10 are indicated as + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.0002$,++++ $p < 0.0001$.

Identification of differentially expressed phosphoproteins

To investigate the altered proteins of RAW264.7 macrophages infected with CHIKV, proteins from control and CHIKV infection were subjected to LC-MS/MS at 24 hpi of MOI 1. A total of 1104 phosphoproteins were identified by phosphoproteomic analysis. Control and infected cells were compared ($p < 0.05$, unpaired *t*-test) based on the criteria of a difference in expression of > 2-fold change (up-regulation) or < −2-fold change (down-regulation). The volcano plot showed all the different phosphoproteins between the two groups, of which 139 and 44 were significantly up-regulated and down-regulated phosphoproteins, respectively (Figure 2). The results suggest that CHIKV infection in RAW264.7 macrophages could induce the alteration of protein and possibly promote viral production.

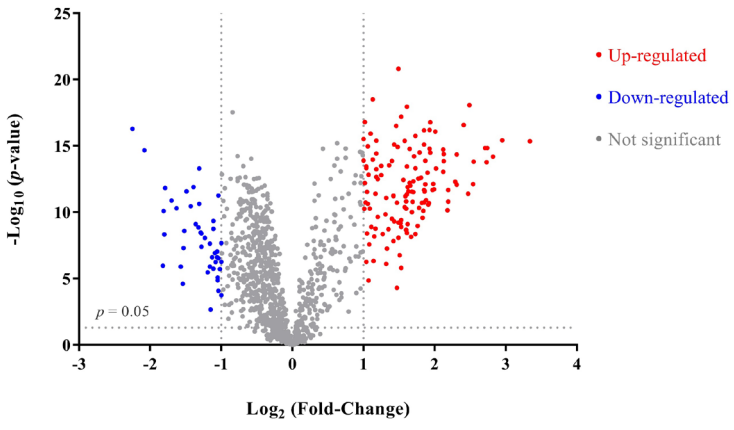

Figure 2 A volcano plot of the phosphoproteins during CHIKV infection in RAW264.7 macrophages. The differential expression was determined as fold-change ratio (infection/control) > 2 or < −2, $p < 0.05$, unpaired *t*-test using GraphPad Prism 7.0. Red dots represent upregulated proteins and blue dots represent downregulated proteins.

Functional classification of differentially expressed phosphoproteins

PANTHER software was used to classify the altered proteins' function and their involvement in biological processes (Figure 3). The results showed that the five major functions of up-regulated phosphoproteins included 31.5% cellular process, 18.2% metabolic process, 11.9% biological regulation, 10.6% response to stimulus, and 8.3% localization (Figure 3A). In down-regulated phosphoproteins, the five major functions consisted of 31.0% cellular process, 21.1% metabolic process, 14.1% biological regulation, 9.9% localization, and 7.0% response to stimulus (Figure 3B). Thus, CHIKV infection induces altered proteins, which are involved in several processes of host cells.

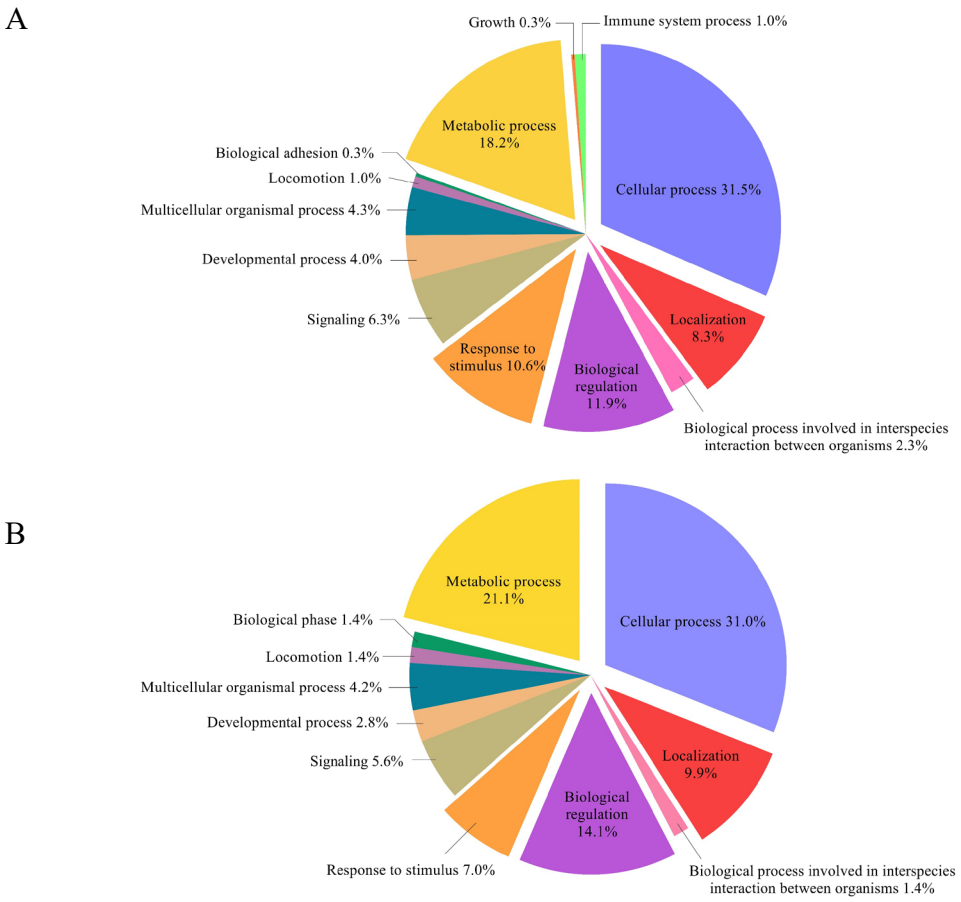

Figure 3 Functional categorization of up-regulated (A) and down-regulated phosphoproteins (B) was performed PANTHER version 17.0.

Protein network analysis

The STRING database is used for protein-protein interactions analysis of up-regulated and down-regulated phosphoproteins. Based on biological processes, a complex protein interaction was indicated as three major networks of up-regulated phosphoproteins: the immune system process, response to virus, and RNA metabolic process (Figure 4A). For down-regulated phosphoproteins, a major network was the macromolecule metabolic process (Figure 4B).

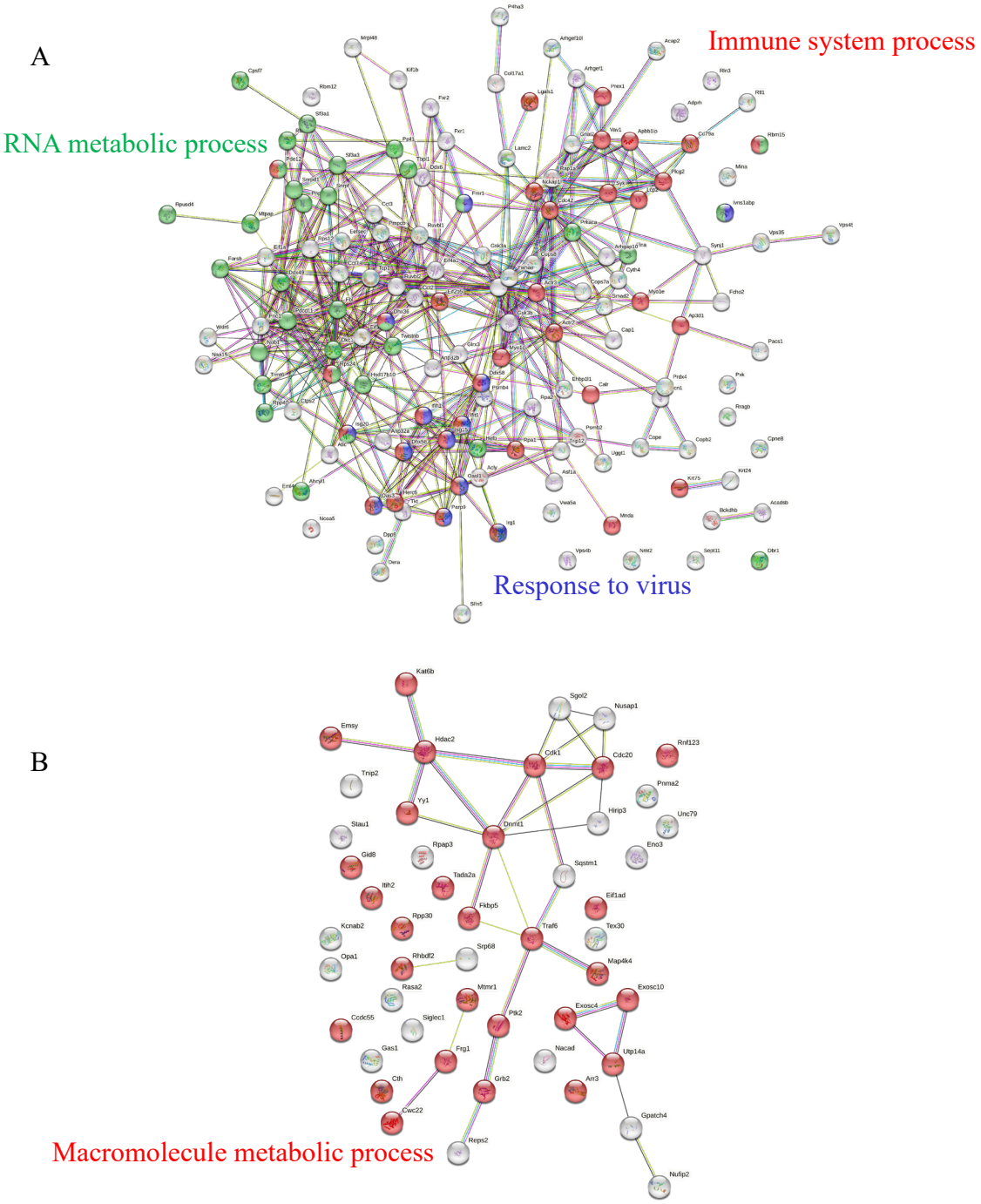

Figure 4 The interaction networks of altered phosphoproteins using STRING database. (A) The three major networks of up-regulated phosphoproteins: immune system process, response to virus, and RNA metabolic process are shown in red, blue, and green, respectively. (B) A major network of down-regulated phosphoproteins was macromolecule metabolic process as shown in red.

DISCUSSION & CONCLUSION

CHIKV has been shown to infect and replicate in various cell types including epithelial cells, endothelial cells, fibroblasts, and immune cells (Lombardi Pereira et al., 2019). Immune cells play an important role in phagocytosis, virus elimination, cytokine, and chemokine production in response to viral infection and inflammation (Nikitina et al., 2018). Previous studies have shown that macrophages are the main reservoir and are susceptible to CHIKV infection, which

may facilitate the spread of the virus throughout the host. (Felipe et al., 2020; Her et al., 2010; Labadie et al., 2010; Sourisseau et al., 2007). Our study provides evidence to confirm that RAW264.7 mouse macrophages are susceptible to CHIKV infection. Among three MOIs investigated to observe viral replication, MOI 1 showed a high viral titer and low level of cell death at 24 hpi. Thus, this condition may be optimal for identifying the proteins involved in viral replication by phosphoproteomics analysis. This is in concordance with a previous study where they predicted treatments in CHIKV-infected U937 cell lines using RNA-sequencing, which showed 234 pathways were significantly affected at 24 hpi (Gray et al., 2022).

Our phosphoproteomics data indicated that CHIKV infection could induce the alteration of host proteins. Among these altered phosphoproteins, 139 and 44 were observed to be significantly up-regulated and down-regulated, respectively. The major functions in biological processes of altered phosphoproteins were classified by PANTHER software. Cellular processes are functions at the cellular level that regulate the cell cycle, cell death, cell division, protein folding, and signal transduction. Our findings are consistent with previous studies which have reported that CHIKV infection can modulate various signaling pathways, including the G-Protein signaling (Abere et al., 2012), MAPK signaling, and chemokine signaling (Abraham et al., 2015), using proteomics analysis. The metabolic process is a key factor that modulates viral infection by activating viral replication. Our results also align with a previous study conducted on CHIKV-infected HEK293 cells, which reported that the virus can modulate the metabolism of carbohydrates, fatty acids, and amino acids in host cells. (Abraham et al., 2015). Another study has shown that the APOL1 protein is involved in cholesterol and lipid metabolic processes upon CHIKV infection (Sukkaew et al., 2020). However, because of the difference in cell types and omics analysis, our results showed the different types of altered proteins. At the phosphoprotein level, we found proteins that are related to signal transduction, such as Prkaca, Rap1a, Plcg2, Syk, Ncoa5, Smad2, Gnai2, and Arhgef1. The proteins involved with metabolic processes such as Acly, Uggt1, Nmt2, Tbpl1, and Acod1 were also detected. Furthermore, our study revealed that the major groups of upregulated phosphoproteins in STRING analysis exhibited a highly significant interaction, consisting of the immune system process, response to virus, and RNA metabolic process. For downregulated phosphoproteins, our analysis identified a very significant interaction within the macromolecule metabolic process. A previous investigation of CHIKV-infected WRL-68 cells revealed the protein networks associated with energy production, cell cycle regulation, gene expression, mRNA metabolism, protein metabolism, and DNA replication (Thio et al., 2013). Another study demonstrated that upregulated protein networks were linked to energy metabolism, transcription and translation, apoptosis, and stress response. (Abraham et al., 2015). However, the protein interaction result in our study differs from other studies. The discrepancy may result from different cell types, MOIs, and incubation times among studies. Although we have identified many interesting proteins upon CHIKV infection by using phosphoproteomic analysis, their biological functions still need validation. Additionally, previous studies reported the ability of CHIKV infection in human cell types. Therefore, further studies need to be performed in primary human cells to investigate the altered proteins of these cells upon CHIKV infection.

In summary, our study demonstrated that RAW264.7 mouse macrophages are susceptible to CHIKV infection, and our phosphoproteomics analysis using LC-MS/MS identified altered proteins during the infection. CHIKV infection affected phosphoproteins which are involved in several processes, including cellular process, metabolic process, biological regulation, response to stimulus, and localization. These findings enhance our understanding of the host protein response to CHIKV infection and provide valuable knowledge regarding the role of phosphoproteins in promoting CHIKV pathogenesis.

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