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THE GROWTH OF ARTHRALGIC ROSS RIVER VIRUS IS RESTRICTED IN HUMAN MONOCYTIC CELLS.

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Running title: Ross River Virus-persistent infection of MM6 cells.

Highlights :

- RRV can infect MM6 monocytes.
- RRV-infected MM6 monocytes induced no interferon or cytokines release.
- RRV-infected MM6 monocytes underwent apoptosis.
- MM6 cells that survived RRV infection carried viral genome days post-infection.

SUMMARY

Alphaviruses such as Chikungunya and Ross River (RRV) viruses are associated with persistent arthritis and arthralgia in humans. Monocytes and macrophages are believed to play an important role in alphaviral arthritides. In this study, we evaluated RRV permissiveness of the human acute leukemia MM6 cell line. Viral growth analysis showed that RRV infection of MM6 cells resulted in a very low virus progeny production with daily output. Using recombinant RRV expressing the reporter gene *Renilla* luciferase, a weak viral replication level was detected in infected cells at the early stages of infection. The infection restriction was not associated with type-I interferon and pro-inflammatory cytokines release. Apoptosis hallmarks (i.e. mitochondrial BAX localisation and PARP cleavage) were observed in infected MM6 cells indicating that RRV can trigger apoptosis at late infection times. The long-term persistence of RRV genomic RNA in surviving MM6 cells identifies human monocytic cells as potential cellular reservoirs of viral material within the infected host.

KEY WORDS

Ross River Virus; alphavirus; persistent infection; arthritis; apoptosis; human monocytic cells.

MAIN TEXT

Ross River virus (RRV) is a mosquito-borne *alphavirus* of medical concern in the South Pacific with thousands of human cases of RRV infection (Aaskov et al 1981). Myalgia and headache are common symptoms in patients suffering from RRV-related disease. Most patients recovered within 6 months after the disease onset without symptoms after 2 years but symptoms can persist for up to 3 years (Fraser 1986). RRV-related disease can be severe at onset with level of disability that is comparable to patients with osteoarthritis, Lyme disease, or chronic rheumatoid arthritis. Inflammatory cell infiltration in joints, muscles, and associated tissues during *alphavirus* infection has been reported in mouse models of RRV infection, suggesting that muscular and articular damages are part of an immunopathological inflammatory disorder (Heise et

al 2000, Lidbury and Mahalingam 2000, Morrison et al 2006). In RRV infections, the cellular infiltrate reaches synovial tissue, evidencing a strong hyperplasia (Gardner et al 2010, Morrison et al 2006, Morrison et al 2011). Monocytes, macrophages, NK cells, and CD4+ and CD8+ T lymphocytes are the main cellular components of the inflammatory infiltrate in animal models, suggesting their possible involvement in the pathogenesis of the RRV-mediated arthritis (Gardner et al 2010, Labadie et al 2010, Lidbury et al 2000, Morrison et al 2006, Morrison et al 2011). It is also interesting to mention that murine macrophage-like cell cultures continued to harbor RRV infection for more than 1.5 month without obvious cytopathic effects (Linn et al 1996, Way et al 2002). In agreement with the data obtained in animal models, macrophages and NK cells have been detected in synovial exudates from patients that have experienced arthralgic alphavirus infection (Fraser et al 1981, Hazelton et al 1985, Hoarau et al 2010, Soden et al 2000). Little is yet known on the susceptibility of human monocytes to arthralgic alphaviruses. The purpose of the present study is to investigate again the RRV permissiveness of human monocytic Mono Mac 6 (MM6) cells, which have been initially claimed as refractory cells to RRV infection (Linn et al 1996).

To evaluate the susceptibility of MM6 cells to laboratory-adapted RRV strain T48 (RRV-T48) and Australian (RRV-AUS) clinical isolate (Welcome Trust, National Collection of Pathogenic Viruses, No:0005281v), MM6 cells were infected with a multiplicity of infection (MOI) of 1 PFU/cell. RT-PCR analysis of total RNA extracted from RRV-infected MM6 cells detected the presence of genomic RNA of both RRV strains as late as 7 days post-infection (p.i) (Fig. 1A). The kinetic of RRV-T48 growth in MM6 cells showed that virus titers slightly increased between 1 day and 3 days p.i. to reach at maximum almost $3.2 \log \text{PFU.mL}^{-1}$ at 3 days p.i. compared to a progeny production upper than $8 \log \text{PFU.mL}^{-1}$ in HEK-293 and VERO cells (Lidbury et al 2011) (Fig. 1B). After 3 days of infection, the virus progeny production daily declined to a basal virus titer of 100PFU.mL^{-1} at day 7 p.i. Taken together, these results show that a low level of RRV growth can occur in MM6 cells giving a new highlight on RRV permissiveness of human monocytic cells.

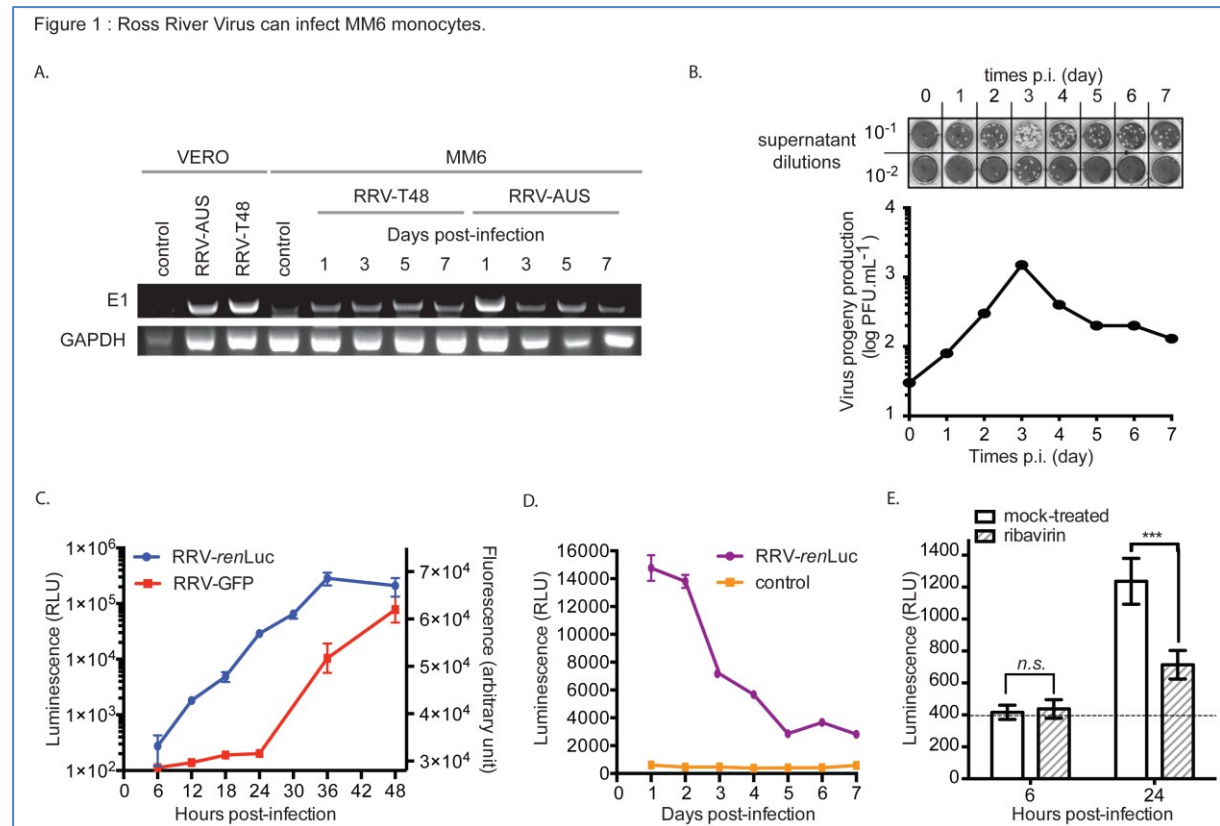


Figure 1: RRV can infect MM6 monocytes. In (A), MM6 cells were infected with RRV Australian strain or T48 molecular clone at MOI of 1. Total RNAs were extracted from MM6 cells each day from day 1 to day 7 p.i. and from VERO cells at 24 hours p.i.. Total RNAs were analyzed for the expression of viral E1 mRNA by RT-PCR. GAPDH mRNA was used as loading control. In (B), MM6 cells were infected with RRV T48 molecular clone at MOI of 1. Culture supernatants were collected each day from day 1 to day 7 post-infection. Viral progeny was determined by plaque assay on VERO cells. Virus titers are presented for one representative experiment. In (C), HEK293T cells were infected with RRV-*renLuc* or RRV-GFP at MOI of 1 or mock-infected. A time course of luciferase activity and fluorescence signal was realized. Results are expressed as the mean (± SEM) of three independent experiments (six replicates per experiment). In (D), MM6 cells were infected with RRV-*renLuc* at MOI of 1 or mock-infected. Cells were collected at each time points and analyzed for luciferase activity. Shown is the mean (± SEM) from six replicates in one representative of four independent experiments. In (E), MM6 cells were infected with RRV-*renLuc* at MOI of 1 or mock-infected. 100 μM Ribavirin was added to culture medium 2 h p.i.. Cells were collected 6 h or 24 h p.i. and analyzed for luciferase activity. Shown is the mean (± SEM) from three replicates in one representative experiment. Dotted line shows the threshold.

Recombinant viruses encoding reporter genes represent useful tools to follow up viral replication and spread in cell culture, but also in animals or mosquitoes (Kummerer et al 2012, Pohjala et al 2008). To better understand the mechanisms of MM6 cells infection by RRV, we decided to generate molecular clones of RRV-T48 containing either the reporter gene *Renilla* luciferase (RRV-*renLuc*) integrated into the non-structural protein region of the genomic RNA or the reporter gene VisGreen GFP (RRV-GFP) into the subgenomic RNA. The *renLuc* gene was inserted in frame between nsP3 and nsP4, using a strategy similar to that previously described with Chikungunya virus (Henrik Gad et al 2012). The codon-optimized VisGreen GFP sequence followed by the *Thosea asigna* virus 2A protease was inserted into the

structural protein region in frame with the first amino acids of RRV E2 protein. Recombinant RRV-*renLuc* and RRV-GFP grown on permissive HEK293T cells allow the monitoring of the early or the late stage of virus replication in RRV infected host-cells, respectively (Fig. 1C). The *renLuc* activity was significantly detected within the 8-12 hours p.i. and increased linearly during the time course of infection to reach a plateau at 36 h p.i. The autofluorescence of GFP was clearly observed within the 24 h p.i and the intensity of signal progressively increased during the course of infection.

MM6 cells were infected with each recombinant RRV at the MOI of 1. In MM6 cells infected 24 h with RRV-*renLuc*, we detected a low level of *renLuc* activity that declined daily to reach a threshold at day 5 of infection (Fig. 1D). To distinguish between the level of *renLuc* activity derived for the initial translation of the input ZIKV RNA and one related to viral replication, we evaluated the effect of antiviral inhibitor Ribavirin on RRV-*renLuc*. (Fig. 1E). Addition of 100 μ M Ribavirin 2 h after RRV-*renLuc* exposure reduced by 65% the level of *renLuc* signal in infected MM6 cells at 24 h p.i. As a control, 100 μ M Ribavirin reduced by at least 50% the *renLuc* activity in VERO cells infected 24 h with RRV-*renLuc* (data not shown). This is consistent with the assumption that the low level *renLuc* activity is attributable to a modest production of intracellular viral RNA in infected MM6 cells. Furthermore, no significant GFP signal was observed in MM6 cells infected with RRV-GFP whatever the time-points of infection examined (data not shown). The absence of GFP expression suggests that no synthesis of subgenomic viral RNA nor translation of structural protein region occurred in the late stages of RRV growth in MM6 cells. We can conclude that RRV replication was severely restricted in MM6 cells leading to a very low virus progeny production.

Therefore, we ask if the inability of RRV to establish a highly productive infection in MM6 cells was associated to marked production of Type-I interferons (IFNs). To determine whether RRV infection results in release of soluble IFN- α/β , the supernatants of infected MM6 cells were collected at different time-points of infection, UV-inactivated and then analyzed on HEK-Blue IFN- α/β cells containing the SEAP reporter gene under the control of IFN- α/β inducible ISG54 promoter (Frumence et al., 2016) (Fig. 2A). No SEAP reporter activity was detected in supernatants of MM6 cells during the time course of infection. The addition of exogenous IFN- α in the

supernatants of RRV-infected cells showed that the absence of SEAP reporter activity was not due to the inhibition of the SEAP or a block in the IFN signaling pathway. These results suggest that IFN-dependent antiviral pathway does not play a role in the restriction of RRV replication in MM6 cells. We investigated whether RRV infection of MM6 cells leads to increase of major cytokines by RT-qPCR (Fig. 2B to C). No significant difference was observed for IL-1 β , IL-6 or IL-8 mRNA. By using a quantitative ELISA assay (Ready-SET-Go!®, e-Bioscience), we failed to detect pro-inflammatory IL-1 β , IL-6, IL-8, TNF- α and chemokine MCP-1 in the supernatants of MM6 cells infected with RRV-T48 (only IL-1 β data shown in Fig. 2D). Altogether, these results show an unresponsiveness of MM6 cells to RRV infection with the absence of pro-inflammatory cytokine and IFN- α/β production.

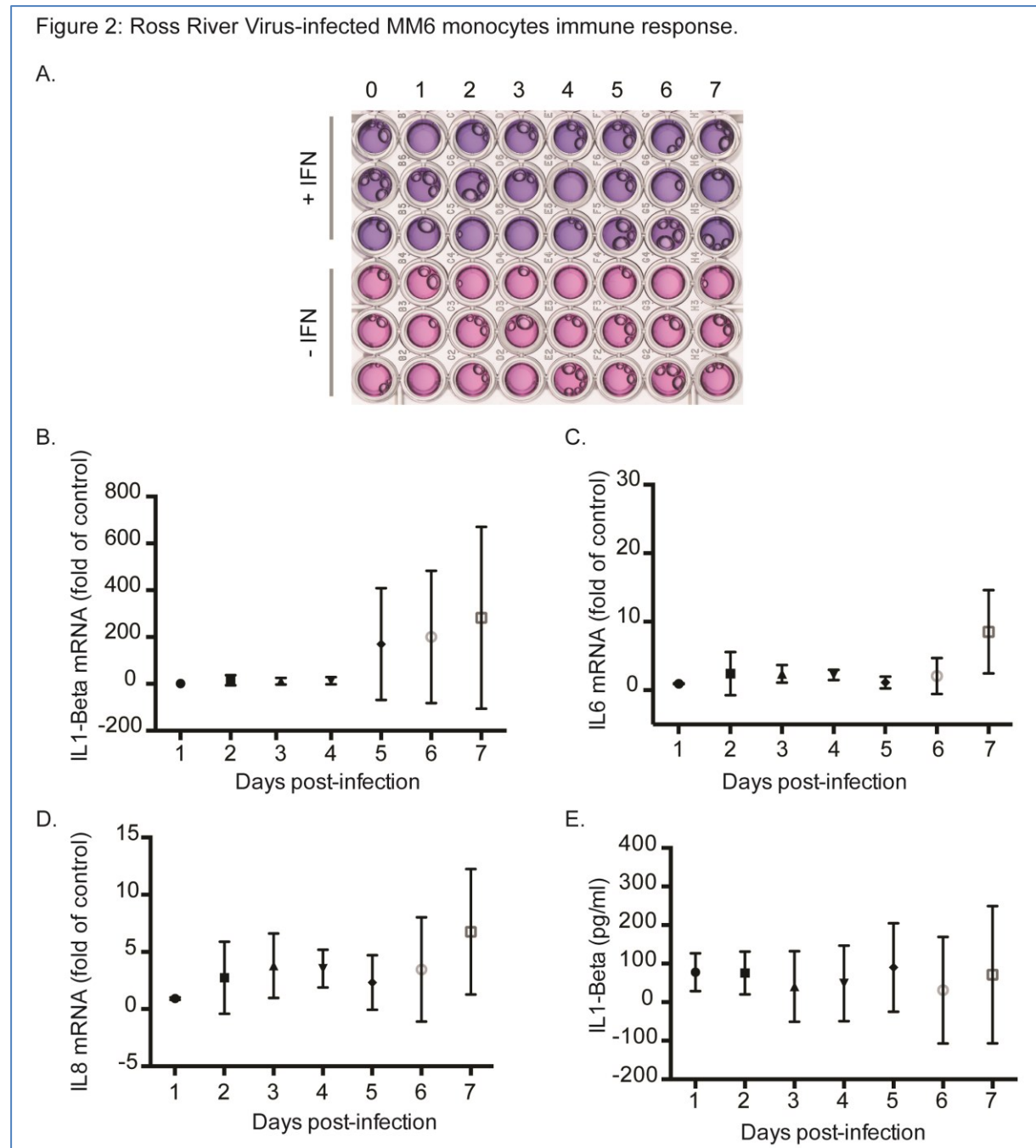


Figure 2 : RRV-infected MM6 monocytes immune response. MM6 cells were infected with RRV-GFP at MOI of 1 or mock infected. Cells or culture supernatants were collected every day from day 1 to day 7 post-infection. In (A), time course of Type 1 IFN release in response to RRV. HEK-Blue™ IFN- α/β cells were incubated 24h with UV-inactivated supernatants of RRV-infected cells and the extracellular activity of released IFN/SEAP was measured using Quanti-blue reagent. Exogenous IFN- α/β was added to the supernatants to confirm that the absence of SEAP reporter activity was not due to the inhibition of the SEAP or to a block in the IFN signaling pathway. One representative experiment is shown. In (B) to (C), cellular mRNA encoding IL-1 beta, IL6 and IL8 were analyzed by qRT-PCR at different times post-infection. Results are the mean (\pm SEM) from two independent experiments performed in triplicates. In (D), ELISA assays for the measure of IL-1 beta production were performed on the supernatants of infected cells at different times post-infection. Results are the mean (\pm SEM) from at least three independent experiments performed in triplicates.

At day 5 post-infection, we observed a loss of viability of MM6 cells infected by RRV compared to control. Given that cells were displaying features of apoptotic death, the

relocalization of the pro-apoptotic mitochondrial factor Bax, a hallmark of apoptosis, was examined by indirect IF using a specific antibody. As shown in Fig. 3A, mitochondrial Bax was positively stained in MM6 cells infected one week by RRV. Immunoblot assays with a specific antibody directed against the 85 kDa-cleaved form of PARP, a hallmark of the executive phase of apoptosis, were performed on lysates of RRV-infected MM6 cells (Fig. 3B). The cleaved form of PARP was detected at 5 days post-infection and then accumulated at the later infection times. The progressive disappearance of the 115 kDa-full length form of PARP coincided with the accumulation of its cleaved form within the 5-7 days of infection. Altogether, these results showed that RRV infection results in a late apoptosis in MM6 cells although viral growth was severely restricted. Despite a very low percentage of RRV-infected cells survived to apoptosis, these cells were cultured with fresh growth medium supply every week for up to 45 days. RT-PCR analysis detected the presence of viral genome up to 45 days post-infection suggesting that RRV could potentially persist in MM6 cells on a long-time period (Fig. 3C).

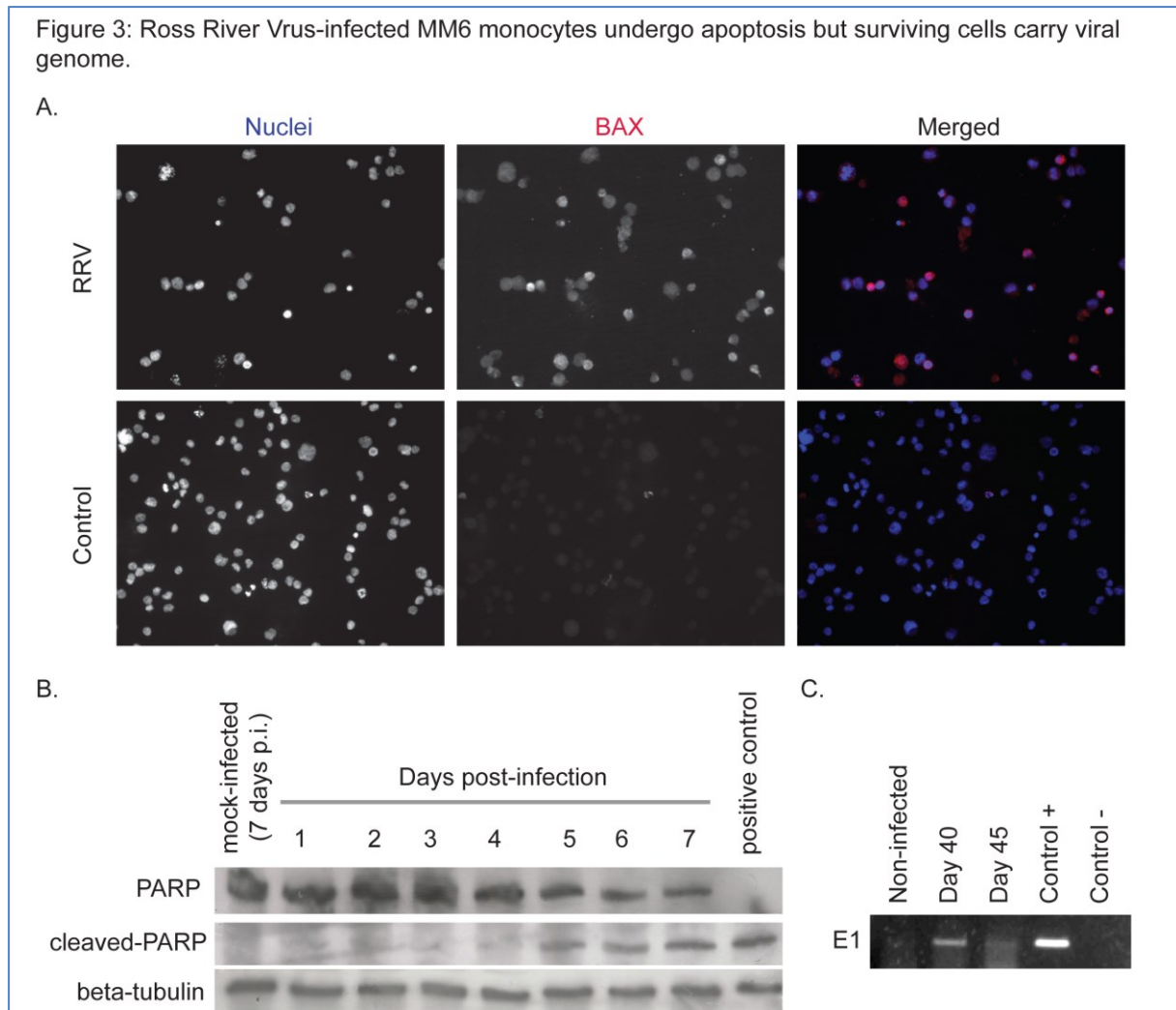


Figure 3 : RRV-infected MM6 monocytes undergo apoptosis but surviving cells carry viral genome. MM6 cells were infected for seven days with RRV-GFP at MOI of 1 or mock infected. In (A), BAX signal in RRV-infected cells was analyzed by immunofluorescence assay using specific antibody. Nuclei were stained with DAPI. Results are presented individually or as merged images. In (B), cells were collected every day from day 1 to day 7 post-infection. The cleavage of PARP in infected cells was analyzed by immunoblot assay using specific antibody against the 85 kDa-cleaved form of PARP. We also visualized total PARP and beta-tubulin as loading control. In (C), Cells were collected at days 40 and 45 post-infection. Total RNAs were extracted and analyzed for the expression of viral E1 mRNA by RT-PCR.

The present study is the first demonstration that RRV has the ability to infect human monocytic MM6 cells although viral growth was poorly efficient with a very low virus progeny production. No release of IFN- β and pro-inflammatory cytokines IL-1 β , IL-6 and IL-8 as well as TNF- α or MCP1 was observed in RRV infected cells indicating that host cells were not able to drive an immune response. For the first time, we demonstrated that MM6 cells underwent apoptosis in response to RRV infection as late as 5 days post infection. Relocalization of mitochondrial Bax was observed in apoptotic MM6 cells suggesting a role for mitochondrion-dependent signalling pathway in induction of apoptosis.

To date, the mechanisms by which RRV can trigger very late apoptosis in human monocytic cells remain to be investigated. In *alphavirus*-infected cells, apoptosis could be initiated at the level of RNA replication or thereafter by the pro-apoptotic factor Bak, which leads to cytochrome *c* release from mitochondria (Urban et al 2008). The production of alphaviral dsRNA molecules that are potent sensing factors for dsRNA signalling pathways, the retinoic acid inducible gene-1 and the melanoma differentiation-associated gene-5 systems, could play an important role in this respect (Sanchez David et al 2016).

CHIKV was shown to infect monocytic cells in patients with a poor virus production (Her et al 2010). Macrophages were also highlighted as a possible cellular reservoir in CHIKV infected patients (Hoarau et al 2010) and in experimentally infected macaques (Labadie et al 2010). Moreover, numerous studies have pointed out the role of macrophages in the RRV pathogeny in either mice or humans (Lidbury and Mahalingam 2000, Linn et al 1996). An unexpected observation was that human monocytic cells that survived to apoptosis triggered by RRV still contained viral genetic material suggesting that such cells could be persistently infected. It is therefore urgent to determine whether the human monocytes can act as potential reservoir seed of arthralgic alphaviruses and thereby contribute to the pathogenesis of the chronic forms of RRV and CHIKV-related arthralgies.

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