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BRIEF REPORT

Inhibition of the PI3K/Akt pathway by Ly294002 does not prevent establishment of persistent Junín virus infection in Vero cells

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Abstract In previous work, we demonstrated that the arenavirus Junín virus (JUNV) is able to activate Akt by means of the phosphatidylinositol-3-kinase (PI3K) survival pathway during virus entry. This work extends our study, emphasizing the relevance of this pathway in the establishment and maintenance of persistent infection *in vitro*. During the course of infection, JUNV-infected Vero cells showed a typical cytopathic effect that may be ascribed to apoptotic cell death. Treatment of infected cultures with Ly294002, an inhibitor of the PI3K/Akt pathway, produced an apoptotic response similar to that observed for uninfected cells treated with the drug. This result suggests that virus-induced activation of the PI3K/Akt pathway does not deliver a strong enough anti-apoptotic signal to explain the low proportion of apoptotic cells observed during infection. Also, inhibition of the PI3K/Akt pathway during the acute stage of infection did not prevent the establishment of persistence. Furthermore, treatment of persistently JUNV-infected cells with Ly294002 did not alter viral protein expression. These findings indicate that despite the positive modulation of the PI3/Akt pathway during Junín virus entry, this would not play a critical role in the establishment and maintenance of JUNV persistence in Vero cells.

Keywords Junín virus · PI3K/AKT · Persistence · LY294002

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The arenavirus Junín virus (JUNV), the etiological agent of Argentine hemorrhagic fever, is able to activate serine/threonine-specific protein kinase B (PKB), also known as Akt, by means of the phosphatidylinositol 3-kinase (PI3K) pathway during virus entry [1]. This early activation, which plays an important role in JUNV uptake, might eventually activate different signaling pathways, contributing to cell survival and, subsequently, virus replication. One of the targets for Akt activity is the eukaryotic initiation factor 4E binding protein (eIF4EBP), which, in its phosphorylated state, releases eIF4E, allowing cap-dependent translation. However, in the case of JUNV, eIF4E seems to be dispensable for translation of viral proteins, suggesting that this pathway is not relevant for this function [2]. On the other hand, it has been demonstrated that many viruses are able to modulate the PI3K/Akt pathway, either by its activation or by its inhibition, in order to facilitate viral replication [3]. Activation of Akt has been reported to be associated with blockage of apoptosis, leading to long-term cellular survival, which allows virus replication and viral protein synthesis [4, 5] and, in some cases, oncogenic transformation, as described for polyomaviruses and human papillomaviruses [6, 7]. Similarly, SARS coronavirus (SARS-CoV) induces weak activation of Akt that is not strong enough to impair apoptosis in response to infection but is nevertheless crucial for the establishment of persistence [8, 9]. In contrast, early activation of Akt by respiratory syncytial virus [10], porcine circovirus type 2 [11], dengue virus (DENV) [12] or influenza virus [13] is enough to prevent infected cells from early apoptotic cell death. This work extends our study about the participation of PI3K/Akt in JUNV replication, emphasizing the relevance of Akt in establishment and maintenance of persistent JUNV infection in Vero cells.

Data published recently demonstrated that *in vitro* JUNV infection leads to apoptotic cell death [14]. We therefore analyzed cell survival in cultures infected with JUNV in order to evaluate if activation of PI3K/Akt during virus entry is sufficient to trigger an anti-apoptotic response favoring virus replication. For this purpose, Vero cells were mock infected or infected with JUNV, and at different times postinfection (p.i.), cell viability was measured using an MTT assay, and nuclear fragmentation associated with chromatin condensation as an apoptotic marker was analyzed by the Hoechst technique. Briefly, cell cultures grown on coverslips were fixed with methanol at −20 °C for 15 min. The methanol was then discarded, and cultures were dried at room temperature. Cells were stained with Hoechst 33342 (5 μg/ml, 20 min) and observed by fluorescence microscopy. As shown in Fig. 1a, the number of apoptotic cells that could be observed in mock-infected cultures was negligible (dark grey bars), and the proportion

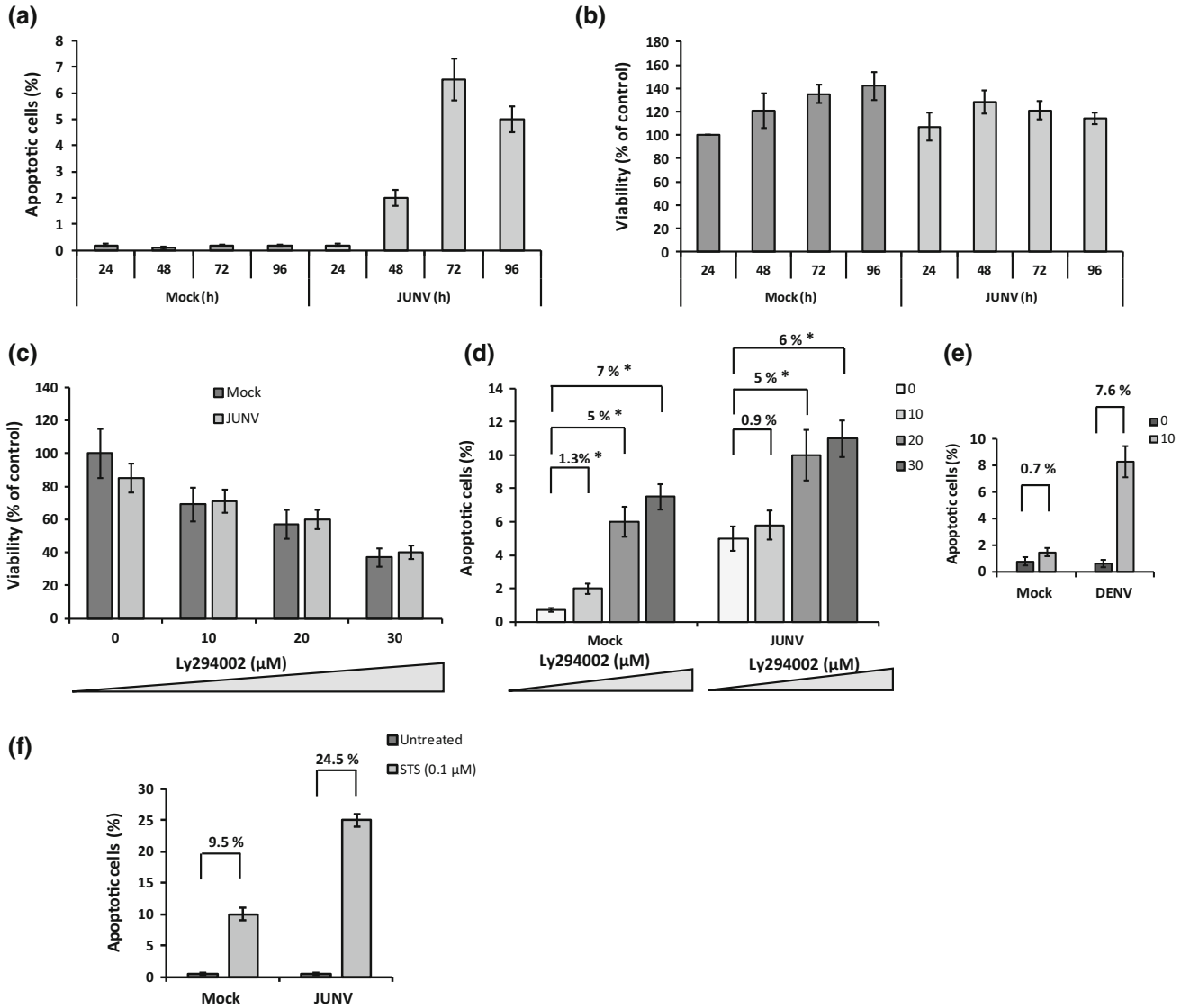


Fig. 1 Serum-starved Vero cells were mock infected or infected with JUNV at an MOI of 1 pfu/cell, and at the indicated hours postinfection apoptosis was evaluated by the Hoechst technique (a) and viability measured by a conventional MTT assay (b). Vero cells were mock infected or infected with JUNV at an MOI of 1 pfu/cell in the presence of different concentrations of Ly294002. At 72 h post-treatment cell viability was measured by MTT assay (c), and apoptosis was evaluated by the Hoechst technique (d). Vero cells were mock infected or infected with DENV-2 at an MOI of 0.1 and treated with 10 μM Ly294002. At 72 h post-treatment, apoptosis was evaluated by the Hoechst technique (e). Vero cells were mock infected or infected with JUNV at an MOI of 1 pfu/cell, and at 0 h p.i. cells were left untreated or treated with 0.1 μM staurosporin (STS). At 18 h post-treatment, apoptosis was evaluated by the Hoechst technique (f). In all cases, the Hoechst technique was used in cells grown on coverslips in a 24-multiwell microtiter plate and viability was determined in cells grown in a 96-multiwell microtiter plate. All samples were analyzed using Student's t-test (*$p < 0.05$)

of apoptotic cells (light grey bars) detected in infected cultures was modest. The viability of JUNV-infected cells (Fig. 1b, light grey bars) remained practically unchanged throughout the experiment when compared to the value obtained at 24 h p.i., whereas mock-infected cells (Fig. 1b, dark grey bars) grew steadily over time. Detection of apoptotic cells was coincident with the appearance of a distinctive cytopathic effect (CPE), characterized by cell rounding and detachment, that could be readily observed from the third day p.i. onwards (data not shown).

Our next approach was to evaluate the participation of the PI3K/Akt pathway in the modulation of the apoptotic state of infected cells. For this purpose, cellular viability and apoptosis were examined in JUNV-infected cells in the presence of the PI3K inhibitor Ly294002 at 72 h p.i. As shown in Fig. 1c, treatment with Ly294002 induced a similar dose-dependent reduction in cell growth for both mock- and JUNV-infected cells. At the same time, JUNV-infected cells treated with Ly294002 showed a larger number of apoptotic cells than did uninfected treated controls (Fig. 1d). However, when the increase in apoptosis was normalized to the corresponding untreated control (0 μM), the percentage values were similar for both mock- and JUNV-infected cultures (Fig. 1d). In view of these results, it may be concluded that the increase in the number of apoptotic cells in JUNV-infected cultures treated with the drug might have been due to an additive effect between virus and Ly294002. To test this hypothesis, we performed a similar experiment with dengue virus serotype 2 (DENV-2), a virus that positively modulates the PI3K/Akt pathway in order to delay the apoptotic response [12]. As shown in Fig. 1e, inhibition of PI3K with Ly294002 in Vero cells infected with DENV resulted in a large increase in the number of apoptotic cells compared with the mock-infected control. Based on these results, activation of PI3K/Akt triggered by JUNV during entry appears not to be a key factor contributing to the modulation of the apoptotic process in infected cells, suggesting that JUNV is not able to counteract apoptosis via upregulation of the PI3K/Akt pathway. This was confirmed when cells were treated with staurosporine (STS), a multi-factorial strong apoptosis inducer. The proportion of cells showing chromatin condensation and nuclear fragmentation was markedly higher for infected cells than for mock-infected cultures (Fig. 1f).

Arenaviruses are able to establish persistent infections *in vivo,* in their natural hosts, as well as *in vitro*, in several types of cell cultures. Persistence *in vivo* is primarily governed by suppression of the immune response. This persistence depends on the long-term infection of fibroblastic reticular cells, which are the primary site of virus replication [15]. Viral targeting of fibroblastic reticular cells contributes to immunosuppression, with PDL-1 and IL-10 being the main effectors for suppression [16, 17]. Thus, looking at long-term infection of a fibroblastic cell line, i.e., Vero cells, is biologically relevant as an approach for understanding *in vivo* persistence.

In view of the results described above, although JUNV modulation of apoptosis via PI3K/Akt appears not to be relevant for survival of the infected cultures, it might be necessary for the establishment of persistence *in vitro*. Thus, our next goal was to investigate the participation of this pathway in the establishment of persistent JUNV infection of Vero cells. To this end, JUNV infection was initiated in the absence or in the presence of Ly294002. Cells infected with JUNV at an MOI of 1 pfu/cell were treated with Ly294002 during the first (0–7), second (7–14), third (14–21) or fourth week (21–28) p.i. or left untreated. The progression of infection was monitored by measuring the expression of the JUNV nucleoprotein N and the production of infectious viruses. All of the cultures were able to recover from the CPE observed during the acute stage of infection, and by day 28 p.i., they became morphologically indistinguishable from uninfected Vero cells (data not shown). As shown in Fig. 2a, treatment of infected cells with Ly294002 altered the pattern of expression of N during the course of infection, in contrast with untreated cultures, in which the protein was steadily synthesized throughout the experiment. Inhibition of virus replication by Ly294002 may be ascribed to the effect of the drug on the adsorption/internalization step. Under these conditions (infection at low MOI), the expression of N is the result of multi-step growth, where adsorption/internalization of progeny virus produced after each cycle is hampered by the drug. On the other hand, treatment with inhibitor was not able to abolish productive infection, although infectivity levels in supernatants were higher for cultures treated during the first, second, and third week p.i. (Fig. 2b). It is tempting to speculate that inhibition of PI3K/Akt would hamper virus replication, thus delaying establishment of persistence. However, it cannot be ruled out that the higher titers detected in treated cultures were the result of the cyclic production of infectivity, which is typical of the early stage of persistence [18, 19]. As shown in Fig. 2c, at 70 days p.i., all cultures synthesized N protein and conserved their capacity to produce infectious virus (Fig. 2b). In fact, an indirect immunofluorescence assay (IFA) confirmed a similar pattern of expression of N as well as the main envelope glycoprotein (G1) in all cultures (Fig. 2d). Characterization was continued up to 120 days p.i., at which time persistently JUNV-infected Vero cells, named V3, became non-virogenic although continuing to express high levels of N. Of note, infectivity levels at 70 days p.i. were higher for treated cultures, whereas at 21–28 days p.i., treated cultures showed a low level of infectivity, similar to that of untreated controls, suggesting that treatment with Ly294002 is effective up to the third

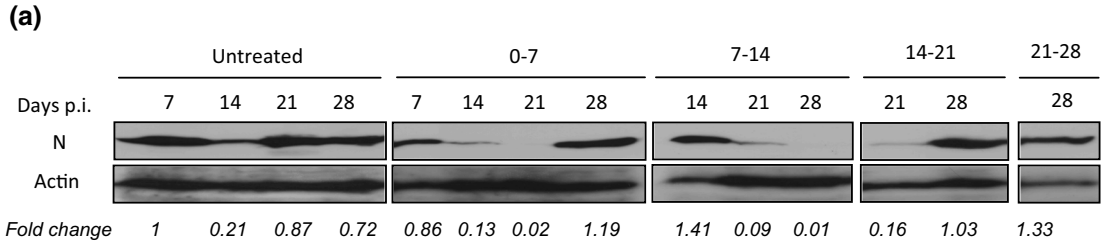
week of infection. This time point is generally accepted as the transition of acute to persistent JUNV infection in Vero cells [18, 19]. At 120 days p.i., all of the cultures expressed high amounts of N, as shown by Western blot (WB) (Fig. 2e) and IFA (Fig. 2f). However, as expected, no infectivity could be recovered from them. In agreement with this observation, G1 expression was not detected (data not shown); however, all cultures showed the capacity to form syncytia upon acidification (Fig. 2f), as described previously [19]. Akt activation has been proposed to contribute to the establishment of persistent infection by DNA viruses such as murine gammaherpesvirus 68 and human herpesvirus 8/Kaposi's sarcoma-associated herpesvirus [20] as well as by an RNA virus, Sindbis virus [21]. For these viruses, persistent infection is promoted by the negative regulation of the lytic infection exerted by the activation of Akt. Our results indicate that inhibition of Akt would not impair the establishment of persistent JUNV infection in Vero cells because all cultures treated with Ly294002 evolved towards persistence in a fashion similar to that observed for untreated cultures.

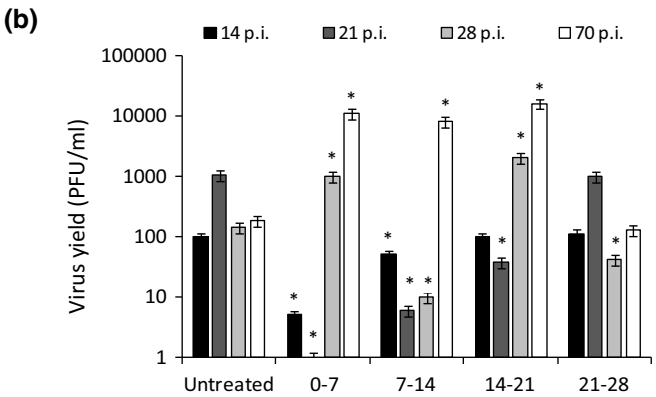
Finally, we investigated whether the PI3K/Akt pathway is needed in the maintenance of persistence. For this purpose, we evaluated the effect of Ly294002 treatment on the viability of V3 cells. Cells were treated with 10 and 30 μM of Ly294002, and at 24 and 48 h post-treatment, cell viability and cell morphology were evaluated by an MTT assay and phase contrast microscopy, respectively. As shown in Fig. 3a, Ly294002 treatment did not significantly reduce the viability or alter the morphology of V3 cells, whereas Vero cells showed reduced survival and altered morphology when compared to untreated controls, suggesting that persistent cells are more resistant to treatment with this drug. We also evaluated the effect of Ly294002 treatment on JUNV protein synthesis in V3 cells. To this end, V3 cells were treated with Ly294002 and processed for WB. As a control for acutely infected cells, Vero cells were infected with JUNV at an MOI of 1 pfu/cell, and after adsorption, cells were treated with the drug. As shown in Fig. 3b, no alteration in the synthesis of N was observed for V3 cells treated with either concentration of Ly294002, while acutely infected cells showed a dose-dependent reduction in the synthesis of the viral protein (Fig. 3c), in accordance with previously reported data [1]. Moreover, overexpression of a dominant negative plasmid of Akt [1] did not significantly alter the expression of N in V3 cells analyzed by IFA. In fact, 10 ± 5 % inhibition was observed for V3 cells transfected with the dominant negative plasmid, whereas 87 ± 12 % inhibition was observed for Vero cells transfected and acutely infected with JUNV. These results support the conclusion that activation of Akt would not be a relevant event in V3 cells, not even for an efficient internalization of virus, in view of their non-

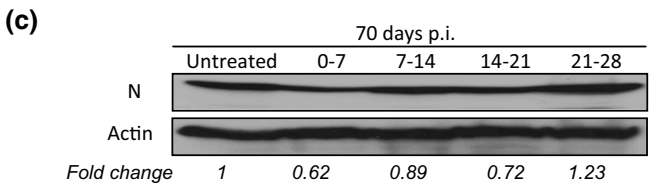
Fig. 2 Serum-starved Vero cells grown in a 24-well microtiter plate ▶ were infected with JUNV at an MOI of 1 pfu/cell and then left untreated or treated with 10 μM Ly294002 during the first (days 0–7), second (days 7–14), third (days 14–21) or fourth (days 21–28) week p.i. The medium was replaced every 3 days and supernatants and cell lysates were harvested every 7 days up to 28 days p.i. in order to evaluate N protein synthesis (a) and virus yield (b). Cultures were maintained weekly, and evaluation of N protein synthesis was determined at 70 days p.i. (c) and 120 days p.i. (d). Expression of N by IFA and syncytium formation after treatment of cells at pH 5.5 was evaluated at 120 days p.i. (e). Alternatively, expression of the viral antigens N and G1 was evaluated by IFA at 70 days p.i. (f). N and G1 were detected using the monoclonal antibodies NA05AG12 and QB03BE08, respectively [24]. Numbers under the panels indicate N expression, calculated as the ratio of N to actin measured with Image J software. Samples in panel b were analyzed using Student's t-test (*$p < 0.05$)

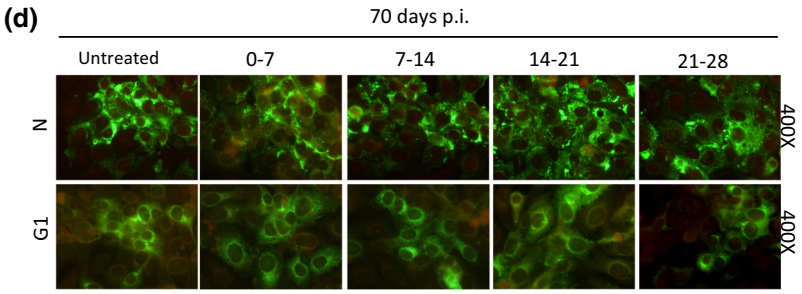
virogenic phenotype. In order to determine the functionality of Akt in persistently JUNV-infected cells, we initially evaluated the uptake of transferrin (Tf), a ligand that depends on this pathway for the efficient recycling of its receptor. Cells were incubated with TRITC-labeled Tf for 30 min in the presence or absence of 10 μM Ly294002 and were then observed by ultraviolet microscopy. As expected, Vero cells showed a characteristic accumulation of labeled Tf around the nucleus when observed at 30 min after addition of Tf (Fig. 3d, left column). This accumulation was also observed in V3 cells, although the level of Tf was lower (Fig. 3d, middle column). Moreover, treatment of V3 cells with Ly294002 induced a scattered pattern of Tf in the cytoplasm, indicating that Tf uptake was efficiently blocked by inhibition of PI3K (Fig. 3d, right column). Taking into account that JUNV enters cells by using the Tf receptor, we attempted to determine if superinfection with JUNV is able to induce activation of Akt by phosphorylation at Ser-473 in V3 cells. Serum-starved V3 cells were superinfected with JUNV at an MOI of 1 pfu/cell or mock infected, and at different times post-superinfection, cells were processed for WB. As shown in Fig. 3e, JUNV superinfection induced a biphasic phosphorylation of Akt, peaking at 15 and 120 min post-virus-cell contact, while mock-superinfected V3 cells showed a negligible level of activation. This activation was similar to the biphasic activation reported for acute infection of Vero cells with this virus [1]. Furthermore, Akt phosphorylation induced by JUNV in V3 cells was susceptible to Ly294002 inhibition (Fig. 3f, lane 9 vs. 10) in a fashion similar to that observed for JUNV infection of Vero cells (Fig. 3f, lane 4 vs. lane 5). Likewise, treatment with Ly294002 also reduced the Akt activation induced by insulin in both Vero (Fig. 3f, lane 2 vs. lane 3) and V3 cells (Fig. 3f, lane 7 vs. lane 8). Together, these results suggest that the PI3K-Akt pathway, which is functional in V3 cells, is not involved in the maintenance of long-term persistence.

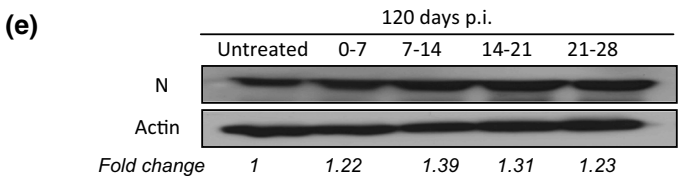
In this work, we have extended our study of the modulation of the PI3K/Akt signaling pathway during JUNV

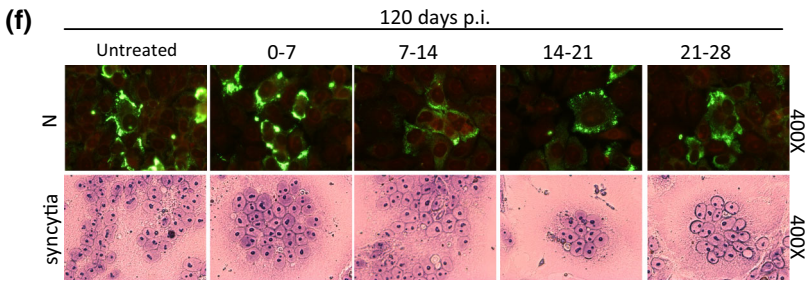

(a)
Untreated
0-7
7-14
14-21
21-28
Days p.i.
7 14 21 28 7 14 21 28 14 21 28 21 28 28
N
Actin
Fold change 1 0.21 0.87 0.72 0.86 0.13 0.02 1.19 1.41 0.09 0.01 0.16 1.03 1.33


(b)
14 p.i.
21 p.i.
28 p.i.
70 p.i.
Virus yield (PFU/ml)
100000
10000
1000
100
10
1
Untreated
0-7
7-14
14-21
21-28


(c)
70 days p.i.
Untreated
0-7
7-14
14-21
21-28
N
Actin
Fold change 1 0.62 0.89 0.72 1.23


(d)
70 days p.i.
Untreated
0-7
7-14
14-21
21-28
N
G1
400X
400X


(e)
120 days p.i.
Untreated
0-7
7-14
14-21
21-28
N
Actin
Fold change 1 1.22 1.39 1.31 1.23


(f)
120 days p.i.
Untreated
0-7
7-14
14-21
21-28
N
syncytia
400X
400X

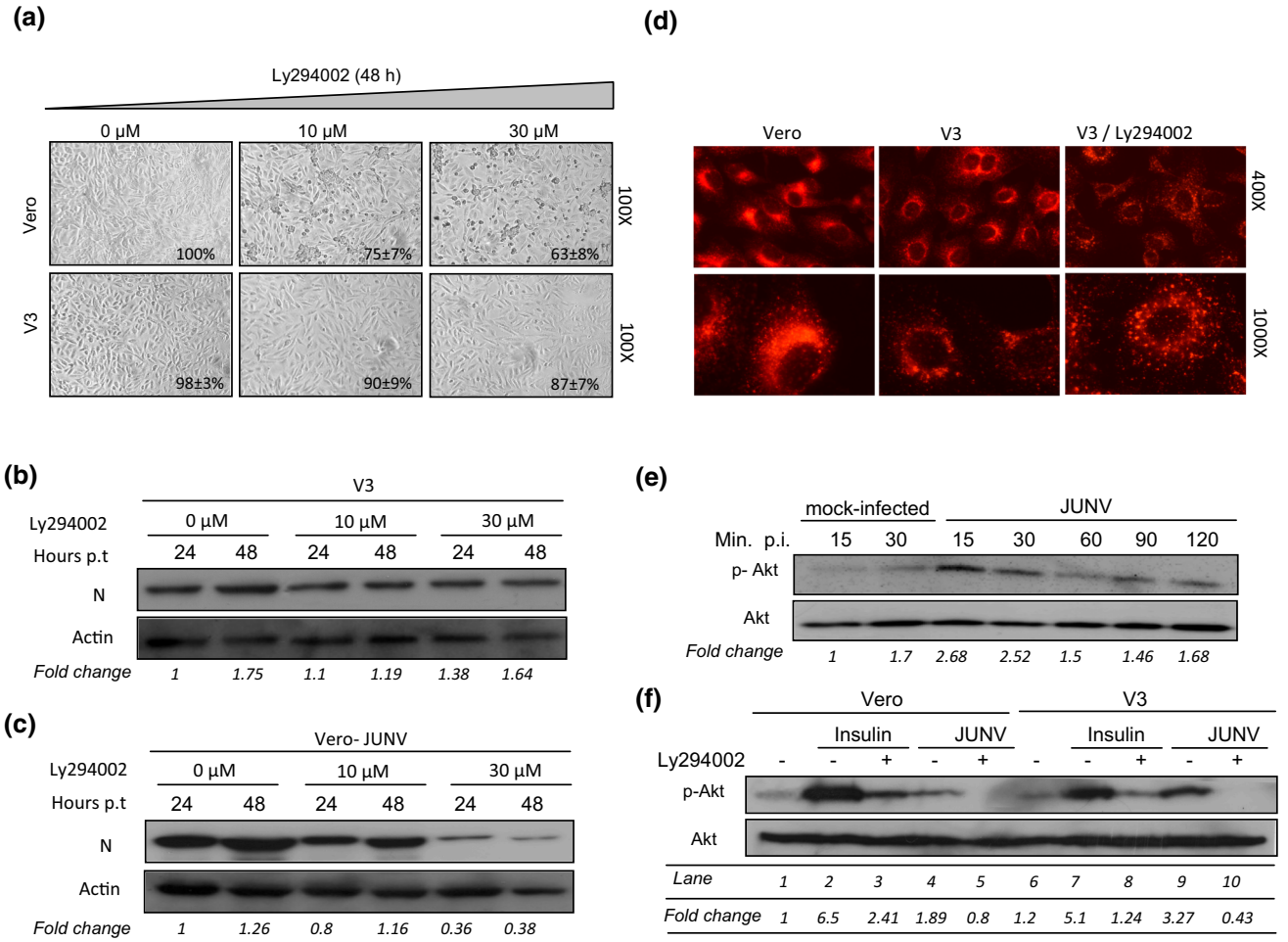


Fig. 3 Serum-starved Vero and V3 cells (150 days p.i.) were left untreated or treated with 10 or 30 µM Ly294002, and at 48 h post-treatment, viability was measured by MTT assay and cell morphology was evaluated by microscopy. Numbers in the pictures indicate the percentage of viability with respect to the control (a). Serum-starved V3 cells (b) and Vero cells infected with JUNV at an MOI of 1 pfu/cell (c) were left untreated or treated with 10 or 30 µM Ly294002. At 24 and 48 h post-treatment (p.t.), cells were harvested and N was detected by WB. Numbers under the panels indicate N expression, calculated as the ratio of N to actin, measured with image J software. Vero and V3 cells were pre-treated with 20 µM Ly294002 and then incubated with transferrin labeled with TRITC in the presence of the drug. Transferrin uptake was evaluated at 15 min by microscopy (d). Serum-starved V3 cells were left uninfected or superinfected with JUNV at an MOI of 1 pfu/cell and at different times p.i. p-Akt (Ser473) was detected by WB (e). Serum-starved Vero and V3 cells were infected with JUNV (MOI 1 pfu/cell) or incubated with insulin (100 nM) in the absence or presence of 20 µM Ly294002. At 15 min post-contact, p-Akt (Ser473) was detected by WB using antibodies from Cell Signalling Technology (f). N was detected using monoclonal antibody NA05AG12 [24]. Numbers under panels indicate the fold change of Akt phosphorylation, calculated as the ratio of p-Akt to Akt-total or of N to actin, measured with Image J software

infection. We found that modulation of this pathway would not influence the apoptotic response to infection, taking into account that the low level of apoptotic cells in JUNV-infected cultures was not increased by inhibition of this survival pathway. A similar situation has been described for SARS-CoV, where activation of Akt by this virus is not strong enough to prevent viral induction of apoptosis [8] but is strong enough to promote the establishment of a persistent infection [9]. In our case, inhibition of the PI3K/Akt pathway with Ly294002 was unable to impair the establishment of persistent infection by JUNV in Vero cells. Also, the PI3K/Akt pathway would not be crucial for the maintenance of JUNV persistence, because treatment of V3 cells with Ly294002 neither reduced cell viability nor impaired viral protein synthesis. In the case of measles virus, the downregulation of the PI3K/Akt pathway exerted by this virus has been suggested as a strategy to interfere with T-cell activation during immunosuppression [22]. Downregulation of Akt, either by reducing expression or inhibiting phosphorylation, was not observed during JUNV persistence. In fact, phosphorylation of Akt could be readily observed in V3 cells superinfected with JUNV or stimulated with insulin. Recent findings point out the participation of the Raf/MEK/ERK cellular survival pathway in JUNV replication during the acute stage of infection of Vero cells [23]. It is tempting to postulate a key role of this

pathway during persistence. Collectively, our results suggest that the PI3K/Akt signaling pathway would not play a critical role in the establishment/maintenance of JUNV persistence in Vero cells.

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