Mammalian orthoreovirus infection is enhanced in cells pre-treated with sodium arsenite

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Running Head: Sodium-arsenite pre-treatment enhances reovirus infectivity

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ABSTRACT

Following reovirus infection, cells activate stress responses that repress canonical cellular translation as a mechanism to limit production of progeny virions. This includes the formation of stress granules (SG) that sequester translationally-stalled cellular transcripts, translation initiation factors, ribosomal proteins, and RNA binding proteins until conditions improve and translation can resume. Work by others suggests that these cellular stress responses, which are part of the integrated stress response, may benefit rather than repress reovirus replication. In agreement with this, we report that stressing cells prior to infection with sodium arsenite (SA), a robust inducer of SG and activator of eIF2α kinases, enhanced viral protein expression, percent infectivity and viral titer in SA-treated cells compared to untreated cells. SA-mediated enhancement of reovirus replication was not strain-specific, but was cell-type specific. While pre-treatment of cells with SA offered the greatest enhancement, treatment of infected cultures as late as 4 h post infection resulted in an increase in the percent of cells infected. SA activates the HRI kinase, which phosphorylates eIF2α and subsequently induces SG formation. Other stresses, such as heat shock (HS) and osmotic shock also activate HRI. Heat shock of cells prior to reovirus infection readily induced SG in greater than 85% of cells. Although HS pre-treatment had no effect on the percentage of infected cells or viral yield, it did enhance viral protein expression. These data suggest that SA pre-treatment perturbs the cell in a way that is beneficial for reovirus and that neither HRI activation nor SG induction is sufficient for reovirus infection enhancement.

SIGNIFICANCE

All viruses rely on the host translational machinery for the synthesis of viral proteins. In response to viral infection, cells activate the integrated stress response resulting in the phosphorylation of eIF2α and translation shutoff. Despite this, reovirus replicates to reduced titers in the absence of this response. In this work, we report that sodium arsenite activation of the integrated stress response prior to virus inoculation enhances virus infectivity, protein expression and titer. Together, these data suggest that modulation of conserved cellular stress responses can alter reovirus replication.

INTRODUCTION

Acute viral infection induces stress within infected cells. The integrated stress response (ISR) is activated in many cells during viral infection [1-3]. The ISR facilitates cellular survival and a return to homeostasis, or initiates cell death signaling under conditions of severe stress or when the initiating stressor is maintained [4]. Four distinct stress kinases, can be activated in response to stress. Although, these kinases may have a number of substrates, they all phosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α) at serine position 51. When eIF2α is phosphorylated, eIF2B is unable to exchange GDP for GTP, preventing eIF2 interaction with the Met-tRNAi and translation initiation [4].

The ISR is activated in response to accumulation of phosphorylated eIF2α. The four cellular kinases that phosphorylate eIF2α are heme-regulated eIF2α kinase (HRI), general control non-depressible 2 (GCN2), double-stranded RNA-dependent protein kinase (PKR) and PKR-like ER kinase (PERK) [4]. Both cell intrinsic and extrinsic stresses can activate these kinases: (i) HRI - heme-deprivation; (ii) GCN2 - amino acid starvation; (iii) PKR - accumulation of double-stranded RNA (as can occur during viral infection) and (iv) PERK - accumulation of unfolded proteins in the endoplasmic reticulum (ER) [4]. However, redundancy does exist amongst the kinases. For example, GCN2 is activated in response to ER stress in cells lacking PERK, and all four kinases can be activated under oxidative stress [5-9].

When the alpha subunit of eIF2 is phosphorylated at position 51, the GDP-loaded eIF2 entraps the limiting amounts of eIF2B leading to a rapid decrease in concentration of functional eIF2.GTP.Met-tRNAi ternary complexes. This results in reduced translation

initiation and inhibition of de novo protein synthesis [4]. In response to the inhibition of translation initiation, stress granules (SG) form in the cytoplasm. SG assembly is mediated by the RNA binding proteins T-cell antigen 1 (TIA-1), TIA-1 related protein (TIAR) and the Ras-GAP SH3-binding protein 1 (G3BP1), and results in the compartmentalization of translationally-stalled mRNA transcripts, RNA-binding proteins, 40S ribosomes, and translation initiation factors [10, 11]. SG are considered to be sites of mRNA triage, protecting mRNA transcripts until a stress is alleviated and the cell returns to homeostasis.

Many viruses prevent activation of the integrated stress response to maintain protein translation and to ensure successful viral infection. To do this, viruses target the initiating kinases and/or the downstream effector mechanisms of the ISR. Cells in which the initiating kinases, such as PKR are inhibited or knocked out are often more permissive for viral replication [12, 13]. However, not all viruses benefit from inhibition of kinase activity and initiation of the ISR. A study examining the role of the PKR-eIF2 pathway during mammalian orthoreovirus (reovirus) infection found some strains had reduced titers in PKR knockout murine embryonic fibroblasts (MEF) [14]. Follow-up studies observed both increased ISR gene expression and reduced levels of the eIF2α kinase inhibitor $P58^{IPK}$ in cells infected with reovirus strains known to robustly interfere with host translation, and these strains replicated less efficiently in MEFs expressing a non-phosphorylatable form of eIF2α [15].

Reovirus infection also modulates stress granule formation that occurs downstream of ISR activation [15, 16]. Early in infection, entering viral core particles localize to stress granules that form within infected cells. However, within 4-6 h after

116 infection, the stress granules have disappeared and viral factories (VFs), the sites of
117 reovirus replication, transcription, translation and assembly, become prominent [16]. In
118 some reovirus-infected cells, the stress granule protein G3BP1 localizes to the margins of
119 the VFs, mediated by an interaction of G3BP1 with the non-structural viral protein σNS
120 [17]; σNS interacts with the nonstructural protein μNS that forms the matrix of VFs [18].
121 Co-expression of σNS and μNS is sufficient to alter the localization of G3BP1 and
122 suppress stress granule induction [17]. The interplay between eIF2α phosphorylation,
123 PKR activation, translational shutoff and G3BP1 induced SG formation is strain-
124 dependent, with SG formation negatively impacting some strains of reovirus [17].
125 Together, these studies suggest a unique role for the ISR during reovirus infection,
126 however the magnitude of this role remains to be elucidated. Most virological studies of
127 the ISR take two approaches: 1) understanding the impact of virus infection on stress
128 responses or 2) understanding how perturbing the ISR during infection affects the virus.
129 Given the previous observation that reovirus replicates to lower titers in cells with an
130 impaired ISR, we hypothesized that reovirus infection would be enhanced in cells in
131 which the ISR has been activated prior to infection. We found that reovirus infection was
132 more efficient (increased infectivity, protein expression, and replication) in cells in which
133 the ISR had been activated by pre-treatment with sodium arsenite prior to virus
134 adsorption. Sodium arsenite-induced enhancement of reovirus infection was observed in
135 all reovirus strains tested but was dependent on cell-type and the time of sodium-arsenite
136 addition. Enhancement of viral infectivity was only observed if sodium arsenite was
137 added to cells within 4 h of inoculation, with maximal enhancement if the addition
138 occurred prior to inoculation, suggesting a relationship between the ISR and early

139 replication events. Furthermore, not all activators of the ISR were equally beneficial as
140 heat shock prior to infection had no impact on viral replication. Taken together, these
141 data suggest a critical role for the ISR during reovirus infection and that activation of the
142 ISR prior to reovirus infection is beneficial in some cell-types.
143
144 **MATERIALS AND METHODS**
145 **Cells and reagents.** CV-1 (ATCC CCL-70) and HeLa cells were maintained in Eagles
146 minimum essential medium (MEM) (CellGro) containing 10% fetal bovine serum (FBS;
147 Hyclone), 100 mM sodium pyruvate (CellGro), and 200 mM L-glutamine (CellGro) at
148 37°C in the presence of 5% CO_2. L929 cells were maintained in MEM containing 8%
149 FBS and 200 mM L-glutamine at 37°C in the presence of 5% CO_2. Human Pancreatic
150 Ductal Epithelial (HPDE) cells (Kerafast H6c7) were maintained in keratinocyte SFM
151 (serum-free medium) supplemented with 25 mg bovine pituitary extract and 2.5 μg
152 human recombinant epidermal growth factor, both provided (Invitrogen) at 37°C in the
153 presence of 5% CO_2. Sodium arsenite was kindly donated by Dr. Shu-bing Qian (Cornell
154 University) and was used at a final concentration of 0.5 mM in all experiments.
155
156 **Viruses.** Reoviruses T1L and T3D laboratory stocks originated from the
157 T1/human/Ohio/Lang/1952 and T3/human/Ohio/Dearing/1955 isolates respectively [19].
158 The superscript $T3D^N$ differentiates a clone obtained from M.L. Nibert (Harvard Medical
159 School) from $T3D^C$ a clone obtained from L.W. Cashdollar (Medical College of
160 Wisconsin). The two clones have been shown to differ in M1 gene sequence and viral
161 factory morphology. All infections were performed with $T3D^N$ (abbreviated T3D). The

prototype reovirus serotype 3 strain Abney (T3A) was a kind gift from Dr. Barbary Sherry (North Carolina State University) and was plaque-purified and passaged twice on L-cell monolayers to generate working stocks.

Infections. CV-1, L929, HeLa or HPDE cells were seeded in 24-well culture plates containing 12 mm glass coverslips or 12-well culture plates the day before to give rise to 50 to 80% confluence prior to infection. Cells were infected with virus at the indicated MOI for 1 h at room temperature (RT) in phosphate-buffered saline (PBS; pH 7.4), supplemented with 2 mM $MgCl_2$, with rocking every 10 min. Following absorption, virus was removed and cells were incubated in growth medium at 37°C and harvested at the indicated time points.

Antibodies. The following commercial primary antibodies were used for immunoblotting: mouse monoclonal anti-G3BP1 (2F3) antibody (H00010146-M01; Novus Biologicals), mouse monoclonal anti-TIAR antibody (sc-398372; Santa Cruz Biotechnology), and mouse monoclonal anti-alpha-tubulin antibody (NB100-690; Novus Biologicals). Reovirus protein expression was assessed using a chicken polyclonal antiserum against μNS prepared against bacterially-expressed purified antigen by Covance. Secondary antibodies used for immunoblotting were as follows: HRP Donkey Anti-Mouse IgG (715-035-150; Jackson ImmunoResearch), and HRP Donkey Anti-Chicken IgY (703-035-155; Jackson ImmunoResearch). Primary antibodies used for immunofluorescence included: mouse monoclonal anti-G3BP1 (611126; BD Biosciences), rabbit monoclonal anti-TIAR (8509S; Cell Signaling Technology), and

chicken polyclonal antiserum against μNS to detect viral factories. Secondary Antibodies used for immunofluorescence assays included Alexa Fluor 594 goat anti-chicken IgG, Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse, and Alexa Fluor 488 goat anti-rabbit (Thermofisher).

Stress granule induction. Stress granules were induced in cells using the following mechanisms: 1) treatment with 0.5 mM sodium arsenite in normal growth media at 37°C for 30 min prior to infection or 30 min prior to harvesting or 2) heat shock in normal growth media in a pre-heated incubator at 44 ℃ for 45 min prior to infection or harvesting. Cells were manually determined to contain stress granules if immunofluorescence revealed a minimum of three granules co-staining for both TIAR and G3BP.

Immunofluorescence. Cells were washed once with PBS supplemented with 2 mM $MgCl_2$ and fixed at room temperature for 10 min with 4% paraformaldehyde in PBS. Fixed cells were washed three times with PBS, permeabilized in 0.1% Triton X-100 in PBS for 15 min and washed three times with PBS. Cells were blocked for 15 min in staining buffer (SB; 0.05% saponin, 10mM glycine, 5% FBS, and PBS) and incubated with primary antibodies diluted in SB for 1 h. Cells were then washed one time with PBS before incubation with secondary antibodies diluted in SB for 1 h. Coverslips were mounted onto glass slides with ProLong Gold Anti-Fade reagent with DAPI (4[prime],6-diamidino-2-phenylindole; ThermoFisher). Images were obtained using an Olympus BX60 inverted microscope equipped with phase and fluorescence optics. Images were

collected digitally with an Olympus DP74 color CMOS camera and cellSens Standard Software (Olympus) and were processed and prepared for presentation using photoshop (CC; Adobe) software.

Immunoblot Assay. Cells were lysed in PBS containing 0.5% NP40, 140 mM NaCl, 30 mM Tris-HCl (pH 7.4), and an EDTA-free protease inhibitor cocktail (04693159001; Sigma Aldrich) for 30 min on ice. Cell lysates were resolved by SDS-PAGE and proteins were detected using antibodies as described above. Images were collected using a C-Digit digital scanner and Image Studio Digits software (Version 4, LiCor). When appropriate, immunoblots were incubated in stripping buffer (200 mM glycine, 3.5 mM SDS, 1% Tween 20, [pH 2.2]) and re-probed.

Plaque Assay. Cells were infected as above before washing with PBS supplemented with 2 mM $MgCl_2$ and incubated in growth medium at 37°C for the indicated times. Cells were subjected to two freeze/thaw cycles prior to the determination of viral titer by the standard reovirus plaque assay in L929 cells [20]. To determine the viral titer in PFU/ml, the following equation was used: PFU/ml = number of plaques / (D × V) where D = dilution factor and V = volume of diluted virus / well.

RESULTS

Infection with reovirus induces stress granule formation. Previous reports have suggested that stress granules form following infection with reovirus, but reports are conflicting as to the timing of stress granule presence in infected cells [15, 16] . To

231 evaluate this further, we first confirmed that stress granules could form in CV-1 cells by
232 treating uninfected cells with 0.5 mM sodium arsenite for 30 min. We then fixed and co-
233 immunostained for TIAR and G3BP, two RNA binding proteins required for stress
234 granule formation. We saw stress granules in ~91% of treated CV-1 cells, confirming that
235 these cells can form stress granules (Figure 1A, upper panels). We next infected CV-1
236 cells with reovirus at a multiplicity of infection (MOI) of 10 and fixed and
237 immunostained cells at the indicated times post-infection (p.i.) for the presence of stress
238 granules (TIAR), and viral factories (μNS) (Figure 1A, lower panels). Stress granules
239 were absent in mock-infected cells and began appearing in infected cells around 2 h p.i.
240 We found that stress granule formation in reovirus-infected CV-1 cells peaked around 6 h
241 p.i. with 6.6% of cells containing stress granules. By 8 h p.i. the percentage of infected
242 cells containing stress granules had dropped to 3.1% and by 18 h p.i., the level was no
243 different from mock-infected cells (Figures 1A-B). A previous report showed that stress
244 granules were present in reovirus-infected human prostate carcinoma DU145 cells at 19.5
245 h post infection [15]. Although we did not see stress granules in CV-1 cells at late times
246 post-infection with reovirus at an MOI = 1, we found that G3BP and TIAR co-localized
247 at the periphery of viral factories at 24 h p.i. when cells were treated with 0.5 mM sodium
248 arsenite for 30 min immediately prior to harvest (Figure 1C). We occasionally saw a
249 similar phenotype at 24 h p.i. in infected cells not treated with sodium arsenite (Figure
250 1C). These findings are consistent with those of Choudhury et al. who found that G3BP
251 localizes to the viral factory periphery during reovirus infection [17]. While reovirus
252 appears to modulate the localization of stress granule proteins at late times post infection,
253 we observed only a modest increase in the expression level of stress granule protein

expression in infected cells as compared to mock that was independent of MOI (Figure 1D). Together, these data suggest that reovirus infection induces stress granules at early, but not late times post-infection in CV-1 cells.

Pre-treatment of cells with 0.5 mM sodium arsenite enhances reovirus infectivity. Following infection with reovirus, the viral protein μNS orchestrates the formation of viral factories, the sites of virus replication, assembly, and translation [21-23]. To facilitate translation, cellular factors including eIF3, eIF4G and ribosomal subunits are recruited to the viral factory [23]. Many of these initiation factors are similarly compartmentalized within stress granules [24]. To date, most studies have focused on the induction of stress granules in response to viral infection, or viral suppression of stress granule formation during an infection. To our knowledge, no studies have been performed to assess the impact of the presence of stress granules on reovirus infection. To explore this, we first examined the effect of stress granule presence on viral protein expression. CV-1 cells were either left untreated (-), or were treated with 0.5 mM sodium arsenite for 30 min before infection at an MOI = 1 (pre) or 30 min before harvest (post). Cell lysates were collected at 0, 6, 10, 18 and 24 h p.i. and the expression level of the non-structural viral protein μNS was determined (μNS was first detectable by 10 h p.i.). At 10, 18 and 24 h p.i., the expression levels of μNS were consistently higher in cells pre-treated with sodium arsenite when compared to untreated cells or cells treated with sodium arsenite 30 min before harvest (Figure 2A).

As the viral protein μNS is necessary for formation of viral factories during reovirus infection, we next explored if the elevated μNS expression was a consequence of

increased size of viral factories or increased numbers of infected cells [25]. We found that in cells pre-treated with 0.5 mM sodium arsenite for 30 min prior to reovirus infection, 67% of cells were infected (contained viral factories) at 18 h p.i., whereas only 33% of untreated cells were infected at 18 h p.i. (Figures 2B and C). We found similar findings at 24 h p.i. (Figure 2C).

Given the increased percentage of infected cells in dishes pre-treated with sodium arsenite, we tested if pre-treatment of cells with 0.5 mM sodium arsenite for 30 min before reovirus infection enhanced viral yield. We found that pre-treatment with sodium arsenite led to a modest but significant 2-3 fold increase in viral titer (PFU/ml) that was independent of multiplicity of infection, but consistent with the increased numbers of infected cells and the increased protein expression (Figure 2D). Together, these data indicate that pre-treatment of cells with sodium arsenite enhances reovirus infectivity by increasing the numbers of virus-permissive cells.

SA-induced enhancement of reovirus infection is cell-type specific. Previous reports have suggested that reovirus-induced stress granule formation is cell-type specific [15, 16]. Therefore, we next sought to determine if the replication enhancement observed in CV-1 cells was seen in other cell types. We assessed the effect of pre-treatment with 0.5 mM sodium arsenite on CV-1, HeLa, L929 murine fibroblasts, and human pancreatic ductal epithelial (HPDE) cells on viral infectivity. In each cell line, a relative multiplicity of infection was chosen such that 20-50% of cells were infected. Consistent with our observations in CV-1 cells, pre-treatment with sodium arsenite in L929 and HPDE cells resulted in a higher percentage of cells exhibiting viral factories than untreated cells

(Figure 3A). However, sodium arsenite pre-treatment did not increase the number of infected HeLa cells (Figure 3A). These data indicate that activation of the stress response pathways prior to infection through pre-treatment with sodium arsenite is beneficial in some, but not all, cell types.

SA-induced enhancement of reovirus infection is strain-independent. The prototypic reovirus strains Type 1 Lang (T1L), Type 2 Jones (T2J), Type 3 Abney (T3A), and Type 3 Dearing (T3D) differ in their capacity to induce host translational shutoff and to induce eIF2α phosphorylation [15, 26]. To determine if the benefit of pre-treatment with sodium arsenite was reovirus strain specific, we infected CV-1 cells with T3D, T1L or T3A and assessed the percentage of infected cells at 18 h p.i. compared to untreated cells. As observed following infection with the T3D strain, pre-treatment with sodium arsenite resulted in nearly a 2-fold increase in the percentage of T1L- and T3A-infected cells (Figure 3B). Sodium arsenite pre-treatment in L929 cells infected with T3D, T1L or T3A yielded similar results, however sodium arsenite pre-treatment had no effect on any strain tested in HeLa cells (Supplemental Figure 1). These data suggest that reovirus strains T3D, T3A and T1L benefit from pre-treatment with sodium arsenite prior to infection in some cell types.

Heat shock prior to infection enhances viral protein expression in T3D-infected L-cells, but does not affect the percentage of infected cells or viral yield. Of the four stress-responsive kinases, sodium arsenite treatment activates HRI kinase. HRI is activated in erythrocytes by reduced heme availability, but it is ubiquitously expressed in

many tissues and, when activated by sodium arsenite, phosphorylates eIF2α and subsequently induces stress granule formation [27]. HRI is the only stress kinase required for translational inhibition in response to arsenite treatment in mouse embryonic fibroblasts [27]. HRI kinase can also be activated by other cellular stresses including osmotic stress and heat shock. To further assess if the enhanced reovirus infectivity following sodium arsenite treatment was a consequence of HRI activation, we assessed viral protein expression, the percentage of infected cells and viral yield in response to heat shock. We used stress granule induction in response to heat shock as an indirect measure of HRI activation (Figure 4A). Our attempts to induce stress granules in CV-1 cells with heat shock were only able to achieve no greater than 25% of cells containing stress granules. Because of this, we assessed the effects of heat shock induction of stress granules in L929 cells. Heat shock for 45 minutes at 44°C resulted in ~88% of L cells containing stress granules (Figure 4A). Similar to our observations in CV-1 cells pre-treated with sodium arsenite, viral protein expression was enhanced at 10 and 18 h p.i. in L cells exposed to a 45 min heat shock prior to infection (Figure 4B). However, heat shock activation had no impact on the percentage of infected cells or viral yield in L cells (Figures 4C-E). These findings suggest that in L-cells, heat shock enhances protein expression, but does not affect either the numbers of infected cells or the per cell yield of virus. Although heat shock activates HRI kinase, its effect on cells differs from sodium arsenite. Together, these data indicate that sodium arsenite pre-treatment perturbs the cell in a way that is beneficial for reovirus compared to other activators of the HRI kinase.

Addition of sodium arsenite prior to 4 hours enhances reovirus permissivity. Activation of the HRI kinase and eIF2α phosphorylation suppress general host translation [27]. We found that the enhancement of viral protein synthesis in cells pre-treated with sodium arsenite treatment was not evident if cells were treated immediately prior to harvest at 10, 18, or 24 h p.i. (Figure 2A). Given that viral protein expression, but not the percentage of infected cells or viral yield, was enhanced by activation of the HRI kinase using heat shock in L cells, we hypothesized that sodium arsenite treatment might enhance viral translation at early times post infection. To test this, CV-1 cells were either left untreated or were treated with 0.5 mM sodium arsenite at -0.5, 0, 1, 2, 4, 6, 8, or 10 h p.i. We then assessed the percentage of infected cells at 18 h p.i.. Consistent with our previous data, pre-treatment (-0.5 h p.i.) with sodium arsenite prior to inoculation with virus resulted in an increase in the percentage of infected cells (Figures 5A-B). Although pre-treatment had the greatest impact on reovirus permissivity, treatment with sodium arsenite at 0, 1, 2 and 4 h p.i. resulted in an increased percentage of infected cells as compared to untreated reovirus-infected cells (Figures 5A-B). Addition of sodium arsenite at 6 h p.i. or later had no effect on the percentage of infected cells at 18 h p.i. (Figures 5A-B). These data suggest that sodium arsenite is most beneficial when added early during the viral life cycle.

Preventing eIF2α de-phosphorylation through the use of salubrinal has no effect on reovirus replication. Growth arrest and DNA damage protein-34 (GADD34) is expressed in response to phosphorylation of eIF2α as part of the ISR and is upregulated in cells following infection with reovirus strains that induce host shut-off [15]. GADD34

368 is a protein phosphatase-interacting protein that, in conjunction with protein phosphatase
369 1 (PP1), acts to dephosphorylate eIF2α at serine 51 during times of cellular stress [28].
370 Our findings thus far are consistent with previous reports suggesting that eIF2α kinase
371 activation is beneficial to reovirus infection. We, therefore, hypothesized that reovirus
372 may be capable of viral protein synthesis in the face of enhanced eIF2α phosphorylation.
373 We reasoned that inhibition of GADD34/PPI dephosphorylation would enhance
374 phosphorylation of eIF2α, but should have no effect on viral protein synthesis. We used a
375 selective inhibitor of GADD34, salubrinal, which prevents the activity of the
376 GADD34/PPI complex without affecting the kinases that phosphorylate eIF2α [29].
377 Immediately following adsorption of reovirus, CV-1 cells were treated with increasing
378 amounts of salubrinal and cells were harvested at 18 h p.i. to determine viral protein
379 expression (Figure 6A). Even at high concentrations, salubrinal had no impact on viral
380 protein expression, suggesting that reovirus tolerates the cellular antiviral activity of
381 eIF2α phosphorylation. Given that sodium arsenite is a known inducer of eIF2α
382 phosphorylation, we also tested the impact of adding salubrinal following reovirus
383 adsorption to sodium-arsenite pre-treated cells (Figure 6A). Again, we observed no
384 negative impact on viral protein expression when cells were pre-treated with sodium
385 arsenite prior to infection and then incubated in the presence of salubrinal for the duration
386 of infection (Figure 6A). Consistent with this, the percentage of virus infected cells was
387 unaffected by the addition of 50 mM salubrinal either in cells left untreated or cells pre-
388 treated with 0.5 mM sodium arsenite (Figures 6B-C). Together, these data suggest that
389 sustained eIF2α-phosphorylation resulting from salubrinal treatment during reovirus
390 infection is not anti-viral.

DISCUSSION

In this report, we find that treatment of cells with sodium arsenite (SA), a potent inducer of the integrated stress response (ISR) and eIF2α phosphorylation, prior to inoculation with reovirus is beneficial to the virus in a strain-independent but cell-type-specific manner. SA treatment induces the formation of reactive oxygen species (ROS) within cells. The increased intracellular ROS in turn lead to activation of HRI, which then phosphorylates eIF2α at serine residues 48 and 51 leading to general translation repression and downstream activation of the integrated stress response. In order to mature and become activated, HRI has to be in a complex with CDC37, PPP5C, and HSP90. In addition to being activated by heme-deficiency, HRI is also activated by heat shock and osmotic shock, but not by ER stress or by amino acid or serum starvation [7]. In contrast, to the enhanced viral replication we saw following pre-treatment with SA, heat shock (HS) pre-treatment did not enhance viral replication. Heat shock also activates HRI, but to a qualitatively lower level than SA and with a different pattern of autophosphorylation of HRI [7]**.** Why pre-stressing cells with SA, but not with heat shock, benefited viral replication is unclear. It is possible that the kinetics, magnitude and timing of eIF2α phosphorylation following these different stressors varied leading to the different outcomes we saw. Alternatively, we cannot rule out the possibility that SA treatment activates responses not mediated by HRI that benefit viral replication.

In addition to inducing eIF2α phosphorylation, SA induces the formation of stress granules (SG). Consistent with the findings of Qin et al, we found that reovirus infection induced SG formation early after infection with the number of SG reducing by 8 h and disappearing late in infection. Our data in CV-1 cells are not consistent with the report by

Smith et al, who detected SGs at 19.5 h post-infection of DU145 cells [15, 16]. While it is likely that different strains of reovirus induce SG to varying extents, and that this is influenced by cell type and MOI, our data using the type 3 Dearing strain suggests that infection with this strain of reovirus results in a low level of SG induction that is absent at late times post infection. The timing of SG appearance (peak at 6 h) and dissolution (beginning at 8 h) in our hands supports the idea put forth by Qin et al that SG induction is linked to VF formation and that the onset of viral translation interferes with SG stability [16]. Smith et al stained reovirus-infected cells at 19.5 h post-infection for the SG protein, TIAR, but did not co-stain for a viral protein, such as μNS, to detect viral factories. We and others have observed that the SG protein G3BP1 localizes to the outer margins of viral factories at late times post infection in a fraction of reovirus infected cells [17]. Therefore, the TIAR-positive punctae seen at 19.5 h post-infection by Smith may in fact have been viral factories that were co-staining for the presence of TIAR [15]. The significance of SG protein relocalization to VFs remains to be determined. Still, these data imply that reovirus has evolved mechanisms to counter the cellular antiviral activity of translation suppression through stress granule induction.

Pre-treatment of cells with SA led to enhanced viral protein expression, increased infectivity, and higher viral titers. Given that reovirus compartmentalizes the translational machinery in VF and that components of the translational machinery are also sequestered within SGs, we previously speculated that SGs may serve as a reservoir of translational machinery for reovirus VFs [23]. However, comparison of reovirus infection following pre-treatment with either SA or heat shock (HS) suggests that the benefit is independent of SG formation, as both treatments resulted in a robust induction of SGs, but only SA

437 pre-treatment led to an increase in viral replication. As we only examined viral
438 replication late during infection, it is possible that we missed early differences.
439 Additionally, since we were unable to achieve robust SG formation following HS in CV-
440 1 cells, viral replication studies were performed in L cells. Therefore, we can't rule out
441 cell-specific influences.

442 Our data is consistent with the findings of Smith et al that reovirus benefits from
443 activation of the ISR. In that study, the authors found that reovirus replicated to lower
444 titers in cells lacking a phosphorylatable eIF2α [15]. SA is a potent activator of the ISR.
445 SA-mediated activation of the HRI kinase leads to phosphorylation of eIF2α and
446 downstream upregulation of ATF4 and other transcriptional mediators of the ISR [27].
447 Given that canonical cap-dependent cellular translation relies on the availability of active
448 GTP.eIF2, it is possible that activation of the ISR allows reovirus mRNAs a competitive
449 advantage for limited translational machinery. The effect of SA was greatest when added
450 before reovirus adsorption but was still beneficial if treatment occurred within the first 4
451 h of infection. Translation of reovirus mRNAs rises sharply at ~ 6 h p.i. and peaks at ~12
452 h p.i. [30]. Given that the rising level of viral protein synthesis is concomitant with
453 continued host protein synthesis, it is possible that SA treatment selectively reduces the
454 translational machinery available for host protein synthesis [30]. It has been postulated
455 that heightened eIF2α-phosphorylation may more strongly affect mRNAs containing
456 long or highly structured 5′ untranslated regions (UTRs) [31, 32]. Reovirus mRNAs have
457 short 5′and 3′ UTRs, possibly providing protection to viral mRNAs during translation
458 [33, 34] . Our findings that reovirus protein expression and infectivity were unaffected

459 when we treated cells with salubrinal to prevent de-phosphorylation of eIF2α further
460 supports this and suggests that reovirus is refractory to salubrinal exposure.

461 It is known that under times of stress and reduced availability of active eIF2, cells
462 can utilize other initiation factors to ensure translation of select mRNAs. These factors,
463 including eIF2A, eIF2D, ligatin, MCT-1/DENR, and N-terminally truncated $eIF5B_{479-1220}$,
464 can promote efficient recruitment of $Met\text{-}tRNA^{Met}i$ to the 40S/mRNA complex under
465 varying circumstances [35-39]. Several viruses take advantage of these alternative
466 initiation pathways including Sindbis virus and poliovirus [36, 39]. We have noted that
467 two of these factors, eIF2A and eIF5B localize to viral factories within infected cells
468 (unpublished observations, JSLP). As yet, we do not know if these factors play a
469 functional role in translation of viral mRNAs within VFs or if they are being sequestered
470 to prevent cellular mRNA translation.

471 We cannot exclude the possibility that pre-treatment of cells with SA has other
472 effects that promote viral replication independently of eIF2α phosphorylation. Reovirus
473 is an oncolytic virus, preferentially infecting cancer cells. This phenomenon has been
474 linked to a mutationally-active Ras pathway, however reports have been conflicting
475 regarding Ras dependency [40-43]. Cancer cells have elevated levels of chaperones that
476 facilitate the high levels of protein synthesis typical of transformed cells. The efficiency
477 of mRNA translation is increased in the presence of supplemental recombinant Hsc70
478 and the rate of translation elongation may be regulated by chaperone availability [44].
479 Treatment with SA also increases Hsc70/Hsp70 chaperone levels in cells [45].
480 Furthermore, Hsc70 is required for HRI activation and blockade of Hsc70 disrupts HRI
481 activation [7]. We have previously reported that Hsc70 is specifically targeted to the viral

482 factory, although why this protein is specifically recruited remains unclear as its
483 recruitment is independent of its chaperone function [46]. It is conceivable that SA pre-
484 treatment could alter the availability of Hsc70 or other protein folding chaperones in a
485 way that is favorable during reovirus replication.

486 Infection with reovirus results in increased expression of stress response genes
487 including Hsc70, and GADD34, the latter of which complexes with protein phosphatase 1
488 to reverse eIF2α phosphorylation. We observed that Type 3 Dearing (T3D) reovirus
489 protein expression was unaffected in the presence of salubrinal, a selective inhibitor of
490 the GADD34/PP1α complex responsible for dephosphorylating eIF2α, even in cells pre-
491 treated with SA to elevate the level of phosphorylated eIF2α. The observation that
492 salubrinal was not anti-viral to T3D reovirus is not unique. Rotavirus, a fellow member of
493 the Reoviride family, as well as other viruses including hepatitis C virus and mouse
494 hepatitis coronavirus, demonstrate continued viral protein synthesis despite heightened
495 eIF2α-phosphorylation [47-49]. While this provides additional evidence that reovirus
496 benefits from the ISR, it is important to note that reovirus-induced expression of stress
497 response genes is not uniform and that host shutoff strains, including C8 and C87 (Type 3
498 Abney, T3A), induce increased expression of these genes compared to T3D, which is not
499 considered a host shutoff strain. While all reovirus strains tested, including T3A,
500 benefited from SA-pre-treatment, host shutoff strains may respond differently from T3D
501 to salubrinal treatment [15].

502 In addition to strain-specific differences in the ability to induce host shutoff, cell
503 differences have also been recorded. For instance, reovirus-induced host shutoff was not
504 observed in HeLa cells but was in L cells [30]. This may be linked to our observation that

505 T3D infection in HeLa cells is unaffected by SA pre-treatment, whereas SA pre-treatment
506 is beneficial in other cell types including L cells, CV-1 and HPDE cells.

507 Overall, this study finds that sodium arsenite-induced activation of the ISR prior
508 to reovirus inoculation results in enhanced viral protein expression, increased infectivity
509 and higher viral yield. Furthermore, HRI kinase activation of the ISR is insufficient for
510 replication enhancement as both heat shock and sodium arsenite activate the HRI kinase,
511 but only sodium arsenite treatment was associated with increased viral infectivity and
512 higher yield. Finally, sodium arsenite-induced enhancement was observed across reovirus
513 strains but was not observed in all cell types suggesting a role for cell-type-specific
514 influences. Understanding the interplay between reovirus and the ISR, and how to
515 modulate it to enhance virus replication, could reveal novel targets to strengthen the
516 oncolytic potential of reovirus.
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718 **FIGURE LEGENDS**

719 **Figure 1. Reovirus infection induces the formation of stress granules at early times**
720 **post infection.** (A) CV-1 cells were mock infected, pre-treated with 0.5 mM sodium
721 arsenite (SA) for 30 min, or infected with T3D at an MOI = 10. At the indicated times,
722 cells were fixed and co-immunostained for G3BP (red) and TIAR (green), upper panels,
723 to detect stress granules (SG) in uninfected cells or TIAR (green) and μNS (red), lower
724 panels, to detect SG in T3D-infected cells. Cell nuclei were stained with DAPI (blue).
725 Mock and SA-treated cells were fixed at 0 h. (B) The percent cells containing SG from
726 panel A was quantified [(# of cells containing SG / total # of cells) × 100] from a
727 minimum of three independent experiments. ** $P < 0.01$, **** $P < 0.0001$; two-tailed
728 unpaired t test. (C) CV-1 cells were infected with T3D at an MOI = 0.5 and at 8 and 24 h
729 p.i. cells were fixed and co-immunostained with G3BP (green) and μNS (red), upper
730 panels. Alternatively, CV-1 cells were infected with T3D at an MOI = 1 and at 23.5 h
731 cells were post-treated with 0.5 mM SA for 30 min. At 24 h p.i. cells were fixed and co-
732 immunostained with G3BP (green) and μNS (red), lower panels. (D) Mock infected (-)
733 or T3D infected cells were lysed at 2 or 18 h p.i and the expression level of the indicated
734 proteins was determined by immunoblotting.

735

736 **Figure 2**. **Pre-treatment with 0.5 mM sodium arsenite enhances reovirus infectivity**.
737 (A) CV-1 cells were mock infected or infected with T3D at an MOI = 1. Cells were left
738 untreated (-), treated with 0.5 mM SA for 30 min prior to infection (pre) or were treated
739 with 0.5 mM SA for 30 min immediately before lysis (post). At the indicated time points,
740 cells were harvested and protein expression was determined by immunoblot. (B) CV-1

cells were infected with T3D at an MOI = 1. Cells were left untreated or treated with 0.5 mM SA for 30 min prior to infection (pre). At 18 h p.i., cells were fixed and immunostained with for μNS (red) and DAPI (nuclei, blue). (C) The percent cells containing viral factories (VF) as represented in panel B was quantified [(# of cells containing VF / total # of cells) × 100] from three independent experiments. (D) CV-1 cells were left untreated or were pre-treated with 0.5 mM SA for 30 min prior to infection with T3D at the indicated an MOI. At 18 h p.i., cells were subjected to the standard MRV plaque assay in L929 cells and plaques were counted from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$; two-tailed unpaired t test.

Figure 3. Pre-treatment with 0.5 mM sodium arsenite enhances permissivity in a cell-type-specific manner across reovirus strains. (A) CV-1, HeLa, L929 or HPDE cells were left untreated (No SA) or were treated with 0.5 mM SA for 30 min prior to infection (Pre-SA). Following this, cells were infected with T3D at an MOI = 1. At 18 h p.i., cells were fixed and immunostained for μNS (red) and DAPI (nuclei, blue). The percent of cells containing viral factories (VF) was quantified [(# of cells containing VF / total # of cells) × 100] from three independent experiments. (B) CV-1 cells were left untreated (No SA) or were treated with 0.5 mM SA prior to infection (Pre-SA). Cells were then infected with reovirus strains T3D, T1L, or T3A such that ~20% of cells were infected. At 18 h p.i., cells were fixed and immunostained for μNS (red) and DAPI (nuclei, blue). The percent of cells containing VF was quantified [(# of cells containing VF / total # of cells) × 100] from three independent experiments. * $P < 0.05$; ** $P < 0.01$; two-tailed unpaired t test.

Figure 4. Heat shock enhances reovirus-protein expression, but not infectivity, in L929 cells. (A) L929 cells were left untreated (unt), were pre-treated with 0.5 mM SA (SA) or were heat shocked at 44°C for 45 min (HS) before fixing and immunostaining for G3BP (green) to detect stress granules (SG). The percentage of SG induced was quantified [(# of cells containing SG / total # of cells) × 100] from three independent experiments. (B) L929 cells were left untreated or were heat shocked as above immediately prior to infection with T3D at an MOI = 1. At the indicated time points, cells were lysed and the expression levels of the indicated proteins was determined by immunoblot. (C) L929 cells were left untreated, treated with 0.5 mM SA for 30 min or were heat shocked as above. Following this, cells were infected with T3D at an MOI = 1. At 18 h p.i, cells were fixed and immunostained for μNS (red) and DAPI (nuclei, blue). (D) The percent cells containing viral factories (VF) as represented in panel C was quantified [(# of cells containing VF / total # of cells) × 100] from at least three independent experiments. (E) L929 cells were left untreated or were heat shocked as above prior to infecting with T3D at an MOI = 1. At 18 h p.i., cells were subjected to the standard MRV plaque assay in L929 cells and plaques were counted from at least three independent experiments. *** $P < 0.001$; two-tailed unpaired t test.

Figure 5. Addition of sodium arsenite prior to 4 h p.i. enhances reovirus permissivity. CV-1 cells were left untreated (Unt), were pre-treated with 0.5 mM SA for 30 min prior to virus inoculation (pre-SA) or were treated with 0.5 mM SA for 30 min at the indicated times post infection. At 18 h p.i., cells were fixed and immunostained for

μNS (red) and DAPI (nuclei, blue). (B) Representative images from each of the conditions above. The percent cells containing viral factories (VF) was quantified [(# of cells containing VF / total # of cells) × 100] from four independent experiments. ** $P < 0.01$; *** $P < 0.001$; n.s. denotes not significant; two-tailed unpaired t test.

Figure 6. Salubrinal treatment does not negatively impact reovirus infection. (A) CV-1 cells were left untreated (-), were pre-treated with 0.5 mM SA for 30 min (SA) or were infected with T3D at an MOI = 10 in the presence or absence of SA pre-treatment. Immediately following infection, salubrinal (Sal) was added to T3D-infected cells at the indicated concentrations. At 18 h p.i., cells were lysed and the expression levels of the indicated proteins were determined by immunoblot. (B) CV-1 cells were left untreated or treated with 0.5 mM SA for 30 min prior to infection with T3D at an MOI = 1. Following infection, 50 mM salubrinal was added where indicated. At 18 h p.i., cells were fixed and immunostained for μNS (red) and DAPI (nuclei, blue). (C) The percent cells containing viral factories (VF) as represented in panel B was quantified [(# of cells containing VF / total # of cells) × 100] from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$; two-tailed unpaired t test.

FIGURE 1

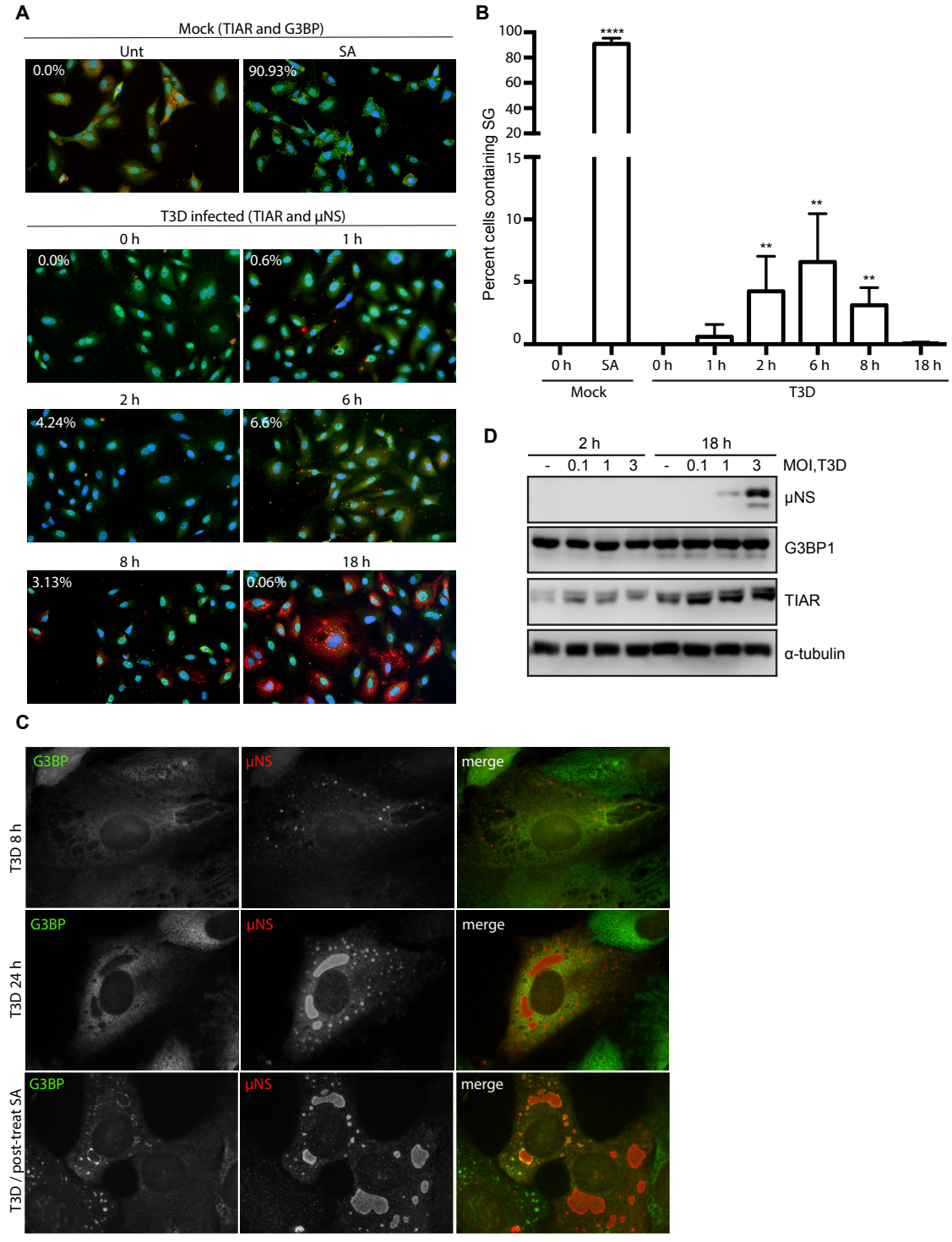

FIGURE 2

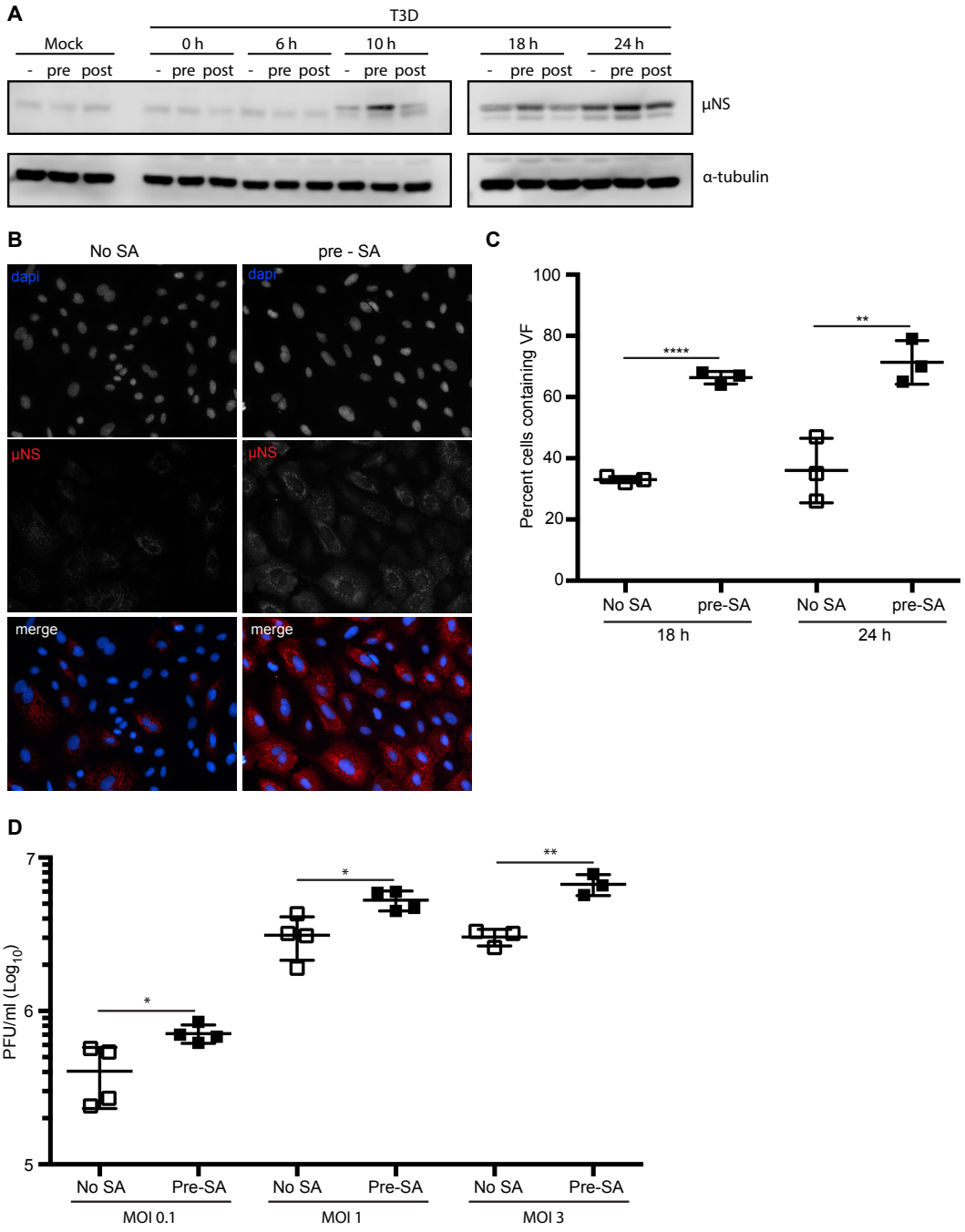

FIGURE 3

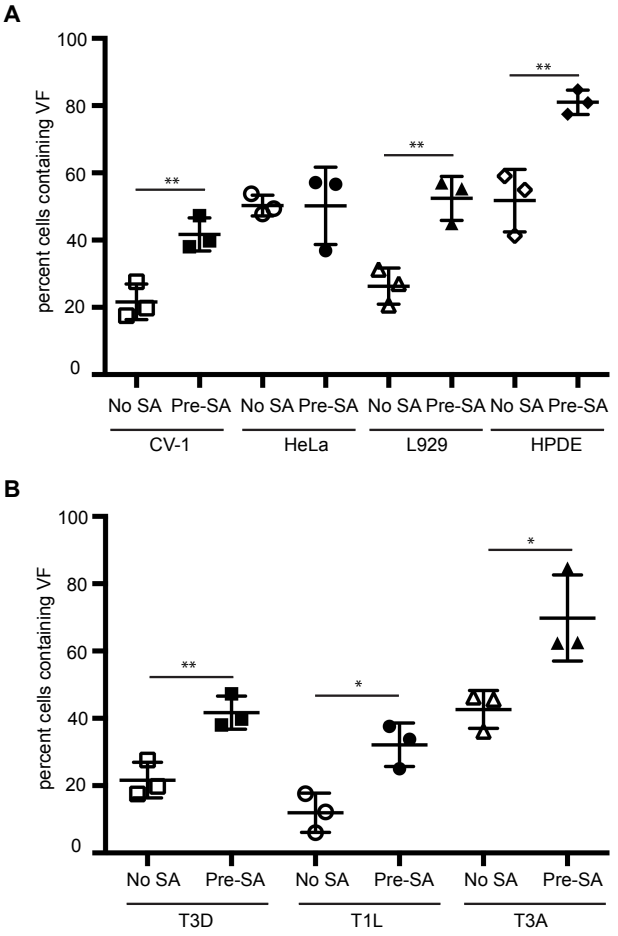

FIGURE 4

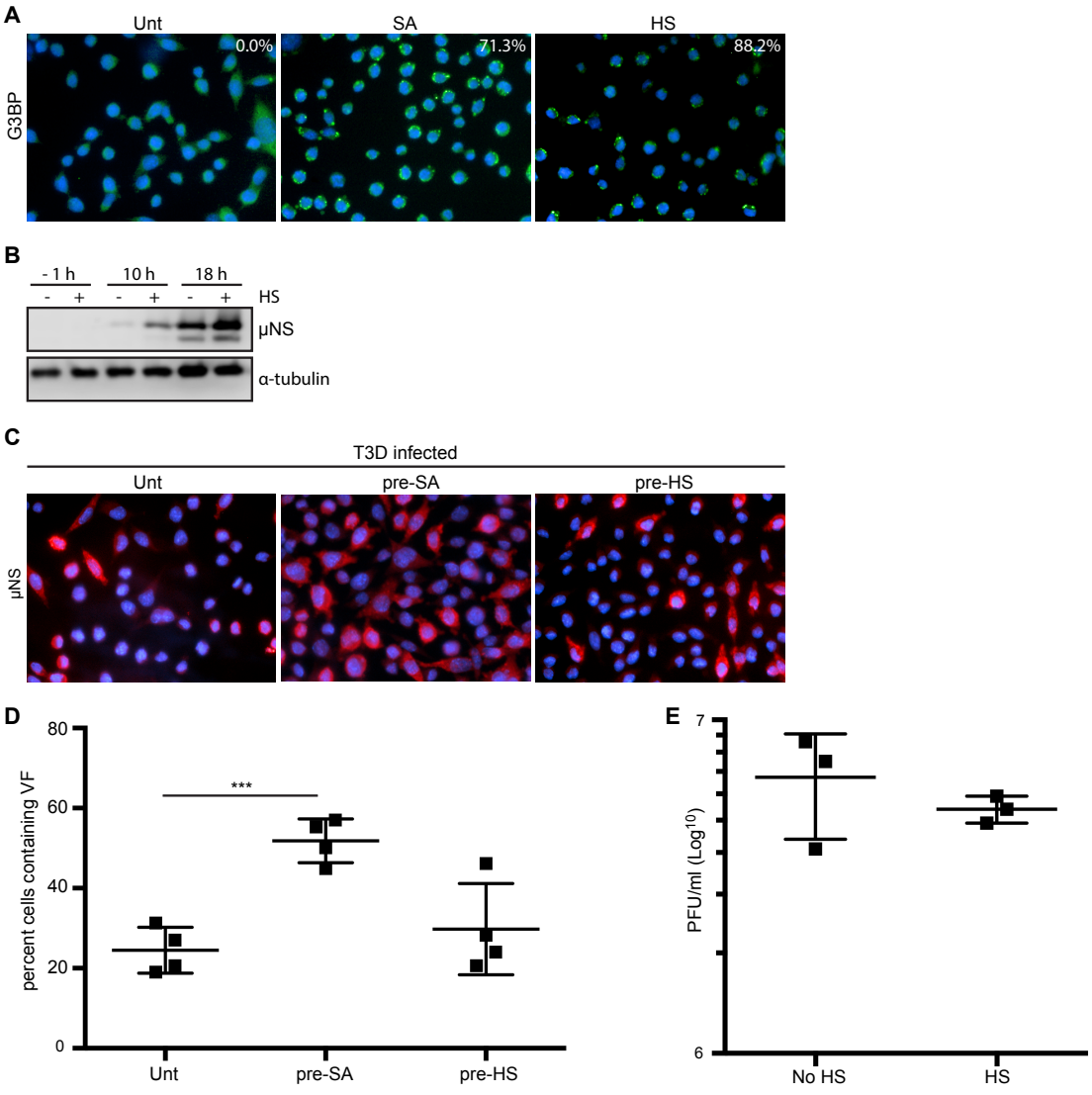

FIGURE 5

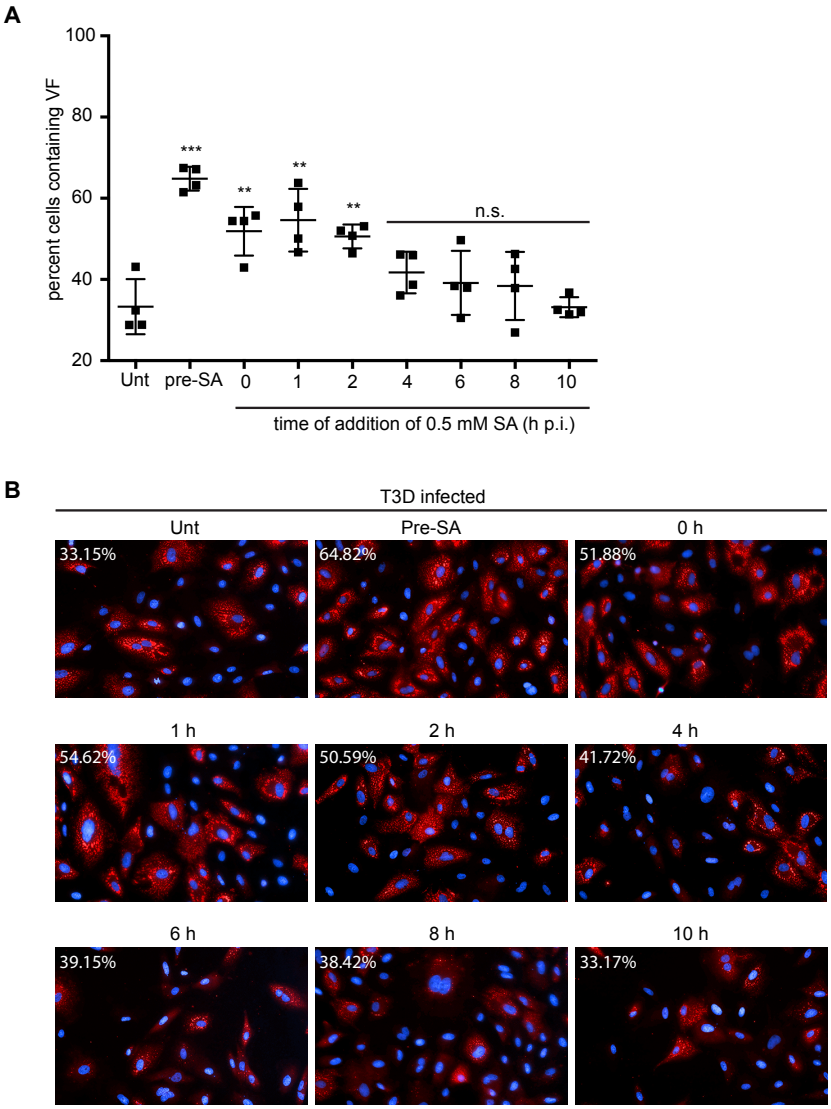

FIGURE 6