# Transient high level mammalian reovirus replication in a bat epithelial cell line occurs without cytopathic effect

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## Abstract:

Mammalian reoviruses exhibit a large host range and infected cells are generally killed; however, most studies examined only a few cell types and host species, and are probably not representative of all possible interactions between virus and host cell. Many questions thus remain concerning the nature of cellular factors that affect viral replication and cell death. In the present work, it was observed that replication of the classical mammalian reovirus serotype 3 Dearing in a bat epithelial cell line, Tb1.Lu, does not result in cell lysis and is rapidly reduced to very low levels. Prior uncoating of virions by chymotrypsin treatment, to generate infectious subviral particles, increased the initial level of infection but without any significant effect on further viral replication or cell survival. Infected cells remain resistant to virus reinfection and secrete an antiviral factor, most likely interferon, that is protective against the unrelated encephalomyocarditis virus. Although, the transformed status of a cell is believed to promote reovirus replication and viral "oncolysis", resistant Tb1.Lu cells exhibit a classical phenotype of transformed cells by forming colonies in semisolid soft agar medium. Further transduction of Tb.Lu cells with a constitutively-active Ras oncogene does not seem cell growth or reovirus effect on these cells. Infected Tb1.Lu cells can produce low-level of infectious virus for a long time without any apparent effect, although these cells are resistant to reinfection. The results suggest that Tb1.Lu cells can mount an unusual antiviral response. Specific properties of bat cells may thus be in part responsible for the ability of the animals to act as reservoirs for viruses in general and for novel reoviruses in particular. Their peculiar resistance to cell lysis also makes Tb1.Lu cells an attractive model to study the cellular and viral factors that determine the ability of reovirus to replicate and destroy infected cells.

## 1. Introduction

Mammalian reoviruses, as their name indicate, exhibit a large host-range and are able to infect most mammalian species and cell lines derived from these animals. Tropism for different cell types is also quite large, resulting in part from the binding to ubiquitous sialic acid and protein receptor JAM-A (reviewed by: Danthi et al., 2010), as well as unidentified sugars and possibly protein receptors (Antar et al., 2009; Chappell et al., 2000). However, proteolytic uncoating of the virus by lysosomal enzymes in the infected cell is often limiting (see for examples: Alain et al., 2007; Golden et al., 2002; Wetzel et al., 1997a,b). Alternatively, the secretion of proteases in the external milieu could likely promote virus infection in some tissues including tumoral microenvironment (Alain et al., 2006 ; Amerongen et al., 1994; Bass et al., 1990; Bodkin et al., 1989).

In the last 20 years, there has been a renewed interest for these viruses due to their ability to preferentially infect transformed cells (Alain et al., 2007; Coffey et al., 1998; Duncan et al., 1978; Hashiro et al., 1977; Marcato et al., 2007; Norman et al., 2004; Rudd and Lemay, 2005; Shmulevitz et al., 2010; Smakman et al., 2005; Strong and Lee, 1996; Strong et al., 1998; reviewed by: Patrick et al., 2005), leading to present clinical studies for their use as virotherapeutic “oncolytic” agents against cancer cells (reviewed among others by: Black et al., 2012; Harrington et al., 2010; Kelly et al., 2009). This preferential replication and cytolysis could result from the presence of a constitutively-active form of the Ras oncogene although other factors leading to cell immortalization and/or transformation are clearly involved, intensive research efforts are presently devoted to further clarify this aspect.

However, as for most viruses, studies of reovirus replication have been mostly performed in a few well-characterized cell types, mostly murine and human-derived. Furthermore, in the last few years, novel strains of reoviruses have been isolated from different animals species, especially wild bats, or in humans in contact with bats (Chua et al., 2007, 2008, 2011; Du et al., 2010; Kohl et al., 2012; Lelli et al., 2012; Pritchard et al., 2006; Thalmann et al., 2010; Wong et al., 2012). These animals present a special interest since they are presently the object of intense studies as important reservoirs for many pathogenic and emerging viruses (reviewed in: Calisher et al., 2006; Hayman et al., 2012; Hughes et al., 2007; Wang, 2011; Wang et al., 2011; Wong et al., 2007). Some of the novel reoviruses are fusogenic and are thus quite different from the classically-studied non-fusogenic mammalian orthoreoviruses. However, other strains are non-fusogenic and are more similar to the previous classical isolates of mammalian reoviruses.

In the present study, the replicative ability of a classical non-fusogenic mammalian reovirus was examined in a bat lung epithelial cell line. Transient replication was observed with production of infectious virus without any apparent cytopathic effect. Virus production rapidly declined although a low level of virus production was maintained over at least two months of cell culture. Infected cells produced and released an antiviral soluble factor that can protect against an unrelated virus, even at times when virus production was reduced to very low levels. Prior uncoating of the virus did not enhance cytopathic effect, indicating that a blockage in entry is not responsible for the lack of cytopathic effect, as expected from high level of virus replication in the absence of prior uncoating. The Tb1.Lu cells exhibit a transformed phenotype, as demonstrated by their ability to form colonies in semisolid medium and further addition of a constitutively active Ras oncogene did not seem to affect virus infection or its effect on the host cells.

## 2. Materials and methods

### *2.1. Cells and viruses*

L929 mouse fibroblasts and Vero cells (African green monkey kidney cells) were originally obtained from the American type culture collection (ATCC) and were grown in minimal Eagle medium (MEM) with 5% fetal bovine serum, with 1% penicillin and streptomycin (P/S) and 1% L-glutamine from commercial stock solutions (Wisent Bioproducts). Tb1.Lu mexican free-tailed bat (*Tadarida brasiliensis*) lung epithelial cells were a generous gift from the laboratory of Heinz Feldmann (Public Health Agency of Canada, Winnipeg, Canada) and were originally from ATCC (ATCC® Number CCL-88™). Tb1.Lu were grown in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum, 1% P/S, 1% L-glutamine and 1% non-essential amino acids from commercial stock solutions (Wisent Bioproducts). Phoenix-ampho packaging cells (a generous gift form Gerardo Ferbeyre, Université de Montréal) were grown in DMEM with 10% fetal bovine serum and 1% P/S and 1% L-glutamine. Mouse NIH-3T3 fibroblasts were originally obtained from Yvan Robert Nabi (Life Sciences Institute of Cell and Developmental Biology, University of British Columbia) and were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 1% P/S, 1% L-glutamine, 1% non-essential amino acids and 1% vitamins from commercial stock solutions (Wisent Bioproducts) .

Clones of Tb1.Lu cells were obtained by by two methods. The method used for clones 1 to 3 is limiting dilution in 96-wells plates. Individual clones resulting from the growth of a single cell were then trypsinized and grown in 35mm plates and propagated before being infected. The other method used for clones 4 and 5 is trypsinisation of well-isolated colonies using small pieces of filter paper wetted with trypsin. Individual colonies were grown in 24-wells plates and propagated before being infected.

Wild-type reovirus used for most experiments was a laboratory stock derived from a pure plaque of reovirus serotype 3 strain Dearing (T3/Human/Ohio/Dearing/55; referred to as T3D); in early experiments, the serotype 1 strain Lang (T1/Human/Ohio/Lang/1953; referred to as T1L) was also used. Both original inocula were obtained from the American Type Culture Collection (ATCC).

For the preparation of ISVPs, L929 cells were infected at a MOI of 2 PFU/cell in the absence of serum; following three cycles of freeze-thaw, chymotrypsin treatment (Sigma Type I-S from bovine pancreas) at 10μg/ml for 30 minutes at 37°C was done by direct addition of chymotrypsin to the virus-containing medium. The reaction was then stopped by addition of 2% heat-inactivated fetal bovine serum.

Wild-type EMC virus (murine encephalomyocarditis virus) was a generous gift from Serge Dea (Institut Armand-Frappier, Laval, Qc, Canada) and was originally obtained from ATCC.

### 2.2. Antibodies

Hybridoma cell lines producing either anti-σ3 (4F2) or anti-μ1 (10F6) have been described (Virgin et al., 1991) and were a generous gift from Kevin Coombs (Winnipeg University).

Hybridoma cells were grown in MEM for suspension culture with 10% fetal bovine serum, proline (20μg/ml) and β-mercaptoethanol (50μM) and antibodies were recovered as previously described (Brochu-Lafontaine and Lemay, 2012). The FITC-conjugated goat antireovirus antibody was obtained from Accurate Chemical & Scientific Corporation (catalog # YV0031-10).

### *2.2. Determination of virus replication*

At different times post-infection, infected cells in petri dishes were frozen directly with culture medium and submitted to three cycles of freeze-thaw before being titrated. Alternatively, medium was removed and separately frozen while fresh medium was added to the cells before being frozen and submitted to three cycles of freeze-thaw, as before.

Virus titers were determined by plaque assay on Vero cells in the presence of chymotrypsin (Sigma Type I-S from bovine pancreas) at 10μg/ml, as previously described (Brochu-Lafontaine and Lemay, 2012).

### *2.3. Immunoblotting*

Infected cells were recovered by scraping in small volume of medium and centrifuged in an Eppendorf tube at 13 000 g for 5 minutes at 4°C. Cell pellets were resuspended in permeabilization buffer (Tris-HCl 10 mM pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40) and left on ice for 5 minutes before centrifugation at maximum speed for 1minute in an Eppendorf centrifuge at 4°C. Proteins were analyzed by SDS-PAGE and immunoblotting. Nitrocellulose membrane (Whatman Protran BA85) was blocked with 2% non-fat dry milk dissolved in TBS (Tris-HCl 10 mM pH 7.5, 150 mM NaCl) and sequentially incubated for one hour at room temperature with the anti-σ3 and anti-μ1 monoclonal antibodies. Antibodies in tissue culture medium were diluted with an equal volume of TBS containing the blocking agent and directly used. The diluted antibody solution was recovered and kept at 4ºC with 1mM sodium azide to be used up to 10 times. Membranes were washed in TBS containing 1% Tween-20. Binding of primary antibody was detected by reaction with peroxydase-conjugated secondary antibody and chemiluminescent substrate, as recommended by the manufacturer (Pierce SuperSignal West Dura Extended Duration Substrate). Images were obtained using Kodak BioMax Light Film.2.3 or on a Typhoon Trio™ imager (GE Healthcare Life Sciences) with Image Quant v2005 software; when necessary, quantitation was done with the same software.

### *2.4. Quantitation of reovirus-infected cells by FACS*

Quantitation of reovirus-infected cells by FACS was done essentially as described by others (Marcato et al., 2007), with modifications. Briefly, cells from a 6-wells plate were collected by trypsinization at 37°C for 5 minutes and resuspended in 1ml of DMEM containing 10% fetal bovine serum before recovery by centrifugation at 4°C for 5 minutes at 1500 g. The pellet was resuspended in 0.25ml of Cytofix/Cytoperm (Becton Dickinson) on ice with gentle agitation and left 20 minutes before centrifugation at 1500 g at 4° for 5 minutes. Cells were then resuspended in the 0.25 ml Perm Wash buffer (Becton Dickinson) centrifuged again and resuspended in 0.035ml of buffer to which 0.015 ml of FITC-conjugated antireovirus antibody was added. Following 30 minutes on ice with occasional gentle agitation, cells were pelleted, washed twice in buffer, and fixed with 4% paraformaldehyde before being analyzed on a BD FACSCalibur cytofluorometer (Becton Dickinson).

### *2.5. Detection of secreted antiviral molecule.*

L929 or Tb1.Lu cells were infected with wild-type reovirus type 3 Dearing at a multiplicity of infection of 5 and the supernatant was recovered 12 hours post-infection. This supernatant (5 ml) was then placed in a 100mm-diameter petri dish and irradiated using the U.V. light of the tissue culture hood for one hour; in these conditions, infectious reovirus titer was reduced to less than the amount that can be detected in the assay used for detection of a secreted antiviral molecule. This irradiated supernatant was then used as medium in EMC virus titration by $TCID_{50}$ on Tb1.Lu cells. Briefly, tenfold dilution of the EMC virus samples were prepared and used to infect one row (12 wells) of a 96-wells microplate of Tb1.Lu cells. For each well, a volume of 50μl of virus dilution in serum-free MEM was used. Plates were left at 4°C for one hour before addition of 100µl per well of the recovered U.V. treated supernatant. Plates were incubated for 3 to 4 days and examined by phase-contrast microscopy for the presence of cytopathic effects. The plates were then fixed with 4% formaldehyde in PBS for one hour before being washed with PBS and stained with methylene blue for one hour. Plates were then washed with PBS and tap water. When dry, methylene blue was solubilized in 100µl of 0.1N HCl for easier visualization.

### *2.6. Introduction of a constitutively-active Ras oncogene in Tb1.Lu and control NIH-3T3 cells.*

Phoenix-ampho packaging cells (Swift et al., 2001) were plated at a density of $4x10^6$ cell per 100mm petri dish. The next day, cells were transfected using the calcium-phosphate precipitation method. PWZL-hygro control vector and PWZL-hygro Ras vector (Ferbeyre et al., 2000) were used at a concentration of 40µg/ml. The next morning, sodium butyrate was added at a final concentration of 10 mM and medium was changed in the afternoon in the transfected phoenix-ampho cells. Twenty-four hours later, supernatants containing the retroviruses encoding the constitutively-active Ras oncogene (H-$Ras^{G12V}$) or the control empty vector control were filtered through a 0.45µm filter and added to Tb1.Lu cells. Polybrene (Hexadimethrine Bromide, Sigma #H-9268) was added at a final concentration of 4µg/ml and 10% of fetal bovine serum was also added to the medium containing the retroviruses. Fresh medium was added to phoenix-ampho cells. The same retroviral transduction was repeated two other times within 12 hours for each infection. Finally, fresh medium was added to transduced Tb1.Lu cells and 200µg/ml of hygromycin (cat no.10843555001, Roche) was added to each petri dish for selection of stably-transduced cells. Selection was pursued three days at 37°C in the $CO_2$ incubator. Tb1.Lu Control and Tb1.Lu Ras-transformed cells were then maintained in the same medium than the original Tb1.Lu cells with periodic addition of 200 µg/ml of hygromycin. Phoenix-ampho cells, PWZL-hygro vectors and protocols were kindly provided by Gerardo Ferbyere, Université de Montréal.

### *2.7. Cell transformation assay: formation of colonies in semisolid medium*

Cells were trypsinized and seeded in 6-wells plates at different cell concentrations (50 000, 10 000 and 2 000 cells per well) by mixing in complete culture medium containing 0.4% Noble agar

(Difco) and overlaying over a preformed 0.8% Noble agar layer, also in complete medium. When medium has hardened, a layer of liquid medium was added on top and was subsequently changed each 3 days. After 14 days of cell growth, liquid medium was removed and replaced for 2 hours with medium without serum before being replaced again with 10% formaldehyde in PBS for cell fixation. Fixative was removed after one hour at room temperature and cell colonies were stained by adding 0.01% crystal violet in PBS for one hour at room temperature, followed by extensive washing in PBS .

## 3. Results

### *3.1. Absence of reovirus-induced cytopathic effect in reovirus-infected Tb1.Lu cells*

Bat cells are poorly studied as *in vitro* models of reovirus infection and replication. Therefore, it was first sought to know if reovirus could replicate efficiently in an epithelial lung cell line (Tb1.Lu cells) which originates from the Mexican free-tailed bat (*Tadarida brasiliensis*). Infection with the wild-type reovirus serotype 3 Dearing in L929 cells, the classical model for reovirus' replication, was compared with that of Tb1. Lu cells. As seen in **figure 1A**, L929 cells infected at an MOI of 3 pfu/cell already showed clear signs of viral-induced cell lysis at 48 hours post-infection and, as expected, were completely killed between 3 to 5 days post-infection at either MOI of 0.3 or 3 pfu/cell (**data not shown**). In contrast, Tb1.Lu cells remained alive and without clear signs of cell lysis or reovirus-associated cytopathic effect at either MOI even at 20 days post-infection (**figure 1B**).

In order to determine whether the absence of cell lysis could be due to an overall resistance of Tb1.Lu cells to viral-induced cytopathic effects, cells were subjected to infection with the unrelated murine encephalomyocarditis virus (EMC), a single-stranded RNA virus of the *Picornaviridae* family. Significant cell death was observed as early as 24h post-infection and increased at 72h post-infection (**Figure 1C**). The resistance of Tb1.Lu cells to reovirus-induced cytopathic effect is therefore somewhat limited to certain viruses and apparently does not reflect an overall resistance of these cells.

### *3.2. Tb1.Lu cells support reovirus replication despite absence of cytopathic effect.*

To determine if reovirus actually infects and replicates in Tb1.Lu cells, cultures of infected cells were recovered at different times post-infection and submitted to three cycles of freeze-thaw before virus titration, as described in Materials and methods. Virus replication was detected from 48 hours, total virus produced stabilizes between 3 and 6 days and total amount remained constant thereafter, suggesting transient replication despite absence of cell lysis (data not shown). In order to further examine virus production, infected cells and their supernatants were separately recovered at different times post-infection before freeze-thaw and virus titration. Since there was no cell passage nor change of medium, this experiment thus examine the accumulation of infectious virus over time. Again, viral replication was clearly observed and virus release in the supernatant was observed despite absence of cell lysis. A peak of infectious virus was observed around 48-72 hours post-infection and then decreased gradually inside the cells and remained constant in the supernatant. Total infectious virus production and final viral titers were similar in L929 cells (after 24 hours) and Tb1.Lu cells at the 48-72 hours peak (data not shown). This confirms that virus replication actually occurs and decreases after peak replication and that the virus present in the supernatant was produced in the first few days (**Figure 2A**).

In parallel of the last experiment, cell lysates from infected cells with either serotype 1 (T1L) and serotype 3 (T3D) virus was recovered for western blot analysis (**Figure 2B**). Viral proteins were easily observed in parallel with the increase in virus titer. Although viral proteins in T1L-infected cells were detected at earlier times, a similar decrease at later times was observed with both viruses and no significant cell death was observed in either cases; this is also consistent with similar amounts of total proteins, as detected by Coomassie blue staining, for either infected or control mock-infected cells. Increasing the multiplicity of infection to 50 did not seem to enhance cytopathic effect in any significant way. Infected cells at either MOI could be kept for up to 50 days without any apparent effect on cell survival (**data not shown**).

### *3.3. Infection of individual Tb1.Lu cells in the cell culture*

Although there was no visible cell death in all previous experiments, the possibility remains that only a small fraction of the cells transiently produce large amount of viruses and are eliminated from the cell culture. To further study this aspect, an intracellular FACS assay was used to determine the percentage of infected cells, producing detectable amounts of viral proteins, as described in Materials and methods.

In a first experiment, a percentage of close to 50% of infected cells was reached by 4 days post-infection (**Figure 3A**). These percentages remained constant if cells were kept without passage but decreased rapidly upon cell passage. The decrease associated with cell passage was also observed at the level of viral proteins by immunoblotting analysis (**Figure 2B** and data not shown). Increasing the multiplicity of infection for 5 to 50 did increase the percentage of infection but not cell death, as previously observed. The relatively high percentage of infected cells suggests that the presence of cellular subpopulations differing in sensitivities to virus-induced cell lysis is unlikely to be responsible for the overall resistance of the cell population.

In order to further verify if the presence of subpopulations of cells could explain that some cells remain uninfected, cell clones were obtained by recovering colonies of well-isolated cells following culture at low cell density, as described in Material and methods, and individually propagated before infection. Although the exact percentage of infected cells was somewhat variable from experiment to experiment, five different clones that were analyzed did not present striking differences in the percentage of infected cells and were also similar to the original cell population when examined in parallel (**Figure 3B**); in addition, there was no evident cytopathic effect in these different cell clones, suggesting that all cells in the culture can be infected but are similarly resistant to virus-induced cytopathic effects.

### *3.4. Limited uncoating is not mainly responsible for the phenotype of Tb1.Lu cells to reovirus.*

As mentioned in the introduction, the ability to uncoat the reovirus virions to generate infectious subviral particles (ISVPs) is often a limiting factor for viral replication. To determine if deficient uncoating could explain the resistance of Tb1.Lu cells, despite obvious viral production in these cells, the infection by virions and *in vitro* generated ISVPs, following chymotrypsin treatment, was compared. Vero cells were used as controls since these cells can be infected by reovirus even though they exhibit a limited capacity to uncoat the virus (Golden et al., 2002); hence, ISVP infects Vero cells more efficiently than virions. At 48 hours post-infection, a similar small percentage of Vero and Tb1.Lu cells was infected with virions while Vero cells infected with ISVPs were essentially all killed (data not shown). At later times (6 days post-infection), most of infected Tb1.Lu cells survived and a higher percentage of cells were infected by ISVP than virions **(Fig. 4A)**. Kinetics of infectious virus produced was also examined and confirmed faster replication of ISVPs, compared to virions, with similar virus titers at later times **(Fig. 4B)**. Faster kinetics of infection by ISVPs compared to virions suggest that inefficient virus uncoating limits reovirus infection in Tb1 cells but only to a certain extent; bypassing the uncoating step with ISVPs is not sufficient to increase cell lysis nor final viral production. Cells initially infected by ISVPs, as well as those initially infected by virions, could be kept for a long time without apparent cytopathic effect.

### *3.4. Secretion of an antiviral molecule by Tb1.Lu cells early after reovirus infection*.

One possible explanation for Tb1.Lu cells resistance to reovirus-induced cytopathic effect, and rapid decrease in virus produced, could be the presence of a strong antiviral mechanism in these cells; one likely possibility is the secretion of an antiviral factor, such as interferon. Since reovirus serotype 1 Lang is known to be more resistant to this cellular defense mechanism both at the level of induction and sensitivity (Jacobs and Ferguson, 1991; Zurney et al., 2009), this will be consistent with the previous observation that this virus isolate was slightly more efficient in infecting Tb1.Lu cells than was serotype 3 Dearing.

The ability of Tb1.Lu cells to secrete an antiviral molecule was thus examined by recovering supernatants of reovirus-infected cells at 12 hours post-infection and testing its antiviral ability on an unrelated virus, namely the murine encephalomyocarditis virus, as an indicator virus that is highly sensitive to interferon. Interestingly, while supernatants of reovirus-infected L929 cells only reduced apparent EMC titer by approximately 4-fold under these conditions, the supernatant from Tb1.Lu cells exhibited a strong antiviral activity, being able to reduce apparent EMC titer by more than a thousandfold **(Fig. 5)**. This suggests the induction and secretion of a strong antiviral factor, most likely interferon, early during infection of these cells, that may be responsible for the rapid decline in synthesis of viral proteins and infectious virus production, possibly also explaining the lack of concomitant cytopathic effect.

### *3.5. Introduction of a constitutively active form of Ras in Tb1.Lu cells does not affect reovirus replication and virus-induced cytopathic effects.*

As mentioned in the introduction, expression of a constitutively-active form of Ras, or activation of Ras signaling pathway, could transform some immortalized nontransformed cells, such as murine NIH-3T3 cells. This results in an increased reovirus replication and/or virus-induced cell lysis or apoptosis (see for examples: Alain et al., 2007; Norman et al., 2004; Marcato et al., 2007; Rudd and Lemay, 2005; Shmulevitz et al., 2010; Smakman et al., 2005; Strong and Lee, 1996; Strong et al., 1998) and forms the basis of the so-called "oncolytic" activity of the virus. One possibility for the resistance of Tb1.Lu cells to reovirus could thus result from lack of Ras activation and non-transformed status of these cells. In order to determine if increased permissivity or sensitivity of Tb1.Lu cells could be achieved by cellular transformation, cells were infected with a retroviral vector encoding the constitutively active H-Ras$^{V12}$, as described in Materials and methods; transduced cells were selected for hygromycin resistance encoded by the vector and will be referred to Tb1.Lu Ras. As a control, cells were similarly transduced with an empty vector (Tb1.Lu ctl).

The cells were first examined for their ability to behave as transformed cells using the soft agar colony formation assay **(Fig. 6)**. Surprisingly, both the Tb1.Lu ctl and Tb1.Lu Ras cells could form colonies in soft agar with similar efficiencies (approximately 30-40% of seeded cells formed visible colonies after 14 days). This contrasts with the classical model of parental NIH-3T3 versus NIH-Ras cell lines that is presented as a comparison; in this case there was no visible colonies after 14 days in cells transduced with the control vector; even under the microscope, most cells were found to remain individual in this case (data not shown). Efficiency of colony formation in both Tb1.Lu-Ctl and Tb1.Lu-Ras was similar to that of NIH-Ras cells. This suggest that the original Tb1. Lu cells were already behaving as transformed cells and that further addition of H-Ras$^{V12}$ does not further affect cellular transformation, at least as assessed by this assay.

The infection by reovirus was nevertheless examined in Tb1.Lu-Ctl and Tb1.Lu-Ras cells. Viral proteins at different times post-infection was examined by immunoblotting and indicates only a small increase, less than twofold upon quantitation, in Tb1.Lu-Ras cells **(Fig. 7)**; furthermore, both cell lines resisted reovirus-induced cytopathic effect and could be passaged for up to two months without any apparent effect on cell survival. This indicates that oncogenic Ras does not have a significant effect on reovirus infectivity, or cell-induced cytopathic effect, in this cell type and lack of activation of Ras signalling pathways is unlikely to explain the resistance of these cells.

### *3.6. Long-term infection of Tb1.Lu cells.*

In order to clarify if the virus is eventually cleared from infected cells, these were kept for up to a month in two different conditions. In one case, medium was changed twice a week but cells were never passaged; it was found that these cells can actually remain viable under these conditions and can then be passaged with a minimum loss of viability. Another culture of infected cells was rather trypsinized twice a week at the same cell concentration each time; again, there was no apparent change in growth properties of these cells and the number of cells remained essentially constant at all time.

Infectious virus production was then measured in the supernatant by virus titration, as well as remaining infectious virus present intracellularly. The amount of infectious virus remained high in the cell culture when cells were not passaged (data not shown), despite the fact that there was no cell killing nor apparent cytopathic effect. In contrast, virus production was reduced by at least a thousandfold compared to acutely-infected cells when cells were regularly passaged, suggesting the need for constant reinfection to maintain the virus in dividing cells **(Fig. 8** , compare panel A and B**)**. Infectious virus was also found to be released in the supernatant of either growing or stationary cells (data not shown). When the same experiment was repeated with either control or Ras-transduced cells, virus production was still observed in both cases even after two months (data not shown).

In order to determine if the cells could be reinfected by the virus, infection was carried out in both the passaged mock-infected and infected cells in parallel with the original Tb1.Lu cells and titers of infectious viruses were determined. Clearly, the presence of the virus, although very reduced in the passaged culture, was sufficient to prevent any further reinfection, probably by maintaining the presence of the soluble antiviral factor **(Fig.8)**.

Virus released from late-infected cells was also recovered and used to infect either L929 or fresh Tb1.Lu cells; while L929 cells were readily killed by the infection, Tb1.Lu cells resisted to this virus, as well as to the original wild-type virus (data not shown). There is thus no evidence that the "adapted" virus has evolved to acquire more cytopathogenicity toward the Tb1.Lu cells.

## Discussion

In the last few years, different reoviruses have been found in various species of bats. In this project, *in vitro* replication of a classical mammalian reovirus was examined in bat cells. These cells differ from most *in vitro* cellular models of reovirus infection since no cytopathic effect was observed despite viral replication and release in the external medium. The mechanism of virus release from these cells remains to be explored. In the closely related avian reovirus, as well as in rotavirus, another member of the *Reoviridae* family, it has been observed that autophagy contributes to virus replication and/or propagation (Meng et al., 2012; Crawford et al., 2012). It cannot be excluded that autophagy could be involved in nonlytic virus release in Tb1.Lu cells, as well as during viral persistence in these and other cell types. Alternatively, recent data indicate a recycling mechanism from endocytic compartments to the cell surface (Mainou and Dermody, 2012) that may be also used in the case of nonlytic virus release.

Bat Tb1.Lu cells were previously shown to support persistent infection with Ebola virus (Strong et al., 2008). However, the absence of cell death and establishment of persistence following virus infection is not a general property of Tb1.Lu cells since they were readily infected and killed by encephalomyocarditis virus.

Among the different cell lines that have been examined over the years, and that can actually support a productive reovirus infection, some of these nevertheless exhibit partial resistance to viral induced cell death at early times post-infection while eventually becoming persistently infected (see for examples: Alain et al., 2006; Danis et al., 1993; Kim et al., 2007; Taber et al., 1976; Verdin et al., 1986). However, detailed data of the kinetics and long-term cultures of infected cells is lacking in most cases and a significant percentage of cell death occurs at early times post-infection in all cases, in contrast with the situation observed with Tb1.Lu cells. The only case where persistent infection was established without a prior phase of actual cell death is a single report in MDCK cells (Montgomery et al., 1991), although the cells still exhibited limited cell growth once infected. Furthermore, in our laboratory, MDCK cells were found to be killed upon reovirus infection (Danis and Lemay, 1993; Bisaillon et al., 1999) . Recent data indicate that the fate of infected MDCK cells depends on postentry events that are regulated by a specific viral protein varying between type 1 Lang and type 3 Dearing virus strains (Ooms et al., 2010), differences between virus stocks could thus possibly explain these conflicting results between laboratories. In the present manuscript, there was no striking difference between the Lang and Dearing strain for the replication in Tb1.Lu cells and most of the present work only used the latter strain. However, it will certainly be interesting to examine different virus mutants for their ability to replicate and eventually kill infected cells.

In most cell lines, long-term infection results in viral persistent infection with resulting virus-cell coevolution (Dermody et al., 1993; Wetzel et al., 1997b; reviewed by: Dermody, 1998). In the few cases examined to date, amino acids substitutions in the σ3 protein were consistently observed in the viruses recovered from persistently-infected cells (Wetzel et al., 1997b; Kim et al., 2010). In the viruses from persistently-infected L929 cells, these substitutions were shown to increase viral uncoating by small amounts of lysosomal proteases, resulting in an ability to infect cells that possess a limiting amount of these enzymes, as observed in the persistently-infected cells (reviewed by: Dermody, 1998). With viruses obtained from “persistently-infected” Tb1.Lu cells, the lack of cytopathic effect of these viruses on Tb1.Lu cells, and the limited impact of prior uncoating of the original virus, suggests that it is unlikely that the virus has actually evolved to acquire a better efficiency of uncoating.

The exact reasons for the resistance of Tb1.Lu cells to cytopathic effects following reovirus infection thus remain elusive. It has been well established that in immortalized yet nontransformed cells, such as NIH-3T3 cells, reovirus replication is blocked, but the ability of Tb1.Lu cells to form colonies in soft agar and lack of effect of retroviral transduction of an activated Ras suggest that the situation is different in Tb1.Lu cells. The lack of effect of Ras transduction could indicate that the Ras signaling pathway is already directly or indirectly activated in these cells or that the cells are transformed by a completely different pathway.

The most probable explanation for the resistance of Tb1.Lu cells remains the production of high amounts of a potent antiviral molecule by the infected cells. The resistance of persistently infected cells to further reinfection is most probably due to the constant secretion of this same molecule, as previously observed in persistently-infected SC1 cells (Danis et al., 1997). The exact nature of this "antiviral molecule" was not established in the present study. However, it is active against both the original inducing virus and an unrelated virus, is secreted from the cells, and is resistant to UV irradiation; altogether this is clearly consistent with interferon. A soluble antiviral-factor, considered to be interferon, has also been previously reported in primary cells of *Tadarida brasiliensis* (Stewart et al., 1969); the bat species from which Tb1.Lu cells originate. Although, there is still relatively few studies of the innate immune response in different bat species (Baker et al., 2012), evidence are now rapidly accumulating for the presence of variety of active immune genes in bat, including pattern recognition receptors, as well as interferons and interferon stimulated genes (see for examples: Biesold et al., 2011; Papenfuss et al., 2012; Zhou et al., 2011). The presence of an active innate immune response is thus likely to be critical in the ability of bats to serve as virus reservoirs of a diverse array of viruses.

Altogether, this work suggests that bat cells possess unusual properties that may be important in the ability of the animals to act as reservoirs for reoviruses, by establishment of persistent productive infection regulated by a soluble antiviral factor; this could well contribute to the emergence of more pathogenic viruses in these animals. The present work also further stresses the need to examine virus replication in a wide range of cells from different species and tissue origins, including different species of bats, as these cells are becoming more widely available (for examples: Crameri et al., 2009; Krähling et al., 2010). The resistance of Tb1.Lu cells also makes them an attractive model to examine the effect of innate immune response and of various signaling pathways on viral replication and virus-induced cytopathic effects.

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## Figure Legends

**Fig. 1.** Tb1.Lu cells are resistant to reovirus-induced cell lysis. (A) L929 cells and Tb1.Lu cells were infected with reovirus serotype 3 Dearing at the indicated multiplicity of infection (MOI) and cells were examined by phase-contrast microscopy 2 days post-infection. (B) Tb1.Lu cells were infected as in panel A and examined 20 days post-infection without cell passage. (C) Tb1.Lu cells were infected with EMC virus at the indicated MOI and cells were examined 3 days post-infection.

**Fig. 2.** Reovirus replication kinetics in Tb1.Lu cells. (A) Wild-type T3D virus was used to infect Tb1.Lu cell at an MOI of 5. Cells were recovered separately from their supernatant at indicated times post-infection without cell passage or change of media. Plaque assay was used for virus titration, as described in Materials and methods. (B) Laboratory stocks of T1L and T3D were used to infect Tb1.Lu cell at an MOI of 5. Cells were passaged 72 hours and 240 hours post-infection. Fresh medium was added at 144 hours post-infection. Proteins were recovered at various times post-infection as indicated and analyzed for the presence of viral proteins by immunoblotting, as described in Materials and methods; position of the major outer capsid protein σ3 is indicated.

**Fig. 3.** Percentage of Tb1.Lu infected cells by reovirus T3D measured by FACS analysis. (A) Tb1.Lu cells were infected at a MOI of 5 and intracellular reovirus antigens were detected by FACS analysis at different times post-infections, as described in materials and methods. (B) Different cell clones, isolated as described in Materials and methods, or the original cell population, were infected at a MOI of 5 and analyzed 72 hours post-infection, the percentage of positive cells detected by FACS analysis is presented.

**Fig. 4.** Infection of Tb1.Lu cells by virions or ISVPs. (A) Tb1.Lu cells were infected at an MOI of 3 with either virions or ISVPs and recovered 144 hours post-infection for FACS analysis. (B) Tb1.Lu cells infected with either virions or ISVPs were recovered at different times post-infection, subjected to three cycles of freeze-thaw and total infectious virus titered, as described in Materials and methods.

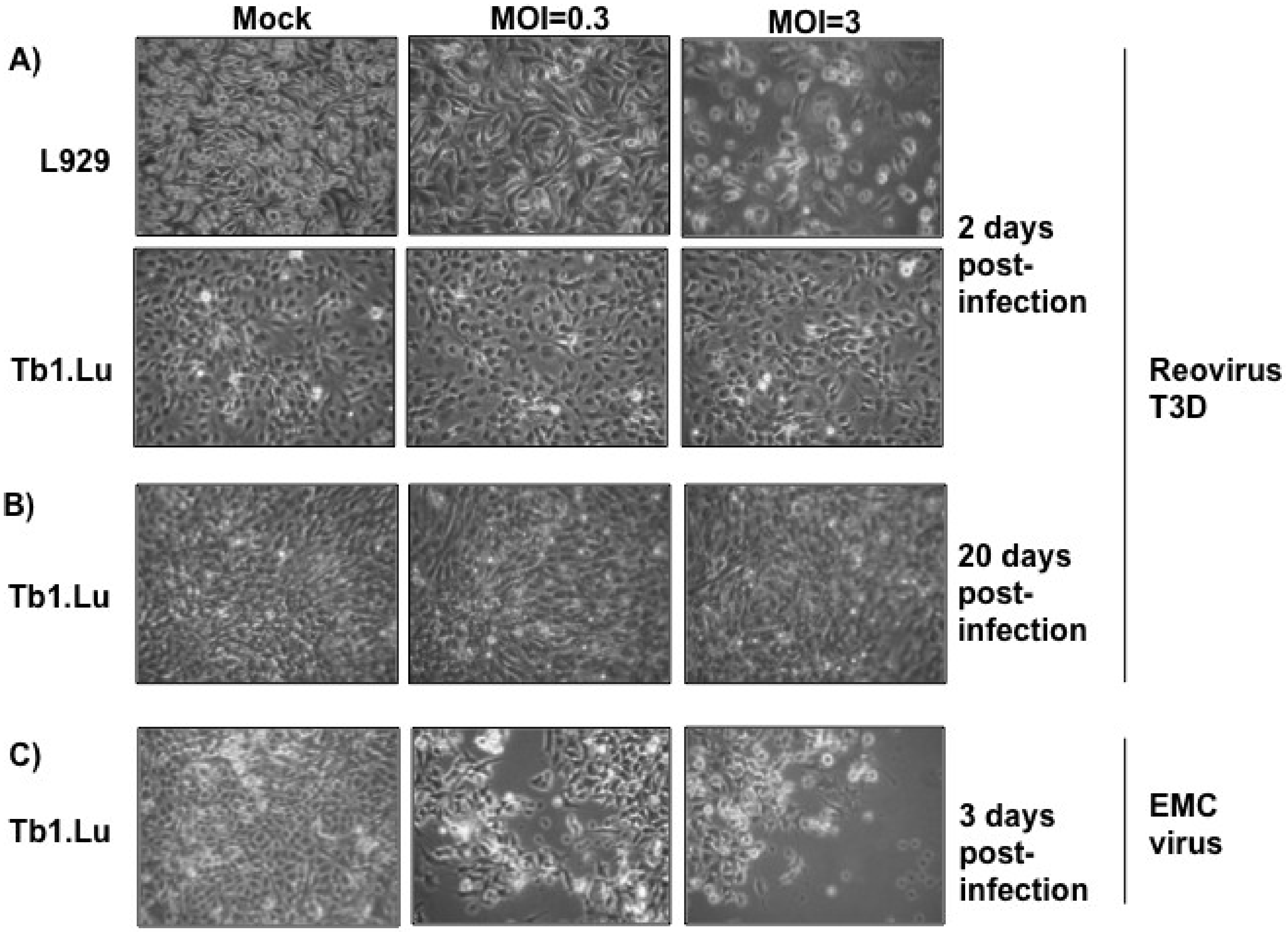
**Fig. 5.** Secretion of an antiviral factor by reovirus-infected Tb1.Lu cells. Tb1.Lu or L929 cells were infected at a MOI of 5 for 12 hours and cell supernatant collected and UV-irradiated to remove infectious reovirus. Uninfected Tb1.Lu or L929 cells in 96-wells microplates were used for $TCID_{50}$ titration of encephalomyocarditis virus using serial tenfold dilution of the EMC virus stock, as indicated. Supernatant of either mock-infected or reovirus-infected cells were added after the EMC adsorption period, as described in Materials and methods, and kept for the whole incubation period. Cells were fixed 3 days post-infection and stained with methylene blue, as described in Materials and methods.

**Fig. 6.** Cell transformation status of control and Ras-transduced Tb1.Lu cells. Tb1.Lu cells and control NIH-3T3 cells were transduced with a retroviral vector encoding constitutively active H-$Ras^{V12}$ or a control empty vector. Stably transduced cells were then plated in 0.4% agar medium at two different cell concentrations, as indicated, and colonies that developed after 2 weeks were stained with crystal violet, as described in Materials and methods.

**Fig. 7.** Effect of transforming Ras on reovirus infection in Tb1.Lu cells. Tb1.Lu ctl and Tb1.Lu Ras cells were at a MOI of 5; cellular proteins were recovered at different times post-infections and analyzed by immunoblotting. Positions of major viral capsid proteins σ3 and μ1 are indicated.

**Fig.8.** Long-term reovirus infection of Tb1.Lu cells. Cells were infected or mock-infected and kept for one month either by changing the medium twice a week (A) or by cell passage at the same cell density twice a week (B). At this point, mock-infected or infected cell stocks were seeded in parallel with the original parental cell stock at the same cell density and infected with wild type reovirus at a MOI of 5 or left uninfected, as indicated. Virus inoculum was removed following virus adsorption, cells were recovered 72 hours post-infection and subjected to three cycles of freeze-thaw before virus titration.

**Figure 1**


Mock
MOI=0.3
MOI=3
A)
L929
Tb1.Lu
2 days post-infection
B)
Tb1.Lu
20 days post-infection
Reovirus T3D
C)
Tb1.Lu
3 days post-infection
EMC virus

## Figure 2

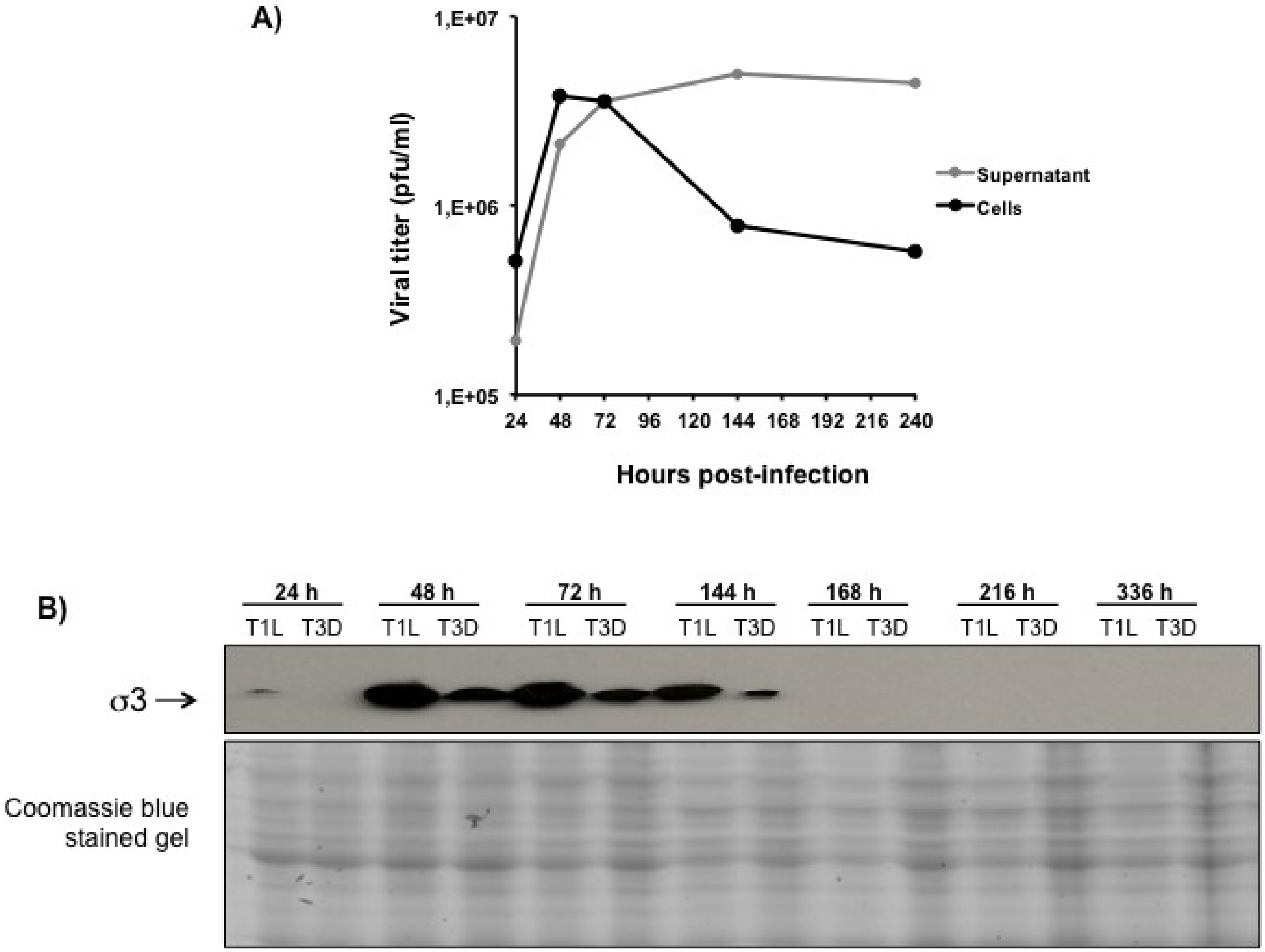
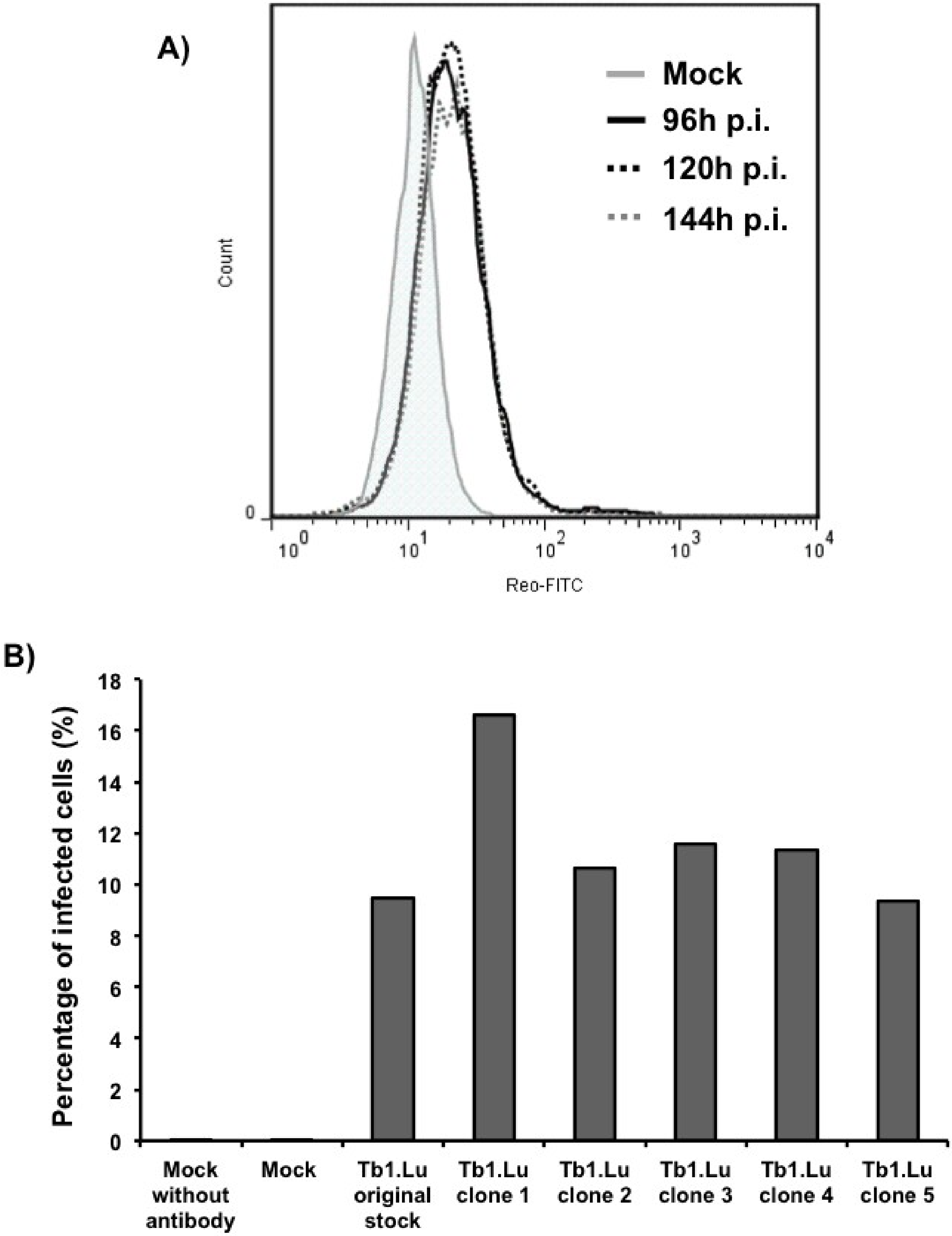

A)
1,E+07
1,E+06
1,E+05
Viral titer (pfu/ml)
24
48
72
96
120
144
168
192
216
240
Hours post-infection
Supernatant
Cells
B)
24 h
48 h
72 h
144 h
168 h
216 h
336 h
T1L
T3D
T1L
T3D
T1L
T3D
T1L
T3D
T1L
T3D
T1L
T3D
T1L
T3D
σ3 →
Coomassie blue
stained gel

**Figure 3**


A)
Mock
96h p.i.
120h p.i.
144h p.i.
Count
0
10^0
10^1
10^2
10^3
10^4
Reo-FITC
B)
Percentage of infected cells (%)
18
16
14
12
10
8
6
4
2
0
Mock without antibody
Mock
Tb1.Lu original stock
Tb1.Lu clone 1
Tb1.Lu clone 2
Tb1.Lu clone 3
Tb1.Lu clone 4
Tb1.Lu clone 5

# Figure 4

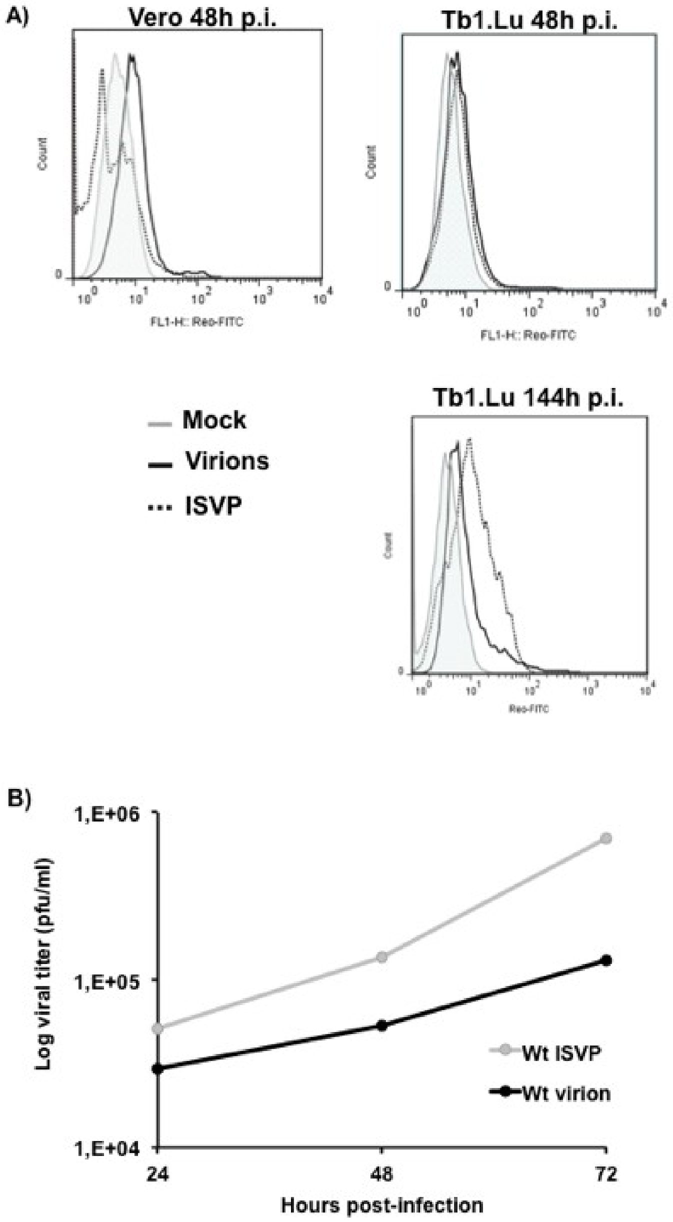

A)
Vero 48h p.i.
Tb1.Lu 48h p.i.
Tb1.Lu 144h p.i.
Count
FL1-H:: Reo-FITC
Reo-FITC
0
10^0
10^1
10^2
10^3
10^4
Mock
Virions
ISVP
B)
Log viral titer (pfu/ml)
1,E+06
1,E+05
1,E+04
24
48
72
Hours post-infection
Wt ISVP
Wt virion

**Figure 5**

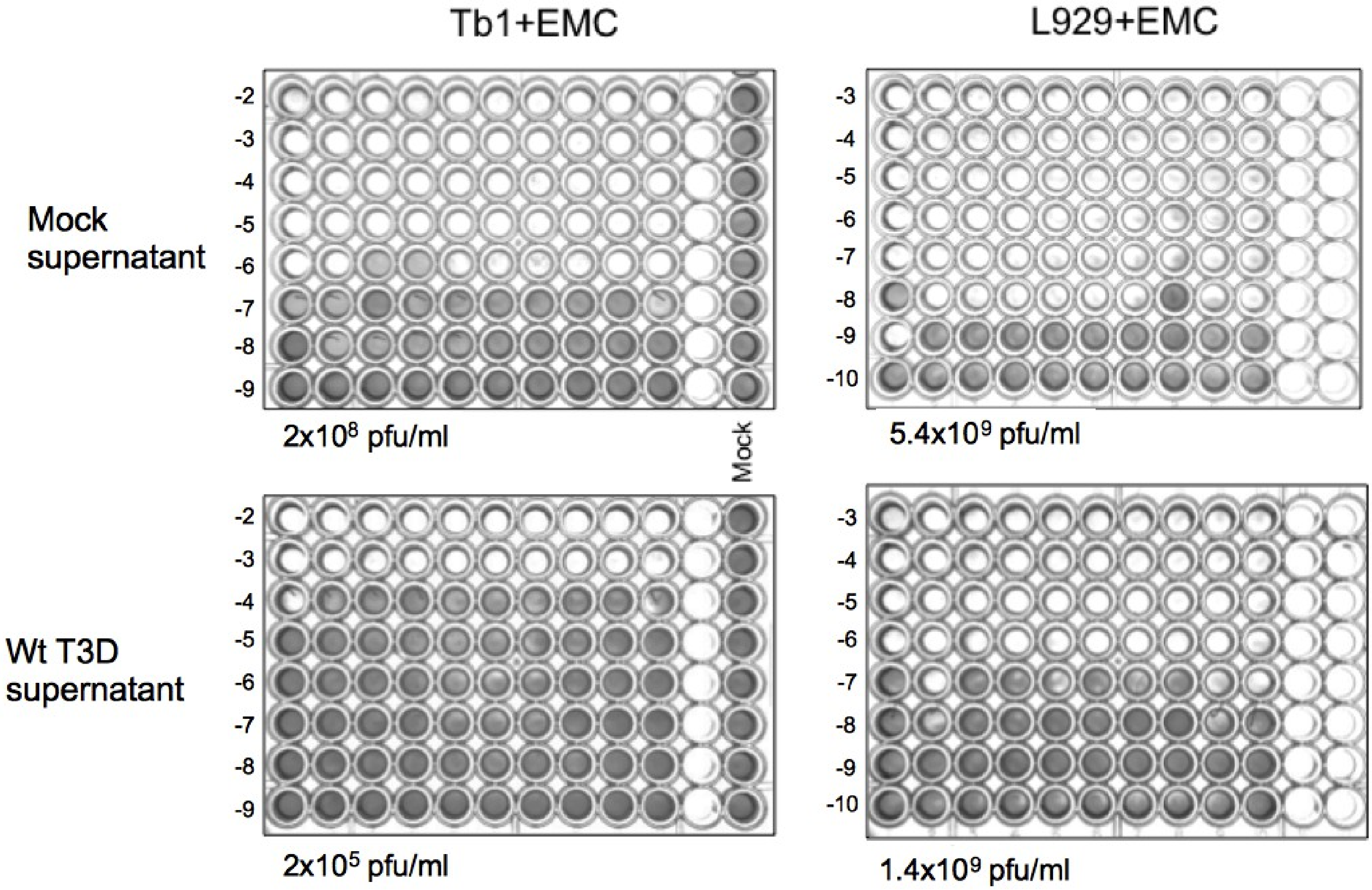
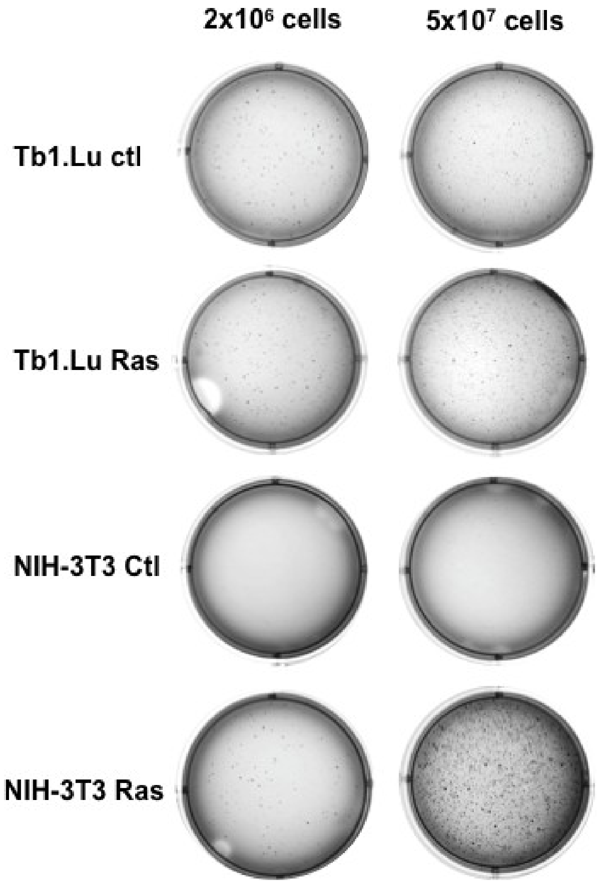

Tb1+EMC
L929+EMC
Mock supernatant
Wt T3D supernatant
-2
-3
-4
-5
-6
-7
-8
-9
2x10⁸ pfu/ml
Mock
-3
-4
-5
-6
-7
-8
-9
-10
5.4x10⁹ pfu/ml
-2
-3
-4
-5
-6
-7
-8
-9
2x10⁵ pfu/ml
-3
-4
-5
-6
-7
-8
-9
-10
1.4x10⁹ pfu/ml

**Figure 6**


2x10⁶ cells
5x10⁷ cells
Tb1.Lu ctl
Tb1.Lu Ras
NIH-3T3 Ctl
NIH-3T3 Ras

# Figure 7

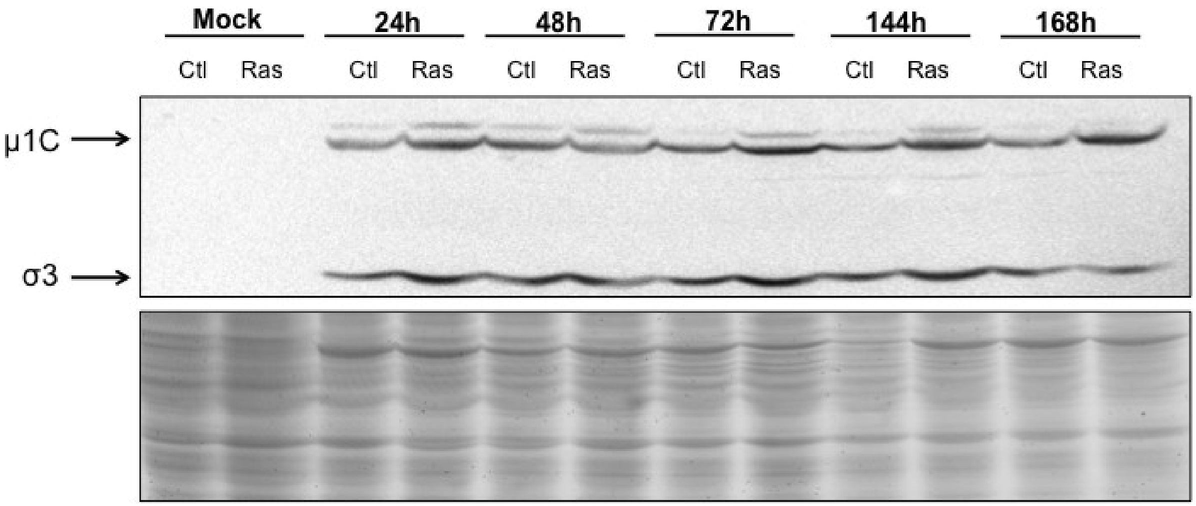
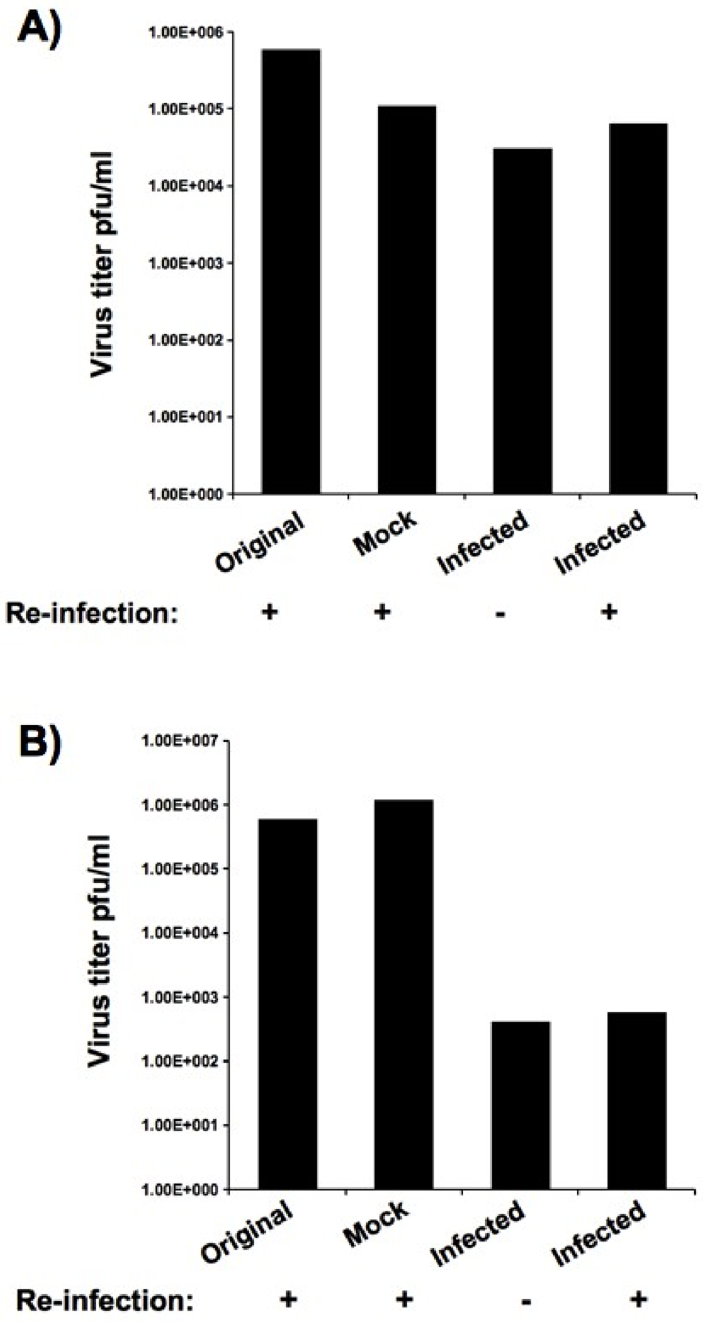

Mock
24h
48h
72h
144h
168h
Ctl
Ras
Ctl
Ras
Ctl
Ras
Ctl
Ras
Ctl
Ras
Ctl
Ras
µ1C
σ3

**Figure 8**


A)
Virus titer pfu/ml
1.00E+006
1.00E+005
1.00E+004
1.00E+003
1.00E+002
1.00E+001
1.00E+000
Original
Mock
Infected
Infected
Re-infection:
+
+
-
+
B)
Virus titer pfu/ml
1.00E+007
1.00E+006
1.00E+005
1.00E+004
1.00E+003
1.00E+002
1.00E+001
1.00E+000
Original
Mock
Infected
Infected
Re-infection:
+
+
-
+