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Cell Entry of Lymphocytic Choriomeningitis Virus Is Restricted In Myotubes

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Abstract

In mice persistently infected since birth with the prototypic arenavirus lymphocytic choriomeningitis viurs, viral antigen and RNA are readily detected in most organs and cell types but remarkably absent in skeletal muscle. Here we report that mouse C2C12 myoblasts that are readily infected by LCMV, become highly refractory to LCMV infection upon their differentiation into myotubes. Myotube's resistance to LCMV was not due to an intracellular restriction of virus replication but rather an impaired cell entry mediated by the LCMV surface glycoprotein. Our findings provide an explanation for the observation that in LCMV carrier mice myotubes, which are constantly exposed to blood-containing virus, remain free of viral antigen and RNA despite myotubes express high levels of the LCMV receptor alpha dystroglycan and do not pose an intracellular blockade to LCMV multiplication.

Keywords

Arenavirus; LCMV; Cell entry; Myoblast; Myotube; Skeletal muscle

INTRODUCTION

Arenaviruses are enveloped viruses with a bi-segmented, negative-strand RNA genome and a life cycle restricted to the cell cytoplasm. Each genome RNA segment, S and L, uses an ambisense coding strategy to direct the expression of two viral polypeptides in opposite orientation, separated by a non-coding intergenic region. The S segment encodes the viral nucleoprotein (NP) and the glycoprotein precursor, GPC that is processed by the cellular site 1 protease to generate the mature virion surface glycoproteins, GP1 and GP2. Trimers of

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GP1/GP2 form the spikes that decorate the virus surface and mediate virus cell entry via receptor-mediated endocytosis. The L segment encodes the viral RNA dependent RNA polymerase, L protein, and the small RING finger protein, Z, which is the counterpart of the matrix protein found in many enveloped negative strand RNA viruses (Buchmeier et al., 2007).

Arenaviruses cause chronic infections of rodents across the world and can infect humans through mucosal exposure to aerosols or by direct contact of abraded skin with infectious material (Buchmeier et al., 2007). Arenaviruses merit interest as both important human pathogens and experimentally highly tractable model systems to study acute and persistent viral infections. Both viral and host factors contribute to a variable outcome of arenavirus infection, ranging from virus control and clearance by the host defenses to chronic infection in the absence of clinical symptoms to severe disease (Buchmeier et al., 2007). Several arenaviruses, chiefly Lassa (LASV) and Junin (JUNV) viruses cause hemorrhagic fever (HF) disease in humans and pose an important public health problem in their endemic regions (Bray, 2005; Buchmeier et al., 2007; Geisbert and Jahrling, 2004). On the other hand, studies with LCMV in its natural reservoir, the mouse, have led to major concepts in virology and immunology that apply universally to other viral infections, including virus-induced immunopathology and MHC restriction (Oldstone, 2002; Zinkernagel, 2002), as well as the contribution to viral persistence of negative immune regulators like PDL-1 (Barber et al., 2006; Brooks et al., 2006) and immune stimulatory molecules (Harker et al., 2011; Yi et al., 2009).

Arenaviruses, including LCMV, are maintained in their natural hosts via vertical transmission and a lifelong chronic infection that is associated with relatively high levels of virus replication in many cell types, a situation that can be recreated by LCMV infection of newborn mice (Hotchin and Cinits, 1958). Replication of the LCMV genome RNA should result in the generation of variety of Pathogen-Associated Molecular Patterns (PAMPs) including double-stranded (ds)RNA, uncapped 5’-triphosphate, small RNA species and single-stranded (ss)RNA molecules that could be recognized by different Pattern Recognition Receptors (PRRs) including both membrane associated Toll-like receptors (TLR) 3 and 7 and cytoplasmic RIG-I-like receptors (RLRs) RIG-I and MDA5 (Borrow et al., 2010). Activated RIG-I and MDA5 (Habjan et al., 2008; Marq et al., 2011; Marq et al., 2010) associate with the IPS-1 (a.k.a. MAVS) adapter (Kumar et al., 2006) to promote activation of the non-classical IKK-related kinases TBK-1 and IKKε that activate IRF3 and NF-κB, which together with ATF2/c-JUN induce production of IFNβ that following interaction with its receptor (IFNAR) leads to activation of the JAK/STAT signaling pathway inducing the expression of hundreds of type I interferon (IFN-I) stimulated genes (ISGs), to produce a cellular antiviral state (Weber and Haller, 2007). Nevertheless, only modest plasma levels of IFN-I are detected in mice persistently infected with LCMV since birth (Bukowski et al., 1983; Kunz et al., 2006), suggesting that similarly to many other viruses (Weber and Haller, 2007), arenaviruses have developed ways to modulate the host IFN-I response to promote their persistence (King et al., 1992; Pircher et al., 1989). Accordingly, we documented that LCMV NP is a potent anti-IFN-I viral factor (Martinez-Sobrido et al., 2009; Martinez-Sobrido et al., 2007; Martinez-Sobrido et al., 2006), which likely contributes to facilitate the LCMV carrier state in mice.

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Early studies documented that in mice persistently infected with LCMV since birth, viral antigen was detected in many different cell types (Accinni et al., 1978; Doyle and Oldstone, 1978; Klavinskis and Oldstone, 1987; Nathanson et al., 1975; Popescu et al., 1979; Rodriguez et al., 1985). Likewise, in these mice viral RNA was readily detected in most tissues but remarkably absent in skeletal muscle (Fazakerley et al., 1991). This observation led to suggest that myotubes lacked the cell entry receptor used by LCMV. However, subsequent studies identified α-dystroglycan (αDG), which is expressed at high levels in skeletal muscle (Ibraghimov-Beskrovnaya et al., 1993), as a primary receptor of Old World arenaviruses including LCMV and LASV (Cao et al., 1998), which raised new questions about the mechanisms underlying the lack of skeletal muscle infection in LCMV carrier mice. To investigate this issue we used LCMV infection of C2C12 cells, a well-established cell system to study differentiation of myoblasts into myotubes (Blau et al., 1983; Yaffe and Saxel, 1977). Here we document that C2C12 myoblasts are readily infected by LCMV, but following their differentiation into myotubes become highly refractory to LCMV infection. Since the LCMV receptor αDG is expressed at high levels on C2C12 myotubes, we reasoned that myotubes restricted a post cell entry step of the LCMV life cycle. Differentiation of C2C12 myoblasts into myotubes is associated with expression of high levels of micro RNAs (miRNAs) 1, 133 and 206 that are also specifically expressed at high levels in skeletal muscle cells *in vivo* (Luo et al., 2013; van Rooij et al., 2008). Notably, inspection of the LCMV genome sequence revealed the presence within the cording region of the LCMV L mRNA of miRNA targeting sequences (miRTS) for miRNA-1, 133 and 206. However, over-expression of each one of these miRNAs in 293T cells did not affect multiplication of LCMV. C2C12 myoblasts persistently infected with LCMV expressed high levels of viral antigen that was not affected by their subsequent differentiation into myotubes, indicating that the myotube intracellular milieu does not restrict LCMV replication and viral gene expression. We found that a recombinant LCMV where the VSV glycoprotein G substituted for the LCMV GPC (rLCMV/VSVG) efficiently infected C2C12 myotubes. Likewise, a recombinant VSV where LCMV GPC substituted for VSV G (rVSV/LCMVGPC) was severely impaired in its ability to infect C2C12 myotubes. We obtained similar results with human myotubes. Our findings indicate that although skeletal muscle cells express high levels of the bona fide LCMV receptor αDG, they are refractory to LCMV infection due to an impaired LCMV GPC-mediated cell entry.

RESULTS

LCMV infection of C2C12 cells

Mouse C2C12 cells have been widely used to investigate differentiation of myoblasts into myotubes (Blau et al., 1983; Yaffe and Saxel, 1977). During the first four days of incubation in the differentiation medium (DMEM containing 2% horse serum-HS-), C2C12 cells fuse and form long-fiber shape multinuclear myotubes. C2C12 myotubes accurately recreate many aspects of bona fide myotubes including morphology and protein and RNA expression profiles (Burattini et al., 2004; Yoshida et al., 1998). To examine whether C2C12 myoblasts and myotubes exhibited different susceptibilities to LCMV infection, we infected non-differentiated (myoblasts) and differentiated (myotubes) C2C12 with rARM and rCl-13 and at 16 h p.i. we examined the degree of LCMV infection by detecting virus NP expression by

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immunofluorescence (IF). Differentiation of C2C12 myoblasts produces a cell population that contains 40–60 % myotubes together with myoblasts that remain non-differentiated, hence differences in susceptibility between C2C12-derived myotubes and their myoblast precursors to LCMV infection cannot be assessed by determining production of LCMV infectious progeny. Strong LCMV NP expression was observed in both rARM and rCl-13 infected C2C12 myoblasts (Fig 1A). In contrast, infection with rARM or rCl-13 of C2C12 myoblasts grown for four days in DMEM with 2% HS to promote differentiation into myotubes resulted in expression of NP predominantly in C2C12 myoblast (arrow heads), whereas C2C12 myobtubes were highly refractory to infection (Fig 1B).

Effect on LCMV multiplication of miRNAs that are expressed at high levels in skeletal muscle cells

To examine the effect of miRNA-1, 133a and 206 on LCMV multiplication we used LCMV to infect (moi = 0.001) 293T cells that also over-expressed, via transfection, miRNA-1, 133a or 206 and monitored production of infectious progeny at 24 h p.i. We first confirmed the functionality of miRNA-1, 133 and 206 under our experimental conditions. For this we co-transfected 293T cells with a plasmid expressing each of the miRNAs and a plasmid expressing *Firefly* luciferase (FL) whose 3'-UTR contained the corresponding miRTS. A plasmid expressing *Renilla* luciferase (RL) was used to normalize transfection efficiencies (Fig 2A). Each miRNA tested affected specifically only expression of the FL that contained the matched miRTS. None of the tested miRNAs had a noticeable effect on LCMV multiplication in 293T cells (Fig 2B).

LCMV replication and gene expression in C2C12 myotubes

We next asked whether LCMV could replicate and express its genome in the context of the myotube gene expression program. For this, we took advantage of the non-cytolytic properties of LCMV to establish LCMV-persistently infected C2C12 myoblasts that were subsequently subjected to the differentiation protocol. The majority of C2C12 myoblasts infected with rCl-13 expressed viral antigen at four days post-infection (Fig 3B). rCl-13 persistently infected C2C12 myoblasts were differentiated for four days and viral antigen expression assessed by IF. NP expression was observed in both myoblasts and myotubes (arrows) (Fig 3C), suggesting that LCMV is able to replicate and express its genome in C2C12 myotubes. Differentiated C2C12 cells contain a mixture of myoblasts and myotubes and therefore we could not rule out that infectious LCMV progeny being continuously generated by infected C2C12 myoblasts could overcome an otherwise restricted LCMV replication in C2C12 myotubes. To rule out this possibility, we used a single-cycle infectious rLCMV that lacks the GPC gene but are pseudotyped with the GPC of Cl-13 strain (rCl-13ΔGPC/Cl-13GPC) (Rodrigo et al., 2011). C2C12 myoblasts were highly susceptible to rCl-13ΔGPC/Cl-13GPC (Fig 4B). Infection of differentiated C2C12 cells with rCl-13ΔGPC/Cl-13GPC resulted in strong NP expression in myoblasts (arrow heads), but negligible levels of NP were observed in myotubes (Fig 4C, After). In contrast, when C2C12 cells were infected with rCl-13ΔGPC/Cl-13GPC before differentiation, NP expression was readily detected in both myoblasts and myotubes (arrows). Since rCl-13ΔGPC/Cl-13GPC-infected cells cannot generate infectious progeny, NP expression in C2C12 myotubes

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indicated that persistent LCMV replication was compatible with the myotube gene expression program.

Resistance of C2C12 myotubes to LCMV infection is mainly determined by an impaired LCMV GPC-mediated cell entry

As LCMV was able to replicate and express its genome in C2C12 myotubes, we reasoned that restricted LCMV infection of myotubes was related to a blockade of one of the multiple processes involved in virus cell entry. To separate cell entry from other steps of the LCMV life cycle, we examined infection of C2C12 cells with a recombinant VSV expressing either its homologous surface glycoprotein G (rVSV-WT), or the GPC of Cl-13 strain (rVSV/Cl-13GPC) (Rojek et al., 2008a). C2C12 myoblasts were infected with either rVSV-WT or rVSV/Cl-13GPC and 7 h later, infected cells were fixed and viral gene expression assessed by IF using an antibody to VSV N. We observed strong VSV N expression in both rVSV-WT- and rVSV/Cl-13GPC-infected C212 myoblasts (Fig 5A, pre-differentiation). We then subjected C2C12 myoblasts to differentiation for four days prior infected them with either rVSV-WT or rVSV/Cl-13GPC and 7 h later we assessed viral gene expression by IF. We observed strong VSV-N expression in rVSV-WT-infected C2C12 myoblasts (arrow heads) and myotubes (arrows), suggesting that in contrast to LCMV infection, C2C12 myotubes are susceptible to VSV infection. Remarkably, rVSV/Cl-13GPC predominantly infected C2C12 myoblasts (arrow heads) with VSV-N expression in C2C12 myotubes being hardly detectable (Fig 5A, post-differentiation), suggesting that LCMV GPC-mediated cell entry was restricted in C2C12 myotubes. We also conducted the complementary experiment using a rLCMV where VSV G substituted for LCMV GPC (rCl-13/VSVG) to infect C2C12 cells. C2C12 myoblasts were highly susceptible to rCl-13/ VSVG (Fig 5B, pre-differentiation). Notably, C2C12 myotubes (arrows) were also very susceptible to rCl-13/VSVG (Fig 5B, post-differentiation).

C2C12 myotubes express high levels of isoform 3 of caveolin (cav-3), and the interaction of cav-3 with DG in myotubes (Kogo et al., 2006) could affect the caveolin-independent cell entry process used by LCMV. To examine this possibility we evaluated whether C2C12 myoblasts overexpressing cav-3 became refractory to LCMV infection. For this we transduced C2C12 myoblasts with recombinant adenoviruses expressing either GFP or cav-3 (Tsutsumi et al., 2008) and subsequently infected them with LCMV (Fig 6). Overexpression of cav-3 or GFP in C2C12 myoblasts did not affect their susceptibility to LCMV infection as determined by detection of NP antigen.

LCMV infection of human myotubes

To assess whether restricted LCMV GPCmediated infection of myotubes was a rather species-specific phenotype we examined LCMV infection of human myotubes. Human derived myoblasts can be differentiated to myotubes in a similar way as C212 myoblasts (Blau and Webster, 1981; Gaster et al., 2001; Henry et al., 1995). We infected human myoblasts, prior or after differentiation into myotubes, with rARM, rCl-13 and rCl-13/ VSVG and 16 h later, viral protein expression observed by IFA. rARM and rCl-13 infected very efficiently human myoblasts (arrow heads), whereas human myotubes were highly refractory to these viruses (Fig 7A). In contrast, rCl-13/VSVG infected very efficiently both

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human myoblasts and myotubes (arrows). To further confirm that restricted LCMV infection of human myotubes was related to a cell entry blockade we used rVSV-WT and rVSV/Cl-13GPC to infect human myoblasts or myotubes. Human myoblasts and myotubes were susceptible to rVSV-WT (arrows Fig 7B), whereas only human myoblasts (arrow heads) were readily infected by rVSV/Cl-13GPC (Fig 7B).

DISCUSSION

Viral antigen and RNA were conspicuously observed in most tissues and cell types in LCMV carrier mice but notably absent in skeletal muscle (Fazakerley et al., 1991). This finding motivated us to investigate the reasons for the restricted multiplication of LCMV in myotubes. To facilitate experimental work aimed at addressing this question we used the C2C12 cells, a validated and widely used cell system for the investigation of myoblasts differentiation into myotubes (Blau et al., 1983; Yaffe and Saxel, 1977). Non-differentiated (myoblasts) C2C12 cells were very susceptible to LCMV infection, whereas following their differentiation into myotubes they became highly refractory to LCMV infection. Because myotubes express high levels of the LCMV receptor αDG we reasoned that the blockade was likely at a post cell entry step of the LCMV life cycle. Differentiation of C2C12 myoblasts into myotubes is associated with high expression levels of miRNA-1, 133 and 206 and intriguingly the corresponding miRTS were found to be present within the coding region of the LCMV L mRNA, raising the attractive possibility that targeting of LCMV L mRNA by miRNA-1, 133 and 206 could contribute to LCMV restricted multiplication in myotubes, a finding that would be consistent with several published studies showing a direct regulatory effect of miRNAs on multiplication of a variety of animal viruses including influenza (Ma et al., 2012; Song et al., 2010), picornavirus and adenovirus (Barnes et al., 2008; Cawood et al., 2009; Kelly et al., 2010; Kelly et al., 2008; Ylosmaki et al., 2008). However, over-expression of miRNA-1, 133 or 206 in 293T cells did not have any noticeable effect on LCMV multiplication (Fig 2), suggesting that highly unlikely these miRNAs played a significant role in the restricted multiplication of LCMV in C2C12 myotubes. Consistent with these results, we found that LCMV replication and gene expression were not affected by the differentiation of LCMV persistently infected C2C12 myoblasts into myotubes. It remains to be determined whether miRTS for miRNA-1, 133 and 206 within the coding region of the LCMV L mRNA are not accessible to miRNA targeting, or whether LCMV can actively counteract the host miRNA-mediated antiviral immunity.

The findings described above ability led us to consider that although myotubes express high levels of the LCMV receptor αDG (Ibraghimov-Beskrovnaya et al., 1993), their resistance to LCMV infection might be related to impaired cell entry mediated by LCMV GPC. Remarkably, we found that resistance and susceptibility of C2C12 myotubes to LCMV and VSV infection, respectively, was drastically influenced by the nature of the virus surface glycoprotein, indicating that impaired LCMV GPC-mediated cell entry critically contributed to myotube's resistance to LCMV infection. DG is translated from a single mRNA and cleaved into αDG and βDG that remain non-covalently associated (Barresi and Campbell, 2006). αDG is an extracellular protein that binds to a variety of extracellular ligands including laminin, agrin and perlecan in muscle tissue (Bowe et al., 1994; Campanelli et al.,

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1994; Gee et al., 1994; Sugiyama et al., 1994). On the other hand, βDG is a transmembrane protein and associates with intracellular proteins including dystrophin and nitric oxide synthase that connect αDG and actin cytoskeleton in muscle tissue (Brenman et al., 1995; Ervasti and Campbell, 1991; Grady et al., 1999; Jung et al., 1995; Yoshida et al., 2000). LARGE-mediated glycosylation of αDG is critical for αDG binding to its extracellular ligands, as well as to its role as a functional receptor for LCMV (Hara et al., 2011; Kunz et al., 2005). Extracellular ligands of αDG in C2C12 cells differentiated into myotubes could potentially hamper the interaction between αDG and GP1 of LCMV, thus interfering with LCMV infection of myotubes. We did not perform studies examining possible differences in virus binding to C2C12 myoblasts versus C2C12 myotubes because differentiation of C2C12 myoblasts into myotubes results in a cell population that contains still a significant percentage (40 to 60 %) of non-differentiating myoblasts, which would make inconclussive results from biochemical assays done at the cell population level. However, LCMV has been shown to be able to compete with extracellular matrix ligands for αDG binding in muscle cells (Han et al., 2009). In muscle cells DG bridges extracellular ligands and intracellular proteins that associate with actin cytoskeleton. These interactions form a solid complex and provide structural stability to the sarcolemma during contraction. It is plausible that the complex structure of which αDG form part in skeletal muscle could pose a barrier for LCMV GPC-mediated cell entry.

Upon receptor binding, LCMV is internalized via receptor-mediated endocytosis, which involves a pH-dependent fusion event between viral and cellular membranes within the acidic environment of the endosome (Borrow and Oldstone, 1994; Castilla et al., 1994). The endocytic vesicles of the LCMV are non-coated and LCMV cell entry process was reported to be cholesterol-dependent but clathrin-, dynamin-, caveolin-, ARF6-, flotillin-, and actin-independent (Borrow and Oldstone, 1994; Quirin et al., 2008; Rojek et al., 2008a; Rojek et al., 2008b; Shah et al., 2006; Vela et al., 2007). Recently, we reported that in A549 human cells LCMV GPC-mediated cell entry is NHE-, actin-, and Pak1-dependent, suggesting a contribution of macropinocytosis to cell entry of LCMV (Iwasaki et al., 2013). These findings indicate that LCMV uses a non-classic endocytic pathway that is distinct of the clathrin-depending endocytic pathway used for cell entry by New World arenaviruses, including JUNV (Geisbert and Jahrling, 2004; Martinez et al., 2007; Rojek et al., 2008a; Rojek et al., 2008b). It is plausible that one of processes required for the endocytic pathway used by LCMV is not operational in skeletal muscle, which could contribute to restricted infection and this could be an explanation of the resistance of muscle to LCMV infection. In this regard DG was reported to be associated with the cav-3 isoform of caveolin in C2C12 myotubes (Kogo et al., 2006), which could affect the caveolin-independent cell entry process used by LCMV. However, overexpression of the interaction between cav-3 and in C2C12 did not affect LCMV infection, suggesting that it is highly unlikely that an interaction between cav-3 and DG was responsible for the resistance of C2C12 myotubes to LCMV infection.

Postnatal growth of skeletal muscle in mice is through hypertrophy and requires the participation of satellite cells, which are skeletal muscle stem that within the first few weeks after birth continue to fuse to grow muscle fibers (Cardasis and Cooper, 1975; Knapp et al., 2006). Mice infected as newborns with LCMV develop a chronic infection where viral

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antigen and RNA are readily detected in most tissues and cell types but strikingly absent in skeletal muscle. Differentiation of LCMV-infected C2C12 myoblasts into myotubes did not affect expression levels of LCMV NP, suggesting that a blockade imposed by the intracellular milieu of myotybes is unlikely the reason for the lack of LCMV infection of skeletal muscle cells. In adult mice satellite cells are in a resting state and resume proliferation and differentiation to muscle cells only in response to exercise or trauma induced damage (Charge and Rudnicki, 2004; Seale et al., 2001). Myoblasts are thought to be an active state of satellite cells and committed to become myotubes (Charge and Rudnicki, 2004; Wozniak et al., 2005; Zammit et al., 2006). In healthy muscle tissue of adult mice satellite cells and myoblasts are present at very low numbers, hence it may be rather difficult to identify in LCMV carrier mice potentially infected satellite cells and myoblasts. LCMV-infected myoblasts in carrier mice have the potential to become muscle cells that, based on our results with C2C12 cells, would allow virus multiplication. However, *in vivo* differentiation of satellite cells into myotubes would occur only under conditions that induce muscle damage, which together with the low numbers of cells involved would make it difficult their detection. On the other hand, the high viremia (10^3 to 10^5 PFU/ml in serum) in LCMV carrier mice together with the existence of an extensive network of capillaries that provide skeletal muscle cells with a large blood flow (1–4 ml/min per 100 g under resting conditions) would predict an easy viral access to skeletal muscle cells. Our finding that C2C12 myotubes are highly refractory to LCMV infection could explain why although in LCMV carrier mice skeletal muscle cells are constantly exposed to blood-containing virus they remain free of viral antigen and RNA.

Conclusions

We have shown that C2C12 cells become highly resistant to LCMV infection upon their differentiation into myotubes. We presented evidence that LCMV replication and gene expression was not restricted in C2C12 myotubes. However, despite the LCMV receptor αDG is expressed at high levels in myotubes, LCMV GPC-mediated cell entry was severely restricted. These findings can explain why in LCMV carrier mice skeletal muscle cells remain free of viral antigen and RNA.

MATERIALS AND METHODS

Cells

C2C12 mouse myoblasts were grown in GlutaMax Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin. To induce myogenic differentiation of C2C12 cells, cell culture medium of a confluent cell monolayer was replaced with fresh medium with 2% horse serum (HS). Human myoblasts were obtained from Lonza (Catalog# CC-2580) and grown in DMEM (Invitrogen) containing 20% FBS, 2 mM L-glutamine, 10 μg/ml insulin, 5.5 μg/ml transferrin, 6.7 pg/ml selenium (ITS, Life Technologies), 100 μg/ml fibroblast growth factor, 500 μg/ml epidermal growth factor, 100 mg/ml streptomycin, and 100 U/ml penicillin. To induce myogenic differentiation of human myoblasts, cell culture medium were replaced with DMEM containing 2% HS, ITS, 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. The cell culture medium for myoblasts and myotubes was replaced every

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two days. BHK-21 and 293T cells were grown in DMEM containing 10% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin.

Generation and titration of Viruses

Recombinant Armstrong (rARM) and Clone 13 (rCl-13) strains of LCMV, as well as rLCMVs expressing GPC from vesicular stomatitis virus (VSV) (rCl-13/VSVG) have been described (Pinschewer et al., 2003). A rCl-13 lacking GPC and expressing *Gaussia* luciferase (rCl-13ΔGPC/Cl-13GPC) was generated by reverse genetics using procedures described (Rodrigo et al., 2011). Briefly, the open reading frame (ORF) of *Gaussia* luciferace was amplified by PCR and substituted for the GPC ORF in pPOLI-S plasmid to generate pPOLI-S NP/Gluc. To rescue rCl-13ΔGPC/Cl-13GPC, we transfected BHK-21 cells with plasmids pPOLI-L (Cl-13 strain backbone) and pPOLI-S NP/Gluc that directed intracellular pol1-mediated synthesis of recombinant L and S genome RNA species, together with plasmids pC-L, pC-NP, and pC-LCMV cl-13 GP to provide the trans-acting factors NP and L and the surface GP1/GP2 complex of Cl-13 strain of LCMV. Rescued virus present in cell culture supernatant of transfected cells was amplified by infecting BHK-21 cells transfected with pC-LCMV cl-13 GP to obtain a high titer virus stock. Wild type rVSV (rVSV-WT) and rVSV expressing GPC of LCMV Cl-13 strain (rVSV/Cl-13GPC) have been described (Rojek et al., 2008a). Titers of rLCMVs and rVSVs were determined by immunofocus (Battegay, 1993) and plaque assay, respectively. Recombinant adenoviruses expressing either GFP or cav-3 have been described (Tsutsumi et al., 2008).

Expression and activity of miRNAs

Plasmids (pCxGb backbone) expressing miRNA-1, 133a or 206, as well as plasmids (pSRlb backbone) expressing *Firefly* luciferase (FL) containing miRTS for miRNA-1+206 and 133a within the 3'UTR, were obtained from Dr. Yoshio Kato. To test the activity of miRNA-1, 133a and 206 we transfected 293T cells seeded in 12-well plates at 4×10^5 cells per well and cultured overnight with pCxGb plasmids expressing each individual miRNA-1 (0.2 μg), miRNA-133a (0.5 μg) or miRNA-206 (0.5 μg) together pSRlb plasmids expressing FL-miRTS-1+026 (0.2 μg), FL-miRTS-133a (0.2 μg), or FL control. A plasmid expressing *Renilla* luciferase (RL) (0.1 μg) was incorporated into the transfection mix to normalize transfection efficiencies. Transfections were done using lipofectamine 2000 (2 μl/μg DNA). At 36 hours post-transfection cell lysates were prepared for the Dual-Glo luciferase assay (Promega).

Immunofluorescence assay (IFA)

Mock-and virus-infected cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). After cell permeabilization by treatment with 0.3% Triton X-100 in PBS containing 3% bovine serum albumin (BSA), cells were stained using an anti-NP rat monoclonal antibody (VL-4) and an Alexa Fluor 568-labeled anti-rat secondary antibody, or an anti-VSV-N mouse monoclonal antibody and an Alexa Fluor 568-labeled anti-mouse secondary antibody. Myosin expression in differentiated myotubes was detected by staining using an anti-myosin heavy chain mouse monoclonal antibody conjugated with an Alexa Fluor 488 (MF20-488). Cav-3 expression was detected by staining using an anti-cav-3

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mouse monoclonal antibody and an Alexa Fluor 488-labeled anti-mouse secondary antibody. Nuclei were detected by 4',6-diamidino-2-phenylindole (DAPI). Stained cells were observed under a confocal microscope (LSM 710, Zeiss).

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- C2C12 myoblasts were highly susceptible to LCMV infection.
- C2C12 myotubes are resistant to LCMV infection.
- LCMV RNA replication and gene expression is not restricted in C2C12 myotubes.
- Cell entry mediated by the arenavirus surface glycoprotein is restricted in C2C12 myotubes.

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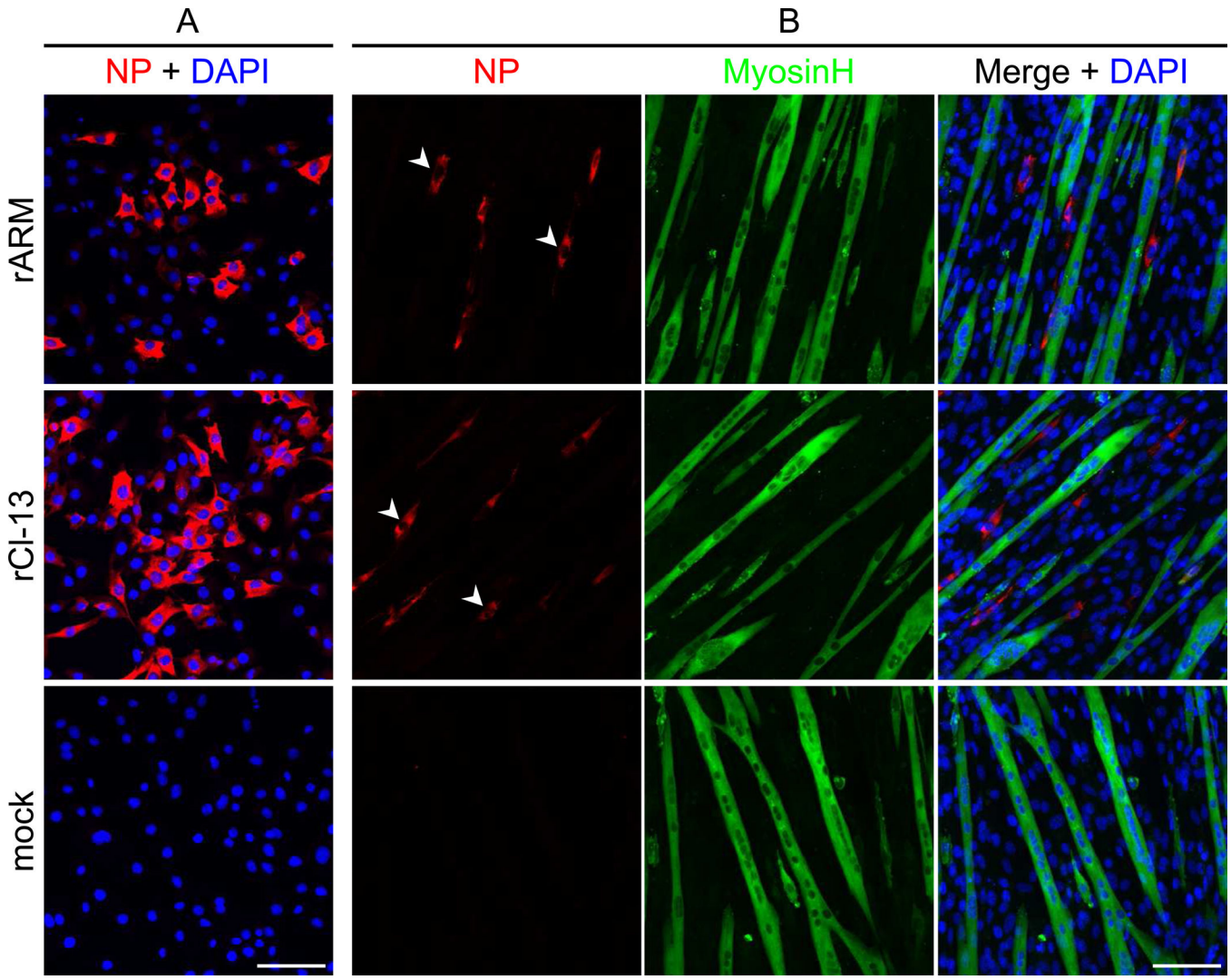

Figure 1. Differentiation of C2C12 cells into myotubes is associated with resistance to LCMV infection

A. C2C12 cells were seeded in 24-well plates onto cover slips (3.5×10^4 cells per well) and cultured overnight. Next day, cells were infected with rARM or rCl-13 using 1×10^5 FFU per well or remained uninfected (mock). At 16 h p.i., cells were fixed with 4% PFA and expression of NP detected by IF using a rat monoclonal ab against LCMV NP (VL-4). Nuclei were detected by DAPI. **B.** C2C12 cells prepared as in A were placed in DMEM containing 2% HS and cultured for four days to allow for their differentiation into myotubes prior infection with either rArm or rCl-13 using 1×10^5 FFU per well. At 16 h p.i., infected cells were fixed with 4% PFA and expression of NP and myosin heavy chain (MyosinH) detected by IF using the rat MAb VL-4 to NP and mouse Mab MF20-488 to MyosinH, respectively. Nuclei were detected by DAPI staining. Arrowheads indicate the C2C12 myoblasts expressing LCMV NP. Bars,100 μm.

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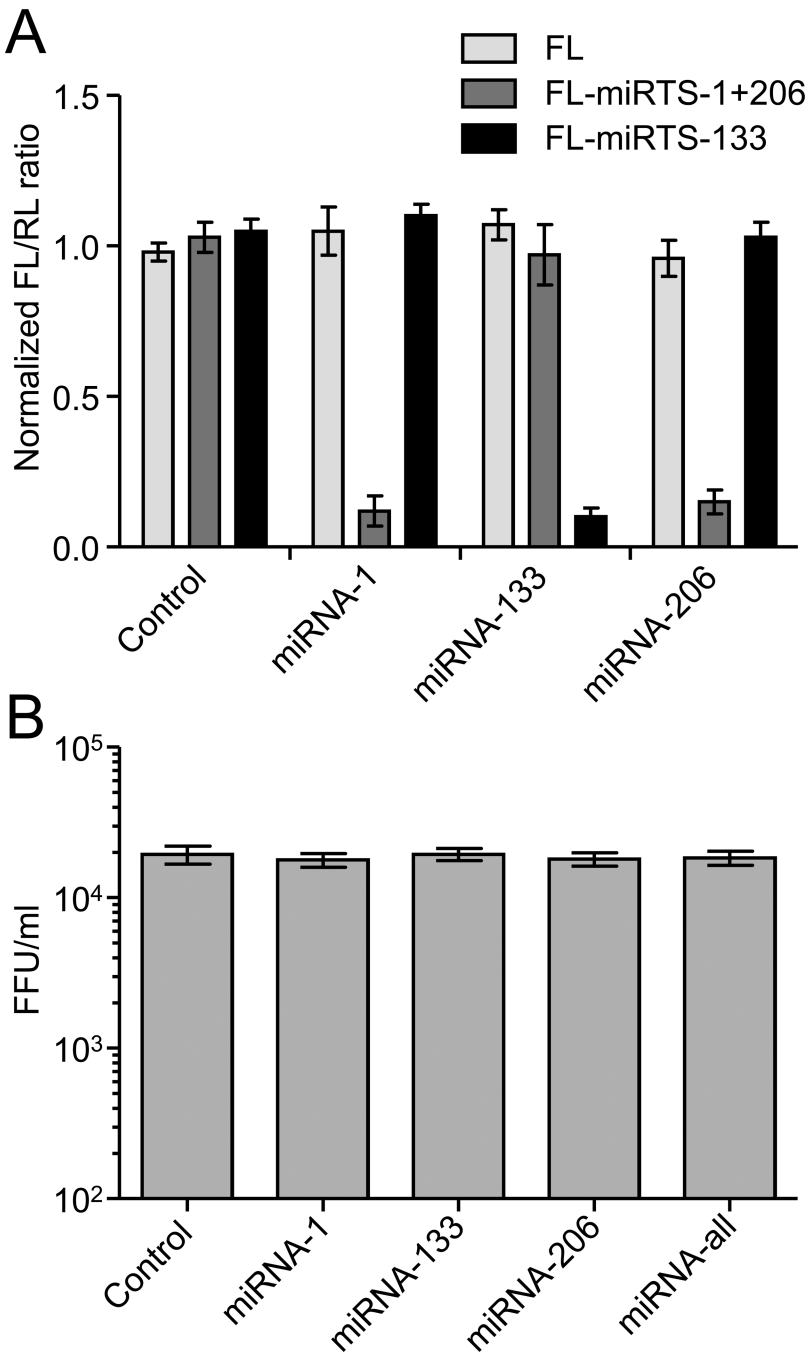

Figure 2. Effect of over-expression of miRNA-1, 133a or 206 on LCMV multiplication

A. Activity of miRNA-1, 133 and 206 in 293T cells. 293T cells seeded on 12-well plates at 4×10^5 cells per well and cultured overnight were transfected with the indicated combination and amounts of plasmids expressing miRNAs and FL containing miRTS within its 3'-UTR, together with a plasmid expressing RL to normalize transfection efficiencies. At 36 h post-transfection cells lysates were prepared for the Dual-Glo luciferase assay. Results represent the average and SD of three independent experiments. **B.** 293T cells were transfected with the indicated miRNA-expressing plasmid (miRNA-all: miRNA-1, 133, and

206) and 24 h later infected (moi = 0.001) with LCMV. Production of infectious progeny in tissue culture supernatants was determined at 24 h p.i. Results represent the average and SD of five replicates.

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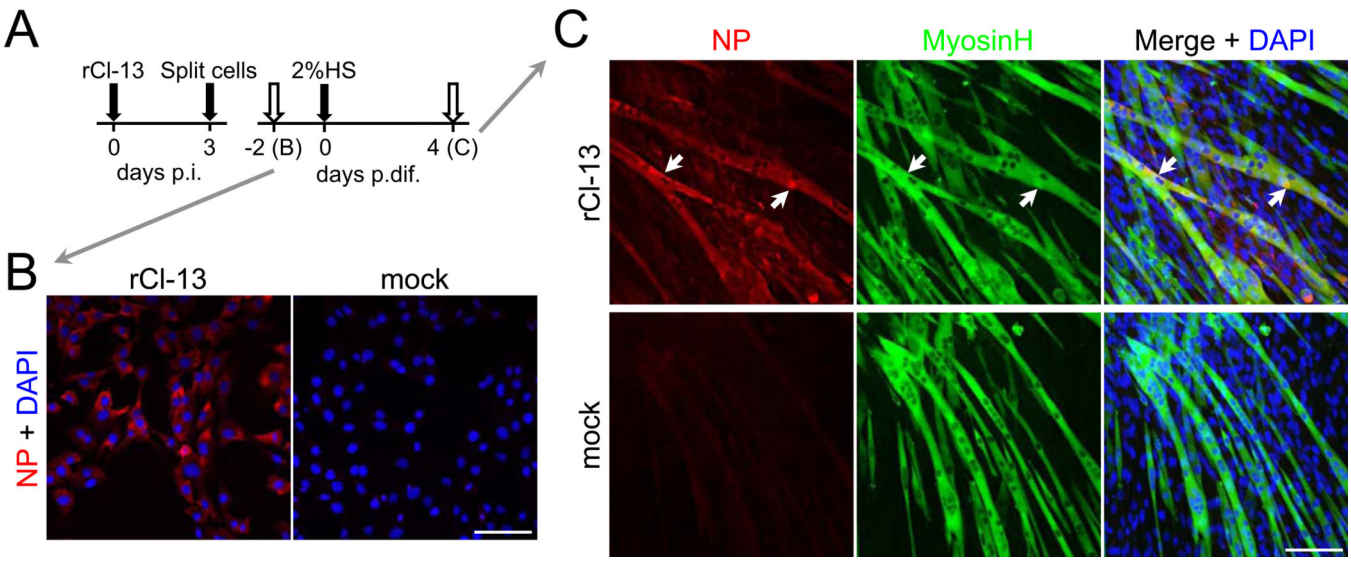

Figure 3. LCMV multiplication and gene expression is not inhibited by the myotube gene expression program

A. Schematic diagram of the experiment. Open arrows indicate the time at which cells were fixed. C2C12 cells were infected with rCl-13 (moi = 0.1). Three days later infected cells were seeded in 24-well plates onto cover slips (3.5×10^4 cells per well). Two days before **(B)** or four days after **(C)** starting differentiation by placing the cells in DMEM + 2 % HS, cells were fixed with 4% PFA and expression of NP and MyosinH detected by IFA using MAb VL-4 and MF20-488, respectively. Nuclei were detected by DAPI staining. Arrows indicate C2C12 myotubes expressing LCMV NP. Bars,100 μm.

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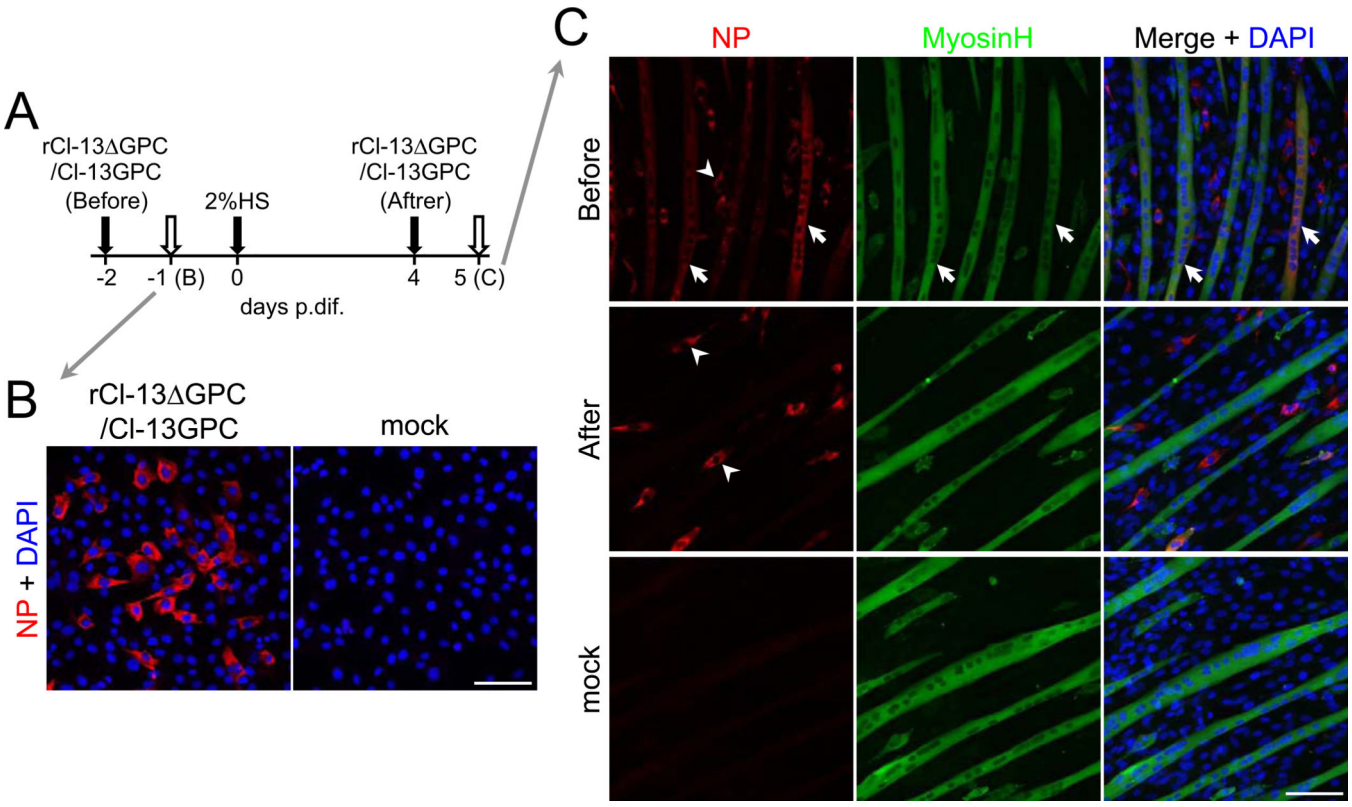

Figure 4. Infection of non-diferentiated (myoblasts) and differentiated (myotubes) C2C12 cells infected with the single-cycle infectious rCl-13ΔGPC/Cl-13GPC

A. Schematic diagram of the experiment. Open arrows indicate times at which cells were fixed and NP expression determined by IF. **B.** C2C12 (myoblasts) were infected with rCl-13ΔGPC/Cl-13GPC and at 16 h p.i. fixed and examined for NP expression by IF using the VL-4 MAb to NP. Nuclei were identified by DAPI staining. **C.** C2C12 myoblasts were seeded in 24-well plates onto cover slips (3.5×10^4 cells per well). Two days before (Before) or four days after (After) starting differentiation into myotubes, cells were infected with rCl-13ΔGPC/Cl-13GPC using 1×10^5 FFU per well. At 24 h p.i., cells were fixed with 4% PFA and stained with VL-4 and MF20-488 for the detection of NP and myosin heavy chain, respectively. Nuclei were detected by DAPI staining. Arrows and arrowheads indicate C2C12 myotubes and myoblasts, respectively, expressing LCMV NP. Bars,100 μm.

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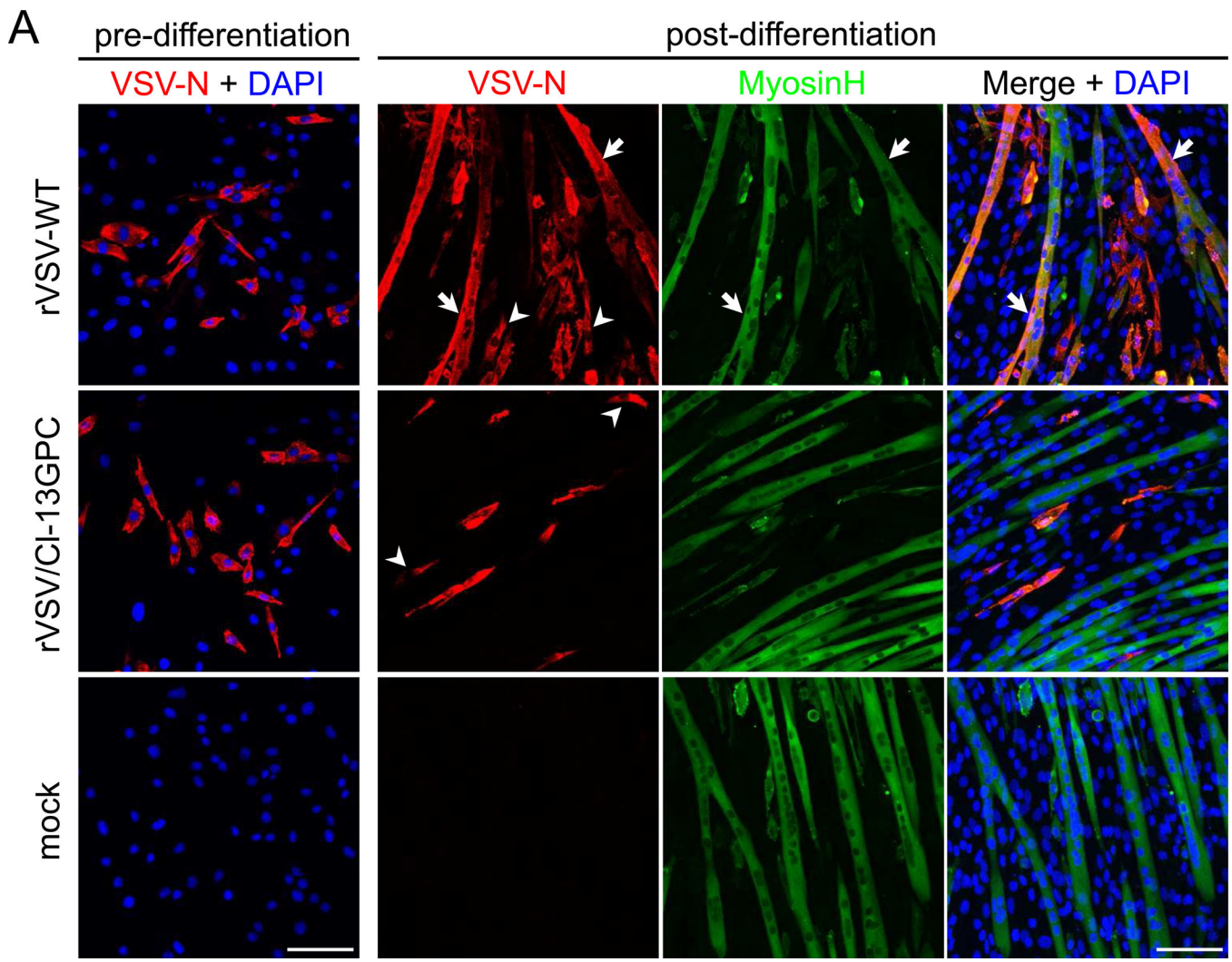

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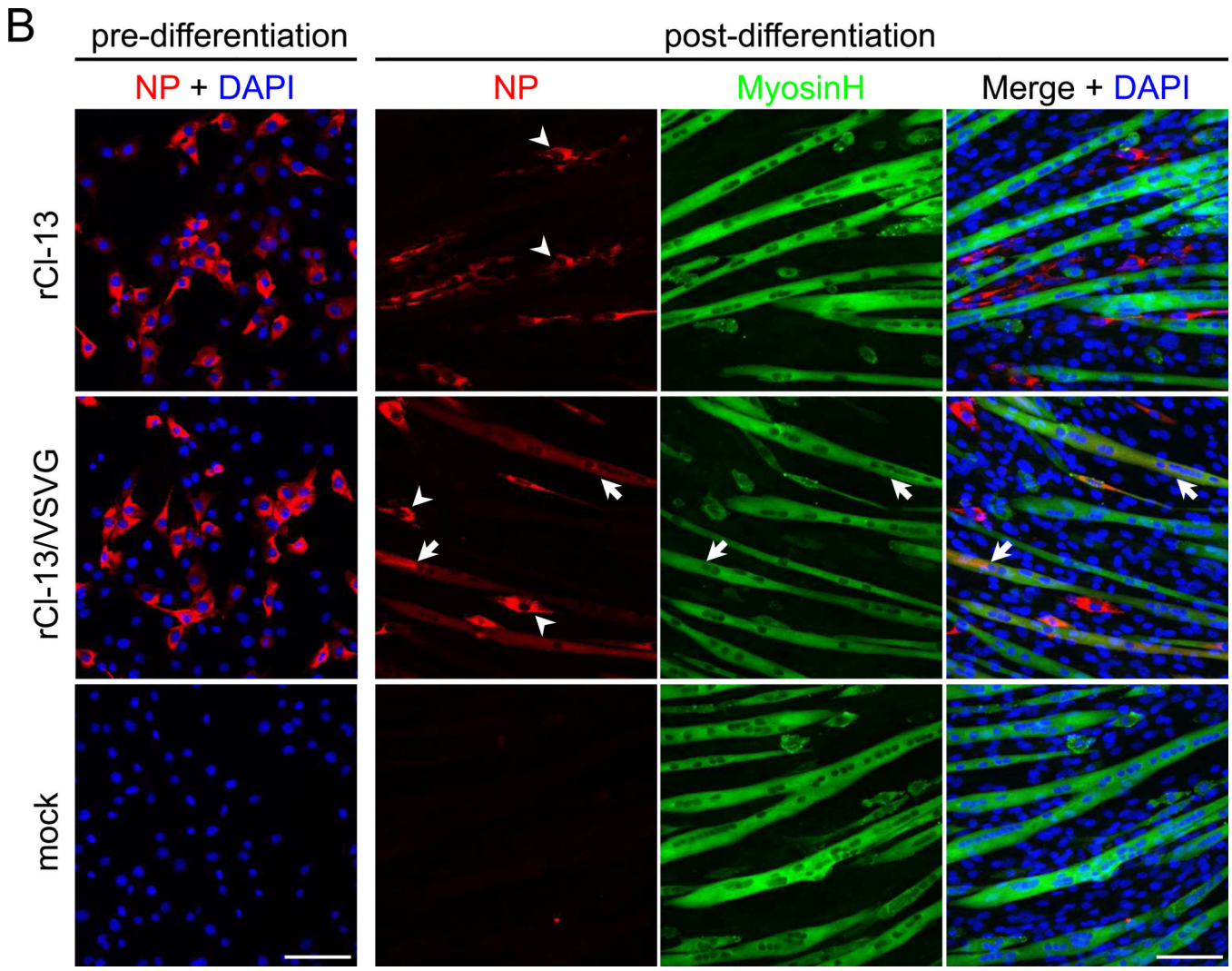

Figure 5. Resistance of C2C12 myotubes to LCMV infection is mainly determined by an impaired LCMV GPC-mediated cell entry

C2C12 myoblasts were seeded in 24-well plates onto cover slips (3.5×10^4 cells per well). Two days before (pre-differentiation) or four days after (post-differentiation) starting differentiation into myotubes, cells were infected with the indicated viruses. **A.** Cells were infected with rVSV-WT or rVSV/Cl-13GPC using 1×10^5 PFU per well. **B.** Cells were infected with rCl-13 or rCl-13/VSVG using 1×10^5 FFU per well. At 7 h (rVSVs) or 16 h (rLCMVs) p.i., cells were fixed with 4% FPA and stained with a MAb against VSV N protein (VSV-N), a rat MAb VL-4 (NP), and also, only post-differentiation, mouse MAb MF20-488 (MyosinH). Nuclei were detected by DAPI staining. Arrows and arrowheads indicate C2C12 myotubes and myoblasts, respectively, expressing LCMV NP. Bars,100 μm.

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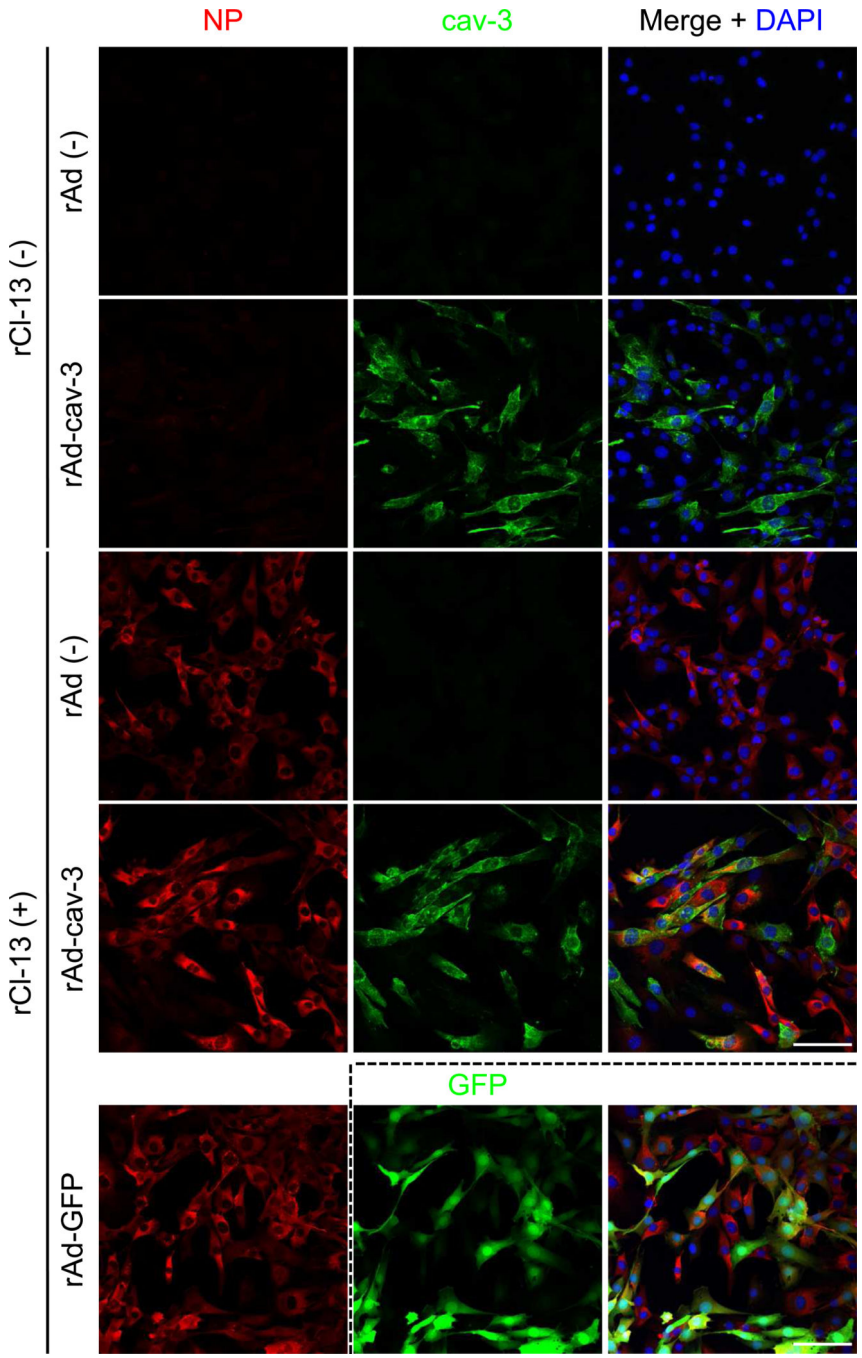

Figure 6. Overexpression of cav-3 does not affect the susceptibility of C2C12 myoblasts to LCMV infection

C2C12 were transduced with recombinant adenoviruses expressing either GFP (rAd-GFP) or cav-3 (rAd-cav3) (moi = 5), or mock-transduced. At 36 h after transduction cells were late infected with rCl-13 (moi = 0.1) and 24 later fixed (4% PFA/PBS) and process for IF using a mouse monoclonal antibody to cav-3 and a rat monoclonal antibody (VL4) to LCMV NP. Expression of GFP was observed directly without the use of antibody staining. Cells transduced with rAd-GFP expressed very high levels of GFP whose fluorescence

signal interfered with the visualization of the NP antigen. To overcome this problem images for GFP expression were collected using a shorter exposure time. Bars, 100 μm.

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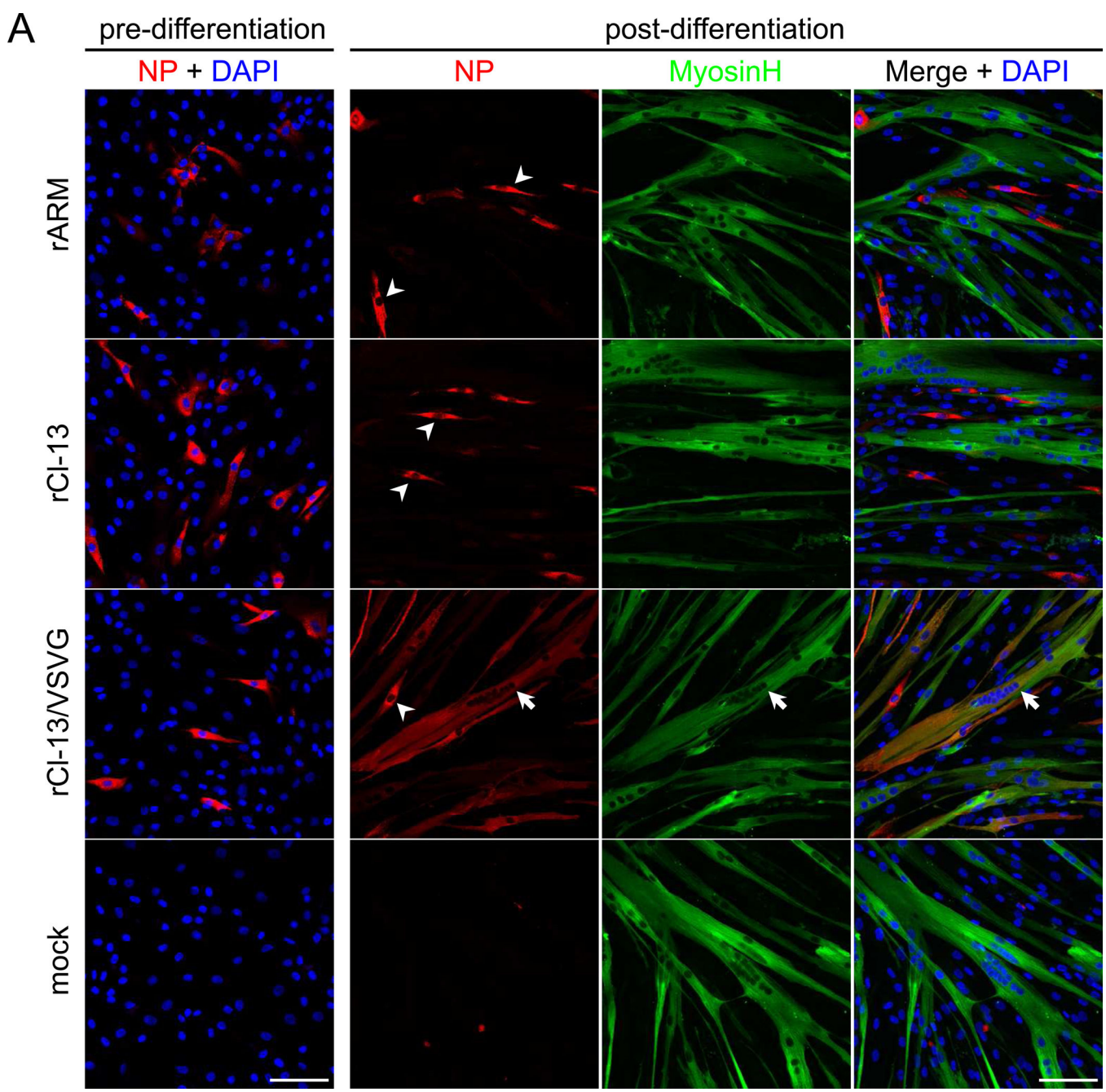

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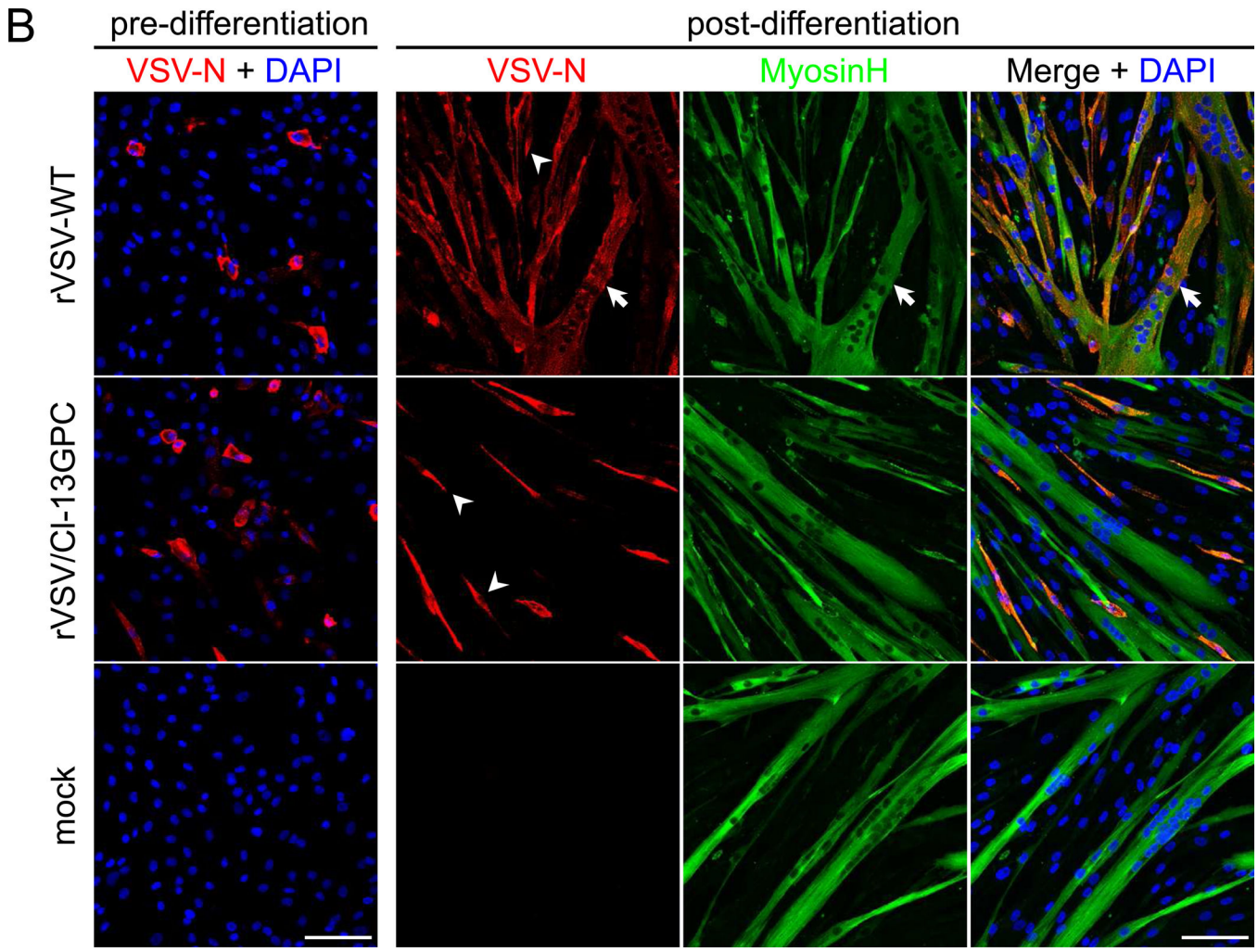

Figure 7. Human myotubes are resistant to LCMV infection

Human myoblasts were seeded on 96-well plates at 2×10^4 cell per well. Two days before (pre-differentiation) or four days after (post-differentiation) starting differentiation, cells were infected with the indicated viruses. **A.** Cells were infected with rARM, rCl-13, or rCl-13/VSVG at 2.5×10^4 FFU per well. **B.** Cells were infected with rVSV-WT, or rVSV/Cl-13GPC at 1×10^5 PFU per well. At 7 h (rVSVs) or 16 h (rLCMVs) p.i., cells were fixed with 4% PFA and stained with mouse MAbs to VSV N protein (VSV-N), LCMV NP (VL-4), or MyosinH (MF20-488). Nuclei were detected by DAPI staining. Arrows and arrowheads indicate human myotubes and myoblasts, respectively, expressing viral proteins. Bars, 100 μm.