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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.

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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 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

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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, 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, 2007, 2006), which likely contributes to facilitate the LCMV carrier state in mice.

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 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

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