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African swine fever virus infects macrophages, the natural host cells, via clathrin- and cholesterol-dependent endocytosis

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ABSTRACT

The main cellular target for African swine fever virus (ASFV) is the porcine macrophage. However, existing data about the early phases of infection were previously characterized in non-leukocyte cells such as Vero cells. Here, we report that ASFV enters the natural host cell using dynamin-dependent and clathrin-mediated endocytosis. This pathway is strongly pH-dependent during the first steps of infection in porcine macrophages. We investigated the effect of drugs inhibiting several endocytic pathways in macrophages and compared ASFV with vaccinia virus (VV), which apparently involves different entry pathways. The presence of cholesterol in cellular membranes was found to be essential for a productive ASFV infection while actin-dependent endocytosis and the participation of phosphoinositide-3-kinase (PI3K) activity were other cellular factors required in the process of viral entry. These findings improved our understanding of the ASFV interactions with macrophages that allow for successful viral replication.

1. Introduction

The invasion of its host cell by a determined virus is a process that can be considered as a sequence of successive steps, including virus binding, internalization, uncoating, early transcription-translation, genome replication, late protein synthesis and virus particle morphogenesis, to finally release the viral progeny. The viral entry mechanism in the cell would determine viral tropism and pathogenesis, then, it is crucial to establish which entry pathway results in a successful productive infection. The main cellular target for African swine fever virus (ASFV) is the porcine macrophage where it was first shown to enter via a receptor-mediated endocytosis (Alcami et al., 1990). However, most studies on ASFV entry have been performed in Vero cells and using viral isolates adapted to this cell line. Early studies first characterized this event as a low pH and temperature-dependent process (Alcami et al., 1989a, 1989b; Valdeira and Geraldes, 1985). Several years later, ASFV entry by endocytosis was characterized as a dynamin- and clathrin-dependent process in Vero and WSL cells (Hernaez and Alonso, 2010) and the effect of a number of inhibitor drugs on ASFV entry in macrophages was reported (Basta et al., 2010). More recent studies found that ASFV induces macropinocytosis (Sanchez et al., 2012). Finally, also the Rho-GTPase Rac1 was found activated upon virus entry (Quetglas et al., 2012; Sanchez et al., 2012).

In fact, few cell lines support a productive ASFV infection, being the natural host cell, the porcine macrophage, the best option to grow ASFV in culture. ASFV is able to enter a wide range of cell types from a number of different species in vitro, but in most of these cells, its entry does not lead to a productive infection (Alcami et al., 1990; Carrascosa et al., 1999). Susceptible porcine macrophages can be of several origins such as peripheral blood, lungs, bone marrow, etc. In principle, a successful ASFV infection is more related to the maturation stage of the macrophages and linked to the expression of the CD163 scavenger receptor. In fact, antibodies to CD163 are able to inhibit both ASFV infection and viral particle binding to alveolar macrophages, highlighting the role of this molecule as a putative receptor for the virus (Sanchez-Torres et al., 2003).

For efficient cell entry, animal viruses employ several endocytosis strategies. Both enveloped and non-enveloped viruses have been shown to use clathrin-mediated pathway. Examples of these are influenza virus (Chen and Zhuang, 2008) and Semliki forest virus (Helenius et al., 1980), between others. The

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plasma membrane (PM) does not present a continuous or homogeneous composition. It contains lipid microdomains, known as lipid rafts, characterized by their high content in cholesterol and sphingolipids (Jacobson et al., 2007). The caveolae, a subset of membrane rafts, are involved in the entry of simian virus 40 (SV40) (Pelkmans et al., 2001) and echovirus type 1 (EV1) (Marjomaki et al., 2002), between others. Dynamin is necessary for the scission of both clathrin- and caveolae-derived endocytic vesicles (Henley et al., 1998). The endocytic pathway is a common route for viruses entering by clathrin- and caveolin-mediated entry processes. Some viruses use macropinocytosis to enter cells, such as vaccinia virus (VV) (Mercer and Helenius, 2008), ebolavirus (EBOV) (Nanbo et al., 2010) and human immunodeficiency virus (HIV) (Marechal et al., 2001). By macropinocytosis, the virus is internalized into vesicular structures formed by membrane protrusions termed "ruffles" in a dynamin-independent manner. Once internalized, macropinocytosis-specific endosomes (macropinosomes) are believed to mature into endocytic vesicles (Mercer and Helenius, 2008).

In this study, we use chemical agents and molecular methods to investigate the cellular mechanism exploited for ASFV entry into the natural target cell. Our data show that virus enters into macrophages by a pH-dependent, dynamin-dependent endocytic pathway involving clathrin, actin and cholesterol. ASFV uses endocytosis to infect host cells and takes advantage of several endocytic pathways to initiate infection.

2. Materials and methods

2.1. Cells and viruses

Vero cells were obtained from the American Type Culture Collection and grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5% heat-inactivated fetal bovine serum (FBS) which was reduced to 2% during viral infection. Swine alveolar macrophages were collected by alveolar lavage with phosphate-buffered saline (PBS) as previously described (Carrascosa et al., 1982) and cultured at 37 °C in RPMI medium containing inactivated 10% swine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 20 mM Hepes and 30 μg/ml gentamycin. The ASFV isolates, BA71V, adapted to grow in Vero cells and the virulent 608 VR13, with a low passage number in Vero cells (Alfonso et al., 2004), were propagated and titrated by plaque assay on Vero cells as described (Enjuanes et al., 1976). Purified ASFV was prepared by ultracentrifugation at 40,000 × *g* through a 40% (w/v) sucrose cushion in PBS 1X for 50 min at 4 °C. Recombinant B54GFP is a recombinant ASFV expressing green fluorescent protein as a fusion protein of viral p54 (Hernaez et al., 2006). Infections with Ba71V, 608 VR13 and B54GFP were performed at a multiplicity of infection (moi) of 1 pfu/cell. The size distribution of virus preparations was between 150 and 200 nm as determined using a filter device and magnetic (QNano, Izon). Vaccinia virus (VV) recombinant vtag2GFP containing the tag2GFP under the control of a strong synthetic VV early/late promoter (Blasco and Moss, 1995) was kindly provided by Dr. R. Blasco (INIA, Spain). VV infections in primary macrophages were performed at a moi of 1 pfu/cell. After viral adsorption at 4 °C (synchronic infection) during 60 min followed by one wash with cold PBS, macrophages were incubated at 37 °C for the times indicated.

2.2. Inmunofluorescence

Swine alveolar macrophages were grown on coverslips at 60% confluence and fixed in 4% paraformaldehyde in PBS for 12 min. After washing in PBS, macrophages were permeabilized or not for 15 min with PBS – 0.1% Triton X-100. Then, the following primary antibodies against macrophage receptors and surface proteins were used undiluted: anti-CD172a, extensively used as the main marker of swine myeloid cells in swine and anti-CD163 and CD169 (also known as Sialoadhesin) as specific and characteristic macrophage markers. Antibodies against the swine leukocyte antigen class I (SLA) and against the lysosome-associated membrane protein 2 (LAMP2 or CD107a) were used as controls. A monoclonal antibody against major virus capsid protein p72 (Ingenasa) was used at a working dilution of 1:1000 and Alexa Fluor 488-conjugated cholera toxin subunit B (Molecular Probes) was used (1 μg/ml) as a marker for lipids raft, membrane microdomains enriched in cholesterol and sphingolipids. A secondary antibody conjugated to Alexa fluor-488 was purchased from Molecular Probes and cell nuclei were detected with TOPRO3 (Molecular Probes) following the Manufacturer's instructions. Coverslips were mounted onto glass slides using ProLong Gold (Invitrogen) and examined in a TCS SPE confocal microscope (Leica).

2.3. Drug treatments

Macrophages were pretreated for 30 min at 37 °C with each of the inhibitors followed by cold synchronized infections. Where indicated, inhibitors were added to previously infected cells at 2 or 3 hpi. Stock solutions were dissolved in DMSO as follows: 10 mM dynasore (Dyn) (Calbiochem), 100 μM bafilomycin A (Baf) (Sigma), 50 mM nystatin (Nys) (Sigma), 50 mM 5-ethylisopropyl amiloride (EIPA) (Sigma), 0.5 mM jasplakinolide (Jasp) (Calbiochem), 0.5 mM latrunculin A (LAT) (Calbiochem), 5 mM cytochalasin D (CytoD) (Sigma), 17 mM blebbistatin (Blebb) (Sigma), 5 mM wortmannin (Wort) (Stressgen) 10 mM LY294002 (LY29) (Gibco) and 10 mM Dyngo4a (Dy4a) (Abcam Biochemicals). Water was used as solvent for 2 mM chlorpromazine (CPZ) (Sigma) and 25 mM methyl-β-cyclodextrin (CD) (Sigma). All inhibitors (except for CD which was removed from cells at 2 hpi), were present throughout all the experiments. Previously, we pursued cell viability and cytotoxicity tests of all inhibitors using the CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega) following the Manufacturer's instructions. This study was required given that we used the highest doses reported in the literature to ensure the efficacy of inhibitors. We also studied the cytotoxic activity of the organic solvent DMSO. Based on these experiments we selected optimal non-toxic working concentrations for infection assays.

For blocking experiments, macrophages were pretreated in serum-free RPMI medium containing dimethyl sulfoxide (DMSO) as control, or 40 or 80 μM Dyn, 0.1 μM Baf, 50 or 100 μM Nys, 50 μM EIPA, 0.1 or 2 μM Jasp, 0.1, 1 or 2 μM LAT, 1, 5 or 10 μM CytoD, 50 or 100 μM Blebb, 5 or 10 μM Wort, 1, 5 or 10 μM LY29, 14 or 30 μM CPZ, 5 or 12 mM CD, 20 or 30 μM Dy4a. These drug-treated cells were subsequently infected with ASFV or VV at a moi of 1 pfu/cell, incubated in growth medium for 6 h at 37 °C and harvested for flow cytometry analyses or Western blotting, or for 16 h at 37 °C and harvested for detection of viral DNA by quantitative PCR, or for 24 h at 37 °C and harvested for viral production by plaque assay. Table 1 shows a description of the each chemical compound used in this work.

2.4. Flow cytometry analysis

Characterization of macrophages by flow cytometry has been based on the set of surface markers described above and previously used for immunofluorescence. Detection of infected cells was also performed by flow cytometry. Swine alveolar macrophages were pretreated with inhibitors at the indicated concentrations in growth medium for 30 min at 37 °C, followed by infection with