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Involvement of lipid raft during feline herpesvirus (FHV-1) infection on permissive cells: A potential target for antiviral treatment

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ABSTRACT

Feline herpesvirus 1 (FHV-1) is a common virus responsible for more than half of feline respiratory syndromes and kitten mortality. Despite recent findings regarding the interactions between FHV-1 and cellular pathways, no information is available on its relationship with lipid rafts, which are cytoplasmic membrane protrusions involved in a variety of cellular functions. The aim of this study was to evaluate the involvement of lipid rafts during FHV-1 infection on permissive cells (CRFK). The use of a specific inhibitor (MβCD) prior to viral adsorption resulted in a significant reduction in viral titers (assessed by $TCID_{50}$) and viral glycoprotein expression (assessed by immunofluorescence and Western blot). Supplying soluble cholesterol to the cells prior to infection replenished the lipid rafts, restoring viral titers and glycoprotein expression to normal. These results were also confirmed by the viability assay, which revealed higher viability in MβCD-treated cells compared to mock. We also observed a decrease in cell viability when cholesterol was replenished, due to cytopathic effects mediated by the virus. The results of this study have highlighted a relevant role for lipid rafts during FHV-1 infection, enriching our understanding of the FHV-1-cell interaction and contributing to rethink them as a potential target for antiviral treatment.

1. Introduction

Orthoherpesviridae are described in several animal species. In addition to causing latent infections, they are generally responsible for skin lesions, abortion, and respiratory signs. Cats are no exception and there is a single serotype of feline herpesvirus type 1 (FHV-1), today renamed *Varicellovirus felidalpha-1* (Thiry et al., 2009). FHV-1, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, is a primary pathogen of cats and other felids as the causative agent of feline viral rhinotracheitis (FVR) (Gould, 2011). This worldwide infection is commonly characterized by upper respiratory and ocular disease, whereas kittens and compromised cats may show neurological disorders and fatal forms (Stiles, 2014). Fever, sneezing, coughing, ocular and nasal discharges, depression, and excessive salivation are some of the clinical outcomes typical of FHV-1 (Thiry et al., 2009). However, in more vulnerable animals, the infection can be more serious, resulting in acute conjunctivitis, stomatitis, respiratory syndromes, and other concurrent diseases. Since the conjunctivae and respiratory mucosa are extensively infected and source of viral shedding, the virus spreads between healthy and diseased cats via the oral or nasal route (Longobardi et al., 2024b). FHV-1 uses the latency mechanism to ensure its survival in the host. Latency is lifelong and during this condition, the virus is present as naked episomal DNA genome within the neuronal nuclei of the trigeminal ganglia, waiting for reactivation (often primed by external stimuli such as stress, immunodepression, or other concurrent infections) (Tai et al., 2010). Several recent investigations have shed light on the pathophysiology of FHV-1 (Lee et al., 2019). It has recently been demonstrated that this virus affects the PI3K/AKT/mTOR axis during its entry into the permissive cells, which involves a pH and dynamin-dependent endocytosis (Ferrara et al., 2023c.; Synowiec et al., 2023). Other scientific evidence supports FHV-1 ability to induce autophagy and apoptosis to promote its own replication (Ferrara et al., 2023a, 2023b). Despite this novel information about virus-host cell interactions, no information is available in the literature concerning the relationship between FHV-1 and lipid rafts.

The plasma membrane includes detergent-insoluble raft microdomains (10–200 nm), called “rafts”, that are rich in sphingolipids and

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cholesterol (Suomalainen, 2002). Rafts are involved in a variety of cellular functions (Ripa et al., 2021). Membrane rafts can concentrate or segregate particular elements into cells while controlling their interactions with the extracellular space. As a result, lipid rafts are critical for maintaining physiological activities such as intracellular lipid and protein trafficking during cellular processes (like migration, apoptosis, etc.), signal transduction, receptor activation, endocytosis, and extracellular vesicle formation (Suomalainen, 2002). Furthermore, lipid rafts play crucial functions in the life cycles of various viral families (especially enveloped viruses), such as virus entrance, assembly, and/or budding processes (Gee et al., 2023; Matveeva et al., 2023). Viruses can exploit the specific features of lipid rafts through interactions mediated by their glycoproteins (Ripa et al., 2021). Several viruses use lipid rafts for their entry into the cell (Bukrinsky et al., 2020). These include human pathogens, such as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), and pathogens of veterinary interest (Li et al., 2021). Herpesviruses, as enveloped DNA viruses, have an obvious link with lipid rafts. Herpes simplex virus (HSV-1), pseudorabies virus (PRV), caprine herpesvirus type-1 (CpHV-1), bovine herpesvirus (BHV-1), and anatid alphaherpesvirus 1 (AnHV-1) are all examples of close viral interaction with lipid rafts (Desplanques et al., 2008; Pratelli and Colao, 2016; Yang et al., 2022). Furthermore, some enveloped viruses can use these plasma membrane components for their egress. Considering the lack of research in the literature on the interaction between lipid rafts and FHV-1, the aim of this work was to shed light on the role of lipid rafts in FHV-1 entry and viral replication.

2. Materials and methods

2.1. Cell culture, virus and reagents

Permissive CRFK cells (ATCC CCL-94) were grown under sterile conditions using Dulbecco Modified Eagle's Medium (DMEM, Corning) supplemented with fetal bovine serum (FBS, Corning) and a specific antibiotic (Penicillin-Streptomycin, Corning). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and when the monolayers showed a confluence between 80 and 90%, they were infected with an MOI of 1 of the FHV-1 strain Ba/91. Infection was performed in different formats (6-well plate, 96-well plate) depending on the type of experiment. Methyl-β-cyclodextrin (MβCD) and cholesterol (Cholesterol-Water Soluble) were supplied by Sigma.

2.2. Lipid raft depletion and cholesterol replenishment

FHV-1 strain Ba/91 was used (MOI 1) to infect CRFK monolayers grown on a 6-well plate using MβCD, a lipophilic chemical that removes cholesterol from membranes, impairs lipid raft formation on cells, and hence prevents biological activities that rely on lipid rafts. MβCD was dissolved (10, 15, 20, 30 mM) directly in DMEM and incubated with cells one hour before viral adsorption. Water-soluble cholesterol was applied (500 μg/mL) for one hour, between MβCD treatment and viral adsorption, to restore cholesterol from cellular membranes following the depletion caused by MβCD. After an incubation of 24 h, supernatants were collected to perform viral titration using Reed and Muench's method, and pellets were utilized for protein extraction and western blot analysis (Longobardi et al., 2025; Montagnaro et al., 2019). After quantification and electrophoresis on polyacrylamide gels, proteins from each sample were transferred to nitrocellulose membranes with a TransBlot Turbo (BioRad). Each membrane was previously blocked with bovine serum albumin (BSA) and incubated with a panel of antibodies, including anti-FHV-1 (Novus Biologicals), β-actin (SantaCruz Biotechonology), Akt (SantaCruz Biotechnology), and p-Akt (Cell-Signaling Technology). Each antibody was incubated in BSA at a 1:000 dilution overnight and, after three washes with 1× Tween-Tris-buffered saline (TBST), replaced with a suitable secondary antibody for an hour (anti-mouse and anti-rabbit, Cell Signaling). The reading was completed using ECL Substrate (BioRad) and a ChemiDoc Blot scanner (BioRad).

2.3. Cholesterol measurement and cell viability assay

The experiment was reproduced in 96-well plates with the same conditions in order to measure the viability of control and infected cells treated with different amounts of MβCD (10, 15, 20, 30 mM) and cholesterol using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (Forte et al., 2021). Cholesterol in CRFK cell membranes was evaluated using the Amplex® red cholesterol assay kit (ThermoFisher Scientific). The Amplex® Red Cholesterol Assay Kit employed a fluorescence microplate reader to accurately quantify cholesterol and compare results to a standard curve (Huang et al., 2011; Martín et al., 2012). The evaluation of absorbance for MTT and for the determination of cholesterol was carried out using a spectrophotometer (ThermoFisher scientific).

2.4. Immunofluorescence assay

Infected and control cells grown on a slide glass in 24-well plates were fixed, permeabilized and incubated at 4 °C overnight (dilution 1:400) with a specific FHV-1 monoclonal antibody (Novus Biologicals) as previously described. A secondary-labeled antibody (Anti-mouse secondary antibody, Alexa Fluor 488) (dilution 1:800) was incubated for one hour at room temperature (Ferrara et al., 2023b). The image acquisition was performed using a ZOE Fluorescent Cell Imaging System (Bio-Rad) apparatus.

2.5. Statistical analysis

All experiments were carried out three times. Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) using multiple *t*-test or one-way ANOVA (multiple comparisons), which were expressed as the mean standard deviation. A p-value <0.05, 0.01, or 0.001 was considered statistically significant and represented by one, two, or three asterisks.

3. Results

The use of MβCD prevented the entry of FHV-1 with a significant reduction in viral titers for all the tested doses, especially when a concentration of 20 or 30 mM was used. The use of these inhibitors led to a reduction of the viral titer from 10^6 to 10^4 $TCID_{50}$ (Fig. 1a). Cholesterol replenishment after MβCD treatment, however, restored viral titers, that appeared similar to those of the infected cells (between 10^5 and 10^6). When these titers were compared with the corresponding MβCD treatment, they were always significantly higher (Fig. 1a). These titers were also compared to those of the infection; no significant differences were found, showing that the titers had returned after the cholesterol replenishment. This result was confirmed by the immunofluorescence assay, as the green fluorescence linked to the presence of viral glycoproteins in infected cells was lower in cells treated with MβCD and comparable to that of infected cells after cholesterol restoration (Fig. 1b).

Western blot analysis highlighted both a reduction in the expression of two viral glycoproteins (gB and gI) and in the phosphorylation of cellular Akt in cells treated with MβCD (Fig. 2). The expression of viral proteins as well as the phosphorylation of Akt returned to basal levels following cholesterol levels restoration (Fig. 2). The decrease in p-Akt was not due to a constitutive decrease in Akt, as the p-AKt/Akt ratio followed a similar pattern (as shown in Fig. 2).

Treatment with MβCD was not cytotoxic with the exception for the highest dose (30 mM), which caused a reduction in viability of approximately 15% (Fig. 3a). The MMT assay was also used to compare the viability of each treatment. Particularly, cell viability increased proportionally with increasing doses of MβCD. Following cholesterol

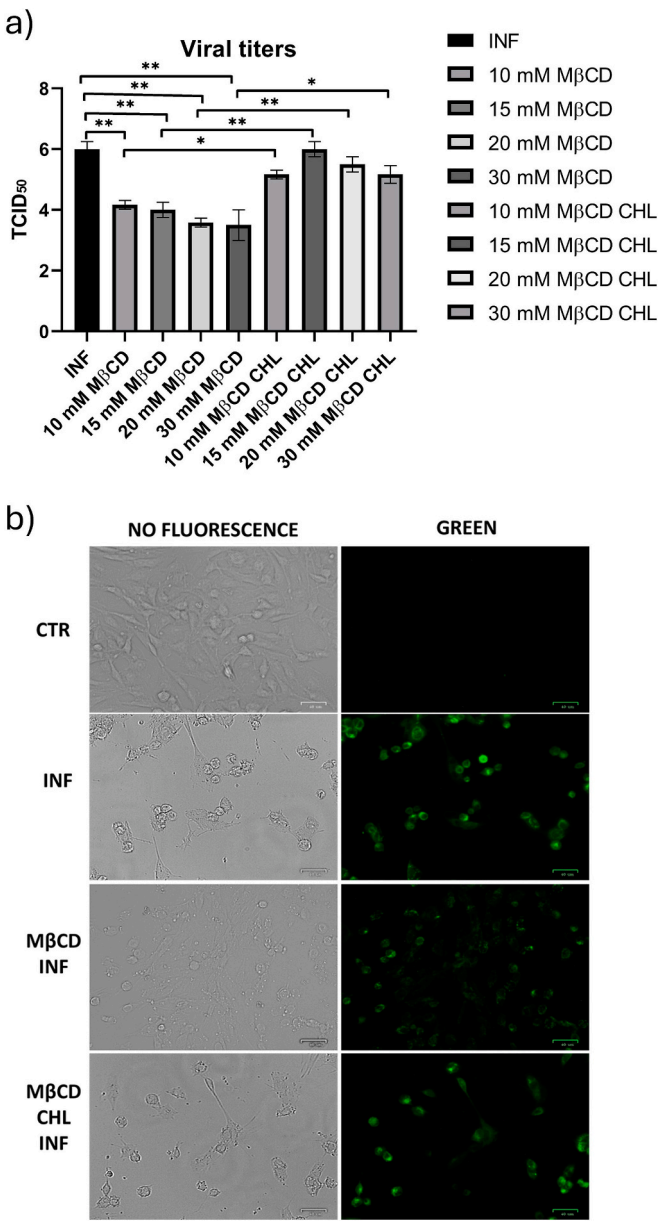

Fig. 1. Evaluation of FHV-1 infection after treatment with MβCD and cholesterol: significant reduction in viral titers after treatment with different dose of MβCD (a). Cholesterol replenishment restored virus titers to normal with no significative difference when compared to the infected cells (a). This occurrence was investigated utilizing immunofluorescence with a specific anti-FHV-1 antibody. Fig. 1b shows the reduction of fluorescence in cells treated with MβCD and the difference with cells that received cholesterol replenishment.

replenishment, we observed a reduction in viability secondary to the cytopathic effect mediated by FHV-1 (except for the 30 mM dose) (Fig. 3a).

Quantification by Amplex® red cholesterol assay kit demonstrated the effective reduction of cellular cholesterol in CRFK cells treated with MβCD and the effective reintegration of cholesterol in the cell membrane when this was added. In addition, a slight reduction in cellular cholesterol content was observed in infected cells (Fig. 3b). Cholesterol concentrations in cells treated with MβCD were consistently lower than those of control and infected cells. Despite being reduced, this disparity remained considerable with the addition of cholesterol, except for treatment with 10 mM MβCD, which entirely restored the cholesterol level to normal.

4. Discussion

The results obtained with this study showed that FHV-1 uses lipid rafts to promote its attachment/entry as well as other members of the *Orthoherpesviridae* family. Interestingly, similar experiments carried out on caprine herpesvirus type-1 (CpHV-1) observed a dose-dependent reduction in viral titers after MβCD treatment (Pratelli and Colao, 2016). Similar findings were described for pseudorabies virus (PRV), Cyprinus herpesvirus 3 (CyHV-3), and Duck enteritis virus (DEV), consistent with cholesterol-rich lipid rafts being likely relevant for the entry/egress of herpesviruses of veterinary interest (Desplanques et al., 2008; Yang et al., 2022). Similarly, this was shown for herpes simplex (HSV-1), where the simultaneous recruitment of cellular receptors (nectin-1) in the rafts mediated by viral glycoproteins (gH) (Azab et al., 2013). Sphingomyelin (the most prevalent sphingolipid in the mammalian cell membrane) was shown to have an important role in the infection of different animal herpesviruses (Pastenkos et al., 2019). The use of specific inhibitors (imipramine or amitriptyline) did not significantly limit BoHV-1 entrance, however significantly decreased PRV entry (Favoreel et al., 2004). These findings suggest that alphaherpesviruses vary in their entrance requirements for cellular sphingomyelin or lipid rafts (Pastenkos et al., 2019).

Dependence on lipid rafts for entry has also been described in viruses other than herpesviruses. For example, three different compounds (MβCD, mevinolin, and filipin), with different mechanisms of action, proved effective in eliminating lipid rafts and simultaneously reducing porcine reproductive respiratory syndrome virus (PRRSV) viral titers (Huang et al., 2011; Yang et al., 2015). The role of rafts for entry was demonstrated in a long list of viruses belonging to different families, including canine distemper virus (CDV), bovine parainfluenza virus (BPIV), Porcine Epidemic Diarrhea virus (PEDV), African Swine Fever virus (ASFV), Canine Parvovirus type 2 (CPV-2), and infectious bursitis virus (IBV) whose entry is likely mediated by a lipid raft-dependent pathway (Guo et al., 2017; Harbison et al., 2009; Imhoff et al., 2007; Li et al., 2017; Wei et al., 2020; Zhang et al., 2023). Similar evidence was described for both human (such as SARS-CoV-2) and animal (such as canine enteric coronavirus, CECoV) coronaviruses (Li et al., 2021; Palacios-Rápalo et al., 2021; Pratelli and Colao, 2015; Roncato et al., 2022).

In the present study, a reduction in Akt phosphorylation was observed following the use of MβCD (also concurrent with a reduction in the p-Akt/Akt ratio). This phenomenon has also been widely described for other antiviral drugs or compounds capable of reducing the replication of FHV-1 with different mechanisms (Longobardi et al., 2024a; Longobardi et al., 2024b; Ferrara et al., 2023c). Interestingly, the cellular levels of p-Akt, appear to correlate with viral entry and severity of infection. In recent studies FHV-1 cell entry causes a significant phosphorylation of Akt as early as three hours after infection, and the use of specific inhibitors may determine a significant reduction in viral titers (Ferrara et al., 2023c.). Further investigations would be instrumental to elucidate whether the rafts are directly associated with the PI3K/Akt/mTOR pathway (as described for other viruses, like coxsackievirus A16), or they represent two distinct pathways involved in cell-viral interaction during FHV-1 replication (Wu et al., 2021). On the other hand, a previous study described that MβCD treatment did not prevent virus binding to cells during CpHV-1 infection (Pratelli and Colao, 2016). Currently, cell receptors for FHV-1 have not been well characterized, and further research is needed to determine if MβCD treatment alters the expression of receptors involved in viral attachment. However, MβCD is extremely toxic to cells (as also evidenced by MTT viability in this study), and its use as a direct therapeutic agent raises significant safety concerns. Conversely, drugs with similar effects on lipid rafts or specific monoclonal antibodies could have a potential antiviral effect against FHV-1 and other viruses. Because lipid rafts are more than just viral entry points, but they are also required for a variety of physiological cellular functions (such as signal transduction, cell adhesion, and membrane trafficking), the design of a therapeutic

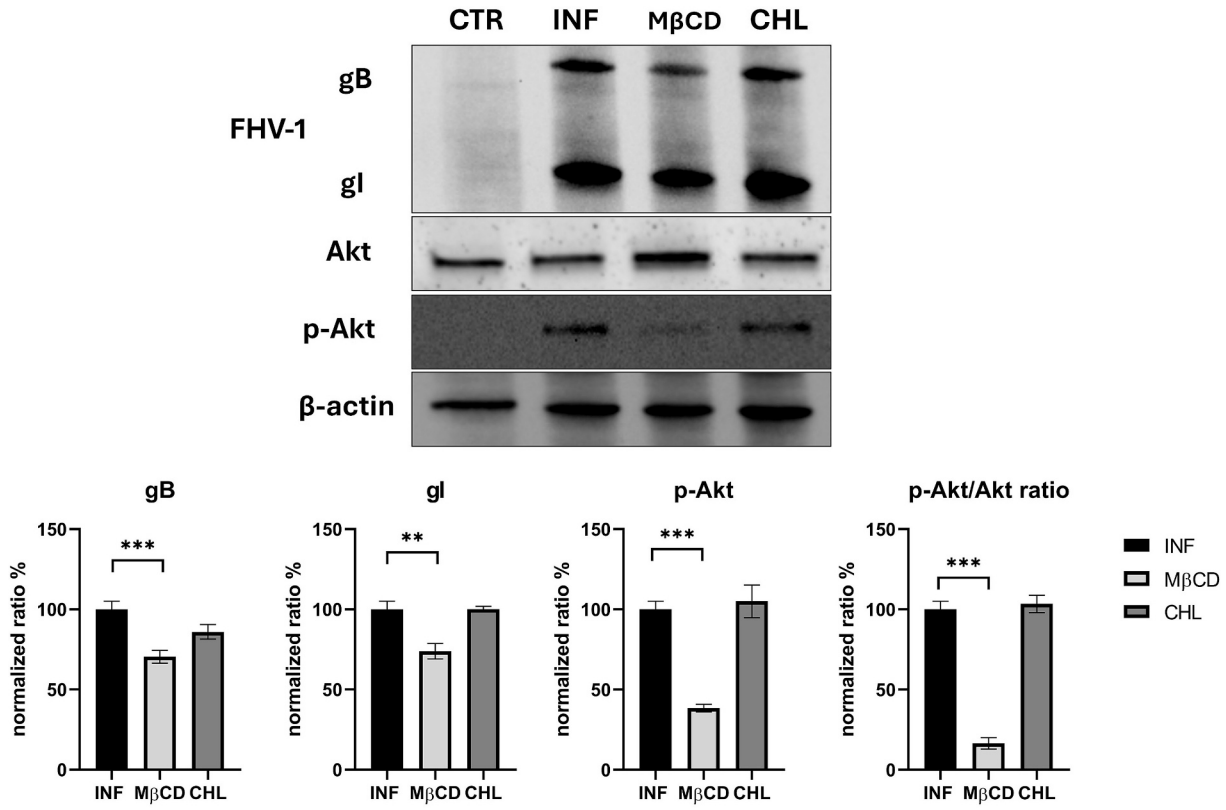

Fig. 2. Western blot analysis of infected cells treated with MβCD and cholesterol: the treatment with MβCD induces a reduction in the expression of viral glycoprotein (gB and gI) and a reduction in the phosphorylation of Akt (p-Akt). The ratio p-Akt/Akt revealed a similar trend. The use of cholesterol reverses these effects, restoring viral glycoprotein expression and p-Akt levels to normal. Full blots are available as supplementary files.

strategy that does not interfere with these critical cellular communication pathways could be difficult.Summarizing the results of recent works focusing on FHV-1 entry, it was shown that virus may access cells (i) via pH and dynamin-dependent endocytosis (as the infection was significantly inhibited by NH4Cl, bafilomycin A1, dynasore, and mitmab); (ii) via caveolin-mediated endocytosis (genistein, nystatin, and filipin treatments, and knock-down of caveolin-1 inhibit the entry of FHV-1, as well as colocalization between FHV-1 and caveolin-1 has been described); (iii) and clathrin-mediated endocytosis (knock-down of the clathrin heavy chain has reduced viral titer, and colocalization of virus particles with clathrin has been demonstrated) (Synowiec et al., 2023). Moreover, the PI3K/Akt/mTOR axis, as well as lipid rafts, might be implicated (Ferrara et al., 2023c).

A further limitation of this study involves the unfeasibility to determine which viral process is dependent on lipid rafts (probably the early stages), but more experiments are needed to determine the exact phase in which these structures play a role in viral replication (attachment, entry, or budding). The absence or the reduction of lipid rafts had a role as well in the release of mature virions in the extracellular compartment in previous works performed on other viruses (Suomalainen, 2002). Further investigation is also required to completely understand the interactions of these membrane components with viruses in order to employ them as antiviral targets.

5. Conclusions

Lipid rafts are cholesterol- and glycosphingolipid-rich microdomains found throughout cells that facilitate cellular communication through signal transduction and membrane trafficking and, at the same time, are employed by pathogens to invade cell membranes. Here we provided solid preliminary evidence consistent with a clear role of lipid raft in the early stages of FHV-1 infection. Future research is needed to determine the involvement of lipid rafts in other specific viral processes, as well as to explore them as a possible target for antiviral treatment.

CRediT authorship contribution statement

Consiglia Longobardi: Resources, Methodology, Investigation, Formal analysis, Data curation. **Ugo Pagnini:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Formal analysis, Conceptualization. **Hyun-Jin Shin:** Visualization, Resources, Data curation, Conceptualization. **Francesco Origgi:** Writing – review & editing, Writing – original draft, Visualization, Resources. **Gianmarco Ferrara:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Ethics approval

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

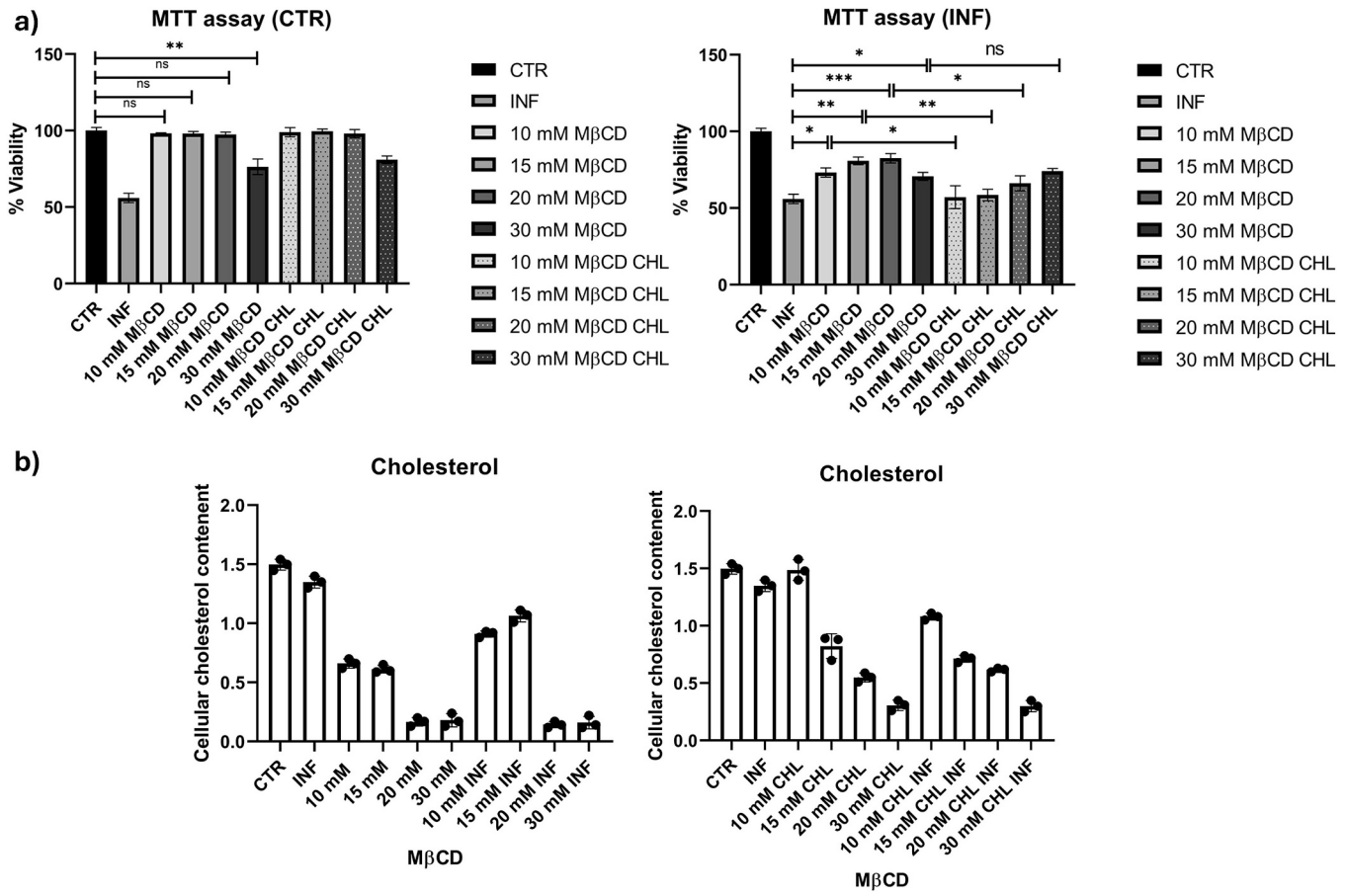

Fig. 3. Evaluation of viability and cholesterol content in infected cells treated with MβCD and cholesterol: treatment with MβCD alone was tolerated at all doses except the highest (a). Cells treated with MβCD had higher viability than the mock. Adding cholesterol reduced viability because it restored viral replication (a). The effects of the different experimental conditions on total cholesterol content were confirmed with the Amplex® Red Cholesterol Assay Kit (b).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2026.106218.

Data availability

Not applicable (no data sets were generated or analysed during the current study).

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