

RESEARCH ARTICLE

The role of lipid rafts in cell entry of human metapneumovirus

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Abstract

Human metapneumovirus (hMPV) is a crucial pathogen in children. A cell entry is the first step for infection. Our previous study indicated that there was an endocytosis pathway for hMPV cell entry. Lipid raft is a specific structure at the cell surface and it has been demonstrated to play an important role in endocytosis process of many viruses. In this study, we investigated whether and how lipid raft can take part in the hMPV entry. The confocal microscope was used to detect colocalization of hMPV and lipid raft marker. We demonstrated that colocalizations were increased along with the viral infection and hMPV particles transferred to the perinuclear region with lipid raft. When specific lipid raft inhibitors: methyl- β cyclodextrin and nystatin were used, hMPV cell entry was inhibited and viral titer decreased dramatically. With the replenishment of exogenous cholesterol, hMPV recovered quickly. These data suggest that lipid raft plays an important role in hMPV endocytosis and maybe one of the pathways for hMPV cell entry.

KEYWORDS

cell entry, human metapneumovirus, lipid raft

1 | INTRODUCTION

Human metapneumovirus (hMPV) was firstly isolated from airway secretions and identified in 2001. Based on viral gene sequence analysis, hMPV belongs to *Metapneumovirus* genus, *Paramyxoviridae* family, and *Pneumovirinae* subfamily.^{1,2} Global epidemiological surveys showed that hMPV is prevalent worldwide with severe bronchiolitis and pneumonia.³ The morbidity of hMPV infection varies from 3% to 14.3% and the predominate strain is subtype A.⁴⁻⁷ The susceptible populations of

hMPV are infants, young children, immunocompromised individuals, and elderly people.⁸⁻¹⁰ Children under 5 years old have almost been infected by hMPV. Recently, respiratory problems caused by hMPV became more and more obvious and cannot be neglected anymore. hMPV could cause lethal infection in posttransplant patients and bring more serious consequences when host coinfects with other virus.^{11,12} However, to date, there is no effective vaccine for prevention from hMPV infection.

There are two main pathways for virus cell entry, fused with cellular membrane (fusion) and endocytosis.¹³⁻¹⁵ Endocytosis is a complex process, in this procedure, the virus binds to specific receptors on cell membrane firstly. Then downstream signaling

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cascades are activated and endocytic internalization is promoted. Finally, virus particle could be endocytosed as an intact form and transported to specific organelles through vesicles. According to different vectors, the main four kinds of endocytic pathways are defined, such as clathrin-, caveolae-, cholesterol-mediated endocytosis, and macropinocytosis.¹⁶ Recently, hMPV had been found to invade BEAS-2B cells via endocytosis¹⁷ and in our previous studies, we also found that hMPV uses endocytosis pathway in Vero E6 and LLC-MK2 cell lines.¹⁸

The cell membrane is the first barrier to prevent the invader. According to the classic liquid mosaic model theory, cell membrane is a homogeneous lipid bilayer embedded with different proteins. Lipid raft plays a critical role in signal transduction and protein trafficking. It is also important to establish cell polarity.^{19,20} Besides, lipid raft has a crucial impact on the viral entry process of many viruses. It was found that lipid raft participated not only in early infection of EV71 virus but also in the binding process of hepatitis C virus. It is not only the key element of human immunodeficiency virus-1 (HIV-1) viral entry, but also the "gateway" in Ebola virus budding.²¹⁻²³

In this study, we have found morphological evidence that hMPV can colocalize with lipid rafts and enter target cells via lipid rafts. When lipid raft inhibitor was used, hMPV cell entry was inhibited.

2 | MATERIALS AND METHODS

2.1 | Cell culture and virus stocks

16HBE cells, purchased from the China Center for Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, CA) in an incubator at 37°C containing 5% CO₂. Besides, the culture medium contains 10% fetal bovine serum (Gibco) and penicillin-streptomycin antibiotics (HyClone, Los Angeles, CA). hMPV used in this study belongs to genotype A (hMPV NL/1/00).³³ Vero-E6 cells were used for virus propagation and the virus particles were released to medium after freeze-thaw at -80°C. After centrifugation to remove cell debris, the supernatant was stored at -80°C.

2.2 | Cell viability assays and cholesterol quantification

To detect whether the two chemical agents affect cell viability, 16HBE cells were seeded at a density of 1×10^4 /mL in 96-well (Corning, NY) plates. Cells were incubated for 1 hour after treating with different concentrations of methyl- β -cyclodextrin (M β CD) and nystatin. Then 10 μ L of Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) solution was added into the medium. After incubated for 60 minutes in the incubator, the fluorescence absorbance at 450 nm was detected by a microplate reader. With a formula, the fluorescence absorbance was used to calculate cell viability.

With or without drugs, Free Cholesterol Assay Kits (Applygen Technologies, Beijing, China) was used to quantify the cellular

cholesterol concentration after treating with 1, 5, 7.5 mg/mL M β CD. According to the experimental manual, the cholesterol concentration was calculated from the standard curve.

2.3 | Cholera toxin B binding (CTB)

16HBE cells were seeded at a density of 2×10^5 /mL in glass bottom cell culture dish (NEST, Hangzhou, China). After washing with phosphate-buffered saline (PBS) three times and incubated at 4°C for 1 hour, Alexa 488-conjugated CTB (Life Technologies, Carlsbad, CA) at 10 μ g/mL and purified hMPV (MOI = 10) was inoculated into the experimental group at the same time. After this, 16HBE cells were placed at 4°C for 1 hour so that CTB and hMPV were attached to the cell surface, but could not initiate endocytosis. Then 16HBE cells were transferred to 37°C for 0, 30, 60, and 120 minutes to initiate the endocytosis of CTB and hMPV. At last, immunofluorescence analysis after washing with PBS three times.

2.4 | Treatment with cholesterol disrupting agents and exogenous cholesterol

16HBE cells were seeded in 24-well plates (Corning) and cell density was 2×10^5 /mL. After washing with serum-free DMEM three times, 16HBE were treated with 1, 5, 7.5 mg/mL M β CD (Sigma, Munich, Germany) and 10, 20, 30 μ g/mL nystatin (Sigma) at 37°C for 1 hour; two pharmacological agents were diluted in serum-free DMEM. In the cholesterol replenishment experiment, 16HBE cells were treated with 5 mg/mL M β CD for 1 hour at 37°C, then water-soluble cholesterol (Sigma) was added to the culture system for 1 hour. The final concentration of cholesterol is 50 and 100 μ g/mL. After the operation which mentioned above were completed, 16HBE cells were inoculated with the same amount of hMPV (MOI = 10). After each step above, 16HBE cells were washed with serum-free DMEM three times to remove the residual drug and unbound virus. Virus titers were detected by quantitative polymerase chain reaction (qPCR) after 24 hours postinfection.

2.5 | Treatment with M β CD after hMPV enter cells

To further investigate the effect of M β CD on virus infection after cell entry, 16HBE was inoculated with hMPV (MOI = 10) 37°C for 120 minutes, washed three times with 0.1 M PBS in 24-well plate, inoculated with different concentrations of M β CD (1, 5, 7.5 mg/mL) and treated at 37°C for 1 hour, the cells were washed three times with PBS after 1 hour. Add 500 μ L of 3% virus maintenance solution and continue to incubate for 24 hours at 37°C. qPCR detected the viral load.

2.6 | CTB as a control for internalization

16HBE cells were treated with 5 mg/mL M β CD at 37°C for 1 hour and washed with PBS for three times. The final concentration of CTB was 10 μ g/mL at 37°C for 2 hours and washed with PBS for three

times, the cell was fixed with 4% paraformaldehyde, while the control group was not treated with M β CD.

2.7 | hMPV cell entry after inhibitors treatment

16HBE cells were cultured in glass bottom dish. After washing with PBS three times, 16HBE cells were treated with 5 mg/mL M β CD and 20 μ g/mL nystatin at 37°C 1 hour, inoculated with the same amount of hMPV (MOI = 10) after washing with PBS three times. After this, 16HBE cells were placed at 4°C for 1 hour so that hMPV was attached to the cell surface but could not initiate endocytosis. 16HBE cells were transferred to 37°C for 120 minutes to initiate endocytosis. Immunofluorescence analysis after washing with PBS three times.

2.8 | hMPV adsorption assay

16HBE cells were seeded in 24-well plates and treated with 1, 5, 7.5 mg/mL M β CD and 10, 20, 30 μ g/mL nystatin at 37°C for 1 hour. Cells were inoculated with the same amount of hMPV (MOI = 10) after washing with PBS three times. The 24-well plates were placed in 4°C for 1 hour to allow the virus binding to the cells. The unbound virus was washed away with iced PBS, and the cells were immediately lysed to tested viral titers.

2.9 | Immunofluorescence assays (IFAs)

16HBE cells were first fixed with 4% paraformaldehyde for 15 minutes at room temperature, permeated by 0.5% Triton X-100 (Sigma) for 15 minutes. Then 16HBE cells were blocked with 1% bovine serum albumin for 1 hour. At last, 16HBE cells were stained with anti-hMPV antibodies (Millipore, MA) at 37°C for 1 hour and Alexa 546 conjugated anti-IgG antibodies (Life Technologies) at room temperature at room temperature for 120 minutes.

2.10 | Quantitative real-time PCR (RT-PCR)

hMPV RNA was extracted from the same amount of 16HBE cells using TaKaRa MiniBEST Viral RNA/DNA Extraction Kit (Takara, Beijing, China) according to the manufacturer. PrimeScript RT reagent Kit (Takara) was used to reverse the hMPV RNA into complementary DNA. The forward primer: GAGCA ATAGC ACTCG GTGTT G; the reverse primer: TCACA AAATC TTCA GCTCT CTCAC; the probe: TTGCC AACAC ACGAA CTCCA TTCCC. The reaction program was 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The reaction was conducted in a CFX96 Fluorescence Thermocycler (Bio-Rad, Hercules, CA), and the data were analyzed with CFX Manager (Bio-Rad) software.

2.11 | Statistical analysis

Results were the percentage of the experimental group and the control group. And data were analyzed by one-way analysis of variance using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA). * is used to represent the *P* value: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

3 | RESULTS

3.1 | hMPV F protein colocalized with lipid raft markers

To determine whether the lipid rafts participate in the process of hMPV cell entry, the morphological analysis was used. CTB subunit is a lipid raft-dependent internalization marker. It binds to its receptor Gangliosides and enter cells through lipid raft.²⁴ As shown in the results, the colocalization of CTB and hMPV were found in cells. The number of colocalization of CTB and hMPV increased in each cell with time increases (Figure 1A). Further analysis showed that the

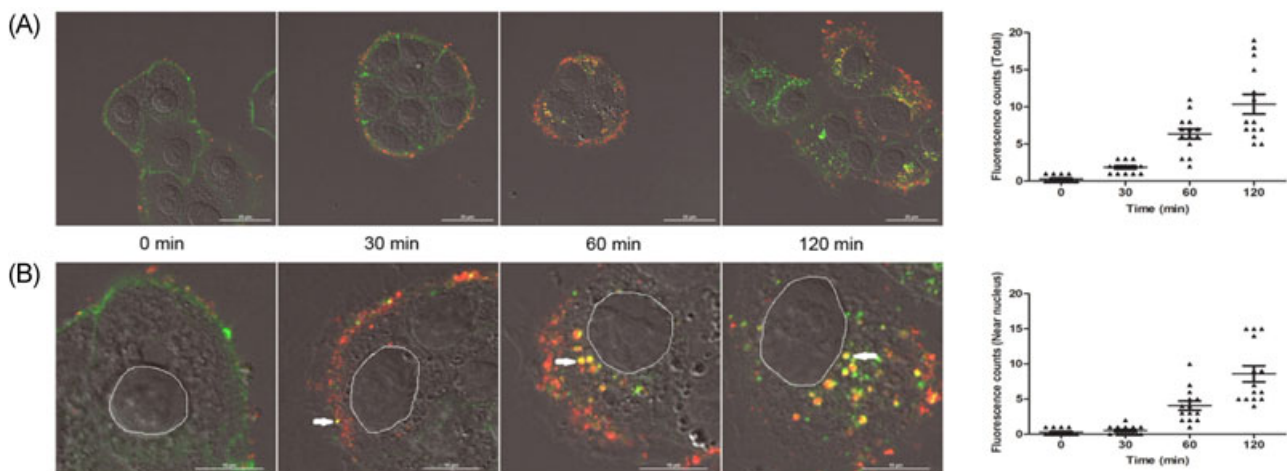


FIGURE 1 hMPV colocalizes with lipid raft markers 16HBE cells were infected with purified hMPV (MOI = 10), and Alexa 488-conjugated CTB of 10 μ g/mL was added at the same time. A, CTB (green) and purified hMPV (red) colocalize in 16HBE cells, the number of colocalization points (yellow) was analyzed by NIS Elements AR 3.2 software. B, The positions of the colocalization points were marked by white arrow and the number of colocalization points near the nucleus was analyzed. CTB, cholera toxin B; hMPV, human metapneumovirus

colocalization points appeared on the surface of cell membrane at 30 minutes, but appeared in the cytoplasm or around the nucleus at 60 and 120 minutes. To facilitate analysis, the cytoplasm was divided into two parts. One part was one-third of cytoplasm closing to the cell membrane, and another was two-thirds of cytoplasm near the nucleus. The number of colocalization points around the nucleus is increasing with time (Figure 1B). These results indicated that hMPV colocalized gradually toward the nucleus with lipid rafts.

3.2 | Cholesterol depletion by M β CD inhibits hMPV infection

M β CD was used to remove cholesterol from the surface of the cell membrane. As a derivative of cyclic oligosaccharides, M β CD can rapidly remove cholesterol from the surface of the cell membrane and this will result in the disruption of lipid raft.²⁵ After treating with three different concentrations of M β CD, virus titer was tested by qRT-PCR. The cells were washed three times with 0.1 M PBS before M β CD and virus inoculation. M β CD could reduce hMPV titer significantly ($P < 0.05$), and hMPV titer decreased gradually with the increase of concentration (Figure 2A). The CCK-8 cytotoxicity assay was used to detect cell viability after drug treatment, and the results showed that M β CD had no effect on the 16HBE cells viability (Figure 2B). The concentration of cholesterol in varying concentrations M β CD-treated 16HBE using Free Cholesterol Assay Kits revealed a dose-dependent decreasing. Compared with the untreated cell, the cholesterol was decreased by 18%, 34%, and 47%, respectively (Figure 2C). The cholesterol concentration was only reduced by 18%, but hMPV reduced by 81% dramatically. These results showed that cholesterol depletion could reduce hMPV titers, and the reduction of hMPV titer was not caused by drug toxicity.

3.3 | Replenishment of exogenous cholesterol restores hMPV infection

After pretreating with M β CD, water-soluble cholesterol was added to the culture system. When exogenous cholesterol was added to the culture system and treated with M β CD, the virus titer had an increasing trend. When exogenous cholesterol concentration is 100 μ g/mL, the decrease of hMPV titer causing M β CD was recovered (Figure 2D).

3.4 | Cholesterol chelating agent, nystatin, inhibits hMPV infection

To exclude a pleiotropic effect of M β CD, another pharmacological agent nystatin was used. Nystatin belongs to polyene macrolide antibiotic and can bind to cholesterol selectively through which the cholesterol in the lipid raft is sequestered.²¹ Three different concentrations of nystatin were used to treat 16HBE cells. The cells were washed three times with 0.1 M PBS before nystatin treatment and virus inoculation. Nystatin could reduce hMPV titers too, and hMPV titers decreased gradually accompanied by concentration increasing (Figure 2E). Compared with the untreated cell, hMPV titer

decreased by 44%, 63%, and 89%, respectively ($P < 0.05$). Nystatin had no toxic effect on the 16HBE at all concentrations (Figure 2F). These results showed that cholesterol chelating agent could reduce hMPV titers, which was not caused by drug toxicity.

3.5 | M β CD had no effect on viral load, when hMPV particles entered cells

M β CD could disrupt lipid raft not only on the cell membrane but also in the cell organelle. To further investigate the effect of M β CD on hMPV infection, cells were treated with M β CD after virus enter cells. Results showed that M β CD had no significant effect on viral load, when hMPV particles had entered cells (Figure 2G). Exogenous cholesterol had no change on viral load when the virus had entered cells (Figure 2H).

3.6 | Reduce hMPV internalization with treatment M β CD and nystatin

After pretreating with M β CD and nystatin, the same amount of virus (MOI = 10) were inoculated into treated or nontreated 16HBE cells. The cells were washed three times with 0.1 M PBS before M β CD, nystatin treatment, and virus inoculation. Then the results were analyzed by IFA after being fixed, permeated, blocked, and stained with anti-hMPV fusion protein (anti-F) antibodies. Fluorescence intensity was analyzed in the drug treatment groups decreased with confocal microscopy (Figure 3A). The fluorescence points of individual cells—represent virus particles—in 10 fields were counted. It was found that the number of fluorescence points in the drug treatment groups was decreased dramatically ($P < 0.05$) (Figure 3C).

3.7 | CTB as a control for internalization

CTB is often used for the internalization and colocalization assay is recommended. It was found that the CTB (green) fluorescence of the M β CD treatment group decreased obviously compared with no M β CD treatment with confocal microscopy. Cells without M β CD showed green fluorescence was a mass-like distribution, while M β CD treatment cells were not obvious and scattered (Figure 3B). The fluorescence intensity of CTB with M β CD treatment decreased significantly ($P < 0.05$) (Figure 3D).

3.8 | Treatment with M β CD and nystatin could not affect virus attachment

To exclude the inefficient virus binding due to cholesterol depletion of M β CD and nystatin, the same amount of virus (MOI = 10) were inoculated into treated or nontreated 16HBE cells. Then, unbound viruses were removed by washing three times with 0.1 M PBS. The number of binding viruses was measured by qPCR. Both M β CD and nystatin treatment groups had no significant difference (Figure 4A and 4B). So, both M β CD and nystatin had no effect on hMPV attachment.

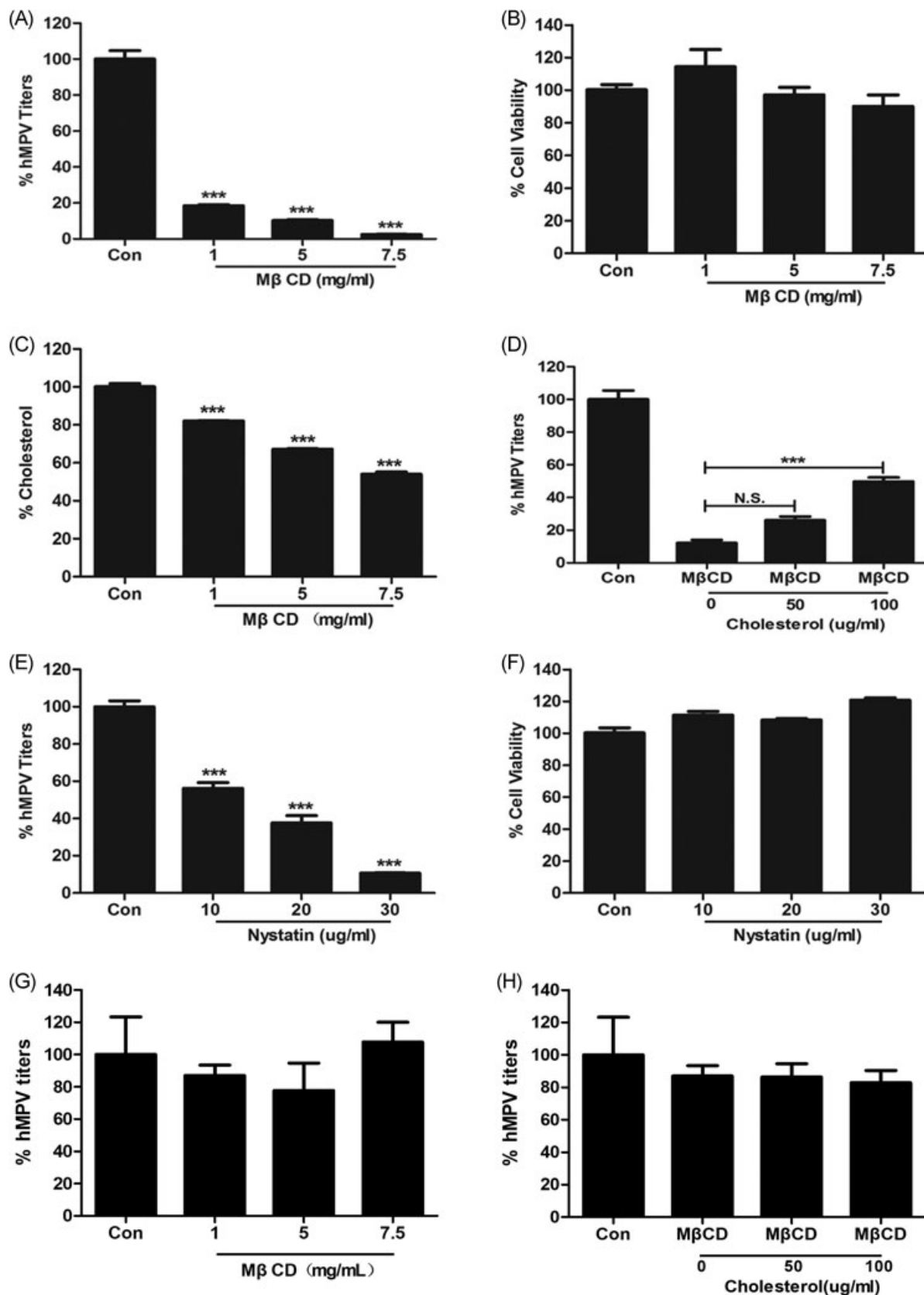


FIGURE 2 MβCD and nystatin inhibit hMPV infection (A) MβCD could reduce hMPV titers, and titers decreased gradually with the increase of concentration. B, Cell viability was measured by CCK-8 assay, and MβCD had no effect on the 16HBE cells viability. C, Cholesterol concentration was measured by Free Cholesterol Assay Kits. D, Replenishment of exogenous cholesterol could recover hMPV infection. E, Nystatin could reduce hMPV titers with the increase of concentration. F, Nystatin had no effect on the 16HBE cells viability. G, Treatment with MβCD had no significant effect on viral load, when hMPV particles had entered cells. H, Exogenous cholesterol had no change on viral load when the virus had entered cells. CCK-8, Cell Counting Kit-8; hMPV, human metapneumovirus; MβCD, methyl-β-cyclodextrin

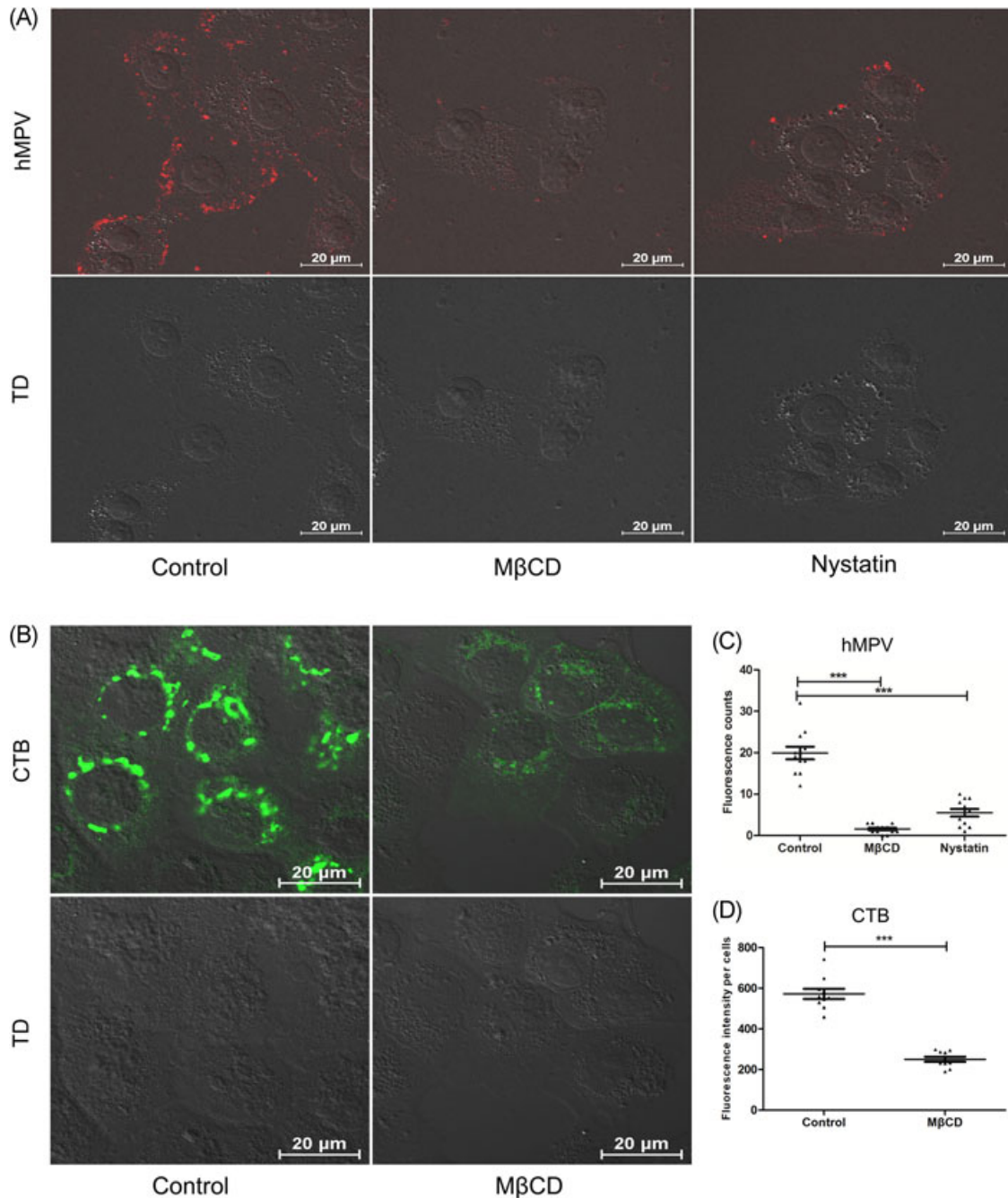


FIGURE 3 Treatment with M β CD and nystatin reduced hMPV internalization (A) 16HBE cells were treated with M β CD or nystatin, hMPV fluorescence points (red) was decreased. B, CTB (green) of control group enter 16HBE in large amount, while CTB entry were inhibited by M β CD. C, The number of hMPV fluorescence points (red) in every two cells was analyzed by NIS Elements AR 3.2 software. D, The mean fluorescence intensity in 10 cells was analyzed, and the M β CD-free group was significantly higher than the M β CD-treated group. CTB, Cell Counting Kit-8; hMPV, human metapneumovirus; M β CD, methyl- β -cyclodextrin

4 | DISCUSSION

Although hMPV was discovered almost two decades, its infectious mechanism is almost dimness and most studies focused on its epidemiology and clinical manifestation. It is still unclear how hMPV enters host cells. The virus cell entry is the key step of an effective infection. hMPV is the one of the enveloped virus and for those

enveloped viruses, membrane fusion is the most common way. But endocytosis way was discovered during viral entry of many enveloped viruses (eg, severe acute respiratory syndrome [SARS] coronavirus).²⁶ For all enveloped viruses, the viral membrane must fuse with cellular membrane or organelle membrane to accomplish infection. These fusion events are considered occur at the cell membrane, therefore, hMPV is regarded as that enters host cells via membrane fusion.

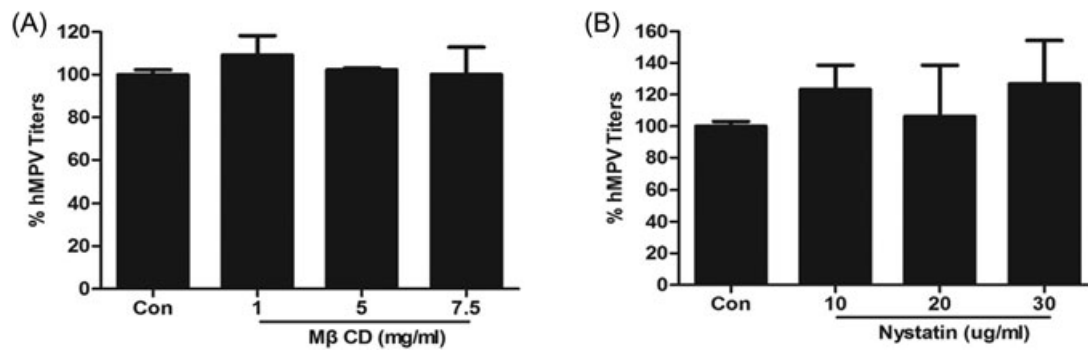


FIGURE 4 MβCD and nystatin could not affect hMPV attachment. MβCD treatment (A) and nystatin treatment (B) had no effect on hMPV attachment. hMPV, human metapneumovirus; MβCD, methyl-β-cyclodextrin

Recently, RGD-binding integrin had been found as a receptor for hMPV cell entry, and hMPV binds to a constant Arginine-Glycine-Aspartate (RGD) motif on the F protein through RGD-binding integrin. Therefore, hMPV F protein also plays an important role in attachment. The binding of hMPV and RGD-binding integrin activates downstream signaling cascades and promotes clathrin-mediated endocytosis.^{17,27,28} Our previous study had showed that hMPV existed whole particles in the cytoplasm and membrane fusion occurred in the cytoplasm.¹⁸ Taken together, these data indicated that hMPV may enter host cells via endocytosis.

There exist many ways of endocytic pathways, such as clathrin-, caveolae-, cholesterol-mediated endocytosis, and macropinocytosis.¹⁶ Lipids in the cell membrane form some specialized lipid structures. These structures are enriched in sphingolipids and cholesterol. This microdomain usually takes emigrant molecules from extracellular microenvironment to intracellular region, so it is named lipid raft.²⁹ Lipid raft plays a critical role in signal transduction and protein trafficking. It is also important to establish cell polarity.^{19,20} In this study, we focused on the effect of lipid raft on virus cell entry. Lipid raft is a specialized membrane microdomain that contains glycosphingolipids and lipoglycoproteins. It is rich in cholesterol, the content of cholesterol in lipid raft is about three to five fold compared with other structure in the cellular membrane. Cholesterol is the key element to maintain lipid raft to keep intact form, so cholesterol depletion could destroy lipid rafts on the surface of cell membrane. Lipid rafts are thought to be involved in the process of HIV invasion of macrophages cells because of cholesterol depletion by MβCD could reduce the infection of HIV, and HIV receptors were found at detergent-resistant membranes (DRMs).²¹ Lipid rafts are important to influenza virus assembly and budding.³⁰ The study of influenza virus found that transmembrane glycoprotein, hemagglutinin, and neuronal ammonia were abundant in the DRMs in the process of influenza virus translation. Besides, influenza virus nucleoprotein could target specifically the apical surface. Because lipid rafts play an important role in virus entry, assembly, and budding, so we have tried to explore the role of lipid rafts in the process of hMPV cell entry.

In this study, the possibility of lipid rafts associating with the process of hMPV cell entry was verified by the morphological study.

CTB subunit is a protein which enters cells via lipid rafts.²⁴ Laser scanning confocal microscope was used to observe fluorescence labeled CTB and hMPV. The colocalization of CTB and hMPV was universal in 16HBE cells, and the colocalization number increased gradually. Morphological research showed a dynamic process of hMPV cell entry. At first, only little colocalizing points were found at 0 minute, and then some colocalizing points were concentrated on the surface of cell membrane at 30 minutes. At last, most of these colocalizing points appear at the site away from the cell membrane at 60 and 120 minutes, hMPV colocalized with lipid rafts moved gradually toward the nucleus with the lipid rafts. This indicated that lipid rafts participate in the transport of hMPV during the process of endocytosis.

Since lipid rafts could be associated with hMPV cell entry, we wonder whether cholesterol depletion could affect hMPV infection. So, MβCD were used to deplete cholesterol in our study. MβCD were most commonly used to deplete cholesterol from cell membrane in the studies of lipid rafts.³¹ In our study, we also used MβCD to remove cholesterol and the results here showed that MβCD can reduce cholesterol in treated cells significantly with dose-dependent. After excluding the influence of cell viability, it is considered that MβCD significant decreased hMPV infection through reducing cholesterol and destroying lipid raft microdomain. To further verify this, we treated 16HBE cells with MβCD following by adding exogenous soluble cholesterol into the culture system. When exogenous cholesterol was added, the replication rate of hMPV was recovered. This result showed that lipid raft played an important role in hMPV infection.

As a pharmacological agent, MβCD has a pleiotropic effect. In addition, to deplete cholesterol from the plasma membrane, MβCD can also inhibit Ca²⁺ entry channels. Besides, it also plays an important role in cell proliferation. Inhibiting the Ca²⁺ entry channels can reduce virus infection and the cholesterol concentration does not affect in the process.³² To exclude this effect of MβCD, we choose another cholesterol chelating-agent-nystatin to verify our results. Nystatin is an antifungal medication and can bond cholesterol very well, it is also used in the study of lipid raft.²¹ After been treated with nystatin, the hMPV infection rate was also decreased in 16HBE cells. When the concentration of nystatin increased, the viral replication

decreased significantly. In other words, hMPV infection declined after the lipid raft was destroyed. It is confirmed that lipid raft played an important role in hMPV infection.

Here we have shown that lipid rafts participate in the process of hMPV infection, and the hMPV could reduce after treating with M β CD and nystatin. However, it is not clear whether the reduction of hMPV titer after treating with chemical inhibitors is associated with hMPV cell process. So hMPV cell entry after treating with chemical inhibitors was further confirmed by IFAs. The numbers of hMPV entering 16HBE cells were reduced significantly. The results were consistent with hMPV titer measuring at 24 hours after inhibitors treatment.

Therefore, hMPV first bind to the receptor before entering the host cell, so the reduction of hMPV attachment resulting by pharmacological agents will also lead to a decrease of hMPV infection. hMPV attachment treating by M β CD and nystatin were detected, and both M β CD and nystatin had no effect on hMPV attachment.

In conclusion, our study had showed lipid rafts play an important role in hMPV cell entry in the human bronchial epithelial cell. The morphological results showed that hMPV colocalized with lipid rafts and moved gradually toward the nucleus with the lipid rafts. The pharmacological agents showed that cholesterol depletion can inhibit the hMPV cell entry and confirmed by IFAs, without affecting attachment. The addition of exogenous cholesterol can recover hMPV infection. Therefore, our results suggest that lipid rafts play an important role in the process of hMPV cell entry. These results have given a better understanding of the mechanisms of hMPV entry and a new way to prevent and treat virus infection.

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