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Brief Report

Enhancement of cytotoxicity against Vero E6 cells persistently infected with SARS-CoV by *Mycoplasma fermentans*

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Summary

We previously reported that cells with persistent severe acute respiratory syndrome coronavirus (SARS-CoV) infection were established after apoptotic events. In the present study, we investigated the cytopathic effects of dual infection with SARS-CoV and *Mycoplasma fermentans* on Vero E6 cells. Dual infection completely killed cells and prevented the establishment of persistent SARS-CoV infection. *M. fermentans* induced inhibition of cell proliferation, but the cells remained alive. Apoptosis was induced easily in *M. fermentans*-infected cells, indicating that they were primed for apoptosis. These results indicated that *M. fermentans* enhances apoptosis in surviving cells that have escaped from SARS-CoV-induced apoptosis.

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Severe acute respiratory syndrome (SARS) is a newly discovered infectious disease caused by SARS coronavirus (SARS-CoV), which became a global health threat due to its rapid transmission and high fatality rate [10, 20]. Vero E6 is a cell line derived from African green monkey kidney cells and is sensitive to SARS-CoV. SARS-CoV induces apoptosis into Vero E6 cells *via* activation of caspase-3 [14]. Activation of p38 mitogen-activated protein kinase (MAPK) induces cell death [14] and inactivation of Akt induces apoptosis [15]. In virus-infected cells, c-Jun N-terminal protein kinase (JNK), extracellular signal-related kinase (ERK)1/2, and 90-kDa ribosomal S6 kinases are also phosphorylated [13, 17]. Four groups, including our groups, independently reported that a small population of virus-infected cells remained alive after the

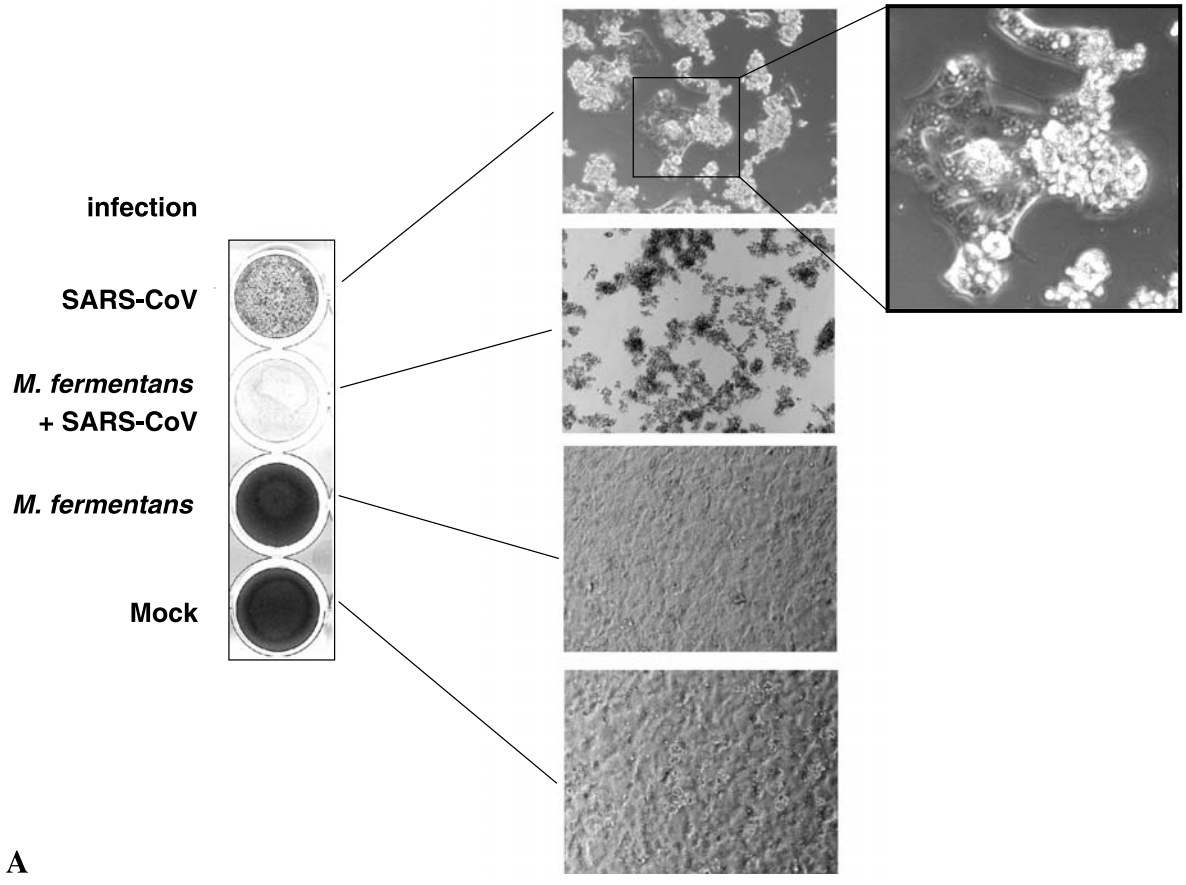
majority of virus-infected cells had died, and these cells grew with virus production [3, 16, 19, 24].

There have been reports of patients who were dual-infected with SARS-CoV and *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, or human metapneumovirus [2, 5, 9, 25]. However, the clinical significance of viral or bacterial co-infection with SARS in patients is still unclear. In the present study, we investigated cytopathic effects of *M. fermentans* on Vero E6 cells, and we investigated the cytotoxicity of dual infection with SARS-CoV and *M. fermentans* in Vero E6 cells. *M. fermentans* is known to enhance human immunodeficiency virus (HIV) replication [1]. Activation of NFκB by infection with *M. fermentans* increased replication of HIV by regulation of the long terminal repeat (LTR) [21, 23]. Thus, it is possible that *M. fermentans* can influence pathogenesis in co-infection with other viruses. *M. fermentans* is detected in approximately 10% of HIV-seronegative individuals [8], suggesting that a certain percentage of the healthy population is infected with *M. fermentans*. The present study was performed to examine whether pathogenicity is increased by co-infection with *M. fermentans* and SARS-CoV using an *in vitro* cell culture system.

Vero E6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 0.2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) fetal bovine serum (FBS), and maintained at 37 °C in an atmosphere of 5% CO_2. In the present study, Vero E6 cells were treated at least three times with MC-210 (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), which is an antibiotic active against mycoplasma. After washing MC-210 from the medium, mycoplasma contamination was checked, and the cells were confirmed to be mycoplasma-free by incubation in glucose-containing PPLO medium. SARS-CoV, which was isolated as Frankfurt 1 and kindly provided by Dr. J. Ziebuhr, was used in the present study. Mycoplasma broth medium consisted of PPLO broth (Difco Laboratories, Franklin Lakes, NJ, USA), yeast extract (Difco), 15% heat-inactivated horse serum, 10% aqueous glucose, and 1000 units/ml penicillin as described previously [7]. In the present study, we used the *M. fermentans* M64 strain, which was isolated from a patient with acute respiratory disease. *M. fermentans* was grown aerobically in glucose-containing PPLO medium at 30 °C for 24 h, and then frozen at −80 °C.

Aliquots of 5×10^7 colony-forming units (CFU) of *M. fermentans* in 250 μl of glucose-containing PPLO medium were added to approximately 2×10^6 Vero E6 cells (100% confluency) in 1 ml of 5%-FBS-containing DMEM in 24-well plates. Mock-infected cultures were prepared with 250 μl of glucose-containing PPLO medium. After 24 h, the cells were infected with SARS-CoV at 2 m.o.i., and the cells were fixed and stained 7 days after virus infection. As shown in Fig. 1A, both *M. fermentans*- and mock-infected cells maintained confluency. No significant morphological changes were observed in the *M. fermentans*- or mock-infected cells when confluent cells were infected. The majority of SARS-CoV-infected cells died by apoptosis at 48 h.p.i., and persistently infected cells were observed at 7 days p.i. (Fig. 1A). Our recent studies indicated that these persistently infected cells grow well and produce viral particles in the medium [16]. On the other hand, all cells died 7 days after dual infection with *M. fermentans* and SARS-CoV. The observation in Fig. 1A raised questions regarding the stage at which *M. fermentans* kills cells that have escaped from apoptosis by SARS-CoV infection. We next examined the effects of *M. fermentans* infection at the late stages of SARS-CoV infection on cells persistently infected with SARS-CoV. Vero E6 cells were infected with SARS-CoV, and almost all cells died by apoptosis at 50 h.p.i. However, a small population of cells survived. At this time point, the cells were infected with *M. fermentans*. The cells were fixed and stained 8 days after SARS-CoV infection. As shown in Fig. 1B, persistently SARS-CoV-infected cells were not observed with additional *M. fermentans* infection. These results indicated that *M. fermentans* killed all surviving cells that had escaped from SARS-CoV-induced apoptosis and prevented the establishment of persistent SARS-CoV infection.

We investigated whether *M. fermentans* shows cytopathic effects on subconfluent Vero E6 cells. Approximately 5000 cells in 96-well plates were



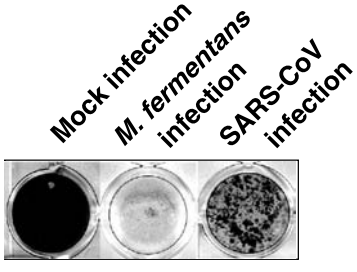


Fig. 1. Enhancement of cytotoxicity by dual infection with SARS-CoV and *M. fermentans*. **A** Confluent Vero E6 cells in 24-well plates were infected with 100 CFU/cell of *M. fermentans* for 24 h, and then the cells were infected with SARS-CoV at 2 m.o.i. A 20% volume of glucose-containing PPLO medium was added to all wells. After incubation for 7 days, surviving cells were observed in SARS-CoV-infected cultures, but not in dual-infected cultures. The cells were fixed with 10% formaldehyde for at least 24 h and stained with 0.1% naphthol blue black for 30 min. After washing with water, the plates were scanned using a GT-9400UF scanner (Epson, Tokyo, Japan). **B** Confluent Vero E6 cells were infected with SARS-CoV for 50 h, and then the cells were infected with *M. fermentans*. The cells were fixed and stained

inoculated with 5×10^6 CFU of *M. fermentans*. The results shown in Fig. 2A indicate that morphological changes in *M. fermentans*-infected cells were observed after day 1. The cells adopted an angular shape following infection with *M. fermentans*. As shown in Fig. 2B, cell growth was suppressed in the *M. fermentans*-infected cells. To clarify why cell proliferation is inhibited in *M. fermentans*-infected cells, Western blot analysis was performed using anti-retinoblastoma (Rb) antibody. Rb is thought

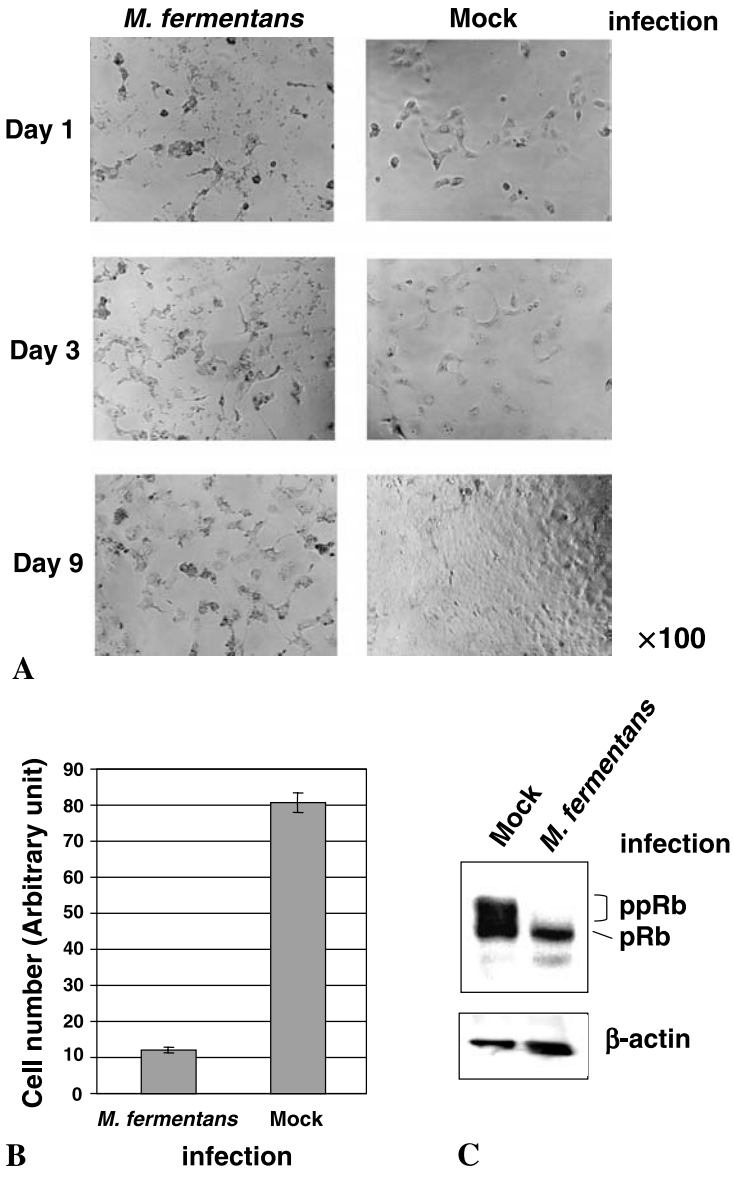


Fig. 2. Inhibition of cell proliferation by *M. fermentans* infection. **A** Subconfluent Vero E6 cells were infected with *M. fermentans* for 1, 3, and 9 days. **B** The cells at 9 days were fixed and stained. Cells were counted using a convenient method described by Everitt and Wohlfart for determination of the actual or relative number of cells in anchorage culture [4]. The dye-protein complexes were released hydrolytically with 0.1 M NaOH and measured spectrophotometrically at 660 nm. **C** Western blot analysis was performed using cell lysate 2 days after infection. Mouse anti-retinoblastoma protein (Rb) monoclonal antibody, which is able to detect the underphosphorylated form (pRb) and hyperphosphorylated from (ppRb), was purchased from BD Biosciences (Franklin Lakes, NJ, USA) and used at a dilution of 1:500. Mouse anti-β-actin antibody was purchased from Sigma and used at a dilution of 1:5000

to play one of key roles in the regulation of the G1 > S phase transition in the cell cycle, and phosphorylation of Rb is an important event in progression at G1 > S [18]. Only the hypophosphorylated form of pRb was detected in *M. fermentans*-infected cells at 2 days (Fig. 2C). The hypophosphorylated form of pRb is largely found in the early G1 phase.

Next, we examined the susceptibility of subconfluent *M. fermentans*-infected cells to apoptosis. Subconfluent cells were infected with *M. fermentans* for 24 h, and cycloheximide (final concentration, 1 mM) was added to the cells to stimulate apoptosis. As the cycloheximide was dissolved in DMSO, the same volume of DMSO alone was added to experimental controls. The subconfluent cells were infected with *M. fermentans* for 21 h, and cycloheximide or DMSO was added to the cells for 2 h. The cells were treated for 30 min with Apopercentage (Biocolor Ltd., Newtownabbey, Northern Ireland), which stains apoptotic cells at the early time stages by changing their color to red, and then the medium was replaced by PBS. As shown in Fig. 3A, the color of *M. fermentans*-infected cells treated with

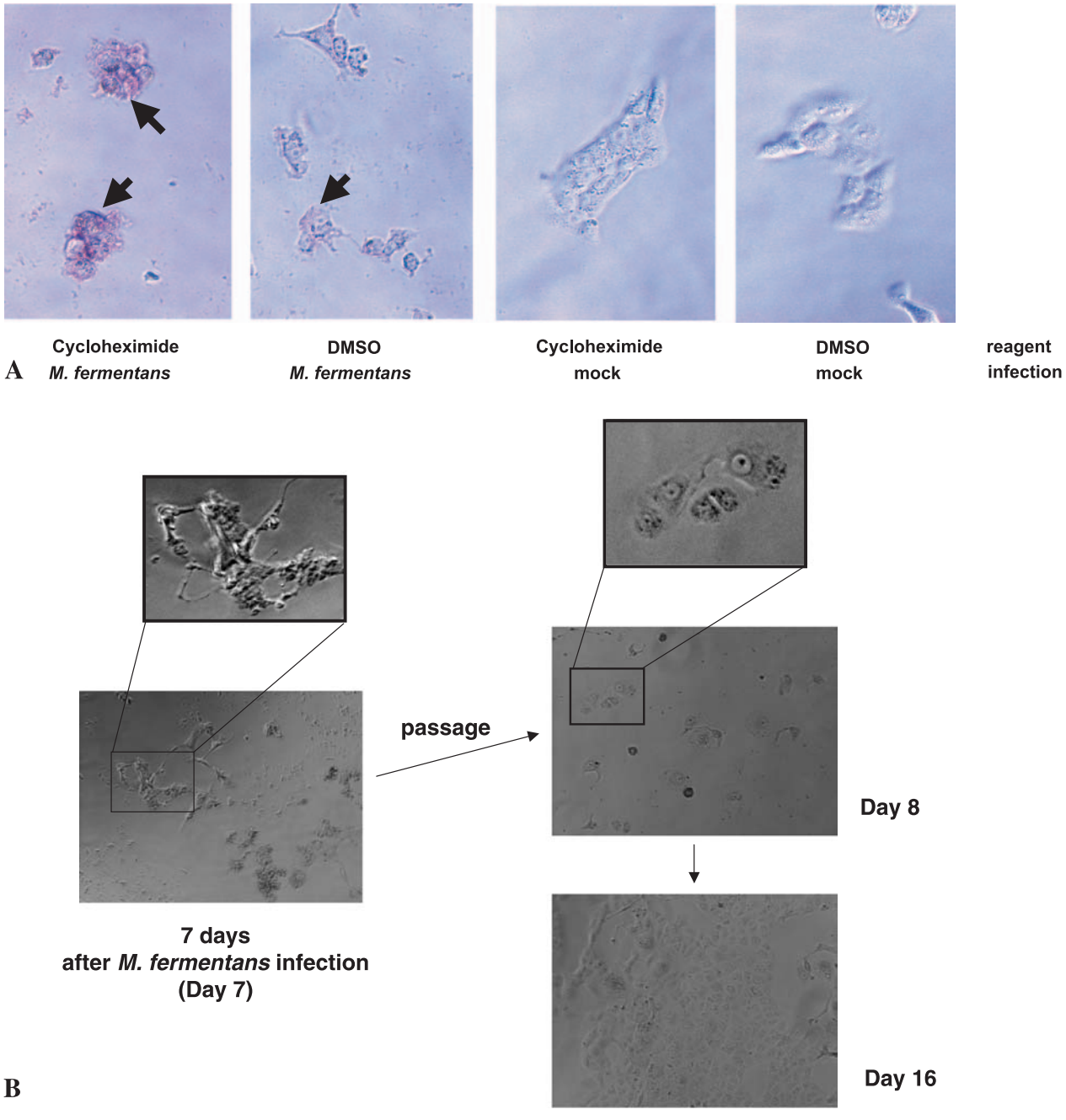


Fig. 3. Viability of *M. fermentans*-infected cells. **A** Subconfluent cells were infected with *M. fermentans* for 21 h, and then the cells were treated with cycloheximide. Apopercentage was used for detection of apoptosis. The arrows indicate apoptotic cells. **B** After 7 days postinfection, cells were passaged and cultured for 1 and 9 days

cycloheximide changed to red, indicating apoptosis, whereas mock-infected cells showed no change in color. However, some DMSO-treated cells infected with *M. fermentans* also changed color to red, but DMSO-treated cells without infection did not, suggesting that stimulation with not only cycloheximide but also DMSO induced apoptosis of *M. fermentans*-infected cells. Therefore, this result

indicated that *M. fermentans*-infected cells were primed for apoptosis. It remained to be determined whether *M. fermentans*-infected cells were alive. Therefore, subconfluent *M. fermentans*-infected cells at 7 days were trypsinized and resuspended in 5%-FBS-containing DMEM with MC-210 to kill the mycoplasma. After one day, almost all cells were attached to the plate, and dividing cells were observed (Fig. 3B). This result indicated that the *M. fermentans*-infected cells were alive and were able to grow when *M. fermentans* was removed.

In the present study, we demonstrated enhancement of cytotoxicity against Vero E6 cells persistently infected with SARS-CoV by *M. fermentans.* As *M. fermentans* processes phospholipase C in the cell membrane [22], the morphological changes caused by infection of Vero E6 cells with *M. fermentans* may be due to partial destruction of cell-surface lipids. In addition, cell death by superinfection of persistently SARS-CoV-infected cells with *M. fermentans* may be induced by phosphorylated p38 MAPK [12, 14], and assembly of the SARS-CoV envelope on the cell surface in surviving cells may also be a trigger of cell death on infection with *M. fermentans*. Akt, JNK, Bcl-2 and Bcl-xL play important roles in the establishment of SARS-CoV-persistent infection [12], and nucleocapsid (N) protein of SARS-CoV using a vaccinia virus expression system [6] is able to induce phosphorylation of Akt and JNK, but not p38 MAPK [12]. Glycogen synthase kinase 3β, which is downstream of Akt, was phosphorylated in N-expressing cells (data not shown). These results suggested that the N protein plays important roles for preventing apoptosis. On the other hand, Vero E6 cells were primed for apoptosis by *M. fermentans* infection in our experimental system, but cell death was not induced by infection. As infection by an excess of *M. fermentans* more than used in this study sometimes kills subconfluent Vero E6 cells, *M. fermentans* itself may be able to kill Vero E6 cells. Therefore, when the number of surviving cells that have escaped from cell death by SARS-CoV infection is very low, it is thought that cell death is enhanced by apoptotic effects of both SARS-CoV and *M. fermentans* infection.

The results of this study demonstrate that cells stressed by infection with *M. fermentans* are subject to further stress after infection with SARS-CoV. This phenomenon is important for understanding clinical pathogenicity, because it is unlikely that patients will be infected with only a single pathogen. There have been no previous reports regarding the pathological implications of dual infection with SARS-CoV and viruses or bacteria using a cell culture system. The findings of the present study have implications for infection control of acute or persistent SARS. Dual infection of the kidney cell line Vero E6 with SARS-CoV and *M. fermentans* also provides important information to further our understanding of renal infection in SARS patients.

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