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The fate of *Treponema denticola* within human gingival epithelial cells

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SUMMARY

Treponema denticola* is one of the major pathogens associated with chronic periodontitis. Bacterial invasion into gingival tissues is a critical process in the pathogenesis of periodontal disease. We recently reported that *T. denticola* can invade gingival epithelial cells. The aim of this study is to determine the fate of internalized *T. denticola* in gingival epithelial cells. Immortalized human gingival epithelial HOK-16B cells were infected with 5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled live or heat-killed *T. denticola* for 24 h, and the presence of bacteria inside the cells was confirmed by confocal microscopy. Live *T. denticola*, but not heat-killed bacteria, invaded HOK-16B cells. Confocal microscopy also revealed that internalized *T. denticola* rarely colocalized with either endosomes or lysosomes. Transmission electron microscopy of infected cells showed that intracellular *T. denticola* was localized inside endosome-like structures. Although a culture-based antibiotics protection assay could not detect viable intracellular *T. denticola* 12 h after infection, a substantial number of bacteria were observed by confocal microscopy and weak expression of bacterial 16S ribosomal RNA was detected 48 h after infection. In addition, flow cytometric analysis of HOK-16B cells infected with CFSE-labeled *T. denticola* showed no loss of fluorescence over 48 h. Collectively, *T. denticola* invades gingival epithelial cells and remains within the host cells for many hours by resisting endolysosomal degradation. These findings may provide new insight into the role of *T. denticola* in the pathogenesis of periodontitis.

INTRODUCTION

Periodontitis is a chronic inflammatory disease caused by polymicrobial infections that leads to the destruction of tooth-supporting tissue (Loesche & Grossman, 2001; Haffajee *et al.*, 2008). Bacterial colonization of the gingival sulcus is an initial step in the establishment of infection (Nishihara & Koseki, 2004). The gingival epithelium, which provides both physical and chemical barriers, is constantly in close contact with the colonized bacteria (Dale, 2002). Bacterial invasion into epithelial cells and gingival tissues is a critical process in the pathogenesis of periodontal disease (Dale, 2002; Feng & Weinberg, 2006). Cellular (Duncan *et al.*, 1993; Lamont *et al.*, 1995) and tissue (Lamont & Yimaz, 2002; Kim *et al.*, 2010) invasiveness of various periodontitis-associated bacteria has been reported repeatedly, and the invasive ability of bacteria is considered an important virulence factor.

Upon bacterial invasion of non-phagocytic cells, bacteria are normally segregated within a membrane-bound vesicle, targeted to the lysosomes, and then ultimately undergo endolysosomal degradation. However, some pathogens evade this endolysosomal degradation pathway using evolved strategies. *Listeria monocytogenes*, a causative pathogen of listeriosis, secretes a pore-forming cytolysin listeriolysin to disrupt the endosomes and escapes into the cytosol where it can replicate (Gouin *et al.*, 1999). Pathogenic group A streptococcus also escapes from endosomes into the cytosol but is recaptured within the autophagosomes and undergoes autophagosomal degradation (Nakagawa *et al.*, 2004). However, *Porphyromonas gingivalis*, *Coxiella burnetii* and *Chlamydia trachomatis* can survive within the autophagosomes (Dorn *et al.*, 2001; Gutierrez *et al.*, 2005). Additionally, mycobacteria inhibit phagosome–lysosome fusion, hence resisting lysosomal killing (Ehrt & Schnappinger, 2009). The ability of pathogens to evade lysosomal degradation is an important mechanism to ensure survival inside hosts and allows persistent infections to occur.

Periodontal pathogens *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* not only survive in the cytosol of epithelial cells but also undergo rapid multiplication and may subsequently spread to adjacent cells (Lamont *et al.*, 1995; Meyer *et al.*, 1996; Yilmaz *et al.*, 2006). Furthermore, direct evidence of the intracellular survival of *P. gingivalis* has been demonstrated in normal and immortalized human gingival epithelial cells (Lamont *et al.*, 1992; Madianos *et al.*, 1996; Meresse *et al.*, 1999; Yilmaz *et al.*, 2004).

Treponema denticola, along with *P. gingivalis* and *Tannerella forsythia*, is one of the major pathogens involved in chronic periodontitis (Socransky *et al.*, 1998; Haffajee *et al.*, 2006). Their virulence factors, such as proteolytic enzymes and outer surface proteins, are well characterized in terms of the pathogenesis of periodontitis (Ishihara & Okuda, 1999a,b; Sela, 2001). In addition, we recently reported that *T. denticola* suppresses human β-defensin expression through inhibition of the Toll-like receptor 2 axis and invades gingival epithelial cells (Ji *et al.*, 2010; Shin *et al.*, 2010). Determining whether it can survive inside the epithelial cells is important to understanding the role of *T. denticola* in the pathogenesis of periodontitis. The aim of this study is to determine the fate of internalized *T. denticola* in gingival epithelial cells.

METHODS

Epithelial cell and bacteria culture

Immortalized human gingival keratinocyte HOK-16B cells were maintained in keratinocyte growth culture medium (KGM from Clonetics, San Diego, CA) containing supplementary growth factors. *Treponema denticola* ATCC 33521 (Jacob & Nauman, 1982) and *Fusobacterium nucleatum* ATCC 25586 obtained from American Tissue Culture Collection (Bethesda, MD) were cultured in an anaerobic atmosphere (5% H_2, 10% CO_2 and 85% N_2) using an appropriate medium for each species: new oral spirochete (NOS) medium for *T. denticola* and brain–heart infusion supplemented with 5 μg ml^{-1} hemin (Sigma, St Louis, MO) for *F. nucleatum*. Bacteria in log-phase growth were harvested and washed twice with phosphate buffered saline (PBS). For the fluorescence studies, bacteria were stained with 5 μM 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Carlsbad, CA) before being used. Heat-killed bacteria were prepared by heating the bacteria at 95°C for 1 h. HOK-16B cells were infected by co-culturing HOK-16B cells and bacteria at a multiplicity of infection of 1000 in KGM medium without antibiotics at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Fluorescence confocal microscopy

For confocal microscopy, HOK-16B cells were plated at a density of 3×10^4 cells cm^{-2} onto 24-mm diameter acid-washed glass cover slips (Fisher Scientific, Houston, TX) 1 day before infection. At 50% confluence, the cells were infected with *T. denticola.* To examine the presence of bacteria inside the cells, cells were infected with live or heat-killed CFSE-labeled *T. denticola* for 24 h and then stained with rhodamine-phalloidin (Sigma) and Hoechst 33342 (Molecular Probes), as previously described (Ji *et al.*, 2010). To examine the colocalization of *T. denticola* with endosomes, HOK-16B cells were infected with unlabeled bacteria for 3.5 or 6 h. After fixing and permeabilizing, the cells were blocked with 1% bovine serum albumin for 30 min and stained with either fluorescein isothiocyanate-conjugated mouse anti-human endosomal antigen-1 (EEA-1) or an isotype control monoclonal antibody (BD Bioscience, San

Diego, CA). Subsequently, the cells were stained with Hoechst 33342 to visualize the *T. denticola*. To examine the colocalization of *T. denticola* with lysosomes, HOK-16B cells infected with CFSE-labeled *T. denticola* for 6, 9 or 11 h were stained with 50 μM LysoTracker Red (Molecular Probes) for 30 min before examination. After washing with PBS twice, the cells were immediately examined by live-imaging confocal microscopy. All confocal images were taken using a FV300 confocal microscope with serial z-sections (Olympus, Center Valley, PA).

Flow cytometric invasion assay

HOK-16B cells were plated at 6×10^4 cells per well in 24-well plates 1 day before infection. At 80% confluence, the cells were infected with CFSE-labeled bacteria for the indicated times. The cells were washed with PBS and detached with trypsin-EDTA (Gibco, Grand Island, NY). After quenching the fluorescence of the bacteria bound on the surface with 500 μl trypan blue (400 mg ml^{-1} prepared in 0.85% saline solution), the cells were analysed using FACSCalibur (BD Bioscience). Non-infected live cells and cells fixed with 3.7% formaldehyde and then exposed to the same amount of CFSE-labeled bacteria served as negative controls. After acquiring 10,000 events, the viability of HOK-16B cells was determined based on their forward scatter and the FL-3 fluorescence of the trypan blue staining, and bacterial invasion was analysed only for the live cells.

To examine the persistence of intracellular bacteria, the bacteria-infected cells were analysed 0, 24 or 48 h after killing extracellular bacteria by antibiotic treatment. *Fusobacterium nucleatum* colocalizes with lysosomes within 4 h of infection (Ji *et al*., 2010). Therefore, HOK-16B cells infected with CFSE-labeled *F. nucleatum* for 4 h were used as a control. Extracellular *T. denticola* was killed by 1 h of culture with 400 μg ml^{-1} gentamicin and 200 ng ml^{-1} amphotericin B, whereas extracellular *F. nucleatum* was killed by 2 h of culture with 50 μg ml^{-1} amoxicillin, 200 μg ml^{-1} gentamicin, and 100 ng ml^{-1} amphotericin B.

Transmission electron microscopy

HOK-16B cells (1×10^6) were plated into a 100-mm dish 1 day before infection and then infected with *T. denticola* for 4 h. Extracellular bacteria were killed by antibiotic treatment. The infected cells were then harvested immediately or after further culturing in fresh medium without antibiotics for 24 h. The cells were pre-fixed in 2.5% glutaraldehyde fixative buffer and post-fixed with 1% osmium tetroxide. After dehydration in an ethanol gradient (50–100%), the fixed cells were embedded in an Epon 812 mixture. Thin sections (70 nm) were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM, JEM-140; JEOL Ltd, Tokyo, Japan). Fourteen HOK-16B cells containing *T. denticola* were photographed at each time point.

Antibiotic protection assay

HOK-16B cells were plated at 2.4×10^5 cells per well in six-well plates 1 day before infection. At 80% confluence, the cells were infected with *T. denticola* for 4 h. After washing with PBS containing antibiotics, extracellular *T. denticola* were killed by antibiotic treatment and the infected HOK-16B cells were further cultured in fresh medium. At the indicated time points, cell lysates were prepared by lysing the cell monolayer with 500 μl sterile distilled water for 1 h. The lysates (100 μl) and the accompanying culture media (100 μl out of 500 μl) were plated onto NOS semi-solid agar and cultured under an anaerobic atmosphere (5% H_2, 10% CO_2 and 85% N_2) for 14 days.

To examine the persistence of intracellular *T. denticola*, HOK-16B cells were infected with CFSE-labeled *T. denticola* for 12 h and then subjected to antibiotic treatment. The infected cells were further cultured in fresh medium without antibiotics for the indicated times and then examined by confocal microscopy and flow cytometry. In addition, total RNA was extracted from the infected cells using RNeasy Plus Mini Kit (Qiagen, Valencia, CA).

Reverse transcription-polymerase chain reaction

To remove the contaminating bacterial DNA, 10 μg total RNA was incubated with RNase free-DNase I (Roche, Mannheim, Germany) at 37°C for 20 min and then the reaction was stopped by heating at 75°C for 10 min. The total RNA (5 μg) was subjected to reverse transcription with $(dT)_{18}$ and Superscript II enzyme (Invitrogen, Grand Island, NY) in a 25-μl

reaction mix at 42°C for 1 h. Bacterial 16S ribosomal RNA (rRNA) was amplified using species-specific primers: 5′-TAATACCGAATGTGCTCATTTACAT-3′ and 5′-TCAAAGAAGCATTCCCTCTTCTTCTTA-3′ for *T. denticola*; 5′-TGTAGTTCCGCTTACCTCTTCAG-3′ and 5′-AAGCGCGTCTAGGTGGTTATGT-3′ for *F. nucleatum*. As a control, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with 5′-CAGCCTCAAGATCATCAGCA-3′ and 5′-CCATCCACAGTCTTCTGGGT-3′. Polymerase chain reaction (PCR) was performed under the following cycling conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 15 s and elongation at 72°C for 30 s. The PCR products were verified by examination in a 3% agarose gel.

Measurement of intracellular reactive oxygen species

HOK-16B cells were plated at 2×10^4 cells per well in 96F Nunclon Delta black micro-well plates (Nunk, Roskilde, Denmark) 1 day before infection. HOK-16B cells were stained with 5 μμ 2′ 7′-dichlorofluorescein-diacetate (DCF-DA; Sigma) at 37°C for 30 min, washed with Hanks' buffered salt solution and then infected with *T. denticola*. The fluorescence of the HOK-16B cells was determined using Fluostar Optima (BMG Lab Tech, Ortenberg, Germany) at 1, 4, 8, 12, 18 and 24 h. Otherwise, HOK-16B cells infected with *T. denticola* for 4 h were subjected to antibiotic treatment and staining with DCF-DA. The fluorescence of cells was measured at 0, 3, 6, 9, 12, and 24 h.

Statistics

Differences between the two groups were analysed using the two-tailed Student's *t*-test. Data were considered statistically significant at a *P*-value of <0.05.

RESULTS

Live but not heat-killed *T. denticola* can invade gingival epithelial cells

We previously reported that *T. denticola* can invade gingival epithelial cells (Ji *et al*., 2010). We tested if dead bacteria can also invade the gingival epithelial cells. HOK-16B cells were infected with CFSE-labeled live or heat-killed *T. denticola* for 24 h. Upon examination by confocal microscopy using serial z-sections, bacterial invasion was confirmed by the presence of *T. denticola* within the cell boundary surrounded by actin filaments through several consecutive sections. In the HOK-16B cells infected with live *T. denticola*, many bacteria were clearly visible around the nuclei. However, heat-killed *T. denticola* remained at the cell boundary (Fig. 1A). These suggest that only live *T. denticola* can efficiently invade gingival epithelial cells. Hence, live bacteria were used in all subsequent experiments.

To examine the kinetics of invasion by *T. denticola*, HOK-16B cells were infected with CFSE-labeled *T. denticola* for 1, 4, 8, 12, 18 or 24 h and analysed by flow cytometry after quenching the fluorescence of the extracellular bacteria. *Treponema denticola* invasion was observed even after 1 h of infection and the maximum percentage of *T. denticola*-containing cells was reached by 12 h of infection. Although the percentage of *T. denticola*-containing cells slightly decreased in infections after 12 h, the mean fluorescence intensity of the total cells continued to increase (Fig. 1B). The viability of HOK-16B cells was not affected by the infection of *T. denticola* (Fig. 1C).

Intracellular *T. denticola* rarely colocalizes with endosomes or lysosomes in HOK-16B cells

To understand the fate of intracellular *T. denticola*, we first examined whether the internalized *T. denticola* was targeted to the endocytic degradation pathway in HOK-16B cells. Colocalization with EEA-1 or lysosomes was examined transiently during endosomal maturation. In our previous study, colocalization of internalized *F. nucleatum* with endosomes and lysosomes was observed at 90 min and 4 h of infection, respectively (Ji *et al*., 2010). However, internalized *T. denticola* remained near the plasma membrane after 90 min of infection and took 3.5 h to migrate from the cell periphery to the region where endosomes are located. Hence, we used 3.5 h and later time-points to examine the colocalization of *T. denticola* with endosomes and lysosomes. To examine colocalization with endosomes, HOK-16B cells infected with *T. denticola* for 3.5 or 6 h were stained with a fluorescein isothiocyanate-conjugated antibody to EEA-1, a marker of early endosomes, and *T. denticola* was visualized using Hoechst 33342.

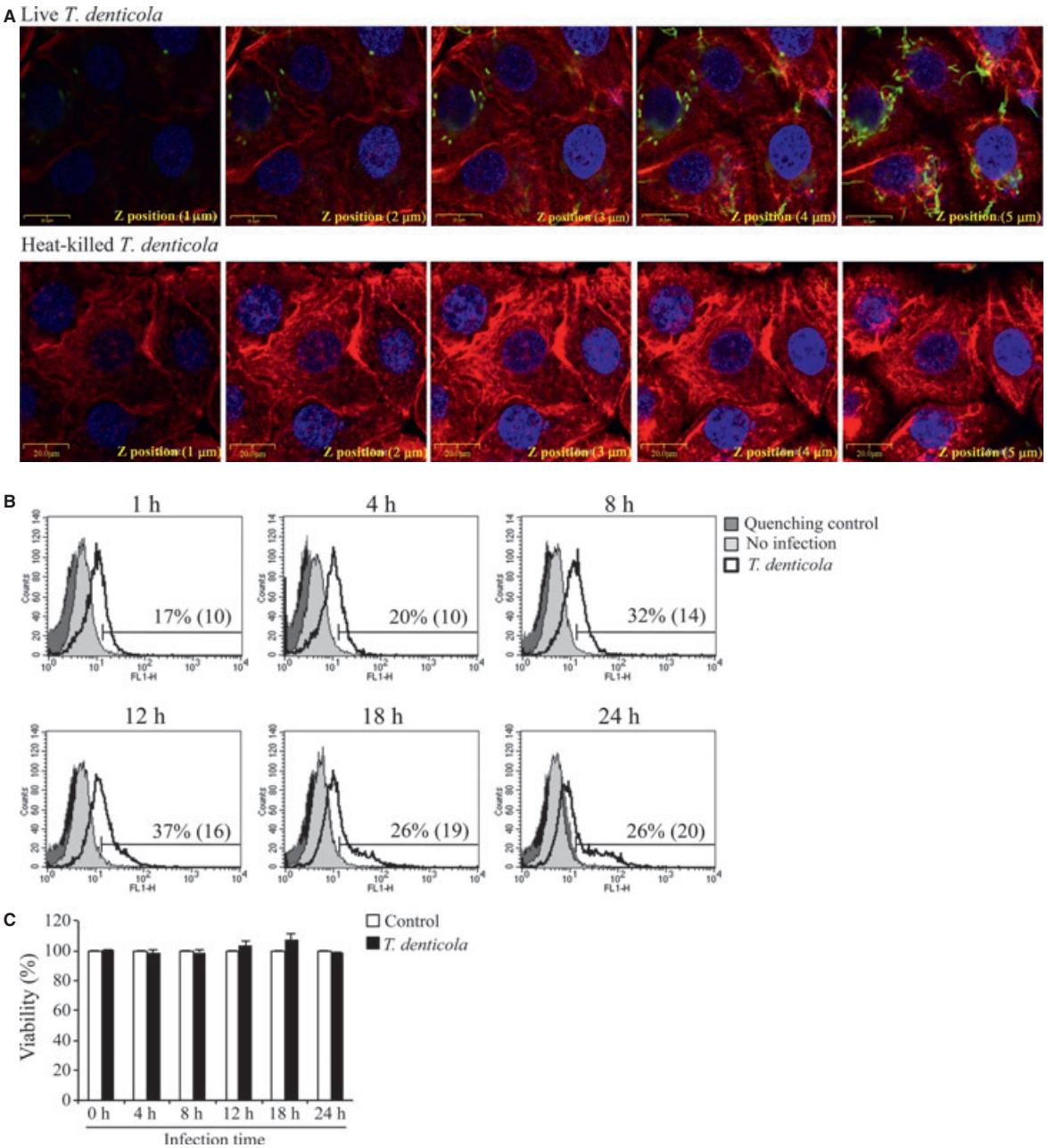


Figure 1 *Treponema denticola* invades human gingival epithelial HOK-16B cells. HOK-16B cells were infected with CFSE-labeled live or heat-killed *T. denticola* for 24 h. The infected cells were stained with rhodamine-phalloidin and Hoechst 33342 and then examined by confocal microscopy using serial z-sections. Scale bar: 20 μm. (B, C) HOK-16B cells were infected with CFSE-labeled live *T. denticola* for 1, 4, 8, 12, 18 or 24 h. After quenching the fluorescence of the bacteria bound to the cell surface with trypan blue, cells were analysed by flow cytometry. The overlay histograms of FL1 present bacterial invasion (B). The data shown are representative of three similar experiments. Filled with dark gray: fixed HOK-16B cells co-cultured with the CFSE-labeled *T. denticola*; filled with light gray: HOK-16B cells alone; solid empty line: HOK-16B cells infected with CFSE-labeled *T. denticola*. The percentage and mean fluorescence intensity (in parenthesis) of bacteria-containing cells are shown. The viability of HOK-16B cells based on their forward scatter and FL3 fluorescence is presented as mean ± standard error of the mean (SEM) of three experiments (C).

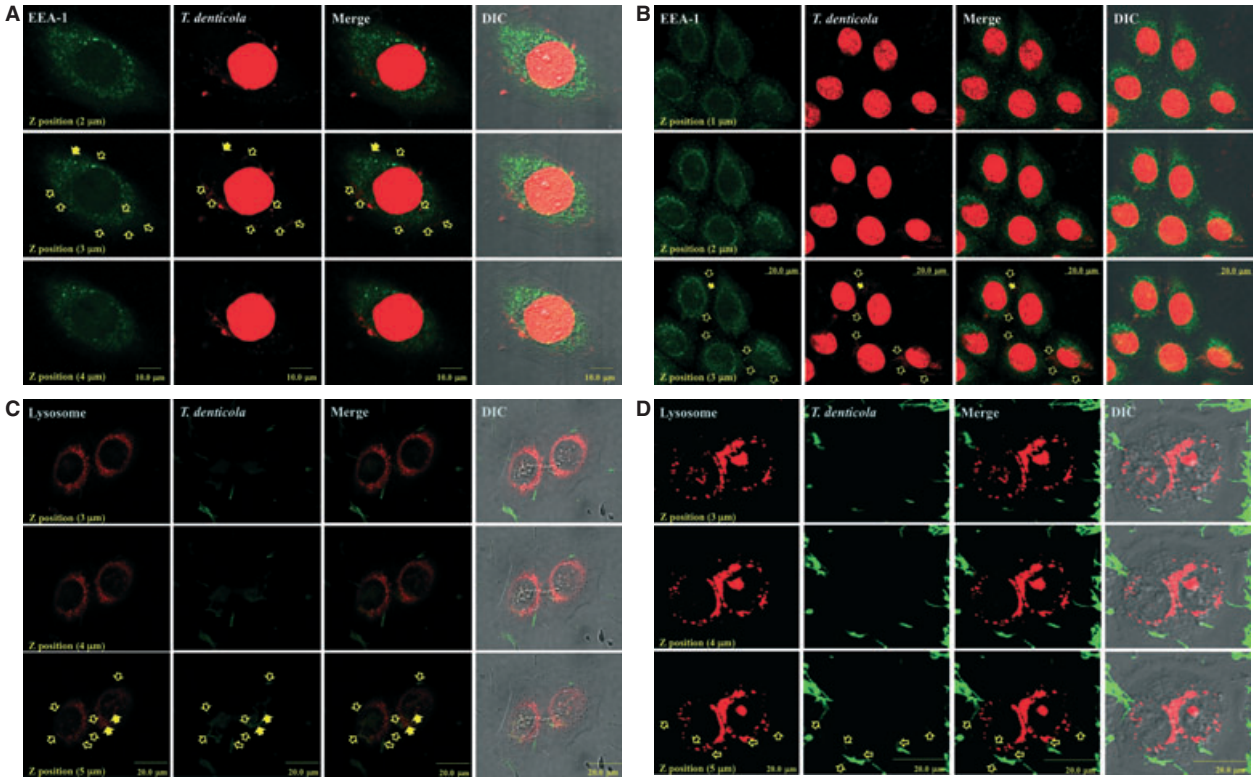


Figure 2 Intracellular *Treponema denticola* rarely colocalizes with either endosomes or lysosomes. (A, B) HOK-16B cells infected with unlabeled *T. denticola* for 3.5 (A) or 6 h (B) were stained with fluorescein isothiocyanate-conjugated mouse anti-human EEA1 (green) and Hoechst 33342 (red) to visualize *T. denticola* and then examined under a confocal microscope using serial z-sections. The data shown are representative of two similar results. (C, D) HOK-16B cells were infected with CFSE-labeled bacteria (green) for 6 (C) or 11 h (D), stained with LysoTracker Red DND-99 (red) for the final 30 min, and examined under a confocal microscope using serial z-sections. The data shown are representative of three similar results. Filled arrow: colocalized *T. denticola*, empty arrow: *T. denticola* that is not colocalized with endosomes or lysosomes.

Among all the examined intracellular *T. denticola*, only a few were observed to colocalize with nearby endosomes (Fig. 2A, B).

Colocalization of CFSE-labeled *T. denticola* with lysosomes was examined using LysoTracker Red. After 6 h of infection, the intracellular *T. denticola* reached the perinuclear region where lysosomes are located but the bacteria were rarely found to colocalize with lysosomes (Fig. 2C). We also examined the colocalization of *T. denticola* with lysosomes after 9 and 11 h of infection and obtained similar results (only 11 h is shown in Fig. 2D). From these results, we concluded that the intracellular *T. denticola* resists endolysosomal degradation.

Intracellular *T. denticola* remains within single membrane-bound structures

Resistance to endolysosomal degradation suggests that the internalized *T. denticola* may escape endosomes and survive in the cytosol. In addition, the subcellular localization of the bacteria is immunologically important because different types of T-cell responses are required to cope with microbes in the vesicles compared with those in the cytosol. To determine the subcellular localization and survival of internalized *T. denticola*, infected HOK-16B cells were exposed to antibiotic treatment and examined by TEM immediately (Fig. 3A) and also after further culturing for 24 h (Fig. 3B). The intracellular *T. denticola* was found at both time points either in the cytosol or in vacuoles. However, a higher magnification revealed that a single membranous structure (arrowhead) tightly surrounded the bacteria in the cytosol. The proportion of bacteria contained in the vacuoles was higher than those in tightly enclosed vesicles at both time points (Fig. 3C). Collectively, these results indicated that intracellular *T. denticola* remains inside single membrane-bound structures.

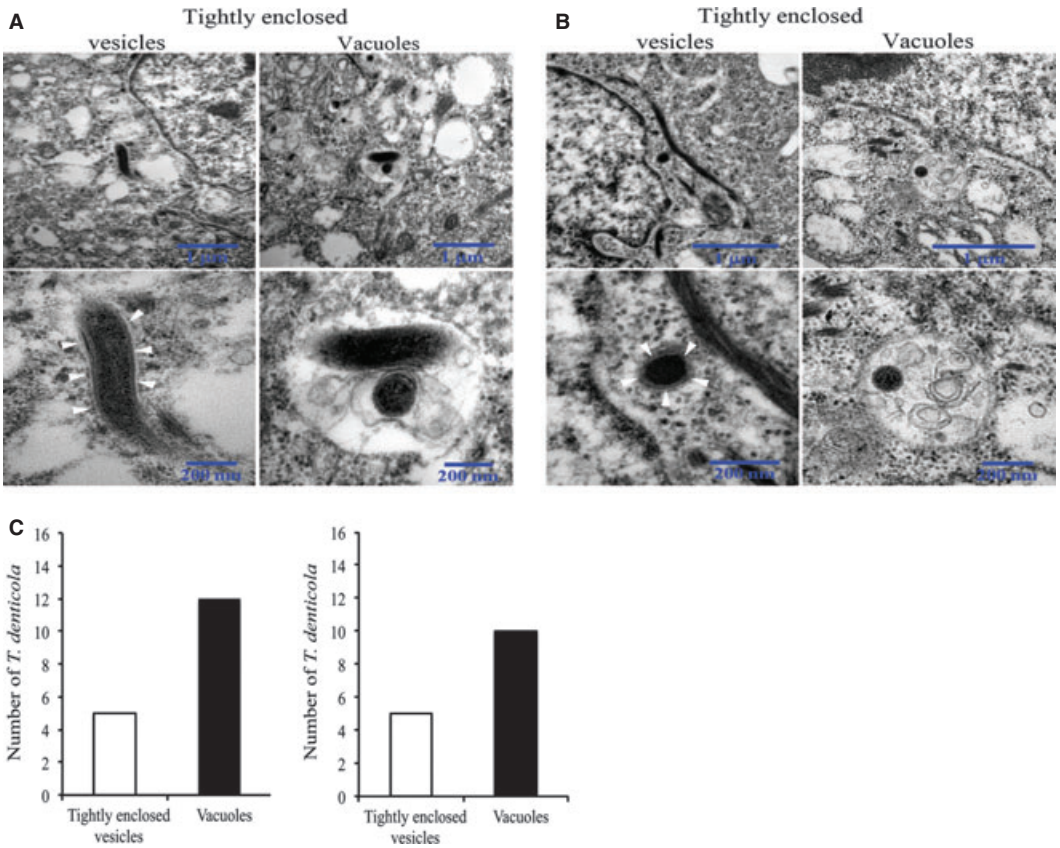
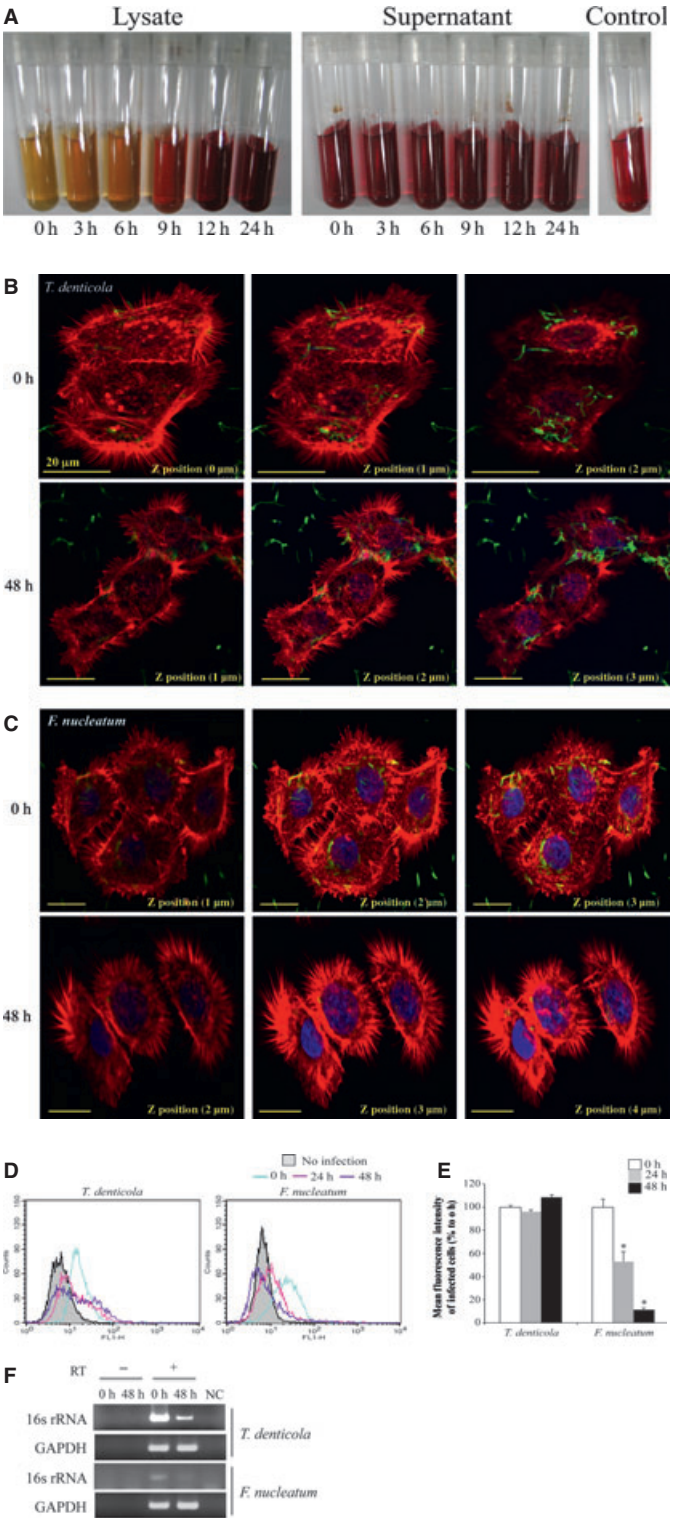


Figure 3 Intracellular *Treponema denticola* remains within single membrane-bound structures. The subcellular localization of the intracellular *T. denticola* was examined by transmission electron microscopy. After infecting with *T. denticola* for 4 h, HOK-16B cells were cultured in the presence of antibiotics for 1 h and then harvested immediately (A) or after 24 h of additional culture (B). (C) Total 14 HOK-16B cells containing intracellular *T. denticola* were photographed at each time point and the number of *T. denticola* in the tightly enclosed vesicles or vacuoles was counted. Left: immediately after antibiotic treatment; right: after 24 h of additional culture.

Intracellular *T. denticola* resists endolysosomal degradation but the number of surviving bacteria decreases over time

To verify the survival of the *T. denticola* that is protected from antibiotics within gingival epithelial cells, we directly cultured the lysate of infected HOK-16B cells on semi-solid NOS agar. Although *T. denticola* does not form colonies on NOS agar, bacterial growth produces metabolites that change the color of phenol red contained in NOS medium (Hunfeld *et al.*, 2000). Immediately after infection and antibiotic treatment, the intracellular *T. denticola* contained in the cell lysate grew efficiently on NOS agar, but the growth decreased to an undetectable level over 12 h of additional culture. Culture supernatants that were harvested along with the cell lysates did not contain bacteria at any time points, suggesting that none of the intracellular bacteria exited the host cells (Fig. 4A).

The failure to cultivate *T. denticola* from the infected cells 12 h after invasion raised the question of whether intracellular *T. denticola* is truly resistant to endolysosomal degradation. To clarify this point, HOK-16B cells infected with CFSE-labelled *T. denticola* were exposed to antibiotics to kill extracellular bacteria and then examined by confocal microscopy after 0 or 48 h of additional culture. A substantial number of *T. denticola* with defined morphology were found within the cells even after 48 h (Fig. 4B). In contrast, almost all of the intracellular *F. nucleatum* disappeared after 48 h (Fig. 4C). Although the fluorescence of CFSE-labelled bacteria can be diluted out by bacterial division, the fluorescence of the infected host cells would not change as long as the bacteria remain within the cells. However, if bacteria



and their fluorescent proteins are degraded within the cells, then the fluorescence of the infected cells would decrease. Therefore, we measured the fluorescence of the infected cells by flow cytometry 0, 24 or 48 h after bacterial infection. Interestingly, a uniform population of *T. denticola*-infected cells with interme-

Figure 4 The number of surviving intracellular *Treponema denticola* decreases despite its resistance to endolysosomal degradation. HOK-16B cells were infected with *T. denticola* for 4 h. After antibiotic treatment to kill extracellular bacteria, the infected cells were further cultured in fresh medium without antibiotics for the indicated times. Cell lysates or culture supernatants were inoculated on semi-solid new oral spirochete (NOS) agar and cultured under an anaerobic atmosphere for 14 days. NOS agar inoculated with control cell lysates without *T. denticola* infection served as a negative control. (B–E) HOK-16B cells were infected with either CFSE-labeled *T. denticola* for 12 h or *Fusobacterium nucleatum* for 4 h to achieve the maximum percentage of infection with each bacterium. The infected cells were subjected to antibiotic treatment to kill extracellular bacteria. (B, C) The cells were examined by confocal microscopy immediately (0 h) or after 48 h of additional culture. The data shown are representative of three similar results. (D, E) The cells were analysed by flow cytometry immediately or after 24 and 48 h. Representative histograms of three experiments (D) and mean fluorescence intensity of the infected cells expressed as a relative percentage to 0 h (E) are shown. (F) Total RNA was extracted from the infected cells immediately or after 48 h of additional culture. The RNA samples without (–) or with (+) reverse transcription were subjected to amplification of bacterial 16S ribosomal RNA and GAPDH of host cells. *Treponema denticola*: cells infected with *T. denticola*, *F. nucleatum*: cells infected with *F. nucleatum*.

diate fluorescence spread to FL-1^{dim} and FL-1^{high} populations after additional culturing (Fig. 4D). However, the overall mean fluorescence intensity of the total cells did not change (Fig. 4E). In contrast, *F. nucleatum*-infected cells lost fluorescence by 50 and 90% after 24 and 48 h, respectively, shifting the histogram to the left (Fig. 4D, E). These results confirmed that intracellular *T. denticola* is resistant to endolysosomal degradation.

Because the culture-based assay underestimates the *T. denticola* cell density (Orth *et al.*, 2010), there is a possibility that a small proportion of intracellular *T. denticola* survives. To explore this possibility, the expression of bacterial 16S rRNA within the infected cells was examined by reverse transcription-PCR. The strong expression of *T. denticola* 16S rRNA observed immediately after antibiotic treatment (0 h) was substantially reduced over 48 h (Fig. 4F) or not detected in two out of five experiments (data not shown). These results indicate slow death of intracellular *T. denticola* within the host cells. In HOK-16B cells infected with *F. nucleatum* a bacterium targeted to the endolysosomal degradation pathway within 2 h, the expression of 16S rRNA was detected at low levels immediately after antibiotic treatment and was hardly detected after 48 h of additional culture (Fig. 4F).

Treponema denticola modulates the production of reactive oxygen species by HOK-16B cells

Along with lysosomal enzymes, reactive oxygen species (ROS) plays a role in killing ingested microbes in phagocytes, the importance of which is evident in patients with an inherited nicotinamide adenine dinucleotide phosphate oxidase deficiency (Song *et al.*, 2011). All cells produce a low level of ROS as by-products of aerobic metabolism, and the low level of ROS may be enough to kill *T. denticola*, an obligatory anaerobe. The amount of intracellular ROS produced by HOK-16B cells upon *T. denticola* infection was examined by DCF fluorescence. HOK-16B cells accumulated intracellular ROS over time. Interestingly, the cells infected with *T. denticola* produced higher levels of ROS than non-infected cells at 1 h of infection but lower levels after 8 h (Fig. 5A). The intracellular ROS was also measured in the setting of an antibiotics protection assay performed in Fig. 4A. The infected cells accumulated intracellular ROS over time but produced consistently lower levels than non-infected cells (Fig. 5B). These results indicate that *T. denticola* modulates the production of ROS by the host cells.

DISCUSSION

In this study, we reported that *T. denticola* invades gingival epithelial cells and that the internalized *T. denticola* resists endolysosomal degradation within the host cells. Confocal microscopy clearly identified the presence of *T. denticola* inside HOK-16B cells. This indicates that *T. denticola* can invade gingival tissue not only through the intercellular space (Lux *et al.*, 2001) but also by directly invading the epithelial cells. In contrast to *F. nucleatum*, which colocalizes with endosomes and lysosomes at the perinuclear region within 3 to 4 h of infection (Ji *et al.*, 2010), *T. denticola* rarely colocalized with endosomes or lysosomes at any of the observed time points. In addition, confocal microscopy revealed that a substantial number of *T. denticola*, but only a few *F. nucleatum*, remained within the host cells 48 h after invasion. Flow cytometry measurements showed no significant reduction in the fluorescence of the cells infected with CFSE-labeled *T. denticola* but a sharp decrease (90%) in that of *F. nucleatum*-infected cells over 48 h, corroborating the fact that *T. denticola* resists endolysosomal degradation.

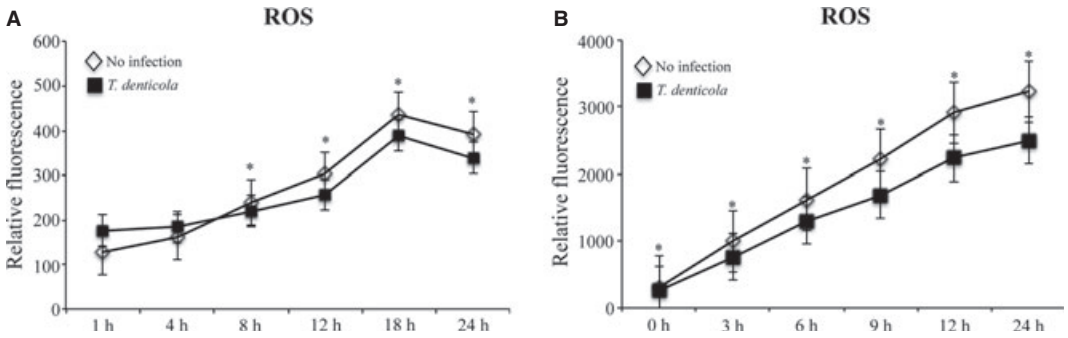


Figure 5 HOK-16B cells infected with *Treponema denticola* modulate reactive oxygen species (ROS) production. HOK-16B cells were stained with DCF-DA and then infected with *T. denticola*. DCF fluorescence was measured at the indicated time points. (B) HOK-16B cells infected with *T. denticola* for 4 h were subjected to antibiotic treatment and staining with DCF-DA. DCF fluorescence was measured at the indicated time points during additional culture for 24 h. The data shown represent the mean ± standard error of the mean of three experiments. $*P < 0.05$.

Pathogens evade the endocytic pathway to lysosomes by escaping into the cytosol, inhibiting fusion with lysosomes, or securing a niche in autophagosomes. *Porphyromonas gingivalis* was reported to be found free in the cytosol or surrounded by endosomal membranes in gingival epithelial cells and to survive within the cells, suggesting the involvement of the first and/or second evasion mechanisms (Sandros *et al.*, 1994; Lamont *et al.*, 1995). In addition, Dorn *et al.* (2001) reported that *P. gingivalis* avoids endolysosomal degradation by trafficking to the autophagosomes in endothelial cells. Several pathogens, such as *Salmonella*, *Mycobacterium* and *Brucella*, can modulate the maturation of the bacteria-containing vacuoles, thereby avoiding lysosomal killing (Alonso & Garcia-del Portillo, 2004). According to our electron microscopy examination, *T. denticola* appears to remain inside endosome-like structures, i.e. tightly enclosed vesicles or vacuoles, rather than escaping into the cytosol. The *T. denticola* may be tightly enclosed within the plasma membrane during internalization, and the tightly enclosed vesicles may acquire some membranous structures, forming vacuoles, but resist further endosomal maturation. Localization of the intracellular *T. denticola* to an autophagosome-like structure was not observed. Collectively, intracellular *T. denticola* remains in endosome-like structures. Hence, the Th1 response is the appropriate immune response to control this intracellular bacterium (Fietta & Delsante, 2009).

Despite its resistance to endolysosomal degradation, the results of a culture-based antibiotics protection assay suggest a decrease in the number of surviving intracellular *T. denticola* over 24 h. Such a decrease in surviving bacteria within the host cells may be attributed to accumulation of intracellular ROS. Interestingly, HOK-16B cells infected with *T. denticola* down-regulated intracellular ROS production at later time points compared with non-infected cells. A review of the *T. denticola* genome sequence revealed many genes encoding proteins to regulate oxidative stress, such as peroxiredoxin, thioredoxin, thioredoxin reductase, flavoredoxin, desulforedoxin, ferritin and ferredoxin, suggesting a potential to tolerate ROS (Seshadri *et al.*, 2004). Actually, upregulation of these genes by 1 h of oxygen exposure has been reported (McHardy *et al.*, 2010). Therefore, a small proportion of intracellular *T. denticola* may adapt to an *in vivo* environment and survive for extended hours, as suggested from the observation that intracellular *T. denticola* express 16S rRNA after 48 h of additional culture. A potential regulation of oxidative stress by *T. denticola* within the host cells warrants further investigation.

The ability to invade host cells is a common feature of periodontal pathogens (Nishihara & Koseki, 2004). We previously showed an association between inflammatory infiltrates with *P. gingivalis* present *in situ* in gingival tissue and proposed that the presence of bacteria within the tissue causes the recruitment of inflammatory cells into the lesion (Kim *et al.*, 2010). The reported persistence of *P. gingivalis* within various types of host cells, such as macrophages and endothelial cells, (Dorn *et al.*, 2001; Wang & Hajishenqallis, 2008) would contribute to the chronic nature of periodontitis. Now we report

that *T. denticola* also invades gingival epithelial cells and remains within the host cells for at least 48 h. Importantly, *T. denticola* is not targeted to a normal endolysosomal degradation pathway. Evasion of endolysosomal degradation has an important implication that *T. denticola* would avoid triggering Toll-like receptor 9 (TLR9) located in the endolysosomal compartment. We recently reported that TLR9 mediates oral bacteria-induced interleukin-8 expression in gingival epithelial cells (Kim *et al.*, in press). Indeed, *T. denticola* does not induce but rather suppresses interleukin-8 expression in gingival epithelial cells (Ji *et al.*, 2007). In addition, *T. denticola* inhibits the TLR2 signaling axis and suppresses human β-defensin expression (Shin *et al.*, 2010). All of these mechanisms would contribute to persistence of *T. denticola* in periodontal lesions.

In conclusion, *T. denticola* invades gingival epithelial cells and remains within the host cells for an extensive number of hours. These findings may provide new insight into the role of *T. denticola* in the pathogenesis of periodontitis.

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