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RESEARCH REPORTS
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Killing of *Treponema denticola* by Mouse Peritoneal Macrophages

ABSTRACT

Treponema denticola has been identified as an important cause of periodontal disease and hypothesized to be involved in extra-oral infections. The objective of this study was to investigate the role of *T. denticola* cell length and motility during mouse peritoneal macrophages *in vitro* uptake. Macrophages, incubated under aerobic and anaerobic conditions, produced a similar amount of TNF-α when stimulated with *Escherichia coli* LPS. The uptake of FlgE- and CfpA-deficient mutants of *T. denticola* was significantly increased compared with the wild-type strain, due to cell size or lack of motility. Opsonization with specific antibodies considerably improved the treponemes' uptake. These results suggest that macrophages, in addition to other phagocytes, could play an important role in the control of *T. denticola* infection, and that the raising of specific antibodies could improve the efficacy of the immune response toward *T. denticola,* either at an oral site or during dissemination.

KEY WORDS: treponemes, phagocytosis, innate immunity, dissemination, immune response, periodontitis.

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INTRODUCTION

Chronic periodontitis is an infectious disease characterized by the presence of a large number of inflammatory cells in the extravascular gingival connective tissue, which eventually leads to the breakdown of periodontal tissue, bone loss, and, finally, tooth loss (Loesche and Grossman, 2001). *Treponema denticola* is an obligate anaerobic oral spirochete frequently involved in the pathogenesis and progression of periodontal diseases (Ellen and Galimanas, 2005). This spirochete was shown to possess various properties leading to periodontal tissue damage (invasiveness associated with motility and the ability to penetrate dense media and epithelial cell layers, high proteolytic activity, adherence to epithelial cells, and cytotoxicity), as well as factors which may inhibit host cell functions (Lux *et al.*, 2001; Sela, 2001; Limberger, 2004; Ellen and Galimanas, 2005).

The possibility that *T. denticola* can escape from the dental compartment and invade different sites of the body has been hypothesized in humans (Riviere *et al.*, 2002; Cavrini *et al.*, 2005), and has been demonstrated in an oral infection model in SCID mice (Foschi *et al.*, 2006). The infiltration of human macrophages into infected tissues has been correlated with a rapid decrease in the number of spirochetes in the anatomical site (Cavrini *et al.*, 2005). The capacity to avoid phagocytosis by macrophages has been hypothesized to be one of the basic mechanisms that allow *T. denticola* to escape the innate immune response.

The aim of this study was to evaluate the influence of *T. denticola* motility and cell size on uptake by mouse peritoneal macrophages, *in vitro*. The anaerobic culture requirements of the treponemes prompted us to design a study to estimate *T. denticola* phagocytosis by macrophages under aerobic and anaerobic conditions. To further assess the relevance of the spirochetes' motility and cell shape during the interaction with phagocytes, we evaluated the capacity of murine macrophages to uptake and kill 2 *T. denticola* mutant strains: One strain lacked motility due to a knock-out of the *flgE* gene, thus resulting in a defect of the flagellar system (Ruby *et al.*, 1997); and the second was characterized by a filamentation phenotype (extremely long cells) associated with a lack of intermediate-like cytoplasmic filament, due to a knock-out of the *cfpA* gene (Izard *et al.*, 2001).

MATERIALS & METHODS

Bacterial Cultures

T. denticola strain ATCC 33520 and mutant strains for the *cfpA* gene (Izard *et al.*, 2001) and for the *flgE* gene (Li *et al.,* 1996; Limberger *et al.*, 1999) were grown as previously reported (Marangoni *et al.*, 2005).

Preparation of Macrophages

Peritoneal murine macrophage cells were obtained as described previously (Rosen *et al.*, 1999). To ensure the viability of macrophages under aerobic and anaerobic (95% CO_2 and 5% N_2) conditions, we incubated the cells in the presence or in the absence of 10 μg/mL of *Escherichia coli* lipopolysaccharides (LPS) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) in RPMI 1640 medium at 37°C. The supernatant from each well was withdrawn at defined time intervals (1, 2, 4, 8, and 24 hrs), and the concentration of released TNF-α was measured by the Mouse TNF-α ELISA kit from Bender Medsystems (Vienna, Austria), following the manufacturer's protocol. Individual wells containing macrophages were stained with Trypan blue for determination of the number of surviving cells.

Preparation of the Anti-major-surface-protein Antiserum

The major surface protein (MSP) of *T. denticola* was extracted as reported previously (Mathers *et al.*, 1996). Polyclonal rabbit antibodies against MSP were obtained by rabbit immunization as previously reported (Giacani *et al.*, 2005). The specificity of the anti-MSP rabbit antiserum was tested by immunoblotting with *T. denticola* cell lysate and a purified control protein, as previously described (Sambri *et al.*, 1999).

Immunofluorescence Assay

The uptake of *T. denticola* by macrophages was assessed by an indirect immunofluorescence assay (IFA) performed with 1:400 diluted anti-MSP rabbit polyclonal antiserum, as previously reported (Sambri *et al.*, 1996). With this method, we were able to detect both extracellular boundaries and internalized bacteria. Briefly, *T. denticola* cells (2.5 x 10^7 cells *per* well) were incubated with adherent macrophages in a final volume of 1 mL (ratio, bacteria/macrophage: 100/1), in RPMI 1640 medium, without any antibiotic for 5, 10, 20, 40, and 60 min at 37°C. After each incubation period, supernatants were removed, without previous plate centrifugation, and were serially diluted in NOS medium. The motile treponemal cells were immediately counted under dark-field microscopy. The cells were washed 3 times with PBS for removal of unbound spirochetes and subsequently fixed in cold methanol for 15 min at -20°C. We obtained the percentage of anti-MSP positive macrophages by counting cells in at least 30 different microscopic fields (400x).

To ensure bacteria viability, we thoroughly rinsed 3 additional wells for each time-point with PBS as described above and scraped off the cells by shaking with glass beads for 4 min. The resulting suspension was cultured in NOS medium at 37°C for up to 14 days, for assessment of the presence of living spirochetes.

In selected experiments performed under anaerobic and aerobic conditions, *T. denticola* strains were opsonized before being challenged with macrophages. This was done by incubation of the treponemes in the presence of heat-inactivated anti-MSP rabbit polyclonal antiserum (diluted 1:100) for 1 hr, in their cultivation media in anaerobic conditions.

Real-time PCR Assay

The uptake experiments were performed as above, with the following difference: The ratios between bacteria and macrophages were 10/1 (2.5 x 10^5 bacterial cells *per* well), 50/1, and 100/1. DNA of the cellular homogenates of phagocytosis was extracted with the NucliSens EasyMag system (bioMérieux, Craponne, France) following the manufacturer's instructions. The real-time PCR was carried out with a LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany) with SYBR Green I dye (Marangoni *et al.*, 2005). The specific primer pairs targeting the *T. denticola* 16S rRNA gene were: DENT1 (5′-TAATA CCGAATGTGCTCATTTACAT-3′) and DENT2 (5′-TCAAAGA AGCATTCCCTCTTCTTCTTA-3′) (Siqueira *et al.*, 2000; Foschi *et al.*, 2005). DNA standards for *T. denticola* quantification were assessed by a 10-fold scalar dilution of a *T. denticola* cell suspension (10^9 treponemes/mL) (Marangoni *et al.*, 2005). The sensitivity of real-time PCR was 10^3 treponemes/mL.

Data Analysis

Means and standard deviation (SD) were calculated for group comparisons within and among experiments. Analysis of variance and Student's *t* test were performed with GraphPad 4.0 software.

RESULTS

Uptake and Killing of *T. denticola*

To validate our approach, we incubated the macrophages in an aerobic atmosphere or under anaerobic conditions. There was no significant difference in the release of TNF-α following the stimulus of macrophages with LPS in either condition (Fig. 1). Trypan blue staining of the cells incubated for up to 2 hrs under each condition demonstrated that at least 95% of cells were viable in all the experiments. After 1 hr of incubation, the treponemal motility ratio (motile/total motile + non-motile) was over 97% for experiments performed under anaerobic conditions, whereas incubation in the presence of normal atmosphere reduced the percentage of motile treponemes to 23%, as observed by dark-field microscopy (Fig. 2). The use of macrophages incubated in anaerobic conditions allowed for the maintenance of an experimental system with the highest possible viability of the spirochetes during the challenge with phagocytes.

The increase in anti-MSP-positive macrophages occurred rapidly in both experimental conditions, as shown by IFA (Figs. 3A, 3B). The difference between the results obtained under aerobic and anaerobic incubation was statistically significant when analyzed by Student's *t* test ($p < 0.001$), with the uptake in aerobic conditions being more efficient. The association of *T. denticola* cells with the macrophages was a rapid process under both aerobic and anaerobic conditions (Figs. 4C, 4D). This process might be saturable for the 50/1 ratio (Fig. 4D). In anaerobic conditions, a decrease in spirochete number was observed after 40 min by real-time PCR (Figs. 4A, 4C), but not by IFA (Fig. 3A).

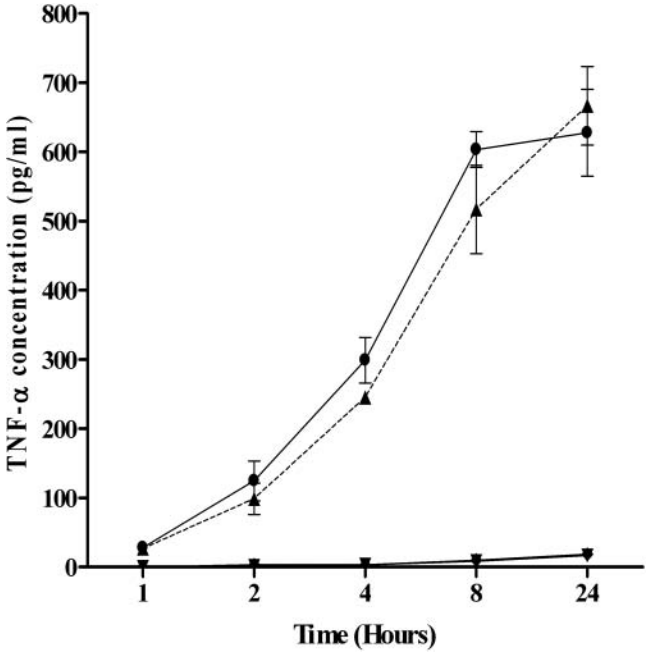

Figure 1. Production of TNF-α by isolated murine peritoneal macrophages stimulated with 10 μg/mL of LPS, for up to 24 hrs, under anaerobic (●) and aerobic (▲) conditions of incubation. Production of TNF-α by isolated murine peritoneal macrophages in the absence of stimulus, under anaerobic (▼) and aerobic (♦) conditions of incubation. The data presented are from 3 independent experiments and are expressed as the means ± standard deviations (error bars) of 3 replicates.

No living treponemes were detected, after 14 days of broth culture, in tubes inoculated with macrophage homogenates obtained at the end of the challenge with *T. denticola,* thus suggesting the effective killing of phagocytized treponemes.

Opsonic Phagocytosis of *T. denticola*

T. denticola treated with anti-MSP antibodies increased the percentage of anti-MSP-positive macrophage cells significantly when compared with the number of positive macrophages obtained with non-opsonized treponemes for each individual time-point ($p < 0.001$). Real-time PCR confirmed (Fig. 4) the significant increase observed by IFA for both anaerobic (Fig. 3A) and aerobic (Fig. 3B) conditions. The Student's *t* test showed that the difference in the uptake of opsonized treponemes in both atmospheric conditions was statistically significant ($p = 0.05$).

Phagocytosis of *T. denticola* Mutant Strains

The observed number of positive macrophages when challenged with either mutant strain was greater than when challenged with the wild-type strain. The number of positive cells, as observed by IFA, increased rapidly (Figs. 3A, 3B) and plateaued after the one-hour time-point (data not shown) in both atmospheric conditions. The real-time PCR analysis showed an increase of *T. denticola* cells associated with the macrophages up to the 40-minute time-point, followed by a decrease, for both atmospheric conditions (Figs. 4A, 4B).

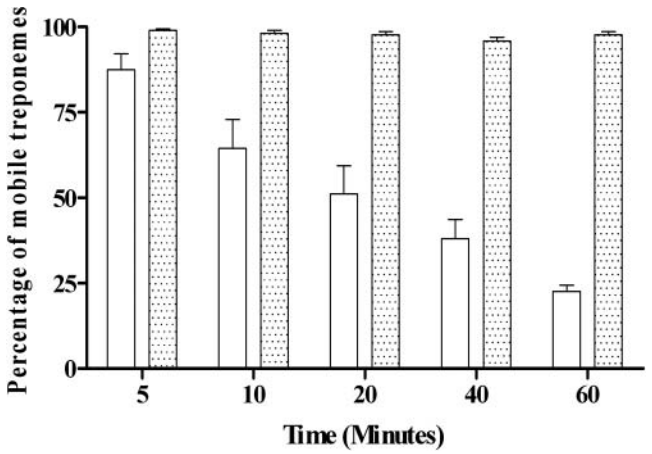

Figure 2. Percentages of motile *T. denticola* cells in the cell culture supernatants after macrophage challenge as observed by dark-field microscopy (400x). Bars indicate the percentages of motile treponemes under anaerobic conditions (dotted bars) and under aerobic conditions (white bars). The data presented are from 3 independent experiments and are expressed as the means ± standard deviations (error bars) for each condition of incubation.

The differences among the percentages of *T. denticola*-positive phagocytes detected when the macrophages were challenged with either the non-motile mutant strain (*flgE* knock-out strain) or the filamentous mutant strain (*cfpA* knock-out strain) compared with the wild-type strain were statistically significant in either incubation condition and at each time-point (Figs. 3, 4).

DISCUSSION

Periodontal diseases are widespread pathological conditions that affect millions of individuals worldwide. The dental biofilm is recognized as one of the major causes of interaction between several different bacteria and the host immune-response. The macrophages, together with the other cells responsible for the innate immunity playing a key role in the infection control, are affected by *T. denticola* (Rosen *et al.*, 1999; Ruby *et al.*, 2007; Magalhães *et al.*, 2008). In this study, the *in vitro* capability of murine peritoneal macrophages to uptake and kill different strains of *T. denticola* was investigated.

For the first time, the interaction of *T. denticola* with phagocytic cells was evaluated in anaerobic conditions. We chose this particular experimental condition to mimic, as far as possible, the *in vivo* conditions of the periodontal pocket, where the level of oxygen is quite low, due to modification of gingival microcirculatory function and the anatomical position of the oral biofilm (Hanioka *et al.*, 2000a,b; Loesche and Grossman, 2001). The isolated mouse macrophages survived under anaerobic conditions, and maintained their responsive functionality to LPS for up to 24 hrs. It is a critical observation that enables us to study organisms that are sensitive to molecular oxygen, such as the treponemes and other anaerobes from oral or gut biofilms.

Once uptaken by macrophages, *T. denticola* were rapidly and effectively killed, within 1 hr, independently of the incubation condition. In contrast, the capability of macrophages to uptake

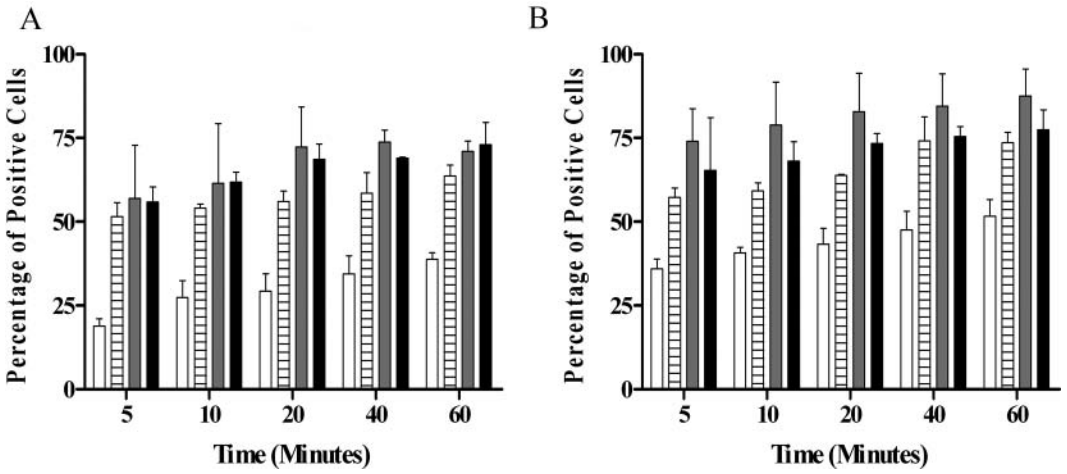

Figure 3. Macrophage uptake of *T. denticola* wild-type and mutant strains analyzed by immunofluorescence in both anaerobic (Part A) and aerobic conditions (Part B). Over-time uptake by isolated peritoneal murine macrophages of the wild-type cells (white bars), of the wild-type cells opsonized with anti-major-surface-protein (MSP) antibodies (bars with horizontal stripes), of the non-motile mutant cells (grey bars), and of the filamentous mutant cells (black bars). The results are expressed as a percentage of macrophages positive for antibodies against MSP. The differences observed between the number of positive macrophages challenged by the wild-type strain and the opsonized wild-type strain ($p < 0.0001$), or the mutant strains ($p < 0.0001$ for either strain), are statistically significant for both anaerobic (Part A) and aerobic conditions (Part B). The results are representative of 3 independent experiments, each done in triplicate, and are expressed as the means ± standard deviations (error bars) of 3 replicates.

T. denticola was influenced by the incubation conditions. When challenge experiments were performed in the presence of atmospheric oxygen concentrations, treponemes lost their motility over time. The number of macrophages showing *T. denticola* intake were greater than those detected in experiments performed under anaerobic conditions. *T. denticola* loss of motility under reduced oxygen conditions was very low, and, consequently, the challenge with macrophages was performed mostly with motile treponemes. These findings suggest that the capability of macrophages to uptake *T. denticola in vitro* is greatly influenced by the mobility and cellular dimensions of the treponemes. Bacterial motility is likely the major factor influencing cell contact and spatial interaction between macrophages and treponemes in the context of cell sedimentation. In addition, these results suggest that the *in vitro* interaction between *T. denticola* and macrophages resembles that of other spirochetes, like *Leptospira interrogans* and *Borrelia burgdorferi,* which are rapidly and efficiently taken up by macrophages, such as by Kupffer cells (Sambri *et al.*, 1999). The proposed anaerobic experimental set-up contributes to a more accurate observation of treponemal behavior in the presence of macrophages.

For further investigation of the influence of motility on macrophage uptake, a mutant strain of *T. denticola* was used in identical conditions. The *flgE* non-motile mutant was uptaken more efficiently than the parental wild-type strain, independently of the incubation atmosphere. Treponemal motility is a major factor in escaping the uptake by isolated murine macrophages.

As expected, another factor that is likely to influence the capability of macrophages to internalize *T. denticola* is the size of the individual bacterial cells. The phagocytosis of a *T. denticola* cytoplasmic filament mutant strain, which produces long cells associated with a filamentation phenotype (also leading to an altered motility), was very efficient.

When the surfaces of living cells are coated with specific antibodies, the uptake of *T. denticola* by isolated macrophages *in vitro* is greatly enhanced. The findings of this study confirmed that the opsonization could indeed increase the ingestion of wild-type treponemes by macrophages, as previously suggested for *T. pallidum* (Alder *et al.*, 1990). The uptake rate increase was independent of the atmospheric condition of the interaction between treponemes and phagocytes. *In vivo*, during an endodontic experimental infection model with *T. denticola*, a greater rate of dissemination from the oral cavity to different organs was observed in Rag1 immunodeficient mice compared with the parental strain (Foschi *et al.*, 2006). These results suggested that the immune response plays a major role in the control of *T. denticola* spreading from the endodontic site to different anatomical locations. The absence of antibody production against the infection, and the resulting absence of opsonization, might have influenced the outcome of the infection and related dissemination. In contrast, opsonization of the 2 mutant strains did not result in an increased uptake (data not shown), due to an extremely high level of uptake of the 2 mutant strains in the absence of opsonization.

Motility and size are capable of influencing the uptake of *T. denticola* by murine macrophage cells, in the *in vitro* system described in this report. Motility greatly facilitates the escape from local immune response, in addition to allowing tissue penetration (Lux *et al.*, 2001) and dissemination to multiple sites (Ehmke *et al.*, 2004).

T. denticola could interact with phagocytic cells, *in vivo*, in two main locations: within the dental pocket and within the blood stream or in extra-oral tissues, after leaving the oral cavity. Based on the results of this study, we can speculate that the presence of a specific antibody response in these sites of infection could greatly contribute to the clearance of the invading treponemes, thus prompting additional investigations devoted to the evaluation of specific antibody production during the course of *T. denticola* extra-oral infections.

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REFERENCES

Alder JD, Friess L, Tengowski M, Schell RF (1990). Phagocytosis of opsonized *Treponema pallidum* subsp. *pallidum* proceeds slowly. *Infect Immun* 58:1167-1173.

Cavrini F, Sambri V, Moter A, Servidio D, Marangoni A, Montebugnoli L, *et al*. (2005). Molecular detection of *Treponema denticola* and *Porphyromonas gingivalis* in carotid and aortic atheromatous plaques by FISH: report of two cases. *J Med Microbiol* 54(Pt 1): 93-96.

Ehmke B, Beikler T, Riep B, Flemmig T, Gobel U, Moter A (2004). Intraoral dissemination of treponemes after periodontal therapy. *Clin Oral Investig* 8:219-225.

Ellen RP, Galimanas VB (2005). Spirochetes at the forefront of periodontal infections. *Periodontol 2000* 38:13-32.

Foschi F, Cavrini F, Sambri V, Montebugnoli L, Prati C (2005). Detection of bacteria in endodontic samples by polymerase chain reaction assays and association with defined clinical signs in Italian patients. *Oral Microbiol Immunol* 20:289-295.

Foschi F, Izard J, Sasaki H, Sambri V, Prati C, Müller R, *et al*. (2006). *Treponema denticola* in disseminating endodontic infections. *J Dent Res* 85: 761-765.

Giacani L, Sambri V, Marangoni A, Cavrini F, Storni E, Donati M, *et al*. (2005). Immunological evaluation and cellular location analysis of the TprI antigen of *Treponema pallidum* subsp. *pallidum*. *Infect Immun* 73:3817-3822.

Hanioka T, Tanaka M, Takaya K, Matsumori Y, Shizukuishi S (2000a). Pocket oxygen tension in smokers and non-smokers with periodontal disease. *J Periodontol* 71:550-554.

Hanioka T, Tanaka M, Ojima M, Takaya K, Matsumori Y, Shizukuishi S (2000b). Oxygen sufficiency in the gingiva of smokers and non-smokers with periodontal disease. *J Periodontol* 71: 1846-1851.

Izard J, Samsonoff WA, Limberger RJ (2001). Cytoplasmic *Treponema denticola* has pleiotropic defects. *J Bacteriol* 183:1078-1084.

Li H, Ruby J, Charon N, Kuramitsu H (1996). Gene inactivation in the oral spirochete *Treponema denticola*: construction of an *flgE* mutant. *J Bacteriol* 178:3664-3667.

Limberger RJ (2004). The periplasmic flagellum of spirochetes. *J Mol Microbiol Biotechnol* 7:30-40.

Limberger RJ, Slivienski LL, Izard J, Samsonoff WA (1999). Insertional inactivation of *Treponema denticola tap1* results in a nonmotile mutant with elongated flagellar hooks. *J Bacteriol* 181: 3743-3750.

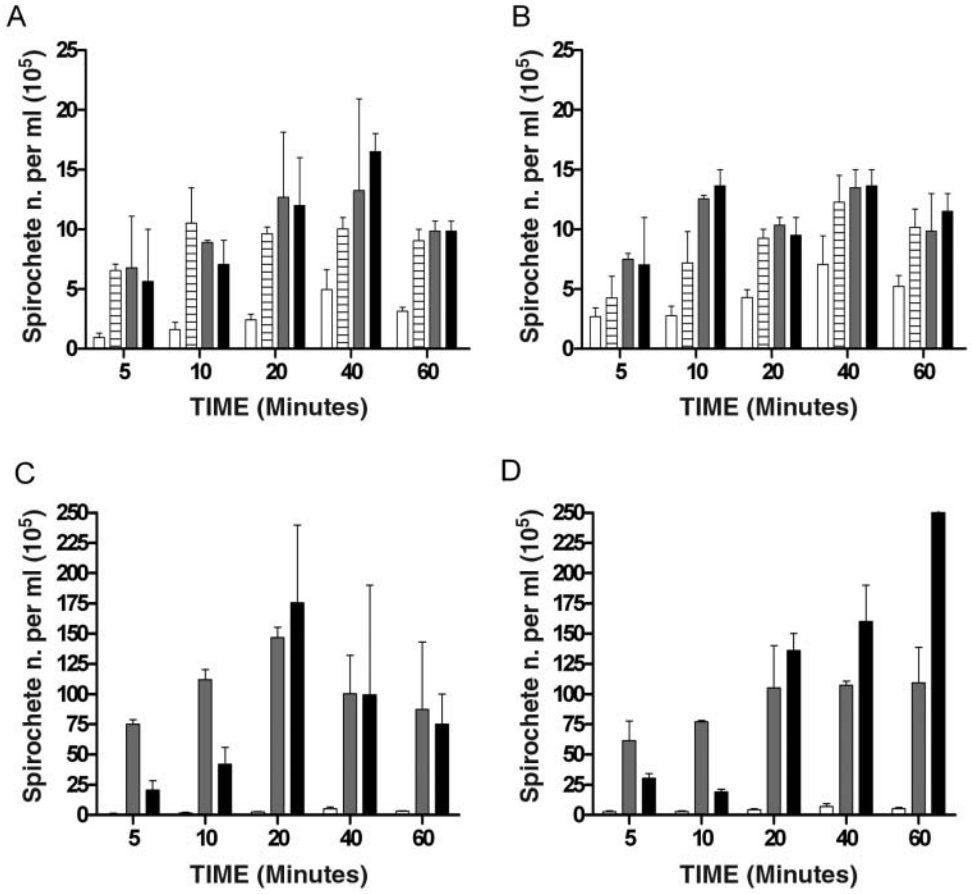

Figure 4. Macrophage uptake of *T. denticola* analyzed by real-time PCR. **(A)** Wild-type and mutant strains under anaerobic conditions. **(B)** Wild-type and mutant strains under aerobic conditions. The bars indicate the quantitative detection of DNA from the wild-type cells (white bars), the wild-type cells opsonized with anti-MSP antibodies (bars with horizontal stripes), the non-motile mutant cells (grey bars), and the filamentous mutant cells (black bars). The differences observed between the number of positive macrophages challenged by the wild-type strain and the opsonized wild-type strain ($p < 0.05$), or the mutant strains ($p < 0.05$ for either strain), are statistically significant in both anaerobic (A) and aerobic conditions (B). **(C)** Effects of different ratios of incubation between macrophages and the *T. denticola* wild-type strain under anaerobic conditions. **(D)** Effects of different ratios of incubation between *T. denticola* wild-type strain and macrophages under aerobic conditions. White bars indicate 10/1 ratio, grey bars, 50/1, and black bars, 100/1. The results are representative of 3 independent experiments, each done in triplicate, and are expressed as the means ± standard deviations (error bars) of 3 replicates.

Loesche WJ, Grossman NS (2001). Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. *Clin Microbiol Rev* 14: 727-752.

Lux R, Miller JN, Park NH, Shi W (2001). Motility and chemotaxis in tissue penetration of oral epithelial cell layers by *Treponema denticola*. *Infect Immun* 69:6276-6283.

Magalhães MA, Sun CX, Glogauer M, Ellen RP (2008). The major outer sheath protein of *Treponema denticola* selectively inhibits Rac1 activation in murine neutrophils. *Cell Microbiol* 10:344-354.

Marangoni A, Sambri V, Cavrini F, Frabetti A, Storni E, Accardo S, *et al*. (2005). *Treponema denticola* infection is not a cause of false positive *Treponema pallidum* serology. *New Microbiol* 28:215-221.

Mathers DA, Leung WK, Fenno JC, Hong Y, McBride BC (1996). The major surface protein complex of *Treponema denticola* depolarizes and induces ion channels in HeLa cell membranes. *Infect Immun* 64: 2904-2910.

Riviere GR, Riviere KH, Smith KS (2002). Molecular and immunological evidence of oral *Treponema* in the human brain and their association with Alzheimer's disease. *Oral Microbiol Immunol* 17:113-118.

Rosen G, Sela MN, Naor R, Halabi A, Barak V, Shapira L (1999). Activation of murine macrophages by lipoprotein and lipooligosaccharide of *Treponema denticola*. *Infect Immun* 67:1180-1186.

Ruby J, Rehani K, Martin M (2007). *Treponema denticola* activates mitogen-activated protein kinase signal pathways through Toll-like receptor 2. *Infect Immun* 75:5763-5768.

Ruby JD, Li H, Kuramitsu H, Norris SJ, Goldstein SF, Buttle KF, *et al*. (1997). Relationship of *Treponema denticola* periplasmic flagella to irregular cell morphology. *J Bacteriol* 179:1628-1635.

Sambri V, Aldini R, Massaria F, Montagnani M, Casanova S, Cevenini R (1996). Uptake and killing of Lyme disease and relapsing fever borreliae in the perfused rat liver and by isolated Kupffer cells. *Infect Immun* 64:1858-1861.

Sambri V, Marangoni A, Olmo A, Storni E, Montagnani M, Fabbi M, *et al*. (1999). Specific antibodies reactive with the 22-kilodalton major outer surface protein of *Borrelia anserina* Ni-NL protect chicks from infection. *Infect Immun* 67:2633-2637.

Sela MN (2001). Role of *Treponema denticola* in periodontal diseases. *Crit Rev Oral Biol Med* 12:399-413.

Siqueira JF Jr, Rôças IN, Favieri A, Santos KR (2000). Detection of *Treponema denticola* in endodontic infections by 16S rRNA gene-directed polymerase chain reaction. *Oral Microbiol Immunol* 15:335-337.