Journal of Microbiology, Immunology and Infection (2013) 46, 164—170

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ORIGINAL ARTICLE

Involvement of Toll-like receptor 2 in apoptosis of *Aggregatibacter actinomycetemcomitans*-infected THP-1 cells

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Received 6 January 2012; received in revised form 31 January 2012; accepted 9 February 2012

KEYWORDS
Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans*;
Apoptosis;
THP-1 cells;
Toll-like receptor 2

Background/Purpose: Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans* is a gram-negative bacterium that has been associated with aggressive periodontitis. *A actinomycetemcomitans* infection induces apoptosis in the human monocytic cell line THP-1, and p38 mitogen-activated protein kinase (p38) activity and tumor necrosis factor-α (TNF-α) production are enhanced by *A actinomycetemcomitans* infection. However, mechanisms governing the recognition of *A actinomycetemcomitans* by monocytes during apoptosis have not been elucidated. *A actinomycetemcomitans* cell wall components stimulate Toll-like receptor (TLR)2 and/or TLR4. The authors examined whether TLR2 and/or TLR4 were involved in the apoptosis of *A actinomycetemcomitans*-infected THP-1 cells.
Methods: A actinomycetemcomitans-infected THP-1 cells were transferred to six-well culture plates and incubated for 0 to 6 hours. In some experiments, THP-1 cells were incubated with anti-TLR2, anti-TLR4, or isotype control antibody (10 μg/mL) for 30 minutes prior to *A actinomycetemcomitans* infection. Expression of messenger RNA (mRNA) was examined by reverse transcription-polymerase chain reaction. Intracellular bacteria recovered from *A actinomycetemcomitans*-infected cells and apoptotic cells were detected by APOPercentage dye (Biocolor Ltd, Northern Ireland, UK) staining. Cellular p38 activity and TNF-α production were assessed with enzyme-linked immunosorbent assay.

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doi:10.1016/j.jmii.2012.02.003

Results: The expression of TLR2 mRNA was increased by *A actinomycetemcomitans* infection. Phagocytosis and apoptosis in *A actinomycetemcomitans*-infected THP-1 cells were inhibited by the addition of anti-TLR2 antibody. Also, anti-TLR2 antibody suppressed the activation of p38 and production of TNF-α in *A actinomycetemcomitans*-infected THP-1 cells.
Conclusion: These results suggest that *A actinomycetemcomitans* induces increased expression of TLR2, leading to phagocytosis and apoptosis of THP-1 cells via p38 activation and TNF-α production.

Introduction

Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans* has been implicated in the pathogenesis of aggressive periodontal disease.[1] *A actinomycetemcomitans* possesses various virulence factors; in addition, *A actinomycetemcomitans* can invade periodontal tissue and survive inside host cells.[2] An earlier study developed an *in vitro* infection model for *A actinomycetemcomitans* to provide evidence for the apoptosis of human epithelial cell line KB and human monocytic cell line THP-1 after infection. The invasion of *A actinomycetemcomitans* into KB cells induced apoptosis.[3,4] This mechanism could occur in gingival epithelial cells, inducing apoptosis without inflammation. We have shown that the induction of apoptosis in *A actinomycetemcomitans*-infected THP-1 cells occurs via the p38 mitogen-activated protein kinase (p38) pathway.[4] The mechanisms governing the recognition of *A actinomycetemcomitans* by monocytes during apoptosis have not yet been elucidated.

Various pattern recognition receptors (PRRs) in innate immune cells recognize specific structures of microorganisms. The target of innate immune recognition is the conserved bacterial pathogen-associated molecular patterns (PAMPs) of microorganisms including lipopolysaccharide (LPS), lipoprotein, peptidoglycans, and lipoteichoic acid.[5] PAMPs are essential for microbial survival and are conserved structures among many pathogens, allowing innate immunity to recognize microorganisms with remitted numbers of PRRs. Among PRRs, Toll-like receptors (TLRs) have been highlighted as key recognition homologues of the innate immune system.[6]

TLRs belong to a family of leucine-rich repeat proteins that are either expressed at the cell surface or in the intracellular compartments of inflammatory cells.[7] Twelve types of TLRs have been identified in humans, each of which recognizes a particular ligand.[6,8] TLR2 and TLR4 are the principal signaling receptors for bacterial cell wall components. In general, LPS of gram-negative bacteria is recognized by TLR4. TLR2 recognizes various bacterial components, including peptidoglycans, lipopeptide, and lipoprotein. [6,7,9,10]

In periodontal tissues, TLR2 and TLR4 are involved in the progression of inflammation and are protective against bacterial infection. [11,12] Increased expression of TLR2 and TLR4 was observed in inflamed gingival tissues.[11] Although the cell wall components of most gram-negative periodontal bacteria exclusively stimulated TLR2, *A actinomycetemcomitans* was capable of stimulating both TLR2 and TLR4.[12] Both TLR2 and TLR4 are expressed on antigen-presenting cells such as macrophages and dendritic cells.[13,14] These TLRs stimulate activation of the cells and the production of cytokines including interleukin (IL)-1β and tumor necrosis factor (TNF)-α, resulting in an adaptive immune response.[15] TLR signaling often induces apoptosis in certain types of cells.[16,17]

We hypothesized that apoptosis in *A actinomycetemcomitans*-infected THP-1 cells is induced by the activation of TLR2 and/or TLR4.

Methods

Cells and growth conditions

A actinomycetemcomitans Y4 strain was grown in Todd-Hewitt broth (Becton Dickinson, Cockeysville, MD, USA) supplemented with 1% yeast extract at 37°C for 1 day in an atmosphere of 5% CO_2 in air. Human monocytic THP-1 cells (JCRB0112.1; JCRB, Tokyo, Japan) were maintained in RPMI 1640 medium (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in 5% CO_2 in air.

In vitro infection procedure

THP-1 cells were infected with *A actinomycetemcomitans* according to the procedures described in our previous study. Briefly, THP-1 cells were suspended in microtubes at a concentration of 2×10^7 cells/mL. *A actinomycetemcomitans*, which was harvested by centrifugation and suspended in RPMI 1640 medium without antibiotics, was added to THP-1 cells in microtubes at a bacterium-to-cell ratio of 1000:1; subsequently, tubes were centrifuged at 1000 $\times$ g for 10 minutes at 4°C. Next, the tubes were incubated in RPMI 1640 medium containing 5% FBS without antibiotics at 37°C for 30 minutes. Infected cells were washed via a series of centrifugations with RPMI 1640 medium supplemented with penicillin, streptomycin, and gentamicin (200 μg/mL) to remove nonadherent bacteria. After transfer to six-well culture plates, the infected cells were incubated in RPMI 1640 medium supplemented with 5% FBS, penicillin, streptomycin, and gentamicin for 0 to 6 hours. Anti-TLR2, anti-TLR4 (Imgenex, San Diego, CA, USA; 10 μg/mL), or isotype control antibody (Sigma-Aldrich;

10 μg/mL) was introduced to THP-1 cells at 30 minutes prior to and during the infection procedure.

Determination of TLR mRNA level

THP-1 cells uninfected and infected with *A actinomycetemcomitans* were cultured for 3 hours; subsequently, the TLR mRNA level was examined by reverse transcription polymerase chain reaction (RT-PCR). Transcribed complementary DNA (cDNA) was amplified with specific primers for human TLR2, TLR4 (TLR-RT-Primers Set; InvivoGen, San Diego, CA, USA), and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Applied Biosystems, Foster City, CA, USA). The amount of DNA amplified was related to the housekeeping gene GAPDH. PCR procedures were as follows: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes; 25 cycles. Subsequently, PCR products were stained with 1% ethidium bromide solution, after which they were separated by electrophoresis on a 2% agarose gel. The expression of TLR2 and TLR4 mRNA was semiquantified in relation to the housekeeping gene GAPDH using the Molecular Imager System (Bio-Rad Laboratories, Hercules, CA, USA).

Phagocytosis of *A actinomycetemcomitans* in THP-1 cells

Infected THP-1 cells were lysed in 1% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes. The lysates, which were appropriately diluted with PBS, were plated on Todd-Hewitt agar containing 0.5% yeast extract. The plates were incubated for 24 hours at 37°C in an atmosphere of 5% CO_2; subsequently, the number of colony-forming units (CFUs) was determined.

Determination of apoptosis

Infected THP-1 cells were stained with APOPercentage dye (Biocolor Ltd, Northern Ireland, UK). Exposure of phosphatidylserine at the exterior surface of the cell membrane has been linked to the onset of the execution phase of apoptosis. Phosphatidylserine transmembrane movement results in uptake of APOPercentage dye (Biocolor Ltd) by apoptotic-committed cells.[18] Upon completion of staining, apoptotic cells were counted and the percentages of apoptotic cells among infected cells were calculated.

Determination of p38 activity

Infected THP-1 cells were incubated for 1 hour. After incubation, cells were collected and lysed with 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma-Aldrich). Lysate samples were used for p38 activity enzyme-linked immunosorbent assay (ELISA; p38 MAP Kinase [pTpY180/182] assay kit; BioSource International, Camarillo, CA, USA); results were expressed as U/mL.

Determination of TNF-α production

Infected THP-1 cells were incubated for 3 or 6 hours. Culture supernatant was collected and assessed for TNF-α levels with a commercial ELISA kit (Human TNF-α ELISA kit; BioSource International).

Statistical analysis

Statistical analyses were conducted using a software program (JMP5.1, SAS Institute Inc., Cary, NC, USA). Results were expressed as mean ± standard deviation. The Tukey-Kramer honestly significant difference (HSD) test was used to evaluate differences with respect to group means.

Results

We observed expression levels of TLR2 and TLR4 mRNA in *A actinomycetemcomitans*-infected THP-1 cells by RT-PCR. The expression level of TLR2 mRNA was significantly higher in the cells at 3 and 6 hours postinfection than in the control cells. No differences in TLR4 mRNA levels were detected between uninfected and infected cells (Fig. 1).

Next, we examined whether TLR2 and TLR4 were involved in the phagocytic capacity of THP-1 for *A actinomycetemcomitans*. The amount of live *A actinomycetemcomitans* recovered from infected THP-1 cells was evaluated by CFUs because phagocytosis was not performed. The effect of anti-TLR2 and anti-TLR4 antibodies on the amount of the *A actinomycetemcomitans* was evaluated. The amount of live *A actinomycetemcomitans* present in infected cells with anti-TLR2 antibody gradually decreased over time, and this amount was significantly lower at all times after infection than in the control cells. Anti-TLR4 and isotype control antibodies did not affect the amount of *A actinomycetemcomitans* present after infection (Fig. 2). The results indicate that TLR2 alone may be involved in *A. actinomycetemcomitans*-induced THP-1 apoptosis. To confirm this hypothesis, we examined whether anti-TLR2 antibody directly inhibited apoptosis. The percentage of apoptotic cells was significantly increased at 3 and 6 hours after *A actinomycetemcomitans* infection. The elevated number of apoptotic cells dropped sharply upon addition of anti-TLR2 antibody, whereas isotype control antibody failed to reduce the percentage of apoptotic cells (Fig. 3).

We previously demonstrated that p38 signaling may regulate apoptotic signaling by *A actinomycetemcomitans* infection, and that TNF-α production was involved in p38 activation.[4] We analyzed the effect of anti-TLR2 antibody on p38 activity and TNF-α production of *A actinomycetemcomitans*-infected THP-1 cells. The p38 activity significantly increased in infected cells; however, the elevated activity was markedly suppressed by anti-TLR2 antibody. The isotype control antibody did not affect p38 activity (Fig. 4). TNF-α production was significantly higher in the *A actinomycetemcomitans*-infected cells than the control cells. The addition of anti-TLR2 antibody decreased

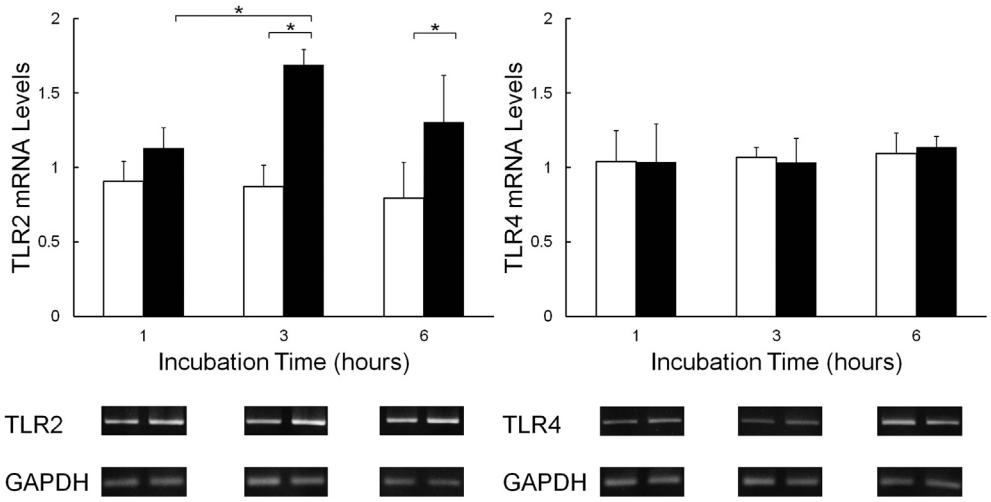

Figure 1. TLR2 and TLR4 mRNA levels in *A actinomycetemcomitans*-infected THP-1 cells. THP-1 cells were infected with *A actinomycetemcomitans* Y4 at bacterium:cell ratios of 0:1 (clear bars) and 1000:1 (black bars), followed by incubation for 1, 3, or 6 hours. Total RNA was isolated from THP-1 cells. Transcribed complementary DNA was amplified via PCR using specific primers for human TLR2 or TLR4. The ratio of TLR2 and TLR4 mRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA. Representative gel images are presented.

the production of TNF-α in *A actinomycetemcomitans*-infected cells. The isotype control antibody did not decrease TNF-α production in infected THP-1 cells (Fig. 5).

Discussion

The current study demonstrated the involvement of TLR2 activation in the apoptosis of *A actinomycetemcomitans*-infected human monocytic cell line THP-1. Monocytes/macrophages play a central role in inflammatory reactions caused by pathogenic bacteria. Many pathogens have evolved specific mechanisms to avoid, alter, or disable the antimicrobial effects of monocytes/macrophages. We have previously shown that *A actinomycetemcomitans* infection induces apoptosis of the murine macrophage cell line J774.1 and the human monocytic cell line THP-1.[4,19,20] These results also indicated that phagocytosis of bacteria might be essential for the induction of apoptosis and that bacterial infection led to p38 activation. It is, however, still unclear what kind of PRRs are involved in the signaling pathways leading to apoptosis.

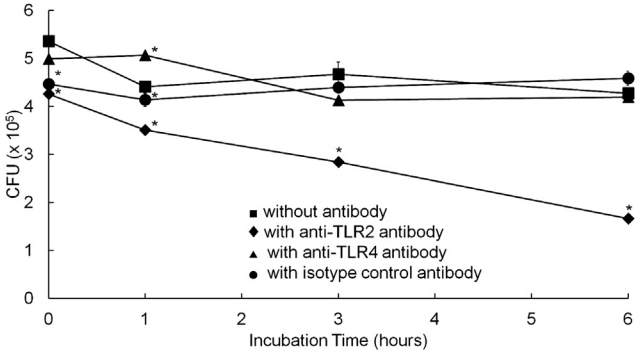

Figure 2. Effects of anti-TLR antibodies on phagocytosis of *A actinomycetemcomitans* in THP-1 cells. THP-1 cells were infected with *A actinomycetemcomitans* Y4 at a bacterium:cell ratio of 1000:1 in the presence of anti-TLR2, anti-TLR4, or isotype control antibody (10 μg/mL). Infected THP-1 cells were collected by centrifugation at 0, 1, 3, and 6 hours post-infection. The amount of intracellular bacteria was expressed as colony-forming unit (CFU) values.

Of the PRRs, TLRs are the main surface links between host innate and adaptive immune defenses. TLR4 mainly recognizes LPS of gram-negative bacteria, whereas TLR2 recognizes lipoproteins expressed by all bacteria and lipopeptides of gram-positive bacteria.[21] *A actinomycetemcomitans* is capable of stimulating both TLR2 and TLR4.[12] In our study, the expression level of TLR2 mRNA was significantly higher in *A actinomycetemcomitans*-infected cells than in the control cells, but this was not the case for TLR4 mRNA.

TLR2 enhanced phagocytosis via augmentation of phagocytic receptors, including scavenger receptors and C-type lectins.[22] We investigated the level of phagocytosis of *A actinomycetemcomitans* by THP-1 cells, and whether TLR2 was involved in a phagocytic capacity. The amount of *A actinomycetemcomitans* phagocytosed by THP-1 cells was measured by counting CFUs. Anti-TLR2 antibody significantly decreased the amount of *A actinomycetemcomitans* in THP-1 cells, indicating that blocking of TLR2 inhibited phagocytic capacity. This finding suggests that TLR2 signaling may be involved in the phagocytic capacity of THP-1 cells, and may also play a part in the apoptotic signaling pathway. Bacterial lipoproteins, which are expressed by all bacteria, activated TLR2, which induced apoptosis.[16] In macrophages infected by *Streptococcus pneumoniae*, TLR2 signaling was active in both phagocytosis and apoptosis.[23] The current study showed that anti-TLR2 antibody inhibited apoptosis of THP-1 cells infected by *A actinomycetemcomitans*. The increased expression of TLR2 may be involved in both phagocytosis and apoptosis of THP-1 cells infected with *A actinomycetemcomitans*.

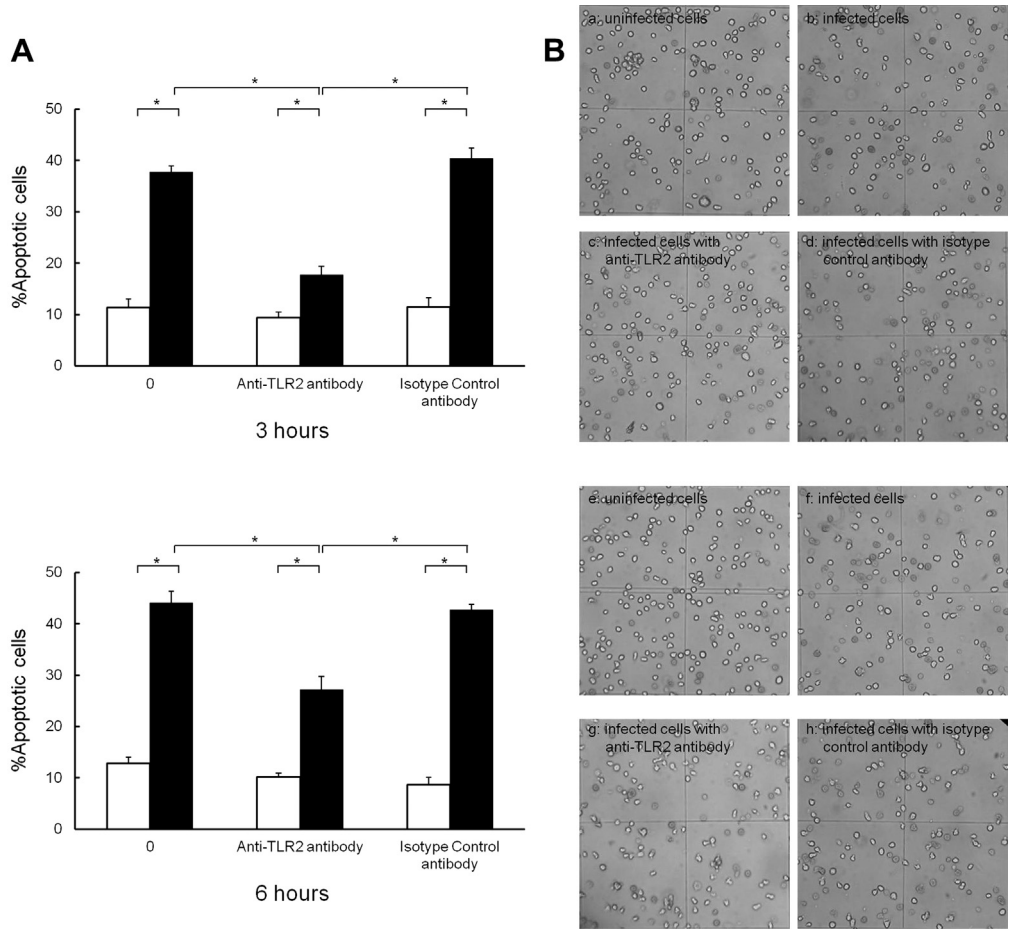

Figure 3. Effect of anti-TLR antibodies on membrane alteration in *A actinomycetemcomitans*-infected THP-1 cells. (A) THP-1 cells were infected with *A actinomycetemcomitans* Y4 at bacterium:cell ratios of 0:1 (clear bars) and 1000:1 (black bars) in the presence of anti-TLR2 or isotype control antibody (10 μg/mL) for 3 or 6 hours. Percentage of apoptotic cells was determined with APOPercentage dye (Biocolor Ltd). (B) APOPercentage dye (Biocolor Ltd) stained in *A actinomycetemcomitans*-infected THP-1 cells for 3 hours (a-d) or 6 hours (e-h). Bar = 50 μm.

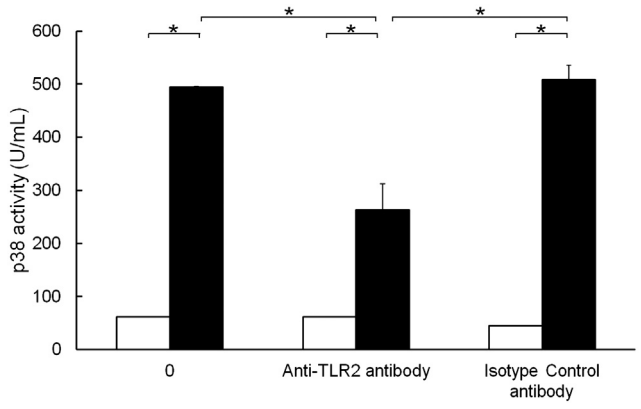

Figure 4. Effect of anti-TLR2 antibody on p38 activity in *A actinomycetemcomitans*-infected THP-1 cells. THP-1 cells were infected with *A actinomycetemcomitans* Y4 at bacterium:cell ratios of 0:1 (clear bars) and 1000:1 (black bars) in the presence of anti-TLR2 or isotype control antibody (10 μg/mL) for 1 hour. p38 activity in cellular extracts was evaluated by enzyme-linked immunosorbent assay.

Our previous study showed that p38 signaling may regulate the apoptosis caused by *A actinomycetemcomitans* infection, and that TNF-α production was involved in p38 signaling.[4] In the current study, we examined whether TLR2 signaling was involved in p38 activation during phagocytosis and apoptosis of THP-1 cells infected by *A actinomycetemcomitans*. The involvement of TNF-α production in the p38 activation was also examined. The addition of anti-TLR2 inhibited p38 activation in THP-1 cells infected with *A actinomycetemcomitans*. Bacterial lipoproteins activated TLR2 and induced apoptosis through MyD88 via a pathway involving Fas-associated death domain protein (FADD) and caspase-8.[16] Recent studies have shown that the outer protein of the bacteria *Yersinia* induced enhanced expression of the IL-1 receptor-associated kinase, FADD, phosphorylation on IκB and p38 in the process of macrophage apoptosis. This apoptosis was inhibited by the addition of p38 inhibitor and neutralization of TLR2 with antibodies, suggesting that TLR2 and p38 may be involved in the apoptotic process.[24] The apoptotic pathway in the current study may be the same as the pathways in these previous reports. Further investigation is needed to clarify this phenomenon. TLRs activate tissue-resident macrophages

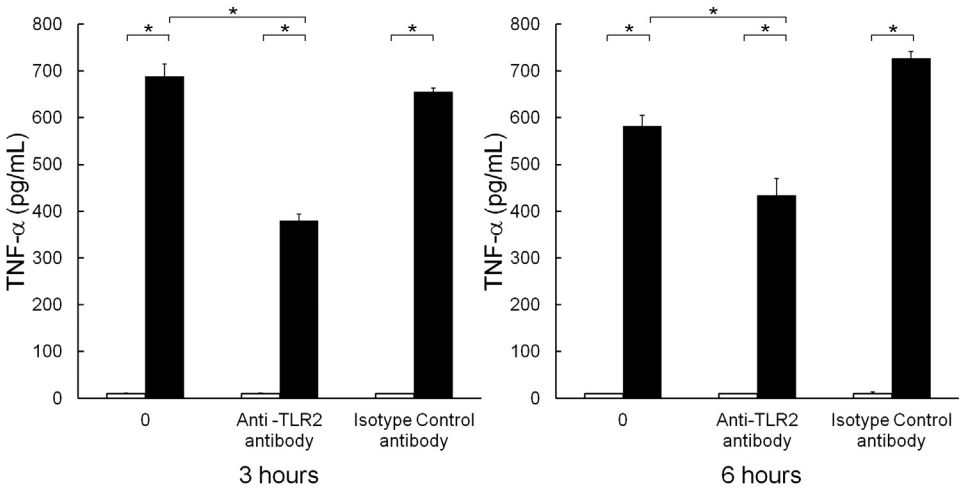

Figure 5. Effect of anti-TLR2 antibody on TNF-α production of THP-1 cells. THP-1 cells were infected with *A actinomycetemcomitans* Y4 at bacterium:cell ratios of 0:1 (clear bars) and 1000:1 (black bars). Tumor necrosis factor-α (TNF-α) levels were measured in the presence or absence of anti-TLR2 or isotype control antibody (10 μg/mL) for 3 or 6 hours.

to produce proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, which coordinate local and systemic inflammatory responses. TLR2 signals lead to TNF-α production and the increased expression of phagocytosis receptors on macrophages.[25] Our results showed that the addition of anti-TLR2 antibody inhibited TNF-α production and p38 activation in THP-1 cells infected with *A actinomycetemcomitans*. These results suggest that *A actinomycetemcomitans* induces increased expression of TLR2, leading to phagocytosis and apoptosis of THP-1 cells via p38 activation and TNF-α production.

Although no increased expression of TLR4 in THP-1 cells infected with *A actinomycetemcomitans* was observed in the current study, *A actinomycetemcomitans* may recognize both TLR2 and TLR4 in the tissues. TLR2- and TLR4-deficient mice developed more severe and less severe periodontitis, respectively, after *A actinomycetemcomitans* infection.[26,27] It was not known how macrophages were involved in these phenomena. We cannot rule out the possibility of involvement of TLR4 expression in macrophages during development of periodontitis caused by *A actinomycetemcomitans* infection. Further investigations are needed to clarify these phenomena.

Another type of eukaryotic cell death was relatively recently proposed as a pyroptosis.[28] The pyroptosis is predicted to be inherently inflammatory and coincides with IL-1β and IL-18 secretion. The pathway of this type of cell death is uniquely depended on caspase-1.[28] Apoptosis pathways are mainly depended on caspase-3, -6, and 7.[28] Apoptosis in another type of macrophage cell line, J774.1, induced by *A actinomycetemcomitans* infection, was involved in the activation of caspase-1, -3, -6, -7, and -9. [29–32] Therefore, cell death might include both apoptosis and pyroptosis pathways. Our previous data showed caspase-3 activity was significantly higher than caspase-1 activity in the cell death of J774.1 infected with *A actinomycetemcomitans*.[29,30] Apoptosis may account for the main aspect of macrophage cell death. Recently, leukotoxin derived from *A actinomycetemcomitans* caused cell death of human macrophages proceeded through pyroptosis.[33] This process might be involved in the purinergic receptor $P2X_7$ but may not be involved in the TLR pathway. The characteristics of cell death caused by *A actinomycetemcomitans* infection requires further investigation.

The results of our study demonstrated that phagocytosis and apoptosis of monocytes/macrophages by *A actinomycetemcomitans* through TLR2 may be one of the relevant mechanisms through which bacteria evade host immune systems and survive in the periodontal tissue. Future examination of signal pathway of the cell death in *A actinomycetemcomitans*-infected immune cells should attempt to clarify the pathogenesis of *A actinomycetemcomitans*, which may lead to the identification of specific molecular targets for periodontal therapy.

Acknowledgments

This work was supported by a grant-in-aid for scientific research (No. 18592267) from the Ministry of Education, Science, Research and Culture of Japan.

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