Oral microorganisms are a common cause of periodontitis, in which accelerated alveolar bone resorption by osteoclasts may ultimately lead to tooth loss. While it is a consensus that periodontal diseases are stimulated by bacterial adherence to the tooth surface, there has been controversy about which bacteria stimulate the irreversible breakdown of periodontal tissues in periodontitis. Among the various species of oral bacteria in humans which have been isolated, cultured and characterized so far, *Porphyromonas gingivalis*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*), *Treponema denticola* and *Actinobacillus actinomycetemcomitans* have been shown to be implicated in infection-driven alveolar bone destruction. The fact that these bacteria are all gram-negative raises the question of whether gram-positive oral bacteria are involved in periodontal diseases. It is well established that bacteria interact with host cells through components of their cell wall by binding to corresponding Toll-like receptors (TLRs). Lipopolysaccharide (LPS), a component of gram-negative bacteria and generally known to be a ligand for TLR4, is the most clearly defined molecule as a potent inducer of osteoclastogenesis and bone resorption. There have been detailed investigations of LPS-induced osteolysis, showing that it enhances bone resorption in many ways, including stimulation of osteoblasts to secrete receptor activator of nuclear factor-κB ligand (RANKL) and IL-6, which consequently induce formation and activation of osteoclasts and support the survival of mature osteoclasts. On the other hand, TLR2 recognizes a variety of bacterial components, LPS, and other bacterial components through its own signaling pathway.

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**Original**

**Pam3CSK4, a TLR2 Agonist, Induces Osteoclastogenesis in RAW 264.7 Cells**

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**Abstract:** To clarify whether Pam3CSK4, a TLR2 agonist, induces the differentiation of osteoclasts, we investigated the osteoclastogenesis and gene expression induced by Pam3CSK4 in RAW264.7 monocyte/macrophage cells. We found that 1 μg/ml Pam3CSK4 induced osteoclastogenesis without adding RANKL exogenously, whereas 1 μg/ml LPS (Re mutant), a ligand for TLR4, failed to produce osteoclasts in RAW 264.7 cells. The number of TRAP-positive multinuclear cells in the Pam3CSK4 group (156.2 ± 26.5 cells/well) was significantly (p<0.01) less than that of 100 ng/ml RANKL (196.5 ± 32.0 cells/well), which was a positive control. Quantitative real-time RT-PCR analysis showed that i) the gene expression levels of TRAP, cathepsin K and matrix metalloproteinase 9, which are osteoclast differentiation markers, were upregulated (p<0.01) by both RANKL and Pam3CSK4, whereas LPS did not increase gene expression of TRAP or cathepsin K, ii) the expression level of RANK was decreased significantly (p<0.01) by both Pam3CSK4 and LPS, but increased by RANKL, iii) the expression levels of TNFα and IL-6, inflammatory cytokines, were upregulated significantly (p<0.01) by both Pam3CSK4 and LPS and iv) the expression level of RANKL was similar to that of other experimental groups in RAW 264.7 cells (p>0.05). Collectively, these results indicate that Pam3CSK4, but not LPS, induces osteoclastogenesis in RAW 264.7 cells in the absence of exogenous RANKL.

**Key words:** Pam3CSK4, osteoclastogenesis, RAW 264.7 cells, TLR2.

Oral microorganisms are a common cause of periodontitis, in which accelerated alveolar bone resorption by osteoclasts may ultimately lead to tooth loss. While it is a consensus that periodontal diseases are stimulated by bacterial adherence to the tooth surface, there has been controversy about which bacteria stimulate the irreversible breakdown of periodontal tissues in periodontitis. Among the various species of oral bacteria in humans which have been isolated, cultured and characterized so far, *Porphyromonas gingivalis*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*), *Treponema denticola* and *Actinobacillus actinomycetemcomitans* have been shown to be implicated in infection-driven alveolar bone destruction. The fact that these bacteria are all gram-negative raises the question of whether gram-positive oral bacteria are involved in periodontal diseases. It is well established that bacteria interact with host cells through components of their cell wall by binding to corresponding Toll-like receptors (TLRs). Lipopolysaccharide (LPS), a component of gram-negative bacteria and generally known to be a ligand for TLR4, is the most clearly defined molecule as a potent inducer of osteoclastogenesis and bone resorption. There have been detailed investigations of LPS-induced osteolysis, showing that it enhances bone resorption in many ways, including stimulation of osteoblasts to secrete receptor activator of nuclear factor-κB ligand (RANKL) and IL-6, which consequently induce formation and activation of osteoclasts and support the survival of mature osteoclasts. On the other hand, TLR2 recognizes a variety of bacterial components, LPS, and other bacterial components through its own signaling pathway.
such as peptidoglycans, triacylated lipoproteins from gram-positive bacteria, mycoplasmal diacylated lipoproteins, and glycosylphosphatidylinositol anchors from *Trypanosoma cruzi*. Among these molecules, osteoclastogenesis by peptidoglycans and diacylated lipopeptide have been investigated extensively. However, these studies were performed in cocultures of osteoblasts and hemopoietic cells and showed that they upregulated RANKL expression in osteoblasts and hence, the direct effects of TLR2 ligation of preosteoclasts on the osteoclastogenesis or bone-resorbing activity had been unclear.

Recently, however, our findings demonstrated for the first time that Pam3CSK4 alone, without adding RANKL exogenously, was able to induce osteoclastogenesis by directly acting on osteoclast precursor cells. To further confirm the Pam3CSK4-induced osteoclastogenesis, we examined whether Pam3CSK4 induces cell fusion in RAW264.7 cells, a murine monocyte/macrophage cell line. Furthermore, since either one of TLR2 and TLR4 recognizes LPS in *Porphyromonas gingivalis*-induced periodontitis, another controversial issue in this field of research, we used LPS purified from *Salmonella enterica* serotype minnesota Re 595, which has been proved to act through TLR4, and compared osteoclastogenesis activity with that of Pam3CSK4, a ligand for the heterodimeric TLR1/TLR2 complex, in the present study.

**Materials and Methods**

**Cell culture**

RAW 264.7 cells (mouse monocyte/macrophage cell line; ATCC, Manassas, VA) were maintained in an α-MEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FCS (Gibco, Invitrogen, NY, USA), 10% GlutaMAX™ (200 mM L-alanyl-L-glutamine, Invitrogen, Carlsbad, CA, USA) and penicillin/streptomycin (Nacalai tesque, Kyoto, Japan) at 37°C in a humidified atmosphere of 5% CO2. For osteoclastogenesis, RAW 264.7 cells (passages 2 to 3) were plated onto 96-well plates at a density of 4.0 × 10^5 cells/well and cultured in α-MEM supplemented with 10% FCS, 10% GlutaMAX™ and penicillin/streptomycin in the presence of 100 ng/ml RANKL (Pepro Tech EC, London, UK), 1 μg/ml Pam3CSK4 (Pam3Cys-SKKKK; EMC microcollections, Tuebingen, Germany) or 1 μg/ml LPS (purified from *Salmonella enterica* serotype minnesota Re 595; Sigma-Aldrich, St Louis, MO, USA) for five days, changing the media every three days at 37°C in a humidified atmosphere of 5% CO2. For quantitative RT-PCR, RAW 264.7 cells (passages 2 to 3) were plated onto 6-well plates at a density of 1.2 × 10^4 cells/well and cultured in α-MEM supplemented with 10% FCS, 10% GlutaMAX™ and penicillin/streptomycin in the presence of 100 ng/ml RANKL, 1 μg/ml Pam3CSK4 or 1 μg/ml LPS for two days at 37°C in a humidified atmosphere of 5% CO2.

**Evaluation of osteoclastogenesis**

RAW 264.7 cell cultures were fixed with 4% paraformaldehyde solution (pH 7.4) overnight, and then stained for TRAP activity by incubating with the mixture consisting of 50 mM sodium tartrate, 45 mM sodium acetate buffer (pH 5.0), 0.01% naphthol AS-BI phosphate (Sigma-Aldrich) and 0.03% fast red violet LB salt (Sigma-Aldrich) at 37°C for 30 min. The cell cultures were observed and photographed on a light microscopy (Axiohot™, Carl Zeiss, Germany) equipped with a camera (AxioCam™ HRc, Carl Zeiss) using software AxioVision™ version 4.6 (Carl Zeiss). The number of TRAP-positive multinucleated (more than three nuclei within a cell) cells was counted on a light microscope.

The intensity of TRAP staining, which is thought to reflect TRAP activity of the cell cultures, was measured at 540 nm on a spectrophotometer Infinite 200™ (Tecan Systems, San Jose, CA, USA) using software i-Control™ version 1.4 (Tecan Systems).

**Quantitative real-time RT-PCR**

Total RNA from RAW 264.7 cell cultures was isolated using the ISOGEN (Nippon Gene, Tokyo, Japan), and one microgram samples were reverse-transcribed
into cDNA using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Then 2.5 μl aliquots were subjected to quantitative polymerase chain reaction in 25 μl reaction mixtures using the specific primers shown in Table 1. Real-time quantitative PCR analysis was performed using Platinum™ SYBR™ Green qPCR SuperMix UDG with ROX (Invitrogen, Carlsbad, CA, USA) on a Applied Biosystems 7900HT Fast Real Time PCR System and SDS software version 2.3 (Applied Biosystems). The reactions were incubated at 50°C for two min, and then heated to 95°C for two min followed by 45 cycles of 15 s at 95°C, and 30 s at 60°C. Specificity of used primer sets was confirmed by the single peak in the dissociation curve drawn by SDS software. The gene expression was compared to expression of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by 2−∆Ct.

Statistical analysis
Levene’s test was used to determine homogeneity of variance and Tukey’s multiple comparison t-tests were performed using Statistical Package for Social Science (SPSS) for Windows™ 11.0.1J (SPSS Japan, Tokyo, Japan).

Results
Pam3CSK4 induces osteoclastogenesis in RAW 264.7 cells
To clarify that Pam3CSK4 induces osteoclastogenesis without adding RANKL exogenously, we examined whether Pam3CSK4 induced cell fusion in RAW264.7 cells, a murine monocyte/macrophage cell line. Our previous results13) showed that Pam3CSK4, ranging from 0.1 to 1 μg/ml, was able to induce osteoclastogenesis dose-dependently in macrophages derived from rat bone marrow in the presence of 20 ng/ml M-CSF. Therefore, 0.1 and 1 μg/ml Pam3CSK4 was examined in the osteoclastogenesis assay in RAW 264.7 cells and shown to possess osteoclast-producing ability at both concentrations (data not shown). As shown in Fig. 1A, 1 μg/ml Pam3CSK4 enhanced the fusion of precursor cells which resulted in the formation of TRAP-positive multinucleated cells (TRAP+MNC) without adding RANKL exogenously, whereas 1 μg/ml LPS failed to induce TRAP+MNC under the same conditions. In seven out of 10 independent osteoclastogenesis experiments, TRAP+MNC formation was observed in response to 100 ng/ml RANKL or 1 μg/ml Pam3CSK4. On the other hand, increase in TRAP activity as measured by the intensity of TRAP staining was observed in 10 out of 10 independent experiments. Although osteoclastogenesis of Pam3CSK4 was confirmed in RAW 264.7 cells without adding RANKL exogenously, Pam3CSK4- induced osteoclastogenesis as well as in rat bone marrow macrophages,13) the number of TRAP+MNC in the Pam3CSK4 group (156.2 +/- 26.5 cells/well; mean +/- SD, n=6) was significantly less than that of 100 ng/ml RANKL (196.5 +/- 32.0 cells/well; mean +/- SD, n=6), which was a positive control (Fig. 1B). Moreover, Pam3CSK4-induced TRAP+MNC

Table 1 Specific primer sequences (5’-3’) for various mouse genes were designed using a software Primer 3™.

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<th>Forward primer</th>
<th>Reverse primer</th>
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<td>CCTGCACCACCCACTGCTTA</td>
<td>TCATGGACCCCTCCACAA</td>
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without adding RANKL exogenously, we examined whether Pam3CSK4 induced cell fusion in RAW264.7 cells, a murine monocyte/macrophage cell line. Our previous results13) showed that Pam3CSK4, ranging from 0.1 to 1 μg/ml, was able to induce osteoclastogenesis dose-dependently in macrophages derived from rat bone marrow in the presence of 20 ng/ml M-CSF. Therefore, 0.1 and 1 μg/ml Pam3CSK4 was examined in the osteoclastogenesis assay in RAW 264.7 cells and shown to possess osteoclast-producing ability at both concentrations (data not shown). As shown in Fig. 1A, 1 μg/ml Pam3CSK4 enhanced the fusion of precursor cells which resulted in the formation of TRAP-positive multinucleated cells (TRAP+MNC) without adding RANKL exogenously, whereas 1 μg/ml LPS failed to induce TRAP+MNC under the same conditions. In seven out of 10 independent osteoclastogenesis experiments, TRAP+MNC formation was observed in response to 100 ng/ml RANKL or 1 μg/ml Pam3CSK4. On the other hand, increase in TRAP activity as measured by the intensity of TRAP staining was observed in 10 out of 10 independent experiments. Although osteoclastogenesis of Pam3CSK4 was confirmed in RAW 264.7 cells without adding RANKL exogenously, Pam3CSK4- induced osteoclastogenesis as well as in rat bone marrow macrophages,13) the number of TRAP+MNC in the Pam3CSK4 group (156.2 +/- 26.5 cells/well; mean +/- SD, n=6) was significantly less than that of 100 ng/ml RANKL (196.5 +/- 32.0 cells/well; mean +/- SD, n=6), which was a positive control (Fig. 1B). Moreover, Pam3CSK4-induced TRAP+MNC
were distinct from those of RANKL morphologically, having edges less sharp than those of RANKL. On the other hand, TRAP+MNC in the LPS group were smaller in size with only several nuclei per cell at most, and the number of TRAP+MNC was 4.1 ± 1.2 (mean ± SD, n=6) cells/well, showing no significant difference from the control (Fig. 1A and 1B). To further quantify the osteoclastogenesis induced by RANKL and Pam3CSK4, TRAP activity was determined by measuring optical density at 540 nm in TRAP-stained cell cultures, which were cultivated independently under the same conditions as in Fig. 1A and 1B. As shown in Fig. 1C, TRAP activity of the RANKL and Pam3CSK4 groups (mean ± SD, n=6; 0.48 ± 0.06 and 0.21 ± 0.03, respectively) was significantly higher than that of the control; however, there was still a significant difference between RANKL and Pam3CSK4 (p<0.01).

Pam3CSK4 upregulates the gene expression of osteoclast markers and inflammatory cytokines in RAW 264.7 cells

To evaluate the differentiation of Pam3CSK4-induced osteoclasts mainly comparing to RANKL, total RNA isolated from RAW 264.7 cells cultured in the presence of 100 ng/ml RANKL, 1 μg/ml Pam3CSK4 or 1 μl/ml LPS for two days, in which TRAP+MNC was not observed yet, were subjected to a quantitative real-time RT-PCR for gene expression. Sequences of the specific primer sets used for detection of key osteoclast markers; TRAP, cathepsin K, matrix metalloproteinase 9 (MMP9) and RANK are described in Table 1. Fold changes of TRAP expression levels normalized to the control group were 110.7 ± 5.1, 21.1 ± 2.8 and 3.5 ± 0.5 (mean ± SD, n=3–4) in RANKL, Pam3CSK4 and LPS, respectively, showing significant upregulation.
Pam3CSK4, a TLR2 Agonist, Induces Osteoclastogenesis

Changes in gene expression levels of osteoclast markers and inflammatory cytokines induced by RANKL, Pam3CSK4 and LPS in RAW 264.7 cells. Total RNA was isolated from RAW 264.7 cells cultured with 100 ng/ml RANKL, 1 mg/ml Pam3CSK4 or 1 mg/ml LPS in α-MEM supplemented with 10% FCS for two days. Complementary DNA, which was reverse-transcribed from total RNA, was subjected to quantitative PCR using Platinum™ SYBR® Green qPCR SuperMix UDG with ROX on an Applied Biosystems 7900HT Fast Real Time PCR System. Expression levels of TRAP (A), cathepsin K (B), MMP9 (C), RANK (D), RANKL(E), TLR2(F), TNFα (G) and IL-1β (H) were expressed as fold changes relative to control. Data represent mean ± SD. *p<0.01 vs control. (I) Since IL-6 expression was not detected (nd) in the control group, fold changes relative to the RANKL group were calculated. Data represent mean ± SD. †p<0.01 vs RANKL.

by RANKL and Pam3CSK4, but not LPS (Fig. 2A). In addition, cathepsin K (Fig. 2B) was increased by RANKL and Pam3CSK4 (mean +/- SD, n=3; 38.5 +/- 3.3 and 6.8 +/- 1.2, respectively) but not by LPS (mean +/- SD, n=4; 2.9 +/- 0.4). Expression of MMP9 was elevated by RANKL, Pam3CSK4 and LPS (mean +/- SD, n=3-4; 53.8 +/- 10.6, 26.5 +/- 7.18 and 34.0 +/- 2.16, respectively, Fig. 2C). In contrast, as shown in Fig. 2D, RANK expression was significantly decreased by both Pam3CSK4 and LPS (mean +/- SD, n=3-4; 0.30 +/- 0.07 and 0.23 +/- 0.02, respectively), but increased by RANKL (mean +/- SD, n=3; 1.53 +/- 0.08). The RANKL expression level by RANKL, Pam3CSK4 and LPS (mean +/- SD, n=3-4; 1.3 +/- 0.3, 1.1 +/- 0.86 and 0.83 +/- 0.35) was similar to the control group (Fig. 2E). The TLR2 expression (Fig. 2F) was elevated by RANKL and Pam3CSK4 (mean +/- SD, n=3-4; 4.9 +/- 3.1 and 13.8 +/- 6.5). Next, we determined gene expression of inflammatory cytokines, TNFα, IL-1β and IL-6, using the specific primer sets described in Table 1,
on the same cDNA used above for osteoclast markers. Expression levels of TNFα and IL-6 were upregulated by Pam3CSK4 and LPS but not by RANKL, whereas IL-1β was upregulated by RANKL, but not by Pam3CSK4 or LPS. Fold changes normalized to the control for TNFα expression were 12.4 +/- 1.9 and 20.0 +/- 2.7 (mean +/- SD, n=3-4) in Pam3CSK4 and LPS groups, respectively. Since IL-6 expression was not detected in the control group, fold changes relative to RANKL were calculated (Fig. 2G), where significant upregulation by both Pam3CSK4 and LPS (mean +/- SD, n=3-4; 775 +/- 171 and 1813 +/- 424, respectively) was demonstrated.

Discussion

The observations in this study provide evidence that, i) Pam3CSK4, a ligand for TLR2, induces differentiation of osteoclasts in the absence of exogenous RANKL, whereas LPS (Re mutant), a ligand for TLR4, fails to produce osteoclasts in RAW 264.7 cells, ii) gene expression levels of TRAP, cathepsin K and MMP9, osteoclast differentiation markers, are upregulated by Pam3CSK4 in RAW 264.7 cells, whereas LPS does not increase gene expression of TRAP or cathepsin K, and iii) gene expression levels of TNFα and IL-6, inflammatory cytokines, are upregulated by both Pam3CSK4 and LPS in RAW 264.7 cells.

Human periodontitis is likely the most common infection-driven chronic inflammatory disease and is characterized by destruction of the supporting tissues of the teeth including alveolar bone. It is widely accepted that many bacteria are capable of stimulating bone matrix loss, and the information available would suggest that each organism possesses different factors which interact with bone in different ways. Tietze et al. have reported that gram-negative and gram-positive bacterial species induce different patterns of immunoregulatory activity, which might be the result of activation of different TLRs. Among these, LPS, a component of gram-negative bacteria and generally recognized as a ligand for TLR4, is the most clearly defined molecule as a potent inducer of osteoclastogenesis and bone resorption. However, in spite of well-defined contribution of LPS from Porphyromonas gingivalis in periodontitis, there has been evidence that LPS fails to activate osteoclasts directly. Both our previous and the present studies also showed that LPS (Re mutant) alone was unable to induce osteoclasts, corresponding to other studies. In contrast, LPS stimulates osteoblasts to secrete IL-1, IL-6, GM-CSF, PGE2 and NO, each of which seems to be involved in LPS-mediated periodontitis. Thus, it is most likely that LPS acts cooperatively with RANKL and inflammatory cytokines in inflammatory conditions exclusively in cocultures and in vivo. On the other hand, the fact that TLR2, in addition to TLR4, was prominently expressed in both precursors and differentiated osteoclasts, raised the possibility that components of gram-positive bacteria, which are known to act through TLR2, are also involved in periodontitis-associated osteolysis.

The development of effective therapies for bacterially induced bone pathology requires an understanding of the cellular and molecular mechanisms involved. Therefore, we sought to investigate osteoclastogenesis and gene expression in response to Pam3CSK4 and LPS, in order to clarify the involvement of TLR2 and TLR4 in osteolysis. Our previous and the present studies showed that Pam3CSK4 stimulated osteoclastogenesis without adding RANKL exogenously in both rat bone marrow macrophages and RAW 264.7 cells, although it was surprising result since RANKL has been well-recognized as an indispensible factor in osteoclastogenesis. However, our results, showing that Pam3CSK4-induced osteoclastogenesis was not accompanied by the elevation of RANK mRNA expression (Fig. 2D), which was consistent with the study by Ji et al., and not impaired by osteoprotegerin clearly revealed that the effects caused by Pam3CSK4 were not dependent on the RANK/RANKL axis.

Furthermore, we have shown that Pam3CSK4 exhibits stimulatory effects distinct from those of RANKL in both rat bone marrow macrophages and RAW 264.7 cells, which can be recognized by morphological changes in mononuclear preosteoclasts, extending pseudopods.
within several hours after the Pam3CSK4 addition, which are characteristics of highly migratory cells. In our preliminary studies on mechanistic evaluation, Pam3CSK4-induced pseudopod extension and cell fusion in preosteoclasts were completely inhibited by the addition of selective TRAF6 decoy peptide, which shows that Pam3CSK4 shares a common molecule with RANKL in the course of the osteoclastogenic process. Most importantly, we need to clarify what causes the difference in RANKL dependency of osteoclastogenesis between TLR2- and TLR4-ligation, since both of these TLRs are known to bind to MyD88, which in turn activates NF-κB through TRAF6 in various cell types. Recently, Harokopakis et al. have reported that Porphyromonas gingivalis fimbrae stimulate monocyte adhesion and transmigration through a pathway involving Rac1, phosphoinositide 3-kinase and TLR2, but not TLR4. This observation, taken together with the fact that Rac1 and phosphoinositide 3-kinase are closely related to cytoskeletal rearrangements, implies that there might be an unidentified pathway which makes osteoclasts cannot be ruled out, because we did not examine the effects of inflammatory cytokine inhibition on the osteoclastogenesis. However, since these cytokines were also upregulated by LPS in RAW264.7 cell cultures, in which no osteoclastogenesis was observed, these cytokines alone, at least, are not likely to contribute to Pam3CSK4-induced osteoclastogenesis.

To clarify the mechanism by which Pam3CSK4 enhances cytoskeletal reorganization, which is a prerequisite for cell fusion to form multinuclear osteoclasts, further studies, especially on the behavior of pro-inflammatory cytokines and chemokine/chemokine receptors, which are considered to be produced by the addition of Pam3CSK4 in osteoclast cultures, are required.

In addition, we indicate that Pam3CSK4 might induce osteoclastogenesis via TLR2 in RANK/RANKL-independent pathways, since RANK expression was significantly decreased by Pam3CSK4, but increased by RANKL in RAW264.7 cells. On the other hand, Zhang et al. demonstrated that Porphyromonas gingivalis abrogates osteoclast differentiation via TLR2 but not TLR4. Thus, further studies are also needed to evaluate the difference of TLRs pathways.

Acknowledgements Authors are grateful to Dr. S. Niida and Dr. S. Moriwaki (National Center for Geriatrics and Gerontology) for providing RAW 264.7 cells which were selected based on the potency to differentiate into large multinuclear osteoclasts. This study was funded by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, Culture and Technology #20592185 (KS).

References


