Role of toll-like receptor 2 in inflammation and alveolar bone loss in experimental peri-implantitis versus periodontitis

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Background and Objective: Peri-implantitis and periodontitis are different entities in immune characteristics even though they share similar features in clinical and radiologic signs. Toll-like receptor 2 (TLR-2), one of the key pathogen-recognition receptors in the innate immune system, plays an important role in the progression of periodontitis. However, the role of TLR-2 in peri-implantitis remains unclear. The objective of this study was to investigate the role of TLR-2 in inflammation and alveolar bone loss in a murine model of ligature-induced peri-implantitis and to compare it with ligature-induced periodontitis.

Material and Methods: Smooth-surface titanium implants were placed in the alveolar bone of the left maxillary molars of wild-type (WT) and Tlr2 knockout (Tlr2-KO) mice 6 weeks after tooth extraction. Silk ligatures were applied to the left implant fixtures and the right maxillary second molars to induce peri-implantitis and periodontitis 4 weeks after implant placement. Two weeks after ligation, bone loss around the implants and maxillary second molars was analysed by micro-computed tomography (micro-CT), and inflammation around the implants and maxillary second molars was assessed at the same time point using histology and TRAP staining, respectively.

Expression of mRNA for proinflammatory cytokines (interleukin-1β [Il1β], tumor necrosis factor-α [Tnfα]), an anti-inflammatory cytokine (interleukin-10 [Il10]) and osteoclastogenesis-related cytokines (Rankl, osteoprotegerin [Opg]) were evaluated, in gingival tissue, using real-time quantitative PCR (RT-qPCR).

Results: The success rate of implant osseointegration was significantly higher in Tlr2-KO mice (85.71%) compared with WT mice (53.66%) (P = .0125). Micro-CT revealed significantly decreased bone loss in Tlr2-KO mice compared with WT mice (P = .0094) in peri-implantitis. The levels of mRNA for Il1β (P = .0055), Tnfα (P = .01) and Il10 (P = .0019) in gingiva were significantly elevated in the peri-implantitis tissues of WT mice, but not in Tlr2-KO mice, compared with controls. However, the gingival mRNA ratios of Rankl/Opg in peri-implant tissues were significantly upregulated in both WT (P = .0488) and Tlr2-KO (P = .0314) mice. Ligature-induced periodontitis exhibited similar patterns of bone loss and inflammatory cytokine profile in both groups of mice, except that the level of Il10 was elevated (P = .0114) whereas the Rankl/Opg ratio was not elevated (P = .9755) in Tlr2-KO mice compared with control mice. Histological findings showed increased numbers of TRAP-positive cells and infiltrated inflammatory cells in ligature-induced peri-implantitis in both WT (P < .01) and Tlr2-KO mice (P < .05), and the numbers of both types of cell were significantly higher in WT mice than in Tlr2-KO mice (P < .01).

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1 | INTRODUCTION

Dental implants have become widely used for edentulous and partially dentate patients because of their high predictability and high success rate.1 However, approximately 30% of patients with dental implants develop peri-implantitis.2 Peri-implantitis is characterized by infection of soft tissue and loss of the surrounding bone, and eventually leads to implant loss.3 It is considered to occur as a result of biofilm formation on the implant surface analogous to periodontitis,4,5 leading to the activation of host immune and inflammatory responses around the implant, which is essential in the pathogenesis of peri-implantitis.6

Although peri-implantitis and periodontitis have many features in common in clinical and radiologic signs, they represent distinct entities7–9 because of their different anatomic and histologic environments, core microbiomes10 and immune characteristics.8,11 Peri-implantitis displays unique features12 and the destruction of tissues in peri-implantitis appears to be of significantly greater severity than occurs in periodontitis.13,14 Quantitative transcriptome analysis indicated that peri-implantitis lesions primarily differ from periodontitis in cell-to-cell adhesion, wound healing, complement activation and innate immune responses.7 It was reported that among 208 transcripts from gingival soft tissue closely related to peri-implantitis and/or periodontitis, transcripts associated with innate immune responses and defense responses were expressed more strongly in peri-implantitis tissue, while in periodontitis tissues, bacterial response genes dominated.7 Some research also revealed differences between periodontitis and peri-implantitis in innate immune responses of the soft connective tissue. Granulation tissue from peri-implantitis sites exhibits stronger expression of mRNA for proinflammatory cytokines compared with granulation tissue from periodontitis sites.15 Moreover, clinical studies have suggested that peri-implantitis represents a more elevated proinflammatory state, with higher levels of interleukin-6, interleukin-8, macrophage inflammatory protein-1b and TIMP-1,11 and a larger number of CD138-, CD68- and myeloperoxidase-positive cells16, compared with periodontitis.

Toll-like receptors (TLRs) are a family of at least 13 proteins that function as key pathogen-recognition receptors in the innate immune system, responding to diverse microbial products and injury-induced endogenous products.17–19 TLR-2 not only mediates cellular responses to a wide variety of pathogens and their products20,21 but also interacts with a wide array of microbial molecules derived from commensal bacteria.22 Previous studies have demonstrated that TLR-2 is required for inflammatory bone loss in periodontitis and TLR-2-dependent osteoclastogenesis can be modulated by Porphyromonas gingivalis through differential induction of the nuclear factor of activated T-cells, cytoplasmic 1 protein and nuclear factor-kappaB.23,24 Furthermore, production of TLR-2-dependent tumor necrosis factor (TNF) is required in macrophage-elicited osteoclastogenesis in response to bacterial stimulation.25,26 However, the role of TLR-2 in peri-implantitis remains unknown. We hypothesized that TLR-2 signaling plays an important role in the pathogenesis of peri-implantitis tissue inflammation and bone loss.

The coexistence of peri-implantitis and periodontitis has become increasingly observed clinically in patients with periodontal disease. It is essential to develop an animal model to incorporate these two diseases in one susceptible host in order to compare their common characteristics with those that are different in the context of microbiota and host immune responses.

The aim of this study was to determine the role of TLR-2 on the inflammation and bone loss in a murine model of experimental peri-implantitis and to compare it with ligature-induced periodontitis. We developed ligature-induced experimental peri-implantitis and periodontitis in the same animal, attempting to simulate the clinical situation of simultaneous development of the two diseases.

2 | MATERIAL AND METHODS

2.1 Mice

Wild-type (WT) C57BL/6 and Tlr2 knockout (Tlr2-KO) mice (B6.129-Tlr2tm1Kir/J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Experiments using these mice were approved by the Institutional Animal Care and Use Committee of the Forsyth Institute. All mice used in the study (4 weeks old; male:female = 2:1) were maintained in specific pathogen-free units of the Forsyth Institute Animal Facility. Mice were fed a soft diet ad libitum for the duration of the experiment.

2.2 Tooth extraction, implant placement and ligature-induced experimental peri-implantitis and periodontitis

The complete procedures are shown in Figure 1. The maxillary first and second molars were extracted on the left side in all mice under general anesthesia by intraperitoneal administration of ketamine (100 mg/kg) and xylazine (5 mg/kg), and the extraction sites were allowed to heal for 6 weeks. Mice were given antibiotics (sulfamethoxazole and trimethoprim, 850 μg/170 μg per mL), dissolved in their drinking water,
for 2 weeks to facilitate healing of the extraction sites. Six weeks later, gingival tissue corresponding to the extraction site was punched manually using a blunt 25G needle (Becton Dickinson Corporation, San Jose, CA, USA). The right maxillary molars were used as a spatial reference. Implants were placed as previously described.27 Briefly, the osteotomy was started by using a 0.3-mm-diameter carbide micro hand drill (D. P. Machining Inc, La verne, CA, USA) by manual rotation into alveolar bone approximately 1 mm in depth. A smooth-surface, screw-shaped titanium implant (1 mm in length and 0.5 mm in diameter; D. P. Machining Inc) was screwed clockwise into the maxillary bone. Implants were allowed to heal for 4 weeks, during which the antibiotics and powder food were given to mice as described above. Four weeks after implant placement, experimental peri-implantitis and periodontitis were initiated. Briefly, a 7-0 silk ligature (Fisher Scientific, Waltham, MA, USA) was placed subgingivally around each implant immediately apical to the implant head on the left side of the maxilla. The right maxillary second molar was tied with a 7-0 silk ligature subgingivally. Two weeks after ligature placement, all mice were killed by CO₂ inhalation. All the procedures, including tooth extraction, implant placement and ligature placement, were performed under optical microscopy (S6D Stereozoom; Leica, Baffalo Grove, IL, USA).

2.3 | Sample preparation

To assess the position and osseointegration of the implant using micro-computed tomography (micro-CT), five mice were killed immediately after implant placement and five mice were killed at the 4-week post-implantation (before ligation) time point in the WT group. Five mice in the WT group and five mice in the Tlr2-KO group were fed for 6 weeks post-implantation without ligation. For each experimental group (ligated or unligated), 10 mice were killed 2 weeks after ligation and were prepared for micro-CT and gene-expression analyses. An additional four mice from each group were prepared for histological evaluation. Maxillae were harvested, the skin and muscle were removed and gingival tissues at the palatal side were collected under surgical microscopy. The gingival tissues were stored at −80°C for future use. The maxillae were defleshed by a dermestid beetle colony. After bleaching with 3% hydrogen peroxide, the bone was scanned with micro-CT. Additional samples were fixed with 10% paraformaldehyde overnight, then decalcified in 10% EDTA for 3 weeks at 4°C with agitation. After complete demineralization, implants were removed manually by rotating counterclockwise. All tissue samples were immersed in 10%, 20% and 30% sucrose solution and then embedded in OCT solution (Tissue-Tek, Sakura Finetek, Torrance, CA, USA). Frozen samples were cut into 8-μm-thick sections along the mesial-distal plane in a cryostat and were collected on Superfrost-plus slides (Fisher Scientific) for histological analysis.

2.4 | Micro-CT analysis

Mice maxillae were scanned using a high-resolution scanner (mCT-40; Scanco Medical, Wayne, PA, USA). Samples were exposed to polychromatic X-rays on a rotating stage at a steep angle of 0.18° over 360°. Measurements were taken at an operating voltage of 70 kVp and 114 mA current and 6 mm isotropic voxel resolution, with an exposure time of 200 ms, and five frames were averaged per view. Quantitative three-dimensional measurements of the implants or teeth were performed using Seg3D software (NIH center for integrative biomedical computing, Salt lake city, UT, USA). Briefly, the same volume of interest (VOI) was chosen for each sample around the second maxillary molar or the implants. A cylinder with a diameter of 1.0 mm and a height of 1.0 mm is defined as VOI from the top surface of implants or a similar position of the natural tooth. A three-dimensional morphometric analysis was conducted to determine the architecture of the bone according to the following: total VOI volume (TV) and total bone volume (BV). The empty space volumes (ESV) surrounding teeth or implants were calculated as TV minus BV. The micro-CT images of implants and natural teeth were converted and collected using Amira software (FEI Visualization Sciences Group, Hillsboro, OR, USA).
2.5 | Real-time quantitative PCR

Palatal gingival tissues were isolated from around both ligatured teeth and ligatured implants, as well as their controls (nonligatured), and were homogenized in lysis buffer using a tissue homogenizer (Omnitron, Carlsbad, CA, USA). Total RNA was extracted using PureLink® RNA Mini Kit (Ambion, Austin, TX, USA). cDNA was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The expression of interleukin-1β, Tnfα, interleukin-10, Rankl and osteoprotegerin (Opg) mRNAs in gingiva were determined by reverse transcription–quantitative real-time PCR (RT-qPCR) using LightCycler® SYBR Green I Master and LightCycler® 480 Instrument (Roche) systems. The sequences of primers are shown in Table 1. The glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene was used as an internal control.

2.6 | Histological analysis

Sections of 8 µm thickness were produced in the mesial–distal plane for hematoxylin and eosin and TRAP staining. Histologic images were captured (DMLS; Leica) and analysed by Image-J software (NIH, Bethesda, MD, USA). For hematoxylin and eosin staining, the number of inflammatory cells in four unit squares (50 µm × 50 µm) of periodontal connective tissue was counted at an objective magnification of 40× and then averaged. For TRAP staining, tissue sections were stained using an acid phosphatase kit (378A; Sigma, St. Louis, MO, USA). After counterstaining with hematoxylin, TRAP-positive cells with three or more nuclei were considered as osteoclasts. A region of interest (ROI) was applied to peri-implantitis samples. It was a 1.5 mm × 1 mm rectangular area of which the longer side aligned with the central long axis of the implant and covered the whole implant length from head to tip. For periodontitis samples, the ROI was the area from the gingiva papilla to the root apex between the second molar and adjacent molars. Each section was acquired under light microscopy at an objective magnification of 40×. Osteoclast numbers either around the implant surface or at the infiltrated connective tissue within the ROI were quantified manually by Image-J.

### TABLE 1 Primers and sequences used for PCR

<table>
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<th>Primers</th>
<th>Sequences</th>
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<tr>
<td>IL-1β</td>
<td>Forward: 5′-ATGCTTCCACAGGCATGT-3′&lt;br&gt;Reverse: 5′-CTGAGCCCTTGGCCG-3′&lt;br&gt;TNF-α</td>
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<th>Variable</th>
<th>Total implants</th>
<th>Lost</th>
<th>Loose</th>
<th>Osseointegrated</th>
<th>SR (%)</th>
<th>SR P-value</th>
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<td>15</td>
<td>4</td>
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<td>.0125</td>
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<td>Tlr2-KO group</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>18</td>
<td>85.71</td>
<td></td>
</tr>
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Tlr2-KO, toll-like receptor 2 knockout.
3.3 Gingival mRNA expression of inflammatory cytokines

In WT mice, the levels of Il1β mRNA expressed in gingiva were significantly increased in the ligated side compared with the control side in both peri-implantitis (P = .0055) and periodontitis (P = .0069), whereas in Tlr2-KO mice, the levels of Il1β mRNA expressed were not significantly changed after ligation compared with controls (Figure 3A,B). The levels of Il1β mRNA expressed in gingiva on the ligated site were significantly higher in WT mice than in Tlr2-KO mice in peri-implantitis (Figure 3A) and periodontitis (Figure 3B). The expression of Tnfα mRNA was similar to that for Il1β mRNA (Figure 3C,D). The levels of Tnfα mRNA expressed in the ligated site were significantly higher in WT mice than in Tlr2-KO mice in peri-implantitis (Figure 3C) and periodontitis (Figure 3D). Meanwhile, the levels of Il10 mRNA expressed in gingiva on the ligated side were significantly higher than those on the control side in WT mice in both peri-implantitis (P = .0019) (Figure 3E) and periodontitis (P = .0218) (Figure 3F). In Tlr2-KO mice, there was no significant difference in the level of Il10 mRNA in gingival tissues around ligated versus non-ligated implants (P = .7899) (Figure 3E), but there was a significant increase in the level of Il10 mRNA in gingival tissues around ligatured teeth compared with those around non-ligated teeth (P = .0114) (Figure 3F). There were no significant differences of Il10 mRNA expression levels in the ligated site in WT mice compared with Tlr2-KO mice in peri-implantitis (Figure 3E) and periodontitis (Figure 3F). The gingival Rankl/Opg relative ratio in peri-implant tissue was significantly elevated after ligation in both WT mice (P = .0488) and Tlr2-KO mice (P = .0314), compared with controls (Figure 3G). The Rankl/Opg ratio in periodontal tissue was significantly increased in WT mice (P = .0133) but was not changed in Tlr2-KO mice compared with the control (P = .9755) (Figure 3H). Also, there were no significant differences of Rankl/Opg ratios on the ligated side in WT mice compared with Tlr2-KO mice in peri-implantitis (Figure 3G) and periodontitis (Figure 3H). Compared with WT mice, a significantly decreased gingival level of Il1β mRNA (P = .0025) (Figure 3A) and Tnfα mRNA (P = .0092) (Figure 3C), but an unchanged level of Il10 mRNA (P = .9730) (Figure 3E) and Rankl/Opg ratio (P = .2139) (Figure 3G), were observed in Tlr2-KO mice after ligature-induced peri-implantitis. Similar results were observed in ligature-induced periodontitis, demonstrating significantly decreased gingival levels of Il1β mRNA (Figure 3B) and Tnfα mRNA (Figure 3D) but an unchanged level of Il10 mRNA (Figure 3F) and Rankl/Opg ratio (Figure 3H). In Tlr2-KO mice, but not in WT mice, the relative level of gingival Il1β mRNA expressed was significantly higher in ligature-induced periodontitis than in ligature-induced peri-implantitis (Fig. S2A). No difference was observed in relative gingival Tnfα mRNA level when comparing ligature-induced peri-implantitis with periodontitis (Fig. S2B). Compared with those from ligature-induced peri-implantitis tissues, gingival Il10 mRNA was significantly increased in ligature-induced periodontitis tissues in both WT and Tlr2-KO mice (Fig. S2C), whereas the Rankl/Opg ratio was significantly decreased in Tlr2-KO mice (Fig. S2D).

3.4 Histological findings

The number of TRAP-positive cells was significantly increased at the ROI in peri-implantitis compared with control in both WT (P < .0001) and Tlr2-KO (P = .0175) mice (Figure 4A,C). Moreover, the increase in the number of TRAP-positive cells in WT mice was significantly greater than that in Tlr2-KO mice (P < .0001) (Figure 4C). The same results were observed in the periodontitis sites (Figure 4B,D) in WT mice (P = .0008) and Tlr2-KO mice (P = .0489), where osteoclastogenesis was also greater in periodontal tissues in WT mice than in Tlr2-KO mice (P = .0019) (Figure 4D). Also, the relative number of TRAP-positive cells at the ligation side was significantly higher in peri-implantitis tissues than in periodontitis tissues in WT mice but not in Tlr2-KO mice (Fig. S3).
Compared with controls, a larger number of inflammatory cells, including plasma cells, macrophages and polymorphonuclear leukocytes, had infiltrated into the periodontal connective tissues in peri-implantitis in WT mice ($P = .0215$) and Tlr2-KO mice ($P = .0143$) (Figure 5A,C). A larger inflammatory infiltrate was found in WT mice than in Tlr2-KO mice ($P = .0144$) (Figure 5C). The same results were observed in the periodontitis sites (Figure 5B,D) in WT mice ($P < .0001$) and Tlr2-KO mice ($P = .0014$), demonstrating a denser inflammatory infiltration in WT mice compared with Tlr2-KO mice ($P = .0047$) (Figure 5D). Moreover, there were no significant differences in the relative number of inflammatory infiltrating cells in ligature-induced peri-implantitis tissue compared with ligature-induced periodontitis tissue in both WT and Tlr2-KO mice (Fig. S4).

**DISCUSSION**

This study showed that alveolar bone loss was alleviated in Tlr2-KO mice compared with WT mice following ligature-induced infection in peri-implantitis, similarly to that observed in periodontitis. This is the first report in a murine model to demonstrate that peri-implantitis bone resorption is associated with TLR-2 signaling analogous to periodontitis.

The Rankl/Opg relative ratio is considered as an accurate diagnostic method for assessing periodontal disease activity.28,29 TLR-2 has been shown to inhibit osteoclastogenesis by downregulating the expression of RANKL.24,30,31 In the present study, we found that the Rankl/Opg mRNA ratio in gingiva was elevated 2 weeks after ligation in peri-implant tissues in both WT mice and Tlr2-KO mice when compared with controls (Figure 3G). These results indicate that TLR-2-mediated peri-implantitis bone loss does not occur through the RANKL/RANK/OPG axis. Indeed, some clinical studies reported that the soluble RANKL/OPG ratio in peri-implant crevicular fluid showed no significant differences between peri-implantitis and healthy controls.32–34 In contrast, in Tlr2-KO mice, no significant difference between ligatured teeth and control was observed (Figure 3H). These results are consistent with our previous findings indicating that TLR-2-mediated periodontal...
bone loss is RANKL dependent. Histological findings further confirmed the molecular profiles we found in this study (Figures 4 and 5). It is noted that because of the limited amount of gingival tissues collected, the protein levels of inflammatory cytokines and RANKL/OPG ratio were not determined, which are warranted to be verified in future studies.

Secretion of proinflammatory cytokines is closely related to the progression of peri-implantitis and periodontitis. Clinical studies have
demonstrated that the levels of interleukin-1β, TNF-α and interleukin-10 in peri-implant crevicular fluid with peri-implantitis were higher than the levels in healthy controls. Our present findings, using a murine model, showed that the expression of Il1β and Tnfα mRNAs were upregulated significantly after ligation in peri-implant tissues (Figure 3A,C) and periodontal tissues (Figure 3B,D) of WT mice but not in those of Tlr2-KO mice, suggesting that the expression of interleukin-1β and TNF-α are commonly regulated through TLR-2 signaling in both peri-implantitis and periodontitis. This observation was supported by our previous study in periodontitis and by other research. Interestingly, upregulated expression of Il10 mRNA in gingiva was observed in WT mice but not in Tlr2-KO mice in ligature-induced peri-implantitis (Figure 3E). However, Il10 mRNA was upregulated in both WT mice and Tlr2-KO mice in ligature-induced periodontitis (Figure 3F). These results suggest that the anti-inflammatory interleukin-10 response in peri-implantitis is TLR-2 dependent, whereas in periodontitis such a response occurs through a TLR-2-independent pathway.

Previous studies have indicated that in osteoblasts TLR-2 signaling was required for cell apoptosis and calcification induced by Staphylococcus aureus and the inhibitory effect of P. gingivalis lip- oids on osteoblast differentiation. Moreover, TLR-2 activation significantly inhibited the migratory response of murine bone marrow stem cells. In agreement with these studies, we found that the os- seointegration in WT mice (53.66%) was significantly lower than that in Tlr2-KO mice (85.71%). Our result suggests that knockout of TLR-2 might benefit implant osseointegration through increasing osteoblast differentiation and downregulating local inflammatory cytokine levels. Furthermore, implant survival was 100% for implants with ligatures in both genotype groups. Pirih et al. reported an osseointegration rate of 82% in WT mice and, at the end of the experiment, implant survival rate was 60%. The difference of implant survival for ligatured implants in WT mice is probably a result of the duration of the experi- mental time period after ligation. In this study, samples were collected 2 weeks after ligation but it was 12 weeks in Pirih’s study.

In the present study, we developed a novel method to evaluate the alveolar bone in peri-implantitis and periodontitis using micro-CT. We defined the VOI as a cylinder (1.0 mm in diameter and 1.0 mm in height) around the implants or natural teeth to standardize the evaluation of bone loss. This method is more accurate for quantifying the irregularity of intrabony bone loss compared with measuring the bone-level value from the implant head in peri-implant and periodontal diseases and can be modified to fit experimental periodontitis models in other species.

Moreover, our study provided a novel animal model to compare peri-implantitis and periodontitis by including the two diseases in the same host environment. The data showed that bone loss was signifi- cantly more severe in peri-implantitis than in periodontitis in WT and Tlr2-KO mice (Fig. S1), and the number of TRAP-positive cells was significantly higher in peri-implantitis than in periodontitis in WT mice but not in Tlr2-KO mice (Fig. S3). These results indicate that peri-implantitis may induce more extensive osteoclastogenesis and bone destruction than occurs in periodontitis under the same host environment and that TLR-2 signaling plays an important role in this process.

In conclusion, the data presented herein demonstrate that TLR-2 signaling mediates bone loss in both peri-implantitis and periodontitis through upregulation of interleukin-1β and TNF-α. However, different molecular features may be involved in TLR-2-mediated bone loss in peri-implantitis (interleukin-10 cytokine response) versus periodonti- tis (RANKL dependent). We provide a useful animal model to dissect pathogenic mechanisms of peri-implantitis and periodontitis simulta- neously, and to explore specific therapeutic targets for each disease.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.
