T Cell Response Mediated by Myeloid Cell-Derived IL-12 Is Responsible for Porphyromonas gingivalis-Induced Periodontitis in IL-10-Deficient Mice

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Periodontal disease is a chronic inflammatory disease in the oral cavity, which culminates in alveolar bone loss. Porphyromonas gingivalis is a consensus periodontal pathogen that has been implicated in adult forms of periodontitis. We previously demonstrated that IL-10-deficient mice exhibit a hyperinflammatory phenotype and are highly susceptible to P. gingivalis-induced periodontitis, indicating an important anti-inflammatory effect of IL-10 in suppressing bone loss. In this study, we analyzed the pathway(s) by which IL-10 deficiency leads to severe P. gingivalis-induced periodontitis. Because Stat3 is essential in IL-10 signaling, immune cell-specific Stat3-deficient mice were subjected to P. gingivalis infection to identify the key IL-10-responsive cells in preventing periodontitis. Myeloid cell-specific Stat3-deficient mice exhibited increased periodontal bone loss \( (p < 0.001) \), whereas T cell- and B cell-specific Stat3 mice were resistant, suggesting that macrophages (MP) and/or polymorphonuclear leukocytes are the key target cells normally suppressed by IL-10. Myeloid cell-specific Stat3-deficient mice exhibited elevated gingival CD40L gene expression in vivo compared with wild-type controls \( (p < 0.01) \), and Stat3-deficient MPs exhibited vigorous P. gingivalis-stimulated IL-12 production in vitro and induced elevated Ag-specific T cell proliferation compared with wild-type MPs \( (p < 0.01) \). Of importance, both IL-12p40/IL-10 and T cell/IL-10 double-deficient mice were resistant to P. gingivalis-induced periodontitis, demonstrating roles for both IL-12p40 and T cells in pathogenesis in a hyperinflammatory model of disease. These data demonstrate that P. gingivalis-induced periodontitis in IL-10-deficient mice is dependent upon IL-12p40-mediated proinflammatory T cell responses. The Journal of Immunology, 2008, 180: 6193–6198.

Materials and Methods

Mice

LysM-Cre transgenic (B6.129P2-LysMcre<em>tm1Mom</em>/J), CD19-deficient (C.129P2-Cd19<em>tm1Cgn</em>/J), IL-10-deficient (B6.129P2-I<em>l10<sup>tm1Cgn</sup></em>/J), IL-12p40-deficient (B6.129S1-Il12b<em>tm1Mom</em>/J), T cell-deficient (B6.129P2-Tcrδ<em>tm1Mom</em>Terδ<em>tm1Mom</em>/J), and C57BL/6J mice were purchased from The Jackson Laboratory. The present study was undertaken to test this hypothesis. Because genetic mutant mice are informative in elucidating the pathways which may contribute to the pathogenesis of periodontitis \( (4, 19–21) \), the key target cell(s) of IL-10 in preventing periodontitis were determined using cell-specific conditional knockouts of Stat3, an essential downstream signaling molecule that transduces anti-inflammatory signals from the IL-10R \( (22) \). In addition, the roles of IL-12 and T cell responses in P. gingivalis-induced periodontitis were also determined using IL-12p40/IL-10 and T cell/IL-10 double-deficient mice.

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Jackson Laboratory. Heterozygotes of CD19-deficient mice express Cre recombinase under the control of the CD19 promoter. Stat3floxflox mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan). Lck-Cre transgenic mice were purchased from The Center for Animal Resources and Development (Kumamoto University, Kumamoto, Japan). These strains were mated to generate macrophage (MP)/polymorphonuclear neutrophil (PMN)-specific Stat3-deficient (LysM-Cre Stat3floxflox), T cell-specific Stat3-deficient (Lck-Cre Stat3floxflox), and cell-specific Stat3-deficient (CD19Cre Stat3floxflox) mice. IL-12p40/IL-10 and T cell-IL10 double-deficient mice. CD19-deficient and Stat3floxflox mice served as wild-type animals in conditional mutant studies. C57BL/6J mice served as wild-type controls in double mutant studies. All animals were maintained under specific pathogen-free conditions in the Forsyth Institute Animal Facility. To prevent background alveolar bone loss caused by food impaction in molars, all animals received a powdered diet beginning at 3 wk after birth until the end of the experiments. All in vivo experiments were completed within 16 wk after birth to avoid any possible effect of age-related alveolar bone loss (4, 23, 24). The use of these animals in this study was approved by the Institutional Animal Care and Use Committee of The Forsyth Institute.

Bacterial culture and infection regimen

*P. gingivalis* W83, a pathogenic strain isolated from a case of human periodontitis, was grown in *Mycoplasma* broth (BD Biosciences), harvested, and resuspended at 10⁸ cells/ml in PBS containing 2% carboxymethylcellulose (CMC; Sigma-Aldrich). Periodontal infection with *P. gingivalis* W83 was conducted following the regimen previously described (4). In brief, 6- to 9-wk-old mice received antibiotic treatment (sulfatrim suspension; 80 mg/L of drinking water) for 4 days to reduce the oral flora, followed by 3 days of an antibiotic-free period, before infection. Animals were then orally inoculated with 10⁸ *P. gingivalis* W83 six times from day 0 at 2-day intervals. Noninfected animals (negative controls) received CMC gavage without *P. gingivalis* W83.

Sample preparation

Animals were killed by CO₂ inhalation on days 0 and 42 relative to the initial oral infection. Mandibles were removed and hemisected. Gingival tissues were isolated under a surgical microscope and stored at −70°C for later RNA extraction. Mandibles were hemisected and defleshed in a demestid beetle colony, bleached, and mounted on a microscope slide for later RNA extraction. Bone loss measurements

Images of mandibular molar teeth and alveolar bone were captured using a digital microscope system and saved as a TIFF file. The area of periodontal bone loss was determined using Adobe Photoshop CS (Adobe Systems). The polygonal area enclosed by the cementoenamel junction, the lateral margins of the exposed tooth root, and the alveolar ridge were measured using ImageJ (W. Rasband, National Institutes of Health, Bethesda, MD). An image of a precise calibrator (Edmund Optics) was captured at the same magnification and was used for the calibration. Results were expressed in millimeters squared.

Quantitative RT-PCR and RT-PCR

Gene expression of CD40L in gingival tissues was assessed by two-step quantitative RT-PCR. Relative CD40L gene expression was determined by the 2−ΔΔCT method (25), where CT is the cycle threshold. The GAPDH gene was used as an internal control based on our preliminary validation of a panel of “housekeeping” genes (data not shown). Total RNA samples were reverse transcribed using the iScript kit (Bio-Rad). Predesigned primers of GAPDH (catalog number QT00309099) and CD40L (QT00104377) and a kit for quantitative PCR were purchased from Qiagen (Quantitect SYBR Green PCR kit). Quantitative PCR was conducted using the iCycler (Bio-Rad) and all reactions were conducted in triplicate. The thermal cycling conditions were as described in the manufacturer’s instruction. *P. gingivalis* W83 colonization in the oral cavity was confirmed by RT-PCR using *P. gingivalis*-specific 16S ribosomal RNA primers (forward: 5’-ATTACGCGGACAAAGTGCAAG-3’, reverse: 5’-TTTACTTACTACACGGTGACACG-3’), as previously described (26). PCR was conducted for 30 cycles, and PCR products (118 bp) were visualized on 1.5% agarose gels containing 1× SYBR Green DNA Gel Stain (Invitrogen).

Macrophage cultures

 Resident peritoneal MPs from MP/PMN-specific Stat3-deficient and wild-type mice were isolated and cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) and 2 mM l-glutamine (Invitrogen) (27). Isolated MPs, plated at 10⁶ cells/well in 96-well plates (n = 4), were stimulated with fixed *P. gingivalis* W83 for 24 h at 37°C in an atmosphere of 5% CO₂/95% air. *P. gingivalis* W83 was grown as described (4), fixed with 0.5% formal saline for 24 h, washed three times in sterile PBS, resuspended in the culture medium, and used to stimulate MPs at 10⁵ bacterial cells/well. After the 24-h incubation, the culture supernatants were collected and stored at −70°C until assay.

Cytokine quantification

Cytokine ELISAs were used to quantify IL-1α, IL-1β, and IL-10 in culture supernatants of the MP cultures. Kits were purchased from R&D Systems (DuoSets) and were used according to the manufacturer’s instructions. Results were expressed as picograms per milliliter.

T cell proliferation assays

Stat3floxflox mice were immunized with fixed *P. gingivalis* W83 (10⁶ cells) by s.c. injection on days −10 and −3. On day 0, animals were sacrificed and submandibular lymph nodes were isolated. T cells were enriched by passing cells through a glass wool and nylon wool column as previously described (28). Adherent splenic APC were isolated from MP/PMN-specific Stat3-deficient and Stat3floxflox mice, and treated with mitomycin C (MMC) as previously described (29). Isolated T cells were cultured (10⁵ cells/well) with MMC-treated APC (10⁶ cells/well) in the presence or absence of fixed *P. gingivalis* W83 (10⁵ cells/well) for 7 days. The proliferation of *P. gingivalis* W83-specific T cells was determined by using CellTiter 96 AQueous (Promega) following the manufacturer’s instructions, and the results were read at 490 nm.

Statistical analysis

The effects of genotypes, *P. gingivalis* W83 infection, and culture conditions on alveolar bone loss, CD40L gene expression, MP cytokine production, and T cell proliferation were evaluated by two-way factorial ANOVA, incorporating genotype and *P. gingivalis* challenge as main effects. Interactions between genotype and *P. gingivalis* challenge were also evaluated. In the presence of statistically significant interactions in the data in Figs. 1–5, comparisons between pairs of means were done by t tests to specifically evaluate 1) *P. gingivalis* infection effects exhibited in differences between means for challenged and nonchallenged groups within the same genotype and 2) genotype effects evaluated by comparisons of means between pairs of genotypes (done separately for challenged and not-challenged animal groups).

Results

The MP/PMN-specific Stat3-deficient mouse is susceptible to *P. gingivalis*-stimulated alveolar bone loss

We previously reported that oral infection with *P. gingivalis* induces significant alveolar bone loss in IL-10-deficient mice (4). To identify the pathway of *P. gingivalis*-induced bone loss in these mice, we first determined the key effector cell type(s) that respond to IL-10 in preventing bone loss. For this purpose, we focused on Stat3, which is a key signal transduction molecule in downstream of IL-10R (30). We used cell-specific conditional knockouts of Stat3, including MP/PMN-, T cell-, and B cell-specific Stat3-deficient mice (n = 10/group). Stat3floxflox mice were used as wild-type controls. All groups were subjected to oral inoculation with *P. gingivalis* W83, and the extent of alveolar bone loss was determined after 42 days. Non-infected animals served as negative controls.

Persistent colonization was confirmed by RT-PCR of the *P. gingivalis*-specific 16S ribosomal RNA gene in gingival tissue samples as indicated in Table I. As shown in Fig. 1, susceptibility to *P. gingivalis*-stimulated alveolar bone loss was observed only in MP/PMN-specific Stat3-deficient mice, which exhibited ~150% of the bone loss compared with noninfected controls (p < 0.0001). In contrast, T cell- and B cell-specific Stat3-deficient mice did not exhibit significant alveolar bone loss after *P. gingivalis* infection, similar to infected wild-type controls. These data demonstrate that...
Table I. Oral colonization of P. gingivalis was confirmed by RT-PCR

| Mouse Strains                  | Noninfected Samples | P. gingivalis-infected Samples |
|-------------------------------|---------------------|-----------------------------|
| Wild type (Stat3<sup>+/+</sup>) | 0/7                 | 8/9                         |
| MP/PMN-specific Stat3 deficient | 0/5                 | 9/9                         |
| T cell-specific Stat3 deficient | 1/4                 | 8/10                        |
| B cell-specific Stat3 deficient | 1/8                 | 9/10                        |

<sup>1</sup> Gingival total RNA samples were subjected to RT-PCR of the P. gingivalis-specific 16S ribosomal RNA gene. Numbers indicate P. gingivalis-positive samples of total samples. Two noninfected animals defined as P. gingivalis-positive exhibited smear-like PCR products compared to other noninfected animals.

expression and increased alveolar bone loss in MP/PMN-specific Stat3-deficient mice is dependent on P. gingivalis infection, and suggests that MP and/or PMNs are key effector cells of IL-10 in suppression of P. gingivalis-induced alveolar bone loss. The lack of bone loss in the other groups is consistent with a previous report that the C57BL/6J wild-type mouse is resistant to P. gingivalis-induced alveolar bone loss (31).

Expression of CD40L gene in gingival tissue

In our previous study, up-regulated T cell activation was observed in gingival tissue during P. gingivalis-stimulated bone loss (4). Accordingly, we determined the expression of the CD40L gene in gingival tissue of MP/PMN-specific Stat3-deficient and wild-type mice by real-time RT-PCR. As shown in Fig. 2, a significant up-regulation of the CD40L gene was observed in P. gingivalis-infected MP/PMN-specific Stat3-deficient mice compared with wild type (p < 0.01). This finding suggests that elevated T cell activation may have an important role in P. gingivalis-stimulated alveolar bone loss in MP/PMN-specific Stat3-deficient mice.

Hyperinflammatory phenotype of Stat3-deficient MPs

In addition to periodontitis, MP/PMN-specific Stat3-deficient mice and IL-10-deficient mice develop enterocolitis. A hyperinflammatory phenotype of MPs, including IL-12 production and a Th1 immune response, appear to induce enterocolitis in these strains. To further assess this pathway in P. gingivalis-infected alveolar bone loss, we determined the response of Stat3-deficient MP to P. gingivalis infection in vitro. As shown in Fig. 3, P. gingivalis stimulation induced a significant elevation in IL-10 in Stat3-deficient MP (>10-fold), supporting our previous finding (27). In addition, Stat3-deficient MP also exhibited a vigorous up-regulation of both IL-12p70 (not detectable in wild-type MP) and IL-10 (8-fold) production compared with wild-type MP following P. gingivalis stimulation. Taken together, these data indicate that endogenous IL-10 is likely a key cytokine in preventing the strong induction of IL-12 by MPs and hence the induction of a vigorous Th1 response.

Elevated P. gingivalis-specific T cell proliferation by Stat3-deficient MPs

We next determined whether Stat3-deficient MP, as APC, induce elevated Ag-dependent T cell proliferation in vitro. MMC-treated Stat3-deficient and wild-type spleen MP were cocultured with T cells, which were derived from P. gingivalis presensitized wild-type animals, in the presence/absence of stimulation with fixed P. gingivalis. As shown in Fig. 4, Stat3-deficient MP induced elevated T cell proliferation compared with wild-type MP (p < 0.01). Fixed P. gingivalis had no mitogenic effect on the proliferation of unsensitized T cells. This result suggests that Stat3 deficiency in
IL-12p40-mediated T cell responses are required for P. gingivalis-induced alveolar bone loss in IL-10-deficient mice

The hyperinflammatory phenotype of Stat3-deficient MP and the increased T cell proliferation in response to P. gingivalis shown above suggest that MP-derived IL-12 may play an important pathogenic role in P. gingivalis-induced alveolar bone loss under conditions of IL-10 signaling deficiency. To directly test this hypothesis, we generated IL-12p40/IL-10-deficient and T cell/IL-10 double-deficient mice, which were then subjected to oral infection with P. gingivalis. As shown in Fig. 5, IL-12p40/IL-10 double-deficient mice exhibited significantly reduced alveolar bone loss compared with IL-10-deficient mice on day 42 after oral infection. In addition, T cell/IL-10 double-deficient mice, in which IL-12p40 is present, also exhibited significantly reduced alveolar bone loss. These data strongly indicate that IL-12p40-mediated proinflammatory T cell responses represent a critical pathway for P. gingivalis-induced alveolar bone loss under conditions of IL-10 deficiency.

Discussion

A number of systemic diseases that possess a hyperinflammatory component, such as IBD, diabetes, and obesity/metabolic syndrome, are associated with periodontal diseases (14, 16, 32–36). IBD and periodontitis are both chronic inflammatory diseases of the alimentary canal, that appear to involve dysregulation of host cytokine expression induced by the resident microbiota. Notably, animals deficient in the key anti-inflammatory cytokine IL-10 are susceptible to both conditions (4, 7).

In the present study, we demonstrated that there is a common pathogenic pathway that underlies periodontitis and enterocolitis when IL-10 is functionally deficient. MP/PMN-specific Stat3-deficient mice, which lack normal IL-10 signaling, are susceptible to P. gingivalis-induced alveolar bone loss, indicating that, similar to enterocolitis (8), myeloid cells are key target cells of IL-10 in preventing infection-stimulated alveolar bone loss. P. gingivalis induced significant up-regulation of CD40L, which occurs after P. gingivalis-mediated initial immune cell suppression (4), in MP/PMN-specific Stat3-deficient mice. Stat3-deficient MPs also exhibited significantly elevated IL-12 and IL-1α production (>10-fold) in response to P. gingivalis, which was similar in magnitude to the overproduction of these mediators in IL-10-deficient MPs (N. Suzuki, H. Sasaki, and P. Stashenko, unpublished observation). Of interest, Stat3-deficient MPs also secreted elevated amounts of IL-10 in response to infection, an apparently compensatory response to the lack of IL-10 signaling. In addition, Stat3 deficiency resulted in significantly elevated P. gingivalis-specific T cell proliferation, supporting previous findings that IL-10 deficiency enhances clonal expansion of specific T cells (37), possibly mediated in part by elevated CD40L gene expression (Fig. 2). Of importance, IL-12p40/IL-10 and T cell/IL-10 double-deficient mice were both resistant to P. gingivalis-induced alveolar bone loss. This pathogenic pathway, which likely involves a highly polarized Th1 response against resident bacteria in the oral cavity, is similar if not identical with that reported previously for enterocolitis (9). Taken together, these data strongly indicate an essential role of IL-12p40-mediated T cell responses in infection-stimulated alveolar bone destruction in an IL-10 deficiency-induced hyperinflammatory state.

Human clinical studies have consistently shown linkages between periodontal disease and IBD including Crohn’s disease and ulcerative colitis (10–13, 17, 18). Patients with active Crohn’s disease had higher gingivitis scores than patients with inactive disease (16). IBD patients experienced a significantly higher prevalence of periodontal disease compared with adults without IBD (14).

A primary role of IL-12 is the induction of Th1 responses (reviewed in Ref. 38) via at least two important effects. First, at the initiation of naïve T cell stimulation, IL-12 enhances differentiation of Th1-type CD4⁺ T cells producing IFN-γ (39). Second is the effect of IL-12 on resting memory T cells. IL-12 inhibits the expression of Th2 type cytokines in allergen-specific human CD4⁺ T lymphocytes from allergic donors by enhancing T cell-derived IFN-γ and IL-2 (40). This effect is maximal when IL-12 is added at the initiation of culture, suggesting an important effect on differentiation of resting memory T cells into Th1-type cells. In previous studies, infection with live P. gingivalis either orally or
s.c. induced a polarized Th1 response, while concomitantly inhibiting production of IL-10 (41, 42). Moreover, preimmunization of BALB/c mice with *P. gingivalis* in the presence of IL-12 resulted in enhanced *P. gingivalis*-specific T cell proliferation, a strongly polarized Th1 immune response including IFN-γ production, and the induction of severe periapical bone loss (43). Although *P. gingivalis* LPS has been reported to induce Th2 effector responses in dendritic cells (44), costimulation of dendritic cells with LPS has significantly enhanced both p35 and p40 IL-12 mRNA, while failing to up-regulate IL-10 mRNA (45). Thus, distinct patterns of polarization of the T cell response may be induced by *P. gingivalis* LPS compared with infection with the live organism.

In patients with periodontal diseases, a negative correlation between disease severity and IL-12 in gingival crevicular fluid has been reported (46). Although this appears to be contradictory to our findings, IL-12 may in fact play a pathogenic role in the initiation and early stages of periodontitis, but could be down-regulated as the disease becomes chronic, a phase that was not examined in these studies. All of the present in vivo studies were completed by 16 wk after birth, so that the age-related effect on alveolar bone loss, which may occur after 30 wk of age and involves other factors such as bone mineral density, was circumvented (23, 24).

There is also evidence that IL-10 and IL-12 may affect bacterial clearance, because IL-10-deficient mice are resistant to tissue destruction in a *P. gingivalis*-induced abscess model, whereas IL-12-deficient mice are susceptible (47). IL-12-deficient mice are defective in IFN-γ production (48), implying that IFN-γ could be involved in the clearance of this pathogen (47). In addition, IL-12 attenuated the persistence and virulence of *P. gingivalis* in experimental mouse periodontitis (49). These findings suggest that IL-10 and IL-12 likely play complex and multifaceted roles in oral inflammation, and that the pathogenic role of IL-12 may be predominantly exerted under conditions of functional IL-10 deficiency.

The present data indicate that IL-10 and IL-12 are key modulators in *P. gingivalis*-induced alveolar bone loss via suppressing/activating the Th1 pathway, and also indicate that IL-10 is functionally dominant over IL-12 in the cytokine network. In this regard, we previously found that individual knockouts of Th1 and Th1-inducing cytokines, including IL-12p40, IL-18, and IFN-γ, exhibited no significant reduction in infection-stimulated bone loss compared with wild-type mice (50). Thus, there are multiple redundancies in Th1-inducing pathways, and the roles of these pathways are not clearly discernable when IL-10 expression is intact. In contrast, double knockouts of IL-12p40/IL-10 or T cells/IL-10 clearly demonstrate the proinflammatory and bone destructive activities of these pathways, when a hyperinflammatory state is created by eliminating IL-10.

There is controversy in the literature concerning whether T cells have a protective homeostatic role in the periodontium (reviewed in Ref. 51) or are predominantly pathogenic/destructive (52, 53). Activated T cells express receptor activator of NF-κB ligand (RANKL), which stimulates osteoclast differentiation and bone resorption, and elevated numbers of receptor activator of NF-κB ligand-expressing T cells are present in chronic human periodontitis tissue (54). However, given that T cells are heterogeneous, their net effect likely reflects the predominant induction of either a pro-(Th1 and Th17) vs anti-inflammatory (TH2 and T regulatory) phenotype. Our findings strongly suggest that periodontal destruction in the hyperinflammatory state is dependent on a pathogenic effect of T cells, either through increases in Th1/Th17 or decreases in Th2/T regulatory. In addition, the finding that *P. gingivalis* infection induced a Th1-dominant response in vivo with a reduction in IL-10 (42) suggests a possible mechanism of *P. gingivalis*-induced immune dysregulation in the pathogenesis of periodontitis in otherwise normal hosts.

In contrast, Th17 cells induce osteoclastogenesis, which links T cell activation and bone resorption (55). Th17 cells may also contribute to the pathogenesis of enterocolitis in IL-10-deficient mice (56). IL-23 induces the differentiation of naïve CD4+ T cells into Th17 cells (57). Because IL-23 is a heterodimer of p19/IL-12p40, IL-12p40 deficiency could also result in the down-regulation of Th17 function (58). These findings suggest that elevated Th17 cells could also be involved in *P. gingivalis*-induced alveolar bone loss in IL-10 deficiency. However, arguing against that possibility is the report that IL-17 inhibits *P. gingivalis*-induced alveolar bone loss via enhancement of neutrophil chemotaxis (19). Additional studies are needed to further clarify these issues, including the key T cell subsets and IL-10-expressing cells in oral inflammation, and how they are regulated following microbial challenge.

Although we focused on periodontitis and enterocolitis in these studies, a similar Th1-dominant state may be induced by other conditions. For example, in obesity and metabolic syndrome, host immune responses are also predisposed to a Th1-dominant profile via IL-12 and leptin (59–61). Recent studies indicate strong clinical associations between metabolic syndrome and periodontitis (33, 34), as well as chronic inflammatory diseases such as arthritis (62), atherosclerosis (63), and diabetes (64). These clinical associations may also be dependent upon the IL-12-mediated Th1 pathway.

In conclusion, the present studies have shown that *P. gingivalis*-induced alveolar bone loss shares the pathogenic pathway of IL-12p40-mediated T cell responses with chronic enterocolitis in IL-10-deficient mice. Our findings suggest that the pathogenesis of these conditions, as manifestations of inflammation in the alimentary canal, may be bidirectional, and that periodontitis may exacerbate systemic hyperinflammatory diseases, and vice versa. IL-12 may thus be useful as a therapeutic target in periodontal diseases, particularly in individuals with associated hyperinflammatory states including IBD (65). Whether this pathway is critical in immunologically normal individuals without associated systemic inflammation remains to be determined.

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The authors have no financial conflict of interest.

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