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Original Article

FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$, and IFN-γ^+CD8$^+$ cell levels in erosive and non-erosive types of oral lichen planus patients

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Background/purpose: Oral lichen planus (OLP) is a localized autoimmune oral mucosal disease. This study evaluated whether different types of OLP patients including erosive OLP (EOLP), major EOLP, minor EOLP, and non-erosive OLP (NEOLP) patients had significantly higher percentages of FoxP3$^+$CD4$^+$ or IFN-γ^+CD4$^+$ cells in total CD4$^+$ cells, and of IFN-γ^+CD8$^+$ cells in total CD8$^+$ cells than healthy control subjects and whether the patient's age had significant influences on these cell percentages in OLP patients.
Materials and methods: Flow cytometry was used to count the FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$, or IFN-γ^+CD8$^+$ cell levels in 183 OLP patients (67 major EOLP, 81 minor EOLP, and 35 NEOLP patients) and 20 healthy control subjects.
Results: Major EOLP patients had a significantly higher FoxP3$^+$CD4$^+$ cell percentage than health control subjects ($P = 0.049$) or minor EOLP patients ($P = 0.008$). Major EOLP patients had a significantly higher IFN-γ^+CD4$^+$ or IFN-γ^+CD8$^+$ cell percentage than healthy control subjects, NEOLP patients, or minor EOLP patients (all P-values < 0.01). In addition, both 61–80 year and 41–60 year OLP patients had significantly higher IFN-γ^+CD8$^+$ cell percentages than

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healthy control subjects or 20—40 year OLP patients (all *P*-values < 0.005).
Conclusion: Major EOLP patients tend to have significantly higher percentages of FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$, and IFN-γ^+CD8$^+$ cells than healthy control subjects, NEOLP patients or minor EOLP patients, suggesting that FoxP3$^+$CD4$^+$ Treg cells are increased to modulate OLP disease activity. Increased number of IFN-γ-producing activated T cell may be involved in oral epithelial cell destruction in OLP patients.

Introduction

Oral lichen planus (OLP) is a T cell dysfunction-induced localized autoimmune and inflammatory oral mucosal disease that affects 1—2% of the population. OLP occurs more frequently in middle-aged and elderly female patients with a female to male ratio of 1.5: 1.[1] However, the female to male ratio of Taiwanese OLP patients is 4: 1 or 5: 1.[1] Previous study showed involvement of both antigen-specific and non-specific mechanisms in OLP. Antigen-specific mechanisms include antigen presentation by basal keratinocytes and antigen-specific keratinocyte killing by CD8$^+$ cytotoxic T cells. Non-specific mechanisms include mast cell degranulation and matrix metalloproteinase activation in OLP lesions.[2,3] Through mast cell/T-cell interactions in OLP lesions, mast cell-released cytokines, chemokines and matrix metalloproteinases can promote T-cell activation, migration, proliferation and differentiation.[4] Histologically, OLP is characterized by liquefaction degeneration of basal epithelial cells and an intraepithelial and subepithelial infiltrate of mononuclear cells which are predominantly CD8$^+$. CD4$^+$ cells can be found mainly in the deep lamina propria.[5,6] An increase in histocompatibility leukocyte antigen (HLA)-DR-positive CD3$^+$ cells in both the local lesional tissues and peripheral blood lymphocytes also indicates T-cell activation in OLP.[6,7]

Although the immune mechanisms involved in OLP remain unclear, considerable researches support the idea that CD4$^+$CD25$^+$ Forkhead-box protein 3$^+$ (FoxP3$^+$) regulatory T cells (Tregs), a subset of T lymphocytes, are involved in the pathogenesis of OLP.[8—10] Treg cells in OLP patients are frequently expanded but functionally deficient.[8—10] This could explain, at least in part, why the increased Treg cells in OLP fail to control the pathogenesis and development of this localized autoimmune disease. Treg cells can suppress the proliferation of conventional T cells and inhibit the cytokine production by these T cells in three different ways: suppression mediated by cell—cell contact, by the secretion of cytokines (such as transforming growth factor β or TGF-β and interleukin 10 or IL-10), and by metabolic disruption. TGF-β plays an important role in down modulation of T cell-mediated immune responses and in controlling autoimmunity.[10,11] FoxP3 is a specific transcription factor for Treg cells and is crucial for the development, differentiation, and suppressive functions of Treg cells. It is the most specific marker of Treg cells, distinguishing Treg cells from other T cells.[11] It has been reported that FoxP3 mRNA expression levels in the explants of OLP lesions and circulating CD4$^+$CD25$^+$ T cells in OLP patients are significantly higher than those in normal control subjects.[10]

IFN-γ can influence naive CD4$^+$ cell differentiation toward a Th1 phenotype and production of IFN-γ is also a characteristic phenotype of activated effector memory CD4$^+$ or CD8$^+$.[12] Langerhans cells are antigen-presenting cells (APCs) in OLP lesions and play a pivotal role in the recruitment of CD4$^+$ and CD8$^+$ cells to the subepithelial region and the subsequent basal keratinocytes apoptosis.[13] IFN-γ can activate CD4$^+$ cells directly and CD8$^+$ cells indirectly to enhance the autoimmune disease activity. The above-mentioned statements indicate that it is very important to measure the FoxP3$^+$CD4$^+$ (Treg) cell levels in OLP patients and to assess the levels of activated CD4$^+$ and CD8$^+$ cells secreting IFN-γ in OLP patients.

OLP more commonly affects buccal mucosa, tongue, and gingiva. It always has a bilateral and symmetric distribution of the oral lesions. Clinically, reticular, papular, plaque-like, atrophic/erosive, ulcerative, and bullous types of OLP can be identified.[1] Moreover, OLP can also be classified briefly into erosive OLP (EOLP; including atrophic/erosive, ulcerative, and bullous types of OLP) or non-erosive OLP (NEOLP; including reticular, papular, and plaque-like types of OLP).[1] In addition, EOLP can be further divided into major EOLP (with erosive or ulcerative oral mucosal lesion ≥ 1 cm in greatest diameter) and minor EOLP (with erosive or ulcerative oral mucosal lesion < 1 cm in greatest diameter).[1,8] EOLP patients usually have oral symptoms such as burning sensation of lesional oral mucosa and sensitivity to spicy and hot food, thus they frequently need specific treatments. However, NEOLP patient may have none of these symptoms or very mild oral symptoms, and they usually need no treatment.[1] Because various types of OLP patients have different disease activities and severities and they may have different autoimmune activities and statuses with different percentages of FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$, and IFN-γ^+CD8$^+$ cells, this study evaluated whether different types of OLP patients including EOLP, major EOLP, minor EOLP, and NEOLP patients had significantly higher percentages of FoxP3$^+$CD4$^+$ or IFN-γ^+CD4$^+$ cells in total CD4$^+$ cells and higher percentages of IFN-γ^+CD8$^+$ cells in total CD8$^+$ cells than healthy control subjects, whether major EOLP patients had higher FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$, and IFN-γ^+CD8$^+$ cell percentages than NEOLP or minor EOLP patients, and whether the age of the OLP patient had significant influences on the

FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$, and IFN-γ^+CD8$^+$ cell percentages in OLP patients.

Materials and methods

Subjects and clinical and histologic diagnoses of oral lichen planus

The study group consisted of 183 OLP patients (42 men and 141 women, age range 20–80 years, mean 54.8 ± 12.2 years) and 20 healthy control subjects (4 men and 16 women, age range 26–71 years, mean 51.9 ± 13.0 years). All the OLP patients and healthy control subjects were seen consecutively, diagnosed, and treated in the Department of Dentistry, National Taiwan University Hospital (NTUH) from August 2010 to July 2013. These 183 OLP patients included 148 EOLP patients (32 men and 116 women, age range 20–80 years, mean 55.3 ± 12.5 years) and 35 NEOLP patients (10 men and 25 women, age range 30–79 years, mean 52.9 ± 10.7 years). They were selected according to the typical clinical presentation of radiating grayish-white Wickham striae, papules and plaques, separately or in combination (NEOLP), and erosion or ulceration on the oral mucosa (EOLP). If the OLP lesions were bilateral, symmetric and typical, no biopsy was needed to confirm the clinical diagnosis. For the uncertainty of clinical diagnosis of OLP, biopsy was performed and the typical histologic features of OLP lesions included hyperkeratosis or parakeratosis, a slightly acanthotic epithelium with liquefaction degeneration of the basal epithelial cells, a pronounced band-like lymphocytic infiltrate in the lamina propria, and the absence of epithelial dysplasia.[14] Those patients with biopsy specimens exhibiting untypical features of OLP were excluded from this study. Moreover, all OLP patients with areca quid chewing habit, autoimmune diseases (such as systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, pemphigus vulgaris, and cicatricial pemphigoid), other inflammatory diseases, or malignancy were also excluded. In addition, all OLP patients with a history of stroke, heavy alcohol use, or diseases of the liver, kidney, or coronary arteries were excluded, too. Healthy control subjects had dental caries, pulpal disease, malocclusion, or missing of teeth but did not have any oral mucosal or systemic diseases. None of the OLP patients and normal control subjects had taken any prescription medication for OLP at least 3 months before entering the study. The blood samples were drawn from OLP patients and healthy control subjects for measurement of the FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$ or IFN-γ^+CD8$^+$ T cell levels. This study was reviewed and approved by the Institutional Review Board at the NTUH.

Human samples and cell isolation

Blood samples were drawn from 183 OLP patients and 20 healthy control subjects. Written informed consent was obtained from each OLP patient or healthy control subject before drawing the blood samples and entering the study. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA). Peripheral blood CD4$^+$ T cells were purified by RosetteSep™ Human CD4$^+$ T Cell Enrichment Cocktail (StemCell Technologies, Cambridge, MA, USA). CD8$^+$ T cells were enriched by negative selection (RosetteSep, Stemcell Technologies).

Flow cytometry analysis (FACS) and intracellular staining

For cell surface staining, cells were stained using the following monoclonal antibodies: CD4 (clone RPA-T4), PerCp-conjugate of CD8 (clone SK1, BD pharmingen, San Diego, CA, USA), and allophycocyanin (APC)-Cy7-conjugate of CD3 (clone HIT3a, Biolegend, San Diego, CA, USA). PBMC (at least 0.2×10^6 cells) were stained with fluorochrome-labeled monoclonal antibodies for 30 min at 4 °C in 100 μl staining buffer (PBS+ 4% Hi-FBS). Appropriate isotype antibody controls were used for each sample. Cells were washed and examined by flow cytometry. For intracellular staining, cells were fixed and permeabilized by Fixation/Permeabilization Kit (eBioscience, San Diego, CA, USA) or Cytofix/Cytoperm™ Kit (BD Biosciences, San Jose, CA, USA) following the manufacture's instruction, and stained using the following monoclonal antibodies: FoxP3 (clone PCH101; from eBioscience) and IFN-γ (clone B27; from BD Biosciences). For cytokine detection, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL), ionomycin (1 μg/mL) (both from Sigma-Aldrich, St. Louis, MO, USA), and monensin (2 μM; eBioscience) for 4 h. Flow cytometry analyses were performed on an LSRFortessa flow cytometer (BD Biosciences), and data were exported as FCS 3.0 for analysis in FlowJo software (Tree Star, Ashland, OR, USA) as we previously described.[11]

Statistical analysis

The percentages of FoxP3$^+$CD4$^+$ cells in total CD4$^+$ cells, of IFN-γ^+CD4$^+$ cells in total CD4$^+$ cells, and of IFN-γ^+CD8$^+$ cells in total CD8$^+$ cells were measured for each OLP patient and normal control subject. Mean percentages (mean $\pm$ standard deviation) of FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$, and IFN-γ^+CD8$^+$ cells were compared firstly between any one group of OLP patients (including 183 OLP, 148 EOLP, 67 major EOLP, 81 minor EOLP, or 35 NEOLP patients) and 20 healthy control subjects and then between any two groups of OLP patients by Student's *t*-test. In addition, the 183 OLP patients were divided into three age groups (including 20–40 year, 41–60 year, and 61–80 year OLP patients). Mean percentages of FoxP3$^+$CD4$^+$, IFN-γ^+CD4$^+$, and IFN-γ^+CD8$^+$ cells were compared firstly between any one age group of OLP patients and normal control subjects and then between any two age groups of OLP patients by Student's *t*-test, too. Differences with the p value < 0.05 were considered statistically significant.

Results

Mean percentages of FoxP3$^+$CD4$^+$ cells in total CD4$^+$ cells, of IFN-γ^+CD4$^+$ cells in total CD4$^+$ cells, and of IFN-γ^+CD8$^+$ cells in total CD8$^+$ cells in different groups of 183 oral OLP patients

Table 1 Mean percentages (mean ± standard deviation) of FoxP3$^+$CD4$^+$ cells in total CD4$^+$ cells, of IFN-γ$^+$CD4$^+$ cells in total CD4$^+$ cells, and of IFN-γ$^+$CD8$^+$ cells in total CD8$^+$ cells in different groups of 183 oral lichen planus (OLP) patients and 20 healthy control subjects.

Patient group	FoxP3$^+$CD4$^+$ cells (%)	IFN-γ$^+$CD4$^+$ cells (%)	IFN-γ$^+$CD8$^+$ cells (%)
OLP patients (n = 183)	4.9 ± 2.4	16.8 ± 10.1	46.0 ± 21.9
[a]*P*-value	0.464	0.211	0.001
EOLP patients (n = 148)	4.9 ± 2.1	17.4 ± 10.3	46.8 ± 22.2
[a]*P*-value	0.406	0.141	<0.001
[b]*P*-value	0.658	0.113	0.285
Major EOLP patients (n = 67)	5.4 ± 1.9	20.7 ± 10.2	57.9 ± 19.9
[a]*P*-value	0.049	0.006	<0.001
[b]*P*-value	0.568	0.003	<0.001
[c]*P*-value	0.008	<0.001	<0.001
Minor EOLP patients (n = 81)	4.5 ± 2.1	14.7 ± 9.7	37.6 ± 19.8
[a]*P*-value	1.000	0.728	0.086
[b]*P*-value	0.249	0.875	0.236
NEOLP patients (n = 35)	5.1 ± 3.4	14.4 ± 8.8	42.4 ± 20.2
[a]*P*-value	0.451	0.826	0.017
OLP patients (n = 183)			
61–80 year (n = 62)	4.8 ± 2.2	17.2 ± 10.1	52.0 ± 21.2
[a]*P*-value	0.563	0.175	<0.001
[d]*P*-value	0.852	0.418	<0.001
[e]*P*-value	0.452	0.856	0.102
41–60 year (n = 99)	5.1 ± 2.6	16.9 ± 10.3	46.3 ± 21.5
[a]*P*-value	0.316	0.214	0.001
[d]*P*-value	0.499	0.478	<0.001
20–40 year (n = 22)	4.7 ± 2.0	15.2 ± 9.3	27.8 ± 15.4
[a]*P*-value	0.700	0.606	0.761
Healthy control subjects (n = 20)'	4.5 ± 1.2	13.9 ± 6.5	29.3 ± 16.3

EOLP = erosive OLP; NEOLP = non-erosive OLP.
[a] Comparisons between any OLP group patients and healthy control subjects by Student's *t*-test.
[b] Comparisons between EOLP, major EOLP, or minor EOLP patients and NEOLP patients by Student's *t*-test.
[c] Comparison between major EOLP and minor EOLP patients by Student's *t*-test.
[d] Comparisons between 61 and 80 year or 41–60 year OLP patients and 20–40 year OLP patients by Student's *t*-test.
[e] Comparison between 61–80 year and 41–60 year OLP patients by Student's *t*-test.

and 20 healthy control subjects are shown in Table 1. We found that major EOLP patients had a significantly higher FoxP3$^+$CD4$^+$ cell percentage (5.4 ± 1.9%) than health control subjects (4.5 ± 1.2%, $P = 0.049$) or minor EOLP patients (4.5 ± 2.1%, $P = 0.008$). Major EOLP patients had a significantly higher IFN-γ$^+$CD4$^+$ cell percentage (20.7 ± 10.2%) than healthy control subjects (13.9 ± 6.5%, $P = 0.006$), NEOLP patients (14.4 ± 8.8%, $P = 0.003$), or minor EOLP patients (14.7 ± 9.7%, $P < 0.001$), Moreover, major EOLP patients had a significantly higher IFN-γ$^+$CD8$^+$ cell percentage (57.9 ± 19.9%) than healthy control subjects (29.3 ± 16.3%, $P < 0.001$), NEOLP patients (42.4 ± 20.2%, $P < 0.001$), or minor EOLP patients (37.6 ± 19.8%, $P < 0.001$). In addition, OLP ($P = 0.001$), EOLP ($P < 0.001$), or NEOLP patients ($P = 0.017$) also had a significantly higher IFN-γ$^+$CD8$^+$ cell percentage than healthy control subjects (Table 1).

If the OLP patients were divided into three age groups of patients: 20–40 year (n = 22), 41–60 year (n = 99), and 61–80 year (n = 82) age groups of patients (Table 1). We found that both 61–80 year and 41–60 year OLP patients had significantly higher IFN-γ$^+$CD8$^+$ cell percentages than healthy control subjects or 20–40 year OLP patients (all *P*-values < 0.005) (Table 1).

Discussion

This study found that major EOLP patients had a significantly higher FoxP3$^+$CD4$^+$ Treg cell percentage than healthy control subjects and minor EOLP patients. Although OLP, EOLP, and NEOLP patients had an elevated FoxP3$^+$CD4$^+$ cell percentage than healthy control subjects, the FoxP3$^+$CD4$^+$ cell percentage did not reach a significantly higher level. These findings indicate FoxP3$^+$CD4$^+$ (Treg) cell number did increase in our OLP patients. OLP is a localized autoimmune oral mucosal disease. The increased Treg cells in OLP patients are supposed to suppress the abnormally higher T cell-mediated autoimmune activity against oral epithelial cells to a lower or normal level and subsequently to modulate the disease severity in OLP patients. It is interesting to know why the increased Treg cells do not possess their inherited function to suppress CD4$^+$ or CD8$^+$ effector memory T cells. One possibility was that these Treg cells had deficits, therefore, they lost their original suppressive function. The other possibility was that these Treg cells were transformed into a non-suppressive phenotype or even become an opposite phenotype to

possess the function on augmentation of the T cell-mediated immunity during the developmental and differentiation process. In fact, a previous study found that although there is a significantly increased number of $CD4^+CD25^+$ Treg cells in OLP patients, these $CD4^+CD25^+$ Treg cells have impaired suppressive function demonstrated by *in vitro* proliferation assay.[8,9] In addition, Schreurs et al.[9] assessed the non-suppressive ($FoxP3^+CD45RA^-CD25^+CD45RO^+$ and $CD15s^-$) and suppressive ($FoxP3^+CD45RA^-CD25^+CD45RO^+$ and $CD15s^+$) phenotypes of $FoxP3^+CD4^+$ cells in biopsied oral lesions of atrophic/erosive and reticular OLP patients. They discovered that oral lesions of atrophic/erosive OLP patients contain higher numbers of $FoxP3^+CD4^+$ cells than those of reticular OLP patients but the main $FoxP3^+CD4^+$ T-cell population in the oral lesions of atrophic/erosive OLP patients is the non-suppressive subset. On the contrary, suppressing phenotypic $FoxP3^+CD4^+$ cells are twice as high in reticular OLP lesions compared to the atrophic/erosive OLP lesions.[9] These findings suggest that the absence of actively suppressing $FoxP3^+CD4^+$ cells may in part explain why the atrophic/erosive OLP lesions have higher disease activity or severity than reticular OLP lesions and why OLP is a remarkably persisting oral mucosal disease, in spite of the presence of substantially high numbers of $FoxP3^+$ $CD4^+$ T cells.[9] Therefore, it is crucial to examine not only numbers but also functional phenotype of $FoxP3^+$ $CD4^+$ T cells in human tissues.

In this study, we also assessed the $CD4^+$ and $CD8^+$ cells isolated from OLP patients could secrete IFN-γ upon activation *in vitro*. We found that both $CD4^+$ and $CD8^+$ cells isolated from OLP patient could produce IFN-γ upon stimulation. Moreover, major EOLP patients had significantly higher IFN-γ^+CD4^+ cell percentage than healthy control subjects, NEOLP patients, or minor EOLP patients. Although OLP, EOLP, minor EOLP, NEOLP patients all had higher IFN-γ^+CD4^+ cell percentage than healthy control subjects, the differences in IFN-γ^+CD4^+ cell percentage did not reach the significant levels. In addition, this study also demonstrated that major EOLP, EOLP, OLP, and NEOLP patients had significantly greater IFN-γ^+CD8^+ cell percentage than healthy control subjects. Furthermore, the severer the OLP disease was, the higher the IFN-γ^+CD8^+ cell percentage was present in OLP patients. When the $CD4^+$ cells in OLP patient produce IFN-γ, the IFN-γ molecules can work together with interleukin 2 to activate $CD4^+$ and $CD8^+$ cells and subsequently enhance $CD8^+$ cell-mediated cytotoxic activity against oral epithelial cells and finally generate OLP lesions in OLP patients. The activated $CD8^+$ cells in this study could also secrete IFN-γ molecules that in turn potentiate the whole vicious cycle against oral epithelial cells in OLP patients. Indeed, a previous study showed that approximately 10% of $FoxP3^+CD4^+$ cells in OLP oral lesions express T-bet, the hallmark transcription factor for IFN-γ-producing T cells, indicating that a distinct subset of $FoxP3^+CD4^+$ cells in OLP lesions or OLP patients are not conventional Treg cells and may produce IFN-γ that enhances autoimmune and inflammatory responses rather than suppress them.

This study found that OLP patients had a significantly higher IFN-γ^+CD8^+ cell percentage than healthy control subjects. Although OLP patients also had higher $FoxP3^+CD4^+$ cell and IFN-γ^+CD4^+ cell percentages than healthy control subjects, the differences were not significant. This study showed no correlation of OLP patients' age with the OLP types or disease severity (data not shown). We further tested whether the age of the OLP patients might influence the $FoxP3^+CD4^+$, IFN-γ^+CD4^+, and IFN-γ^+CD8^+ cell percentages in OLP patients. We discovered significantly higher IFN-γ^+CD8^+ cell percentages in both 61—80 year and 41—60 year OLP patients than in healthy control subjects or 20—40 year OLP patients. Both 61—80 year and 41—60 year OLP patients also had higher $FoxP3^+CD4^+$ and IFN-γ^+CD4^+ cell percentages than healthy control subjects or 20—40 year OLP patients, but the differences were not significant. Moreover, of 183 OLP patient, 99 (54.1%) patients of 41—60 year age group had the highest $FoxP3^+CD4^+$ cell percentage, followed by 62 (33.9%) patients of 61—80 year age group, and 22 (12.0%) patients of 20—40 year age group. The above-mentioned age-related findings of increased activated effector memory T cells in older OLP patients were comparable with the specific OLP clinical features showing the common occurrence of OLP in middle-aged and elderly patients with a highest OLP prevalence in patients between 41 and 60 years of age.

We conclude that major EOLP patients tend to have significantly higher $FoxP3^+CD4^+$, IFN-γ^+CD4^+, and IFN-γ^+CD8^+ cell percentages than healthy control subjects, NEOLP patients, or minor EOLP patients. These findings suggest a significant association of increased IFN-γ^+CD4^+ and IFN-γ^+CD8^+ cell levels with OLP disease activity or severity. However, the increased $FoxP3^+CD4^+$ cells in OLP patients may not be the conventional Treg cells that have inherited function to modulate the disease activity. Both $CD4^+$ and $CD8^+$ cells in OLP patients can produce IFN-γ molecules that are subsequently responsible for the T cell activation and further oral epithelial cell destruction or alteration in OLP patients.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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