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Helicobacter pylori Vacuolating Cytotoxin Inhibits Activation-Induced Proliferation of Human T and B Lymphocyte Subsets

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Helicobacter pylori are Gram-negative bacteria that persistently colonize the human gastric mucosa despite the recruitment of immune cells. The H. pylori vacuolating cytotoxin (VacA) recently has been shown to inhibit stimulation-induced proliferation of primary human CD4+ T cells. In this study, we investigated effects of VacA on the proliferation of various other types of primary human immune cells. Intoxication of PBMC with VacA inhibited the stimulation-induced proliferation of CD4+ T cells, CD8+ T cells, and B cells. VacA also inhibited the proliferation of purified primary human CD4+ T cells that were stimulated by dendritic cells. VacA inhibited both T cell-induced and PMA/anti-IgM-induced proliferation of purified B cells. Intoxication with VacA did not alter the magnitude of calcium flux that occurred upon stimulation of CD4+ T cells or B cells, indicating that VacA does not alter early signaling events required for activation and proliferation. VacA inhibited proliferation of purified primary human CD4+ T cells, but did not reduce the mitochondrial membrane potential of T cells. In this study, we investigated effects of VacA on the proliferation of various other types of primary immune cells. The major effectors of the adaptive immune response, may contribute to the ability of H. pylori to establish a persistent infection in the human gastric mucosa.

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5 Abbreviations used in this paper: VacA, vacuolating cytotoxin; DC, dendritic cell; SEB, staphylococcal enterotoxin B.
Although effects of VacA on primary human CD4+ T cells have been documented (24–26), possible effects of VacA on other types of primary human immune cells have not yet been investigated in detail. In the current study, we demonstrate that VacA can suppress the proliferation of primary human CD8+ T cells and CD4+ Th cells. We show that VacA inhibits proliferation of Th cells regardless whether the cells are stimulated by TCR/CD28 Abs, PMA/ionomycin, or APCs. Moreover, we show that VacA can act directly on B cells and thereby inhibit B cell proliferation. We propose that VacA may enhance the capacity of *H. pylori* to establish a persistent infection by suppressing the activity of CD4+ and CD8+ T cells, as well as B cells, in the gastric mucosa.

Materials and Methods

**Purification of VacA**

*H. pylori* strains were grown as described previously (17, 27). *H. pylori* 60190 is a wild-type strain and strain AV452 secretes a mutant form of VacA (*VacA*Δ–27) that is defective in membrane channel formation (27). Oligomeric forms of VacA were purified from broth culture supernatants of *H. pylori* as described previously (17). All experiments were performed using acid-activated preparations of VacA or acidified buffer control (PBS), unless stated otherwise (16). The final VacA concentration was 10–20 μg/ml for all experiments, as indicated.

**Cell culture and primary cell isolation**

Blood was obtained from healthy human adult volunteer donors, in accordance with procedures approved by the Vanderbilt University Medical Center Institutional Review Board. PBMC were isolated from blood using a Ficoll-Hypaque gradient. Resting (i.e., nonproliferating) CD4+ and CD8+ human T cells were purified as previously described (28). B cells were purified using a CD19 AutoMACS sort (Miltenyi Biotec) using positive selection on the EasySep Human B cell enrichment kit (StemCell Technologies), as described by the manufacturer. The purified cells were 90–99% pure as assessed by staining and flow cytometric analysis. For the generation of dendritic cells (DC), CD14+ monocytes were isolated from PBMC as previously described (28) and cultured in RPMI 1640 complete medium (10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin) supplemented with IL-4 (50 μg/ml; R&D Systems) and GM-CSF (50 μg/ml; R&D Systems) for 5 days. Maturation of the DC was subsequently induced by addition of LPS (100 ng/ml; Sigma-Aldrich) for 1–2 days. Maturation of DC was assessed by staining cells with Abs to CD14, CD83, CD86, and HLA-DR (all from BD Biosciences).

**Proliferation assay and FACS analysis**

For proliferation assays, cells were labeled with CFSE (Molecular Probes) and proliferation of the cells was monitored by CFSE partitioning 5 days poststimulation, as previously described (24). Cell populations were analyzed using a FACSCalibur flow cytometer (BD Biosciences), using anti-CD3-Cy5.5, anti-CD4-biotin, anti-CD8-biotin, anti-CD19-biotin Abs and streptavidin-allophycocyanin. The proliferation index was calculated as the sum of the cells in all generations divided by the estimated number of original parent cells. The estimated number of original parent cells was calculated by dividing the final number of cells in each generation by the squared generation number. Forward and side scatter profiles as well as propidium iodide staining were monitored to evaluate the level of cell death. In all experiments described in this study, VacA treatment of cells did not cause a significant increase in cell death compared with that of cells treated with PBS, as shown previously (21, 24). In most experiments, the tested agents (VacA or various controls) were added only once to cells and were not removed from the cultures, unless stated otherwise.

**Stimulation of primary human PBMC and T cells**

Stimulation-induced proliferation of CFSE-labeled primary human T cells (CD4+ and CD8+) was accomplished by plating 10^5 T cells on a 96-well plate containing plate-bound anti-CD3 (OKT3; American Type Culture Collection) and soluble anti-CD28 Abs (BD Biosciences), which we termed TCR/CD28 stimulation, as previously described (24). Cells were removed from the activation signals after 48 h and expanded in medium supplemented with recombinant human IL-2 (200 U/ml; Chiron) for a total of 5 days. Proliferation of CFSE-labeled PBMC was induced by TCR/CD28 stimulation as described. CFSE-labeled PBMC were also stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich), and after 48 h, the cells were expanded in RPMI 1640 complete medium supplemented with IL-2 as described. The PMA/ionomycin remained present for the duration of the experiment. As controls to inhibit TCR/CD28 stimulation or IL-2-driven stimulation, T cells were treated with cyclosporine A (10 μM; Alexis Biochemicals) or rapamycin (300 ng/ml; Alexis Biochemicals), respectively. Proliferation of T cells and PBMC was monitored by CFSE partitioning 5 days poststimulation, as described.

DC-induced activation of CD4+ T cells was accomplished by mixing CFSE-labeled CD4+ T cells with DC (10:1 ratio) in a 96-well plate, followed by stimulation with staphylococcal enterotoxin B (SEB, 10 ng/ml; Sigma-Aldrich). T cell proliferation was monitored by CFSE partitioning 5 days poststimulation as described.

**Stimulation of primary human B cells**

T cell-induced B cell proliferation was accomplished by treating purified primary human CD4+ T cells with 50 μg/ml mitomycin C (Sigma-Aldrich) for 30 min at 37°C. T cells were then washed twice to remove the mitomycin C. The mitomycin C-treated T cells were then mixed with CFSE-labeled B cells (2:1 ratio). The cell mixtures were stimulated with TCR/CD28 Abs and expanded in the presence of IL-2 as described. B cell proliferation was monitored by CFSE partitioning 5 days poststimulation as described.

Stimulation-induced proliferation of CFSE-labeled purified primary human B cells was accomplished by stimulating the B cells with PMA (5 ng/ml) and anti-IgM (BD Pharmigen) in medium supplemented with recombinant human IL-2 (200 U/ml; Chiron). The stimulus remained present for the duration of the experiment and the stimulation-induced proliferation was monitored by CFSE partitioning 5 days poststimulation as described.

**Calcium mobilization and mitochondrial membrane potential**

Calcium flux was measured using the Fluo-4 NW Calcium Assay kit from Invitrogen Life Technologies/Molecular Probes. Briefly a stock solution of the Fluo-4 dye was prepared by resuspending the powdered dye in 10 ml of the provided assay buffer and 100 μl of 250 mM propanicid stock solution. T cells or B cells (200,000 cells) were pretreated with VacA, and were then pelleted and resuspended in 200 μl of the Fluo-4 solution for 30 min at 37°C in 5% CO2, followed by incubation for 30 min at room temperature before analysis by FACS. Cells were analyzed on a FACSCalibur for 20 s to establish a baseline, and the cells were then briefly removed from the FACS machine. B cells were stimulated with anti-IgM (10 μg/ml) and anti-CD40 (10 μg/ml). The cells were vortexed and quickly placed back on the FACS machine and analyzed for 60 s. The cells were removed again, 10 μg/ml goat-anti-mouse Ab was added to cross-link bound primary Abs (anti-IgM and anti-CD40), and the cells were analyzed for an additional 70 s. T cells were analyzed in a similar manner, following stimulation with anti-CD3 and anti-CD28. As a positive control, cells were removed after the treatment, washed with 100 ng/ml ionomycin, and analyzed by FACS for an additional 50 s. Cells were analyzed by monitoring fluorescence of the Fluo-4 dye in the FL1 channel (520 nm) over time.

The effect of VacA intoxication on mitochondrial membrane potential was done as previously described (25). Briefly, the Mito Flow reagent (Cell Signaling Technology) was used according to the manufacturer’s instructions. Activated T or B cells (1 × 10^5 cells) were used for the Mito Flow assays. The Mito Flow data were collected by FACS and analyzed using flow cytometry (FACSCalibur; BD Biosciences). Live cells were gated based on forward and side scatter profiles and analyzed using the FlowJo (Tree Star) program. T cells were stimulated with anti-CD3 and anti-CD28, and B cells were stimulated with PMA (5 ng/ml) and anti-CD40 in medium supplemented with recombinant human IL-2 (200 U/ml; Chiron) as described.

**Results**

VacA inhibits stimulation-induced proliferation of primary human PBMC

We showed recently that VacA can inhibit the activation-induced proliferation of purified primary human CD4+ Th cells (24). To investigate whether VacA can inhibit the activation-induced proliferation of other primary human immune cells, we first isolated human PBMC from the blood of healthy adult donors and labeled the cells with CFSE. Labeled cells were then intoxicated with wild-type VacA. As a control, cells were also treated with a VacA mutant protein (*VacA*Δ6–27) that fails to form membrane channels (24, 27), or with control buffer. Cells were then stimulated...
with anti-CD3/CD28 Abs (TCR stimulation) or with a combination of chemical agonists (PMA and ionomycin) and cultured for 5 days. Intoxication of PBMC with wild-type VacA resulted in inhibition of PBMC proliferation, with varying degrees of inhibition detected depending on the activation stimulus (Fig. 1). In contrast, PBMC intoxicated with VacAΔ6–27 proliferated similarly to cells treated with the buffer control (Fig. 1). These data demonstrate that VacA is able to inhibit the stimulation-induced proliferation of PBMCs.

VacA inhibits the activation-induced proliferation of CD4+ T cells, CD8+ T cells, and B cells within PBMC

To investigate which subsets of cells within primary human PBMC are affected by VacA, resting PBMCs from healthy adult donors were labeled with CFSE and intoxicated with acid-activated wild-type VacA, a mutant VacA protein (VacAΔ6–27), or control buffer. Cells were then activated via TCR/CD28 stimulation and cultured for five days. The proliferation of individual cell types

FIGURE 1. Effects of VacA on activation-induced proliferation of PBMC. A, Resting PBMC were labeled with CFSE and treated with wild-type VacA (WT-VacA, 10 μg/ml), a mutant VacA (VacAΔ6–27, 10 μg/ml), or control buffer for 8 h. T cells were then activated by either TCR/CD28 Abs or PMA/ionomycin stimulation. After 48 h, cells were removed from the activation signals and expanded in medium supplemented with recombinant human IL-2 (200 U/ml) for a total of 5 days. Cells were harvested and analyzed for CFSE content by flow cytometry. B, Data representative of proliferation indices of cells from A. Representative results are from one of three independent experiments, each done at least in triplicate, using different adult blood donors. *, p < 0.05 compared with buffer-treated control cells, analyzed by two-tailed t test.

FIGURE 2. Effects of VacA on proliferation of CD4+ T cells, CD8+ T cells, and B cells. A, Resting PBMC were labeled with CFSE and treated with wild-type VacA (WT-VacA, 10 μg/ml), a mutant VacA (VacAΔ6–27, 10 μg/ml), or control buffer for 8 h. T cells were then activated with TCR/CD28 Abs. After 48 h, cells were removed from the activation signals and expanded in medium supplemented with recombinant human IL-2 (200 U/ml) for a total of 5 days. Cells were harvested, stained with the indicated Abs, and analyzed by flow cytometry. The CFSE content of individual cell populations was determined by gating on CD3+/CD4+ (CD4+ T cells), CD3+/CD8+ (CD8+ T cells), and CD19+ (B cells) (top). Data represent proliferation indices of the cells (bottom). B, Resting PBMC were labeled with CFSE and treated with different additives as described in A. The cells were then activated with PMA/ionomycin. After 48 h, cells were expanded in medium supplemented with recombinant human IL-2 (200 U/ml) for a total of 5 days. Cells were harvested, stained with the indicated Abs, and analyzed by flow cytometry (top). Data represent the proliferation indices (bottom). Results represent one of three independent experiments, each done at least in triplicate, using different adult blood donors. *, p < 0.05 compared with PBS-treated cells, analyzed by two-tailed t test.
VacA inhibits superantigen-induced proliferation of T cells

These experiments demonstrated that VacA can inhibit proliferation of PBMC when the cells are stimulated in vitro using cross-linking Abs (i.e., anti-CD3/CD28 Abs) or chemical agonists (PMA/ionomycin) (Fig. 2). To investigate VacA-mediated inhibition of human T cell proliferation in a more physiologically relevant context, we determined whether VacA is capable of inhibiting the proliferation of T cells stimulated by APCs. We first investigated whether VacA can inhibit the stimulation-induced proliferation of T cells induced by superantigen SEB. In the presence of professional APC, SEB induces polyclonal stimulation of primary human T cells, resulting in T cell proliferation and cytokine production (29–31). PBMC from healthy adult donors were labeled with CFSE as before. Cells were then intoxicated with wild-type VacA, the mutant VacAΔ6–27, or control buffer, stimulated with SEB, and cultured for 5 days before FACS analysis. Similar to cells treated with control buffer, cells intoxicated with the mutant VacAΔ6–27 proliferated in a SEB-dependent manner (Fig. 3A). In contrast, SEB-induced proliferation of PBMC was inhibited by wild-type VacA (Fig. 3A). Furthermore, VacA inhibited SEB-mediated proliferation of both CD4+ and CD8+ T cells within the PBMC population (Fig. 3B).

VacA inhibits DC-induced proliferation of primary human CD4+ T cells

To begin dissecting how VacA inhibits SEB-mediated proliferation of T cells, we investigated whether VacA could inhibit the ability of purified primary human myeloid DC to activate primary human TH cells. CFSE-labeled resting CD4+ T cells, CD8+ T cells, or B cells within the PBMC population (Fig. 2A). As an alternate method for stimulating cell proliferation, the PBMC were stimulated with PMA/ionomycin instead of anti-CD3/CD28 Abs. Similar to the effect observed following VacA treatment of TCR/CD28-stimulated cells (Fig. 2A), VacA also inhibited the proliferation of CD4+ T cells, CD8+ T cells, and B cells within the PBMC population when stimulated with PMA/ionomycin (Fig. 2B).

VacA inhibits T cell-induced proliferation of primary human B cells

As shown in Fig. 2, VacA inhibited the proliferation of B cells within the PBMC population when cells were stimulated with TCR/CD28 Abs or with PMA/ionomycin. In the experiments shown in Fig. 2, B cells were activated indirectly by CD3/CD28-activated TH cells. Thus, inhibition of B cell proliferation by VacA could either be due to the suppression of TH cell activation or through direct effects of VacA on B cells. To differentiate between these possibilities, we performed experiments using purified B cells and purified CD4+ T cells. We labeled purified B cells with CFSE and treated the purified TH cells with mitomycin C, a drug that inhibits TH cells proliferation without adversely affecting the up-regulation of surface activation markers (such as CD40L) and cytokine secretion (32). CFSE-labeled B cells and mitomycin C-treated TH cells were mixed, treated with wild-type VacA, mutant VacAΔ6–27, or control buffer, and the cocultures were activated using TCR/CD28 Abs (Fig. 5A). Intoxication of the cocultures with wild-type VacA or treatment with cyclosporine A, a drug that inhibits T and B cell activation, each resulted in inhibition of B cell proliferation (Fig. 5A). We then repeated these experiments using a protocol in which either mitomycin C-treated TH cells or CFSE-labeled B cells were treated separately with the different additives before coculture (Fig. 5B). After treatment, cells were washed to remove unbound additives, mixed, and stimulated using TCR/CD28 Abs. Pretreatment of the primary human TH cells with VacA did not inhibit the ability of these...
VacA directly inhibits proliferation of primary human B cells

Further studies were then undertaken to investigate in more detail whether VacA directly targets primary human B cells and inhibits their proliferation.

VacA directly inhibits proliferation of primary human B cells

Further studies were then undertaken to investigate in more detail whether VacA can directly target B cells. We tested the ability of several agents (i.e., PMA/ionomycin, PMA/anti-IgM, anti-IgM/anti-CD40) to stimulate in vitro growth of primary human B cells. Stimulation with PMA/anti-IgM resulted in a low level of B cell proliferation, whereas the other agents had no detectable effect (data not shown and Fig. 6). Intoxication of primary human B cells with wild-type VacA, but not the mutant VacA/H9004_6–27 or control buffer, markedly inhibited PMA/anti-IgM-induced B cell proliferation (Fig. 6). These data provided further evidence that VacA can directly inhibit the proliferation of primary human B cells.

To obtain insight into the mechanism by which VacA inhibits B cell proliferation, we first investigated whether VacA interfered with early events that occur upon B cell activation. As an initial
approach, we investigated whether VacA had any effect on calcium fluxes that are induced upon stimulation of lymphocytes. Calcium is known to play a crucial role in lymphocyte activation, primarily due to its role in the activation of the NFAT. Intoxication of primary human B and Th cells with VacA did not result in a significant change in the magnitude of the calcium fluxes upon stimulation (Fig. 6B). These data provide further evidence indicating that VacA does not have an effect on early signaling events required for lymphocyte activation and proliferation (25).

We recently showed that VacA causes mitochondrial membrane depolarization in primary human Th cells (25). Therefore, we investigated whether VacA has a similar effect on primary human B cells. As expected, intoxication of primary human Th cells resulted in a significant increase in the percentage of Th cells with a low mitochondrial membrane potential (Fig. 6C). In contrast, VacA intoxication of B cells did not result in a reduction in membrane potential, and actually increased the mitochondrial membrane potential of the B cell population (Fig. 6C). These data suggest that there are differences in the susceptibility of T cells and B cells to VacA-induced mitochondrial membrane depolarization.

**Discussion**

In this study, we show that VacA can inhibit the activation-induced proliferation of multiple types of primary human immune cells, including CD4$^+$ and CD8$^+$ T cells, as well as B cells (Fig. 1). The inhibitory effect of VacA on lymphocytes was observed when cells were stimulated with either cross-linking Abs or chemical agonists (i.e., TCR/CD28 Abs or PMA/IONOMYCIN) (Figs. 1 and 2). In addition, we show that VacA inhibits proliferation of CD4$^+$ T cells that are stimulated by APC.

Our data demonstrate that VacA inhibits Th cell-mediated B cell proliferation when the B cells are individually pretreated with VacA, but not when the Th cells are pretreated with VacA (Fig. 5). Thus, VacA intoxication of primary human CD4$^+$ T cells does not perturb the ability of Th cells to activate B cells, a process that requires T cells to become fully activated. In contrast, treatment of primary human Th cells with cyclosporine A, an NFAT-activation inhibitor, potently inhibited T cell-mediated B cell proliferation. These data demonstrate that, in contrast to the effects of VacA on Jurkat cells (21, 23), VacA does not interfere with TCR-mediated activation of primary human T cells (24). Furthermore, we show that VacA inhibits PMA/anti-IgM-induced proliferation of primary human B cells, which provides further evidence that the inhibitory effects of VacA on B cell proliferation are due to direct effects of VacA on B cells (Fig. 6). To our knowledge, an inhibitory effect of VacA on stimulation-induced B cell proliferation has not been described previously.

Another important finding in the current study is that VacA can inhibit the proliferation of primary human Th cells that are activated by APC and a superantigen (Fig. 4). Our data indicate that inhibition of APC/superantigen-induced Th cell proliferation is due to the ability of VacA to target Th cells, rather than APC (i.e., DCs) (Fig. 4). Intoxication of DC with VacA did not inhibit superantigen-induced Th cell proliferation, suggesting that VacA does not alter the expression of cell surface proteins or cytokine signals derived from DC, which are required for T cell activation and proliferation (Fig. 4). In a previous study, VacA was shown to interfere with the process of Ag presentation in B cells by specifically inhibiting the B-dependent pathway of Ag presentation mediated by newly synthesized MHC class II molecules (33).
previously observed effect of VacA on Ag presentation is consistent with the ability of VacA to alter endocytic trafficking (5, 34). In the current study, the absence of detectable effects of VacA on DC can be explained by the fact that superantigen presentation requires MHC class II expression but does not require Ag processing.

Despite the presence of numerous B cell and plasma cells in the gastric mucosa of infected individuals, H. pylori are able to persistently colonize the human stomach. One possible consequence of the VacA-mediated inhibition of B cell proliferation observed in this study may be a reduction in secretory IgA synthesis in the stomach. In support of this hypothesis, plasma cells containing IgA have been detected in gastric biopsy specimens from patients with H. pylori-associated gastritis (35). The formation of crystalline inclusions in plasma cells is thought to reflect altered production, storage, or secretion of IgA. Inhibited production or secretion of IgA could be a factor that contributes to the persistence of H. pylori in the stomach.

Because VacA inhibits activation-induced proliferation of both T and B lymphocytes, it seems possible that a similar mechanism of action might account for the effects of the toxin on the two cell types. We recently showed that VacA does not inhibit the activation of important signal transduction components, including the signal transducer and activator of transcription STAT3 and STAT5, ERK, and p38 MAPK, in primary human T cells (25). In contrast, we showed that VacA inhibits the activation of regulatory proteins required for G1 cell cycle transition (25). In the current study, we show that VacA does not have a detectable effect on stimulation-induced calcium fluxes in either T cells or B cells (Fig. 6B). This finding, together with the observation that VacA inhibits proliferation regardless whether the cells are stimulated with TCR/CD28 Abs or with PMA/ionomycin, suggests that VacA has an effect on a cellular process downstream of receptor signaling in both T and B lymphocytes. Interestingly, we demonstrate in the current study that VacA causes a reduction in the mitochondrial membrane potential of T cells but not B cells (Fig. 6C). This observed difference in the susceptibility of T cells and B cells to VacA-induced mitochondrial depolarization suggests that there may be differences in the VacA target sites in these two cell types.

Membrane channel formation by VacA seems to be required for inhibiting the proliferation of both B and T cells. Specifically, based on the observation that VacAΔΔΔ2–27 is defective in formation of membrane channels (27) and fails to inhibit proliferation of T cells or B cells (24, 25), it can be surmised that membrane channel formation by VacA has an important mechanistic role in the process by which VacA inhibits lymphocytes proliferation.

Patch clamp studies indicate that VacA can form membrane channels in the plasma membrane of epithelial cells (36), and there is evidence that VacA also may form channels in the membranes of endosomes and mitochondria (5, 34, 37). Further investigation will be required to determine the relevant site of VacA channel formation in Th and B cells and to more completely elucidate the specific mechanism by which VacA exerts its inhibitory activities against these primary human lymphocytes.

Gastric biopsies from H. pylori-infected humans consistently demonstrate infiltration of T lymphocytes, plasma cells, DCs, mononuclear phagocytes, B cells, and neutrophils into the lamina propria (38–40). Despite the development of humoral and cellular immune responses and a gastric mucosal inflammatory response, H. pylori are able to evade the immune response and establish a life-long persistent infection (4). VacA is potentially an important bacterial factor that contributes to H. pylori persistence. Although it is not known what concentrations of VacA are present in the gastric mucosa of H. pylori-infected humans, we speculate that the local VacA concentration adjacent to sites of H. pylori colonization could be relatively high, and that VacA may target several types of immune cells, including B and T lymphocytes, that are recruited to such sites. We propose that VacA might inhibit the clonal expansion of infiltrating T cells, as well as B cells, that have been activated by H. pylori Ags. Effects of VacA on proliferation of T cells and B cells may effectively attenuate the host immune response, and thereby facilitate the establishment of a life-long persistent H. pylori infection.

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Disclosures

The authors have no financial conflict of interest.

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