Exercise training protects against an acute inflammatory insult in mouse epididymal adipose tissue

Laura Castellani, Jared Root-Mccaig, Scott Frendo-Cumbo, Marie-Soleil Beaudoin, and David C. Wright

Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

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Exercise training reduces systemic and adipose tissue inflammation. However, these beneficial effects seem to be largely tied to reductions in adipose tissue mass. The purpose of the present study was to determine if exercise training confers a protective effect against an acute inflammatory challenge. We hypothesized that the induction of inflammatory markers, such as interleukin 6 (IL-6), suppressor of cytokine signaling 3 (SOCS3), and TNF-α by the beta-3 adrenergic agonist CL 316,243 would be reduced in adipose tissue from trained mice and this would be associated with reductions in transient receptor potential cation channel 4 (TRPV4), a protein recently shown to regulate the expression of proinflammatory cytokines. Exercise training (4 wk of treadmill running, 1 h/day, 5 days/wk) increased markers of skeletal muscle mitochondrial content and the induction of PPAR-gamma coactivator 1 alpha in epididymal adipose tissue. The mRNA expression of IL-6, SOCS3, and TNFα were not different in subcutaneous and epididymal adipose tissue from sedentary and trained mice; however, the CL 316,243-mediated induction of these genes was attenuated ~50% in epididymal adipose tissue from trained mice as were increases in plasma IL-6. The effects of training were not explained by reductions in lipolytic responsiveness, but were associated with decreases in TRPV4 protein content. These results highlight a previously unappreciated anti-inflammatory effect of exercise training on adipose tissue immunometabolism and underscores the value of assessing adipose tissue inflammation in the presence of an inflammatory insult.

adipose tissue; exercise; inflammation; TRPV4; mice

OBSERVATIONS AND CONCLUSIONS

Obesity and impairments in glucose homeostasis are associated with increased inflammation in white adipose tissue (14). Studies in which adipose tissue inflammation (macrophage infiltration and the expression of inflammatory cytokines such as IL-6, TNF-α, etc.) is reduced by means of targeted genetic manipulations provide evidence for a causative role of adipose tissue inflammation in the development of insulin resistance. Specifically, these studies demonstrate that reducing adipose tissue inflammation can prevent diet-induced impairments in glucose tolerance in mice fed a high-fat diet (23, 24, 26, 33).

Observational data from several large cohort studies have demonstrated an inverse relationship between physical activity levels and markers of inflammation (1, 9, 32). However, it is difficult to discern if the beneficial effects of exercise on inflammation are direct, or are secondary to alterations in adipose tissue mass. For example, exercise training protects against the development of high-fat diet-induced increases in markers of adipose tissue inflammation in parallel with attenuated increases in adipose tissue expansion (2, 34). Similarly, exercise training that is initiated after the development of obesity and insulin resistance leads to decreases in adipose tissue inflammation and reductions in fat pad mass in mice (28, 29). Likewise, exercise training in lean, healthy animals that do not experience marked weight loss would appear to have little effect on reducing markers of adipose tissue inflammation (2).

Collectively, the above mentioned studies would suggest that the beneficial effects of exercise training on reducing markers of inflammation in adipose tissue, especially in the setting of diet-induced obesity and insulin resistance, is largely secondary to changes in body weight and adipose tissue expansion. With this being said we cannot rule out the possibility that exercise training may confer a protective effect against the induction of adipose tissue inflammation. In support of this theory, some (4), but not all (5), have shown that prior exercise training protects against endotoxin-induced systemic inflammation. However, at this point it is not known if prior exercise training confers a protective effect against inflammatory insults at the level of adipose tissue.

Within this framework the purpose of the present study was to determine if prior exercise training provides a protective effect against an acute inflammatory insult in adipose tissue. To address this question we used CL 316,243, a beta-3 specific adrenergic agonist, to induce inflammation. Long-term treatment with CL 316,243 has been shown to have insulin-sensitizing effects in obese animals (7, 10, 27) that is likely related to reductions in food intake (7, 12), increases in oxygen consumption (12), and subsequent decreases in fat pad mass (7, 10, 27). On the other hand, short-term treatment with CL 316,243 rapidly and robustly induces the expression of markers of adipose tissue inflammation [i.e., interleukin-6 (IL-6) and TNF-α] (19, 22). This is likely due to the proinflammatory effects of the large increases in fatty acid release caused by CL 316,243 (19). A second aim of this study was to examine potential mechanisms that could account for a protective effect of prior exercise training. We were particularly interested in examining the effects of training on transient receptor potential cation channel 4 (TRPV4), a membrane associated calcium channel recently shown to regulate the expression of proinflammatory genes (35). We hypothesized that exercise training would provide a protective effect against CL 316,243-mediated inflammation in adipose tissue in conjunction with reductions in TRPV4 content in mouse adipose tissue.

MATERIALS AND METHODS

Materials

CL 316,243 (cat. no. C5976) and free glycerol reagent (cat. no. F6428) were purchased from Sigma Aldrich (Oakville, ON). Nonesterified free fatty acid kits were from Wako Chemicals (Richmond, VA). Enzyme-
linked immunosorbent assay (ELISA) kits for IL-6 (cat. no. EZMIL6) and TNF-α (cat. no. EZMNTFA) were ordered from EMD Millipore (Billerica, MA). Molecular weight marker, reagents, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON). ECL Plus was a product of Amersham Pharmacia Biotech (Arlington Heights, IL). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Antibodies against total hormone-sensitive lipase (HSL) (cat. no. 4107), phospho-serine 660 HSL (cat. no. 4126), total AMPK alpha (cat. no. 2603), phospho-threonine 172 AMPK alpha (cat. no. 2531), phospho-extracellular regulated kinase 1/2 (ERK1/2) (cat. no. 9101), total ERK1/2 (cat. no. 9102), and PPARγ (cat. no. 2430) were purchased from Cell Signaling (Danvers, MA). Antibodies against β-actin (cat. no. ab8227), tubulin (cat. no. ab 7291), and COXIV (cat. no. ab14744) were from Abcam (Cambridge, MA). TRPV4 (cat. no. ACC-034) antibody was purchased from Alomone Labs (Jerusalem, Israel). Oligo(dT), SuperScript II Reverse Transcriptase, and dNTP were from Invitrogen (Burlington, ON). Taqman gene expression assays for mouse interleukin-6 (IL-6) (Mm00446190_m1), GAPDH (AB55932E), SOCS-3 (Mm00545913_s1), PGC-1α (Mm01208835_m1) and TNF-α (Mm00443258_m1) were from Applied Biosystems (Foster City, CA).

Animals

Ten-week-old male C57BL6 mice were purchased from Charles Rivers and housed one per cage. Animals had free access to water and standard rodent chow and were maintained on a 12:12-h light-dark cycle. All protocols were approved by the University of Guelph Animal Care Committee and conformed to the Canadian Council on Animal Care (CCAC) guidelines (19a).

Training protocol. Prior to beginning the training protocol mice were acclimated to treadmill running during two sessions consisting of 10 min of running at 15 m/min at a 5% incline. Exercise training began 48 h following the last acclimation session. The training protocol consisted of treadmill running 5 days/wk, 1 h/day, for 4 wk. The treadmill speed increased progressively each week as follows: 20, 22, 23, and 25 m/min. Likewise the treadmill incline increased from 10% to 15% to 20% on weeks 1, 2, and 3, respectively, and remained at 20% for week 4.

CL 316,243 treatment. Forty-eight hours following the last bout of exercise mice were injected intraperitoneally with either the beta-3 adrenergic agonist, CL 316,243 (1 mg/kg body wt), or an equivalent placebo. Exercise mice were injected intraperitoneally with either the beta-3 adrenergic agonist, CL 316,243 (1 mg/kg body wt), or an equivalent placebo. Exercise mice were injected intraperitoneally with either the beta-3 adrenergic agonist, CL 316,243 (1 mg/kg body wt), or an equivalent placebo.

Plasma IL-6, TNF-α, glycerol, and NEFA

Plasma was analyzed for glycerol and NEFA concentrations using commercially available colorimetric kits. IL-6 and TNF-α concentrations were determined using mouse specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s directions. Samples were run in duplicate. The coefficient of variation for these assays in our laboratory is <10%.

Western Blotting

Samples were homogenized in 3 vol of cell lysis buffer, supplemented with phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma). Homogenized samples were centrifuged at 4°C for 10 min at 5,000 g, the infranatant was collected, and protein content was determined using a bicinchoninic acid assay (25). Samples were prepared to contain equal concentrations of protein that were then separated on 10% SDS-PAGE gels. Protein was transferred onto nitrocellulose membranes using a wet transfer technique at 200 mA/transfer unit. Membranes were blocked in Tris-buffered saline/0.1% Tween 20 (TBST) prepared with 5% nonfat dry milk for 1 h, then incubated at 4°C in the according primary antibody overnight. Primary antibodies were diluted in TBST/5% bovine serum albumin. Following incubation in primary antibody, membranes were rinsed with TBST and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies were diluted in TBST with 1% nonfat dry milk. Following incubation with secondary antibody membranes were washed a final time with TBST. Signals were detected using enhanced chemiluminescence and were subsequently quantified by densitometry using a FluorChem HD imaging system (Alpha Innotech, Santa Clara, CA).

Real-Time PCR

Changes in mRNA expression of IL-6, SOCS3, and TNF-α were determined using real-time qPCR as described in detail previously by our laboratory (30, 31). Briefly, RNA was isolated from epididymal and inguinal subcutaneous white adipose tissue using an RNaseasy kit according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized from total RNA (1 μg) using SuperScript II Reverse Transcriptase, dNTP, and oligo(dT). Real-time PCR was carried out using a 7500 Fast Real-Time PCR system (Applied Biosystems). Samples were loaded in duplicate using a 96-well plate layout. Each well contained a total volume of 20 μl comprised of 1 μl gene expression assay, 1 μl cDNA template, 10 μl Taqman Fast Universal PCR Master Mix and 8 μl RNase-free water. For GAPDH, each 50 μl reaction contained 25 μl PCR Master Mix, 2.5 μl gene expression assay, 1 μl of cDNA template, and 21.5 μl RNase-free water. GAPDH was used as our housekeeping gene, and relative differences in gene expression between groups were determined using the 2-ΔΔCT method (18). The PCR efficiency was similar between GAPDH and our genes of interest. The experimental manipulations did not alter the expression of our housekeeping gene (GAPDH).

Statistical Analysis

Comparisons between two groups were made using unpaired, two-tailed t-tests. Differences in CL 316,243-stimulated signaling and plasma glycerol, fatty acid, and IL-6 levels in sedentary and trained mice were determined using a 2 × 2 ANOVA with LSD post hoc analysis. In some cases data were logarithmically transformed to ensure equal variance among groups. Significance was set at P < 0.05.

RESULTS

Exercise Training Adaptations in Muscle and Adipose Tissue

To assess the efficacy of the exercise training protocol, differences in body weight and fat pad mass, expression of PGC-1α mRNA in epididymal adipose tissue and skeletal muscle COXIV protein content were measured. As shown in Fig. 1, exercise-trained mice weighed significantly less (Fig. 1A) and had reduced epididymal adipose tissue mass (Fig. 1B) compared with sedentary control mice. The mRNA expression of PGC-1α in epididymal adipose tissue (Fig. 1C) and the protein content of COXIV in vastus muscle (Fig. 1D) were increased in trained compared with sedentary mice.
Exercise Training Does Not Reduce Markers of Inflammation

IL-6 and TNF-α are classic markers of adipose tissue inflammation. Similarly, the expression of SOCS3 is increased in adipose tissue from obese mice (20) and can be used as a useful marker of inflammation. The mRNA expression of IL-6, SOCS3, and TNF-α were not different in either epididymal or subcutaneous adipose tissue from trained and sedentary mice (Table 1).

CL 316,243 Induces Adipose Tissue Inflammation

Although the expression of inflammatory genes was not reduced in adipose tissue from trained mice we reasoned that prior exercise training could confer a protective effect onto adipose tissue when mice were presented with an acute inflammatory insult. CL 316,243 is a beta-3 adrenergic agonist that rapidly and robustly induces the expression of proinflammatory genes, such as IL-6 and TNF-α in epididymal adipose tissue (19, 22). To confirm these findings mice were treated with CL 316,243 (1 mg/kg body wt ip) or an equivalent dose of sterile saline and tissue collected 4 h postinjection. Treatment of CL 316,243 increased the epididymal adipose tissue mRNA expression of IL-6, TNF-α, and SOCS3 mRNA. CL 316,243 treatment resulted in an ~7 fold increase (P < 0.05) in circulating plasma IL-6 levels (Fig. 2).

Table 1. mRNA expression of inflammatory markers in inguinal subcutaneous (SQ) and epididymal (eWAT) adipose tissue from sedentary and trained mice

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Trained</th>
<th>P Value</th>
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<tbody>
<tr>
<td>SQ IL-6</td>
<td>1.2 ± 0.3</td>
<td>0.7 ± 0.3</td>
<td>0.27</td>
</tr>
<tr>
<td>SQ SOCS3</td>
<td>1.1 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.15</td>
</tr>
<tr>
<td>SQ TNF-α</td>
<td>1.7 ± 0.6</td>
<td>1.4 ± 0.6</td>
<td>0.15</td>
</tr>
<tr>
<td>eWAT IL-6</td>
<td>1.3 ± 0.3</td>
<td>1.6 ± 0.5</td>
<td>0.63</td>
</tr>
<tr>
<td>eWAT SOCS3</td>
<td>1.1 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>0.34</td>
</tr>
<tr>
<td>eWAT TNF-α</td>
<td>1.4 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>0.34</td>
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</table>

Values are means ± SE; n = 5–6 SQ, and n = 9–10 eWAT.
Prior Exercise Training Dampens CL 316,243-Induced Inflammation in Epididymal Adipose Tissue

Having confirmed the proinflammatory effects of CL 316,243, we next examined if prior exercise training would confer a protective effect against this inflammatory challenge. As shown in Fig. 3, prior exercise training attenuated the CL 316,243-mediated induction of IL-6, SOCS3, and TNF-α in epididymal adipose tissue (P < 0.05). The induction of IL-6, SOCS3, and TNF-α by CL 316,243 was similar in subcutaneous adipose tissue from trained and sedentary mice. CL 316,243 significantly increased circulating plasma levels of IL-6 in sedentary mice, but not in trained mice (Table 2). Circulating concentrations of TNF-α were below the detectable limit of the assay in all groups examined.

As fatty acids are thought to mediate CL 316,243-induced inflammation (19) it was necessary to determine if blunted beta-adrenergic signaling could explain, in part, the attenuated induction of inflammatory genes in adipose tissue from trained mice. Therefore we measured plasma glycerol and fatty acid content as well as epididymal adipose tissue HSL and ERK signaling. Treatment with CL 316,243 resulted in similar increases in plasma glycerol and fatty acid levels, and HSL and ERK phosphorylation in epididymal adipose tissue from sedentary and trained mice (Fig. 4).

TRPV4 Protein Content Is Reduced in Epididymal Adipose Tissue from Trained Mice

To further elucidate the mechanism that could mediate the changes in expression of inflammatory markers between sedentary and trained conditions in response to an acute inflammatory challenge, we evaluated changes in proteins (TRVP4, PPARγ, AMPK) thought to be involved in regulating the inflammatory state of adipose tissue. Reductions in TRPV4 have been previously linked to a pronounced attenuation of proinflammatory cytokine expression in adipocytes (35). Our results demonstrate a significant reduction in TRPV4 content in epididymal but not subcutaneous adipose tissue from sedentary and trained mice. Reductions in TRPV4 content (sedentary 1.00 ± 0.07, trained 0.78 ± 0.08 arbitrary units) and phosphorylation (sedentary 1.00 ± 0.22, trained 0.82 ± 0.06 arbitrary units) of AMPK alpha in epididymal adipose tissue from sedentary and trained mice were not different.

DISCUSSION

Adipose tissue inflammation is associated with, and is thought to play, a causal role in the development of insulin resistance (as reviewed in 14). For instance, targeted genetic
manipulations that attenuate adipose tissue inflammation prevent the development of insulin resistance in mice fed a high-fat diet (23, 24, 26, 33). Exercise training initiated at the onset of a high-fat diet, or following the development of obesity, protects against and reduces adipose tissue inflammation and macrophage infiltration in mice (2, 15, 16, 28, 29). However, the interpretation of these studies is complicated as it is difficult to discern whether the attenuation in inflammatory markers is the result of exercise-induced adaptations in adipose tissue biology or secondary to changes in adipose tissue mass. The results presented in the current study are the first to demonstrate that prior exercise training protects against the deleterious effects of an acute inflammatory challenge in adipose tissue.

To investigate the role of exercise training in modulating inflammation in adipose tissue we utilized the pharmacological...
agent CL 316,243, a specific beta-3 adrenergic agonist. Recent work with this compound has shown a rapid induction of IL-6 and TNF-α, independent of increases in macrophage infiltration, in mouse epididymal adipose tissue in vivo (22). While the specific mechanism through which CL 316,243 induces inflammation has not been fully elucidated, it is likely associated with increases in fatty acids, as the inflammatory effects of CL 316,243 are attenuated when HSL is inhibited (19). In the present study, we have confirmed the ability of CL 316,243 to induce the expression of inflammatory markers (IL-6, TNF-α, and SOCS-3) in epididymal adipose tissue and have expanded upon this, demonstrating an induction of IL-6 and SOC3 mRNA in inguinal subcutaneous adipose tissue, although to a much lesser extent. We have further shown that the induction of inflammatory markers is not explained by alterations in markers of adipose tissue lipolysis. CL 316,243 increased plasma glycerol (A) and nonesterified fatty acid (NEFA) (B) levels to a similar extent in sedentary (S) and trained (T) mice. Increases in the serine 660 phosphorylation of hormone sensitive lipase (HSL) (C) and phosphorylation of ERK1/2 (D) were not different in epididymal adipose tissue from sedentary and trained mice treated with CL 316,243 (1.0 mg/kg body wt ip). Representative Western blots are shown in E. Data are means ± SE for 4–6 per group. Representative blots are shown to the right of the quantified data in C. *P < 0.05 compared with the saline-treated condition from the same group.

**Table 2. Plasma IL-6 concentrations in sedentary and trained mice treated with saline or CL 316,243**

<table>
<thead>
<tr>
<th></th>
<th>Sedentary-Saline</th>
<th>Sedentary-CL 316,243</th>
<th>Trained-Saline</th>
<th>Trained-CL 316,243</th>
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<tr>
<td>IL-6, pg/ml</td>
<td>36 ± 10</td>
<td>236 ± 91*</td>
<td>45 ± 13</td>
<td>98 ± 20</td>
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Values are means ± SE for 4–8 samples/group. *Significantly different from the corresponding saline value from the same group. P < 0.05.
of adipose tissue IL-6 mRNA expression is reflected in large increases in circulating IL-6.

Consistent with previous reports (2, 17), exercise training did not lead to reductions in markers of adipose tissue inflammation or circulating levels of IL-6. However, for the first time, we have uncovered a previously unappreciated anti-inflammatory effect of prior exercise training. We are the first to show that exercise training attenuates the induction of IL-6, SOC3, and TNF-α in response to an acute inflammatory challenge. Interestingly, this attenuated response was observed in epididymal but not subcutaneous adipose tissue from exercise-trained mice. Epididymal adipose tissue displays greater lipolytic responsiveness in vivo than inguinal subcutaneous adipose tissue (21), and thus the depot-specific attenuation of the induction of inflammatory markers could be related to the greater lipolytic stimulation and metabolic perturbation that occurs in epididymal adipose tissue during exercise.

The blunted induction of these inflammatory markers is likely due to a training effect, and not the residual effects of the last bout of exercise, as these experiments were completed ~48 h following the last training session. Although epididymal fat pad mass was reduced with training, this likely does not explain the attenuated induction of inflammatory markers by CL 316,243 as the expression of IL-6, SOC3, and TNF-α was not different in sedentary and trained mice treated with equivalent doses of CL 316,243. Although we cannot discount the possibility that differences in the species of lipids released from adipose tissue from trained mice might be different from those released from adipose tissue from sedentary mice and could perhaps explain a portion of the attenuated induction of inflammation, our findings suggest that the protective effects of prior exercise were not secondary to reductions in lipolytic responsiveness.

To further elucidate the mechanisms which could be linked to the protective effects of prior exercise training against an acute inflammatory challenge, we examined signals implicated in mediating inflammation in adipose tissue. Recent work from Galic et al. (8) demonstrated that hematopoietic deletion of the AMPK β1 subunit led to increased adipose tissue macrophage inflammation when mice were challenged with a high-fat diet (8), thus providing evidence of a potential role for AMPK in inflammation. However, our results show that the content and phosphorylation of the catalytic alpha subunit of AMPK is not altered in adipose tissue with training, indicating that AMPK may not be playing a role in the protective effects of prior exercise training. Similarly, the protein content of PPARγ, a member of the nuclear receptor superfamily that possesses noted anti-inflammatory properties (as reviewed in 3), was not increased in adipose tissue from trained mice.

TRPV4 is a membrane-associated calcium channel that is expressed in adipose tissue (35) and is a potential target mediating the protective effects of training in adipose tissue. The deletion of TRPV4 in 3T3 adipocytes has recently been shown to reduce the expression of proinflammatory genes, while at the same time increasing the expression of genes involved in thermogenesis such as PGC-1α (35). Likewise, markers of inflammation in adipose tissue from TRPV4 knockout mice, or mice treated with GSK205, a TRPV4 inhibitor, are reduced when these mice are fed a high-fat diet (35). Our findings build upon this and we provide novel data demonstrating an exercise-mediated reduction in TRPV4 protein content in association with increases in the mRNA expression of
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PGC-1α in epididymal adipose tissue. Interestingly the protective effects of prior exercise training against CL 316,243-induced inflammation was only present in epididymal adipose tissue, the fat depot in which TRPV4 protein content was reduced. These findings provide evidence suggesting that the training-induced reductions in TRPV4 protein content could mediate the protective effects of prior exercise training against an acute inflammatory insult.

In summary, this paper has been the first to explore the effects of prior exercise training on modulating the effects of an acute inflammatory insult. Training did not reduce markers of adipose tissue or systemic inflammation; however, a protective effect was uncovered when mice were challenged with an acute inflammatory stress. These results highlight a previously unappreciated anti-inflammatory effect of exercise training on adipose tissue immunometabolism and underscores the value of assessing adipose tissue inflammation in the presence of an inflammatory insult. Further, results from this study indicate a potential role of TRPV4 in mediating the protective effect that exercise training has on adipose tissue when presented with an inflammatory insult.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.C., J.R.-M., S.F.-C., M.-S.B., and D.C.W. interpreted results of experiments; L.C. and J.R.-M. designed and planned the research; L.C., J.R.-M., S.F.-C., M.-S.B., and D.C.W. edited and revised manuscript; L.C., J.R.-M., S.F.-C., M.-S.B., and D.C.W. contributed to data collection; L.C., M.-S.B., and D.C.W. interpreted results of experiments; L.C. and J.R.-M., S.F.-C., M.-S.B., and D.C.W. contributed to conception and design of research; L.C., J.R.-M., S.F.-C., M.-S.B., and D.C.W. edited and revised manuscript; L.C., J.R.-M., S.F.-C., M.-S.B., and D.C.W. approved final version of manuscript.

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