Cigarette smoke exposure promotes differentiation of CD4\(^+\) T cells toward Th17 cells by CD40–CD40L costimulatory pathway in mice

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**Purpose:** This study aimed to investigate the impact of cigarette smoke exposure upon CD40–CD40L ligation between bone marrow-derived dendritic cells (BMDCs) and CD4\(^+\) T cells, and to examine the effects of cigarette smoke exposure upon differentiation of CD4\(^+\) T cells toward Th17 cells through blockade of CD40–CD40L pathway in mice.

**Methods:** The study was processed in vivo and in vitro. In vivo, Th17 cells, CD40, interleukin (IL)-17A, and IL-27 in the lung tissues were quantified and compared between mice with and without cigarette smoke exposure. In vitro, Th17 cells, IL-17A, and IL-27 yielded by multiple cell cultivations in which BMDCs from mice with or without cigarette smoke exposure were fostered with CD4\(^+\) T cells from healthy mice spleens in the presence of antagonistic CD40 antibody and/or cigarette smoke extract (CSE) were quantified and compared. The flow cytometry was used to detect expressions of Th17 cells and CD40, and the liquid chip was used to detect levels of IL-17A and IL-27.

**Results:** Both in vivo exposed to cigarette smoke and in vitro to CSE, CD40 expressions noticeably escalated on the surfaces of BMDCs. The presence of Th17 cells, IL-17A, and IL-27 in the lung tissues prominently increased in mice exposed to cigarette smoke. In the in vitro culture of CD4\(^+\) T cells and BMDCs significantly enhanced the differentiation of CD4\(^+\) T cells toward Th17 cells and secretions of IL-17A and IL-27 in the case that BMDCs were produced from mice exposed to cigarette smoke or the culture occurred in the presence of CSE. Usage of antagonistic CD40 antibody evidently reduced the number of Th17 cells, IL-17A, and IL-27 that increased due to cigarette smoke exposure.

**Conclusion:** The CD40–CD40L ligation is associated with the quantities of Th17 cells and relevant cytokines in the context of cigarette smoke exposure. Reducing the number of Th17 cells via the usage of antagonistic CD40 antibody can be an inspiration for pursuing a novel therapeutic target for immune inflammation in COPD.

**Keywords:** cigarette smoke extract, BMDC, CD4\(^+\)IL-17A T cell, CD40–CD40L pathway, IL-17A, IL-27

**Introduction**

COPD is a globally common lung disease with high morbidity and mortality, low life quality, and heavy disease burden.\(^1\) Profound immune inflammation has been well documented as one of the crucial pathophysiologic mechanisms associated with occurrence and development of COPD. Patients with COPD manifested typical signs of immune inflammation, including noticeable infiltration of inflammation cells in airway and in lung tissue, obviously increased number of myeloid dendritic cells (mDCs) in lavage fluid of bronchoalveolus and airway,\(^2\)\(^-\)\(^4\) and excessive presence of Th1 cells, Th17 cells, and CD8\(^+\) T cells.\(^5\)\(^-\)\(^7\) Th17 cells actively engage in the immune inflammation occurring...
The CD40–CD40L cross-talk combining CD40 on the surface of dendritic cell (DC) and CD40L on the surface of activated T cells provides the crucial costimulatory molecules on the surfaces of mDC1 and mDC2. The CD40–CD40L cross-talk combining CD40 on the surface of dendritic cell (DC) and CD40L on the surface of activated T cells provides the crucial costimulatory molecules on the surfaces of mDC1 and mDC2. Th17 cells along with relevant cytokines in the mice lungs were evaluated. In addition to as the main bearer of CD40, DC functions as a powerful antigen-presenting cell (APC) required for optimal activation of naive T cell. It induces proliferation and differentiation of CD4+ T cells and engages in the maintenance of effective immune defense and tolerance. According to the aforementioned literature over roles of CD40–CD40L pathway and DC in human immune response, potential connections amid CD40–CD40L pathway, DC, and physiological activities of Th17 cells as well as relevant cytokines have been strongly suggested and also served as the fundamental hypothesis of our study.

We conducted an animal study containing an in vivo experiment and an in vitro experiment to investigate the hypothetical effects of CD40–CD40L and DC over the differentiation of CD4+ T cells toward Th17 cell. First, a mouse model of cigarette smoke exposure was performed to imitate lung immune inflammation in vivo and quantities of CD40 and Th17 cells along with relevant cytokines in the mice lungs were evaluated. Second, in vitro experiment, CSE was used to continue the environment of cigarette smoke exposure and bone marrow-derived dendritic cells (BMDCs) of mice with or without cigarette smoke exposure were cultured with CD4+ T cells of healthy mice in the presence of antagonistic CD40 antibody. Then, Th17 and relevant cytokines yielded from the culture of BMDCs and CD4+ T cells were examined.

**Methods**

**Ethics**

The Laboratory Animal Ethics Committee of Guangxi Medical University approved all the in vivo and in vitro experiments of this study. "Regulation on the Administration of Experimental Animals (2017 edition)" and "Guideline on the Good Treatment of Experimental Animals (2006 edition)", both issued by the Ministry of Science and Technology of our country, had been followed to deal with all animals involved in this study.

**Animals**

The research subjects used in this study were 90 male Balb/c mice, aged 6–8 weeks, weight 20–22 g, specific pathogen free, and purchased from the Animal Experiment Center of Guangxi Medical University (Nanning, China).

**Cigarette smoke exposure**

Of the 90 healthy Balb/c mice, 60 mice were randomly assigned into two groups by random number table, such as the control group (A group) and the cigarette smoke exposure group (B group) (n=30). Two identical glass boxes marked as “No 1” and “No 2”, respectively, were self-made in the size of 90×55×40 cm, and two holes at the diameter of 2 cm were made on each one for ventilation. The A group was put inside No 1 glass box and allowed to move freely inside for 30 minutes a day, 5 days per week up to 4 weeks. The B group was placed into No 2 box and exposed whole bodies to cigarette smoke produced by 10 Marlboro cigarettes (unfiltered cigarette: 12 mg of tar, 0.9 mg of nicotine, and 12 mg of carbon monoxide; Longyan Tobacco Co, Ltd, Nanning, China) on fire for 30 minutes a day, 5 days per week up to 4 weeks. Mice tolerated cigarette smoke exposure without evidence of toxicity (carboxyhemoglobin levels 10% and no weight loss). The rest 30 healthy Balb/c mice were used to attain spleen T lymphocytes.

**Preparation of lung mononuclear cells and production of lung homogenate**

Mice of both the A group and the B group underwent cervical dislocation and, then, were positioned with the back on the operating board and sterilized three times with iodophor. Both lungs were perfused through right ventricle by 10 mL of phosphate buffer solution (PBS) containing 0.6 M ethylene diamine tetraacetic acid (EDTA) in order to fend off any remaining circulating mononuclear cells. The left lower lung and the right upper lung were removed from the mice chest cavity, rinsed with PBS at 4°C, diced into pieces in 1–2 mm of diameter, and digested in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) including 5% of fetal calf serum and 330 U/mL of collagenase IV in a constant temperature oscillator for 30 minutes. After digestion, lung tissues were filtered through a 200 mesh stainless steel filter and centrifuged at low temperature and 1,000 rpm for 5 minutes. Residual red blood cells from lungs were lysed with 15 mL of red blood cell (RBC)
lysis solution. The cell suspension was resuspended twice with 2 mL of ice-cold PBS and centrifuged at low temperature for 5 minutes at 1,000 rpm to attain lung mononuclear cells. Th17 cells and CD40 expressed on the surfaces of lung mononuclear cells were quantified by the flow cytometry.

The left upper lung was fetched out of the mice chest, washed with ice-cold PBS, weighed on a scale, immersed in ice-cold PBS, and diced into pieces. Smashed lung tissues were placed into glass homogenate tubes and grinded in a homogenizer. A total of 10% of lung homogenate were centrifuged at low temperature for 10 minutes at 3,000 rpm and restored in a refrigerator at −80°C. Levels of IL-17A and IL-27 were quantified in the lung homogenate by the liquid chip.

Preparation of BMDCs

The thigh bones and tibias of mice from both the A group and the B group were taken out. The bone marrow cell suspension was collected by repeatedly rinsing the bone marrow cavities, centrifuged at 1,500 rpm for 10 minutes, then overlying mouse monocyte lymphocyte separation in twice volume, and centrifuged at 2,000 rpm for 20 minutes. The myeloid mononuclear cell belt was sucked out, washed with PBS twice, and suspended in the RPMI 1640 complete medium containing 150 mL/L of fetal calf serum (FCS), 40 ng/mL of recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF) (PeproTech, Rocky Hill, CT, USA), and 10 ng/mL of recombinant mouse interleukin 4 (rmIL-4) (PeproTech). The myeloid mononuclear cell suspension was adjusted to 10⁶/L, distributed in six-well plates, and fostered in the RPMI 1640 complete medium containing 150 mL/L of fetal calf serum (FCS), 40 ng/mL of rmGM-CSF, and 10 ng/mL of rmIL-4. Adherent cells and semiadherent cells were collected at the sixth day of the fostering and cultured with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD11c Monoclonal Antibody (R&D Systems, Inc., Minneapolis, MN, USA). The positive incidence of CD11c+ cells detected by the flow cytometry exceeded 70%.

Cigarette smoke extract (CSE) production

CSE was produced through the process reported by Li et al. A total of 15 burning cigarettes (unfiltered cigarette: 12 mg of tar, 0.9 mg of nicotine, and 12 mg of carbon monoxide) were put inside a glass 50 mL syringe that was connected by a rubber tube with a plastic 50 mL syringe containing 10 mL of PBS. CSE was collected by repeated suction of the plastic 50 mL syringe, purified through a sterile strainer, and measured for the concentration by ultraviolet spectrophotometry.

The in vitro cultures of CD4+ T cells and BMDCs

Spleens were fetched out from the rest 30 healthy mice, treated with RPMI 1640 medium, diced into pieces, and filtered by 200 mesh stainless steel filter. The spleen cells' suspension was added with PBS, centrifuged at 1,500 rpm for 10 minutes, overlying mouse monocyte lymphocyte separation in twice volume, and centrifuged at 2,000 rpm for 20 minutes. The spleen mononuclear cells were sucked out of centrifuge tubes, rinsed with PBS twice, and centrifuged at 1,000 rpm for 5 minutes. The mononuclear cells were purified by CD4+ T-negative immunomagnetic beads (Thermo Fisher Scientific) and examined for purity by the flow cytometry. The CD4+ T cells were put into 96-well plates, 200 μL unit in each well, and fostered with BMDCs in 24-well plates in the following seven groups: 1) the CD4+ T group; 2) the CD4+ T + CSE group in which CD4+ T cells were cultured in the presence of 10 mL/L CSE; 3) the CD4+ T + BMDC group in which CD4+ T cells were cultured with BMDCs of healthy mice; 4) the CD4+ T + smoke BMDC group in which CD4+ T cells were cultured with BMDCs of mice exposed to the cigarette smoke for 4 weeks; 5) the CD4+ T + BMDC + CSE group in which CD4+ T cells were cultured with BMDCs of mice exposed to the cigarette smoke for 4 weeks along with 10 μg/mL of antagonistic CD40 antibodies (BD Pharmering, San Diego, CA, USA); and 7) the CD4+ T + BMDC + CSE + CD40 antibody group in which CD4+ T cells were cultured with BMDCs of healthy mice along with 10 mL/L CSE; 6) the CD4+ T + smoke BMDC + CD40 antibody group in which CD4+ T cells were cultured with BMDCs of mice exposed to the cigarette smoke for 4 weeks along with 10 μg/mL of antagonistic CD40 antibodies; and 7) the CD4+ T + BMDC + CSE + CD40 antibody group in which CD4+ T cells were cultured with BMDCs of healthy mice in the presence of 10 μg/mL of antagonistic CD40 antibodies and 10 mL/L CSE (Figure 1). Each well was inoculated 10⁵/mL of cells and fostered in serum-free mediums for 24 hours.

A range of concentrations of CSE, including 5, 10, 15, and 20 mL/L, were used to sift out the optimal one, to which BMDCs were exposed and showed more active proliferation measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay than to other three concentrations. All absorbance values of BMDCs detected at 490 nm at 12, 24, 48, and 72 hours culminated with 10 mL/L CSE in comparison with other three concentrations of CSE (Table 1). Hence, the 10 mL/L CSE was applied to the cultures of CD4+ T cells and BMDCs in vitro. Before these in vitro cultures started, parts of BMDCs produced from the A group were cultured...
with 10 mM/L CSE in 24-well plates for 24 h. Then, the flow cytometry was used to evaluate CD40 expression on the surfaces of BMDCs that were sampled from ones prepared from the A group, from the B group, and from the A group and cultured with CSE. Th17 cells produced by cultivations of CD4+ T cells and BMDCs were quantified by the flow cytometry, and IL-17A and IL-27 were quantified by the liquid chip.

**Flow cytometry**

Cells were stained with FITC-labeled CD11c+ mAb for the detection of expression of CD11c+, with PE-labeled CD40 mAb (R&D Systems, Inc.) for the quantification of CD40, with peridinin chlorophyll protein (PerCP)-labeled antirat CD4 mouse mAb (R&D System, Inc.) for purification of CD4+ T cells, and with PE-labeled antirat IL-17 mAb (R&D System, Inc.) for Th17 cells count according to each manufacturer’s instruction. The stained cells were analyzed on a FACSCalibur Four Color Fluorescence Flow Cytometer (BD Pharmingen).

**Liquid chip**

The concentrations of IL-17A and IL-27 in mice lung homogenate and in the above seven groups of cultivations of CD4+ T cells and BMDCs were measured by Luminex liquid phase chips (ProcartaPlex Mouse-Il-27 and IL-17A-Simplex 96; eBioscience, Dummer, Germany) through a Luminex 200 standard high-throughput detection technology platform (LianKe Bio, Hangzhou, China).

**Statistical analysis**

The SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data were expressed as \( \bar{x} \pm s \). The independent sample t-test was used to examine the difference between two groups. Repeated measures analysis of variance with post hoc Bonferroni procedure were used for comparisons of multiple groups over normally distributed samples. In the case of sample with heteroscedasticity, nonparametric Kruskall–Wallis test and Mann–Whitney test.

### Table 1 BMDCs proliferation exposed to varied concentrations of CSE (A_{395nm} \pm s)

| Time (hours) | Control group | 5 mL/L CSE | 10 mL/L CSE | 15 mL/L CSE | 20 mL/L CSE |
|-------------|---------------|------------|-------------|-------------|-------------|
| 12          | 0.531±0.084   | 0.549±0.053| 0.599±0.041 | 0.391±0.041 | 0.274±0.034  |
| 24          | 0.584±0.064   | 0.591±0.061| 0.715±0.090 | 0.428±0.036 | 0.320±0.053  |
| 48          | 0.621±0.041   | 0.619±0.070| 0.693±0.057 | 0.278±0.045 | 0.133±0.038  |
| 72          | 0.671±0.031   | 0.603±0.044| 0.602±0.054 | 0.148±0.014 | 0.157±0.017  |

**Notes:** *P<0.01 vs control group. *P<0.01 vs 10 mL/L CSE. *P<0.05 vs control group.

**Abbreviations:** BMDCs, bone marrow-derived dendritic cells; CSE, cigarette smoke extract.
were used for measuring differences. The differences were considered to be statistically significant when $P \leq 0.05$.

**Results**

**In vivo: Th17 cell, IL-17A, IL-27, and CD40 on the surface of lung mononuclear cell**

Mice exposed to cigarette smoke for 4 weeks indicated significantly higher quantity of CD4+$\cdot$IL-17$^+$ T cells (2.81±0.58) (Figure 2A–C) and levels of IL-17A (484.00±74.08) and IL-27 (25.30±3.74) in the lung than that of the controls (Th17 cell: 1.12±0.36, $P<0.05$, IL-17A: 220.31±83.09 and IL-27: 7.22±1.18, $P<0.01$). As well, CD40 expressions on surfaces of lung mononuclear cells of mice with cigarette smoke exposure noticeably increased (43.67±7.03) in comparison with that of those exempt from smoke exposure (15.86±3.84, $P<0.01$; Figure 3A and B).

**In vitro: CD40 on the surface of BMDCs**

CD40 expression obviously escalated on the surfaces of BMDCs that were sampled from ones prepared from the B group (51.61±2.23, $P<0.01$), and from the A group and fostered with 10 mL/L CSE (44.93±5.71, $P<0.01$), when compared to that from the A group without exposed to CSE (10.39±2.81). (see Table 2 and Figure 4A–C).

**In vitro: Th17 cells, IL-17A, and IL-27**

All the CD4+ T + CSE group (Th17: 3.85±0.73, IL-17A: 93.83±14.71), the CD4+ T + BMDC group (Th17: 3.91±0.42, IL-17A: 109.29±16.09), the CD4+ T + smoke BMDC group (Th17: 5.35±1.03, IL-17A: 313.13±49.35), and the CD4+ T + BMDC + CSE group (Th17: 7.78±0.76, IL-17A: 329.36±45.18) exhibited prominent increases in a comparison with the CD4+ T group (Th17: 1.39±0.48, IL-17A: 38.42±9.41) with respect to the number of Th17 cells and IL-17A. Besides no values of IL-27 showed in either the CD4+ T group or the CD4+ T + CSE group, values of IL-27 (Table 4) indicated a similar trend as that of Th17 and IL-17A among the CD4+.
CD40 expression on the surfaces of BMDCs

| Groups                        | CD40 expression |
|-------------------------------|-----------------|
| BMDCs from the A group \(^a\) | 10.39±2.81      |
| BMDCs from the B group \(^b\) | 51.6±2.23 \(^c\) |
| BMDCs from the A group +10 m/L CSE \(^d\) | 44.93±5.71 \(^e\) |

Notes: \(^a\)The A group in which mice were exempt from cigarette smoke exposure. \(^b\)The B group in which mice were exposed to cigarette smoke for 4 weeks. \(^c\)P<0.01 vs the A group. \(^d\)BMDC from the control group and exposed to 10 m/L CSE for 24 hours. Data presented as mean ± standard deviation.

Abbreviations: BMDCs, bone marrow-derived dendritic cells; CSE, cigarette smoke extract.

Discussion

Among the studies focusing on the role of CD4+IL-17+ T cells in the pathogenesis and development of COPD, this study is one of the first attempts to explore the impacts that DCs and CD40–CD40L costimulatory pathway generated on CD4+IL-17+ T cells under the circumstance of cigarette smoke exposure. As demonstrated in the “Results” section, regardless of in vivo or in vitro, cigarette smoke exposure always enhanced the differentiation of CD4+ T toward CD4+IL-17+ T cells, significantly expanding the expression of CD4+IL-17+ T cells. Our previous study similarly indicated the excessive presence of CD4+IL-17+ T cells in the lungs of enrolled COPD patients and smokers and further demonstrated that IL-17 cells positively correlated with the size of alveolar space or thickness of pulmonary artery wall or the number of lung CD4+ and CD8+ cells while negatively related to FEV\(_1\)%pred of patients.\(^{10}\) CD4+IL-17+ T cells, also called Th17 cells and generally identified as one subpopulation of helper T cell, are characterized with promoting the expression of transcription factor ROR-\(γ\) and yielding a variety of cytokines mediating immune response.\(^{19}\) Hence, Th17 cells are indispensable to immune response targeting antigens and immune toleration in normal situations. Activated IL-17 triggers recruitments of neutrophils and macrophages, induces releases of CXCL8 and CXCL10 from bronchial epithelial cells and macrophages, and, in consequence, promotes airway chemotaxis of neutrophils and mononuclear cells.\(^{20–23}\) IL-17 is mainly secreted by Th17 cells and IL-27 produced by activated DCs. However, there is evidence showing increased expression of IL-17A/F in the cultivation of lung tissue cells and CSE in healthy population and COPD patients\(^{24}\) and elevated level of IL-27 in sputum and blood plasma of COPD patients,\(^{25}\) which is consistent with our study conducted on mice that indicated significantly elevated levels of IL-17A and IL-27 in vivo or in vitro after cigarette smoke exposure or CSE stimulation.

DC is the most powerful APC in human body and acts as a bridge connecting innate immunity and acquired immunity. DCs not only capture and present antigens but also activate native T lymphocytes. Cigarette smoke exposure induces proliferation and maturation of DCs in the lung tissues and drives DCs immigrating to the lung lymphoid tissue. Toll-like receptor located in the DCs functions as a pattern

![Figure 4 CD40 expression on surfaces of BMDCs](Image)

Notes: The A group in which mice were exempt from cigarette smoke exposure. The B group in which mice were exposed to cigarette smoke for 4 weeks. (A) CD40 on BMDCs in A group, red line as blank control. (B) CD40 on BMDCs in B group, red line as blank control. (C) CD40 on BMDCs in A group exposed to 10 m/L CSE, red line as blank control.

Abbreviations: BMDCs, bone marrow-derived dendritic cells; CSE, cigarette smoke extract; PE, phycoerythrin.
Table 3 Th17 cell expressions in the in vitro cultures of CD4+ T cells and BMDCs

| Group                        | CD4+ T-17 T lymphocytes |
|------------------------------|-------------------------|
| CD4+ T group                 | 1.39±0.48               |
| CD4+ T + CSE group           | 3.85±0.73               |
| CD4+ T + BMDC group          | 3.91±0.42               |
| CD4+ T + smoke BMDC group    | 5.35±1.03               |
| CD4+ T + smoke BMDC + CSE group | 3.27±0.80             |
| CD4+ T + BMDC + CSE group    | 7.78±0.76               |
| CD4+ T + BMDC + CSE + CSE group | 3.88±0.41             |

Notes: *P < 0.01 vs the CD4+ T group. **P < 0.01 vs the CD4+ T + BMDC group. ***P < 0.01 vs the CD4+ T + smoke BMDC group. ****P < 0.01 vs the CD4+ T + BMDC + CSE group. Data presented as mean ± standard deviation.

Abbreviations: BMDCs, bone marrow-derived dendritic cells; CSE, cigarette smoke extract.

Table 4 Concentrations of IL-17A and IL-27 in the in vitro cultures of CD4+ T cells and BMDCs

| Group                        | IL-17A (pg/mL) | IL-27 (pg/mL) |
|------------------------------|----------------|---------------|
| CD4+ T group                 | 38.42±9.41     | –             |
| CD4+ T + CSE group           | 93.83±1.71     | –             |
| CD4+ T + BMDC group          | 109.29±16.09** | 8.91±1.98     |
| CD4+ T + smoke BMDC group    | 313.13±49.35*  | 34.92±5.03*   |
| CD4+ T + smoke BMDC + CD40 antibody group | 174.24±35.21*** | 14.37±4.95** |
| CD4+ T + BMDC + CSE group    | 329.36±45.18** | 40.07±8.54*   |
| CD4+ T + BMDC + CSE + CD40 antibody group | 141.18±29.27** | 13.75±5.11** |

Notes: *P < 0.01 vs the CD4+ T + BMDC group. **P < 0.01 vs the CD4+ T + BMDC + CSE group. ***P < 0.01 vs the CD4+ T + CSE group. ****P < 0.01 vs the CD4+ T group. **P < 0.01 vs the CD4+ T + BMDC + CSE + CSE group. Data presented as mean ± standard deviation.

Abbreviations: BMDCs, bone marrow-derived dendritic cells; CSE, cigarette smoke extract.
Conclusion

DCs and CD40–CD40L costimulatory pathway are associated with the development of immune inflammation caused by cigarette smoke exposure. The blockade of CD40–CD40L pathway with antagonistic CD40 antibody effectively restrains differentiations of T lymphocytes toward Th17 cells and, in consequence, substantially cuts down excessive presence of Th17 cell, IL-17A, and IL-27 due to cigarette smoke exposure. Depressing the CD40–CD40L ligation between DCs and T cells can be an inspiration for pursuing a new therapeutic target for immune inflammation in COPD.

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**Author contributions**

XZ made substantial contributions to concept and design of this study, data interpretation, and critical modifications of some important intellectual contents. YL and YS equally made substantial contributions over drafting this article, data acquisition, and data interpretation. LK, GZ, and LZ were significantly involved in data acquisition and data analysis. JZ managed some important modifications over the content. JL substantially contributed to data analysis. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

**Disclosure**

The authors report no conflicts of interest in this work.

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