Interleukin-1β stimulates the secretion of thymic stromal lymphopoietin (TSLP) from endometrioma stromal cells: possible involvement of TSLP in endometriosis

Yoko Urata, Yutaka Osuga*, Gentaro Izumi, Masashi Takamura, Kaori Koga, Miwako Nagai, Miyuki Harada, Tetsuya Hirata, Yasushi Hirota, Osamu Yoshino, and Yuji Taketani

Department of Obstetrics and Gynecology, Faculty of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan

*Correspondence address. Tel: +81-3-3815-5411; Fax: +81-3-3816-2017; E-mail: yutakaos-tky@umin.ac.jp

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STUDY QUESTION: Is thymic stromal lymphopoietin (TSLP) involved in the pathophysiology of endometriosis?

SUMMARY ANSWER: TSLP is up-regulated by interleukin (IL)-1β and may be involved in the development of endometriosis.

WHAT IS KNOWN ALREADY: Endometriosis is a chronic inflammatory disease in which the Th2 immune response is activated and has been suggested to promote the disease. TSLP is a master cytokine that drive Th2 immune response.

STUDY DESIGN, SIZE, DURATION: A laboratory study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Primary cultures of endometrioma stromal cells (ESCs) were treated with IL-1β, a typical inflammatory cytokine associated with endometriosis. Gene expression of TSLP in ESCs and secretion of TSLP protein from ESCs were studied using quantitative PCR and a specific ELISA. Interferon γ (IFN-γ), a typical Th1 cytokine, and IL-4, a typical Th2 cytokine, were added to the culture to evaluate their effect on the IL-1β-induced secretion of TSLP. Inhibitors of p38 mitogen-activated protein kinase (MAPK), p42/44 MAPK and stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK) were added to the culture to examine intracellular signals involved in IL-1β-induced TSLP secretion. The expression of TSLP in endometrioma tissue was examined by immunohistochemistry. The concentration of TSLP in the serum and peritoneal fluid (PF) of women with or without endometriosis was measured with a specific ELISA.

MAIN RESULTS AND THE ROLE OF CHANCE: IL-1β stimulated the expression of TSLP mRNA and secretion of TSLP protein from ESCs. IL-4 enhanced the IL-1β-induced TSLP secretion from ESCs, while IFN-γ reduced it. Inhibitors of p42/44 MAPK, p38 MAPK and SAPK/JNK suppressed the IL-1β-induced secretion of TSLP from ESCs. Positive immunostaining of TSLP was observed in the stroma of endometrioma tissue. TSLP concentrations in the serum and PF were both higher in women with endometriosis compared with those without endometriosis.

LIMITATIONS, REASONS FOR CAUTION: The present study was only in vitro. The samples used for culture were endometrioma tissues, not including other types of endometriosis. Therefore, the present findings should be interpreted with caution.

WIDER IMPLICATIONS OF THE FINDINGS: This study provided new insights in the Th2 immune response-related mechanism in endometriosis.

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Key words: endometriosis / thymic stromal lymphopoietin / interleukin-1β / Th2 immune response / inflammation
Introduction

Endometriosis is an enigmatic chronic inflammatory disease. It causes painful symptoms that remarkably deteriorate women’s health (Giudice, 2010). It also induces infertility/subfertility through multiple mechanisms including tubal dysfunction, ovulatory dysfunction, reduced fertilization and implantation failure (Gupta et al., 2008; de Ziegler et al., 2010). In addition, recent findings indicate that endometriosis increases the risk of ovarian cancer when it forms a cystic lesion in the ovary (ovarian endometrioma; Giudice, 2010; Kobayashi et al., 2011). Its etiology is generally believed to be that endometrial cells or tissues in retrograde menstruation implant and grow in the pelvic peritoneum. However, only a minority of women are affected with endometriosis despite the fact that retrograde menstruation is observed in most women. This means that other pathogenic events are required to develop the disease. Inflammation and immune responses are among the events involved (Lebovic et al., 2001; Osuga et al., 2002; Khan et al., 2008; Osuga, 2008; Osuga et al., 2011); however, how they drive the development of endometriosis remains unclear.

Interleukin (IL)-1β is a versatile inflammatory cytokine that may promote the disease. IL-1β stimulates endometriotic cells to produce various cytokines and growth factors that play roles in adhesion, growth, invasion, inflammation and angiogenesis in endometriotic tissues (Lebovic et al., 2000; Akoum et al., 2002; Lavoie et al., 2007; Takemura et al., 2007; Kao et al., 2011; Yoshino et al., 2011). The levels of IL-1β are increased in the peritoneal fluid (PF) and peritoneal macrophages (Mori et al., 1992) in women with endometriosis and in endometriotic tissues (Bergqvist et al., 2001). On the other hand, IL-1 receptor antagonist, a competitive antagonist for IL-1β, is decreased in the PF (Zhang et al., 2007) or absent in endometriotic tissues (Sahakian et al., 1993). Another antagonist for IL-1β, soluble IL-1 receptor type II, is also decreased in the PF of women with endometriosis (Akoum et al., 2008). Consequently, the increased level of IL-1β can act without restriction on endometriotic tissues and promote the disease.

The Th2 immune response, as well as the effect of IL-1β, has been suggested to play multiple roles in the disease (Osuga et al., 2011). Specific features of the Th2 immune response are the release of IL-4 and the induction of allergy. The levels of IL-4 are increased in the peripheral blood and peritoneal cells of women with endometriosis (Hsu et al., 1997; Antsiferova et al., 2005). Another report shows an increased ratio of IL-4 to IFNγ, a cytokine released in the Th1 immune response, in the PF of women with endometriosis (Podgaec et al., 2007). IL-4 stimulates the proliferation of endometriotic stromal cells (Ouyang et al., 2008) and secretion of eotaxin from endometriotic stromal cells (Ouyang et al., 2010), suggesting its pleiotropic roles in endometriosis. Clinically, women with endometriosis are at higher risk of diseases that associate with the Th2 immune response, such as allergies and asthma (Snaid et al., 2002). In contrast, women with endometriosis have diminished the Th1 immune response in the peritoneum (Mier-Cabrera et al., 2011).

The Th2 immune response is under the control of thymic stromal lymphopoietin (TSLP). TSLP was initially identified as a growth-promoting factor secreted in the supernatants of a murine thymic stromal cell line (Friend et al., 1994). Later studies revealed that TSLP polarizes dendritic cells to stimulate the differentiation of inflammatory Th2 cells that secrete IL-4, IL-5, IL-13 and TNF-α (Liu, 2006; Ziegler and Liu, 2006). TSLP has been implicated in the development of allergic diseases, such as asthma (Al-Shami et al., 2005) and atopic dermatitis (Ziegler and Liu, 2006); serum TSLP levels are increased in children with atopic dermatitis (Lee et al., 2010). TSLP levels are also increased in the synovial fluid of patients with rheumatoid arthritis, indicating its role in non-allergic inflammation (Koyama et al., 2007). TSLP is expressed in a range of cell types, including epithelial cells, fibroblasts, keratinocytes, airway smooth muscle cells, mast cells and dendritic cells (He and Geha, 2010; Kashyap et al., 2011). In the reproductive organs, TSLP expression in trophoblast cells and decidual epithelial cells and its possible involvement in maternal–fetal tolerance have been reported (Li and Guo, 2009; Guo et al., 2010). While various stimuli including inflammatory cytokines and Toll-like receptor ligation have been reported to regulate the expression of TSLP (He and Geha, 2010), we noticed that IL-1β increases TSLP expression in airway epithelial cells (Lee and Ziegler, 2007) and hypothesized that similar effect may be observed in endometriotic cells.

Given the notion that both inflammation and the Th2 immune response promote endometriosis, there might be a molecule that integrates these two events. In the present study, first we studied whether IL-1β stimulates TSLP secretion in endometriotic cells and whether cytokines involved in the Th1 and Th2 immune responses have any effect on it. Next, the presence of TSLP in the serum, PF and endometriotic tissue was examined.

Materials and Methods

Patients and samples

This study was conducted under a protocol approved by the institutional review board of the University of Tokyo. Written informed consent was obtained from all the patients before collecting the samples. All the patients had regular menstrual cycles and had not received hormonal treatment for at least 3 months before surgery. Endometrioma tissues for primary culture experiments (n = 17) and for immunohistochemistry (n = 10) were obtained from patients with ovarian endometrioma (Supplementary data, Table S1). The serum and PF were obtained from the patients with endometriosis [serum, n = 23, age, 39.0 ± 7.1 (mean ± SD) years; PF, n = 24, 39.0 ± 6.9] or with other benign gynecological conditions (n = 11, 35.6 ± 3.9; n = 11, 35.6 ± 3.9). These benign conditions were dermoid cyst (n = 7), serous cystadenoma (n = 1), serous cystadenoma and uterine leiomyoma (n = 1), infertility (n = 1) and hydrosalpinx (n = 1). The serum was collected the day before the surgery and PF was collected from the cul-de-sac at the beginning of the laparoscopic surgery. The stage of endometriosis was determined according to the revised American Society for Reproductive Medicine (r-ARSM) classification. The collected serum and PF were stored at −80°C before use. The collected endometriotic tissues were transported to the laboratory in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Invitrogen, Rockville, MD, USA) on ice under sterile conditions. The tissues were processed for the generation of primary cell culture.
Isolation and culture of endometrioma stromal cells

The isolation and culture of human endometrioma stromal cell (ESCs) were performed as described previously (Hirata et al., 2010; Saito et al., 2011). Fresh endometriotic tissue collected in the sterile medium was rinsed to remove blood cells. The tissue was minced into small pieces and incubated in DMEM/F-12 containing collagenase (0.25%, Wako, Osaka, Japan) and deoxyribonuclease I (250 IU/ml, Invitrogen) for 80 min at 37°C. The resultant dispersed endometriotic cells were separated by filtration through a 70-μm nylon cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ, USA). Stromal cells remaining in the filtrate and resuspended in phenol red-free DMEM/F-12 containing 3% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml amphotericin B. The ESCs were plated onto 100 mm dishes and incubated in DMEM/F-12 containing collagenase (0.25%, Wako, Osaka, Japan) and deoxyribonuclease I (250 IU/ml, Invitrogen) for 120 min at 37°C. The resultant dispersed endometriotic cells were collected in the sterile medium was rinsed to remove blood cells and allowed to adhere at 37°C in a humidified 5% CO₂. At the first passage, the cells were plated onto 12-, 24-, 48-well plates at 2 × 10⁵ cells/ml in medium containing 5% FBS. Once the cells reached confluence, in 2 or 3 days, they were used for experiments. The purity of ESCs was >95%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin, CD45 and von Willebrand factor.

Treatment of ESCs

First, the dose effect of IL-1β on the secretion of TSLP was evaluated by incubating the cells in 24-well plates with different concentrations of IL-1β (R&D systems, Minneapolis, MN, USA) for 24 h. Second, the time-course effect of IL-1β on the expression of TSLP mRNA was assessed by incubating the cells in 12-well plates with or without IL-1β (1 ng/ml) for 0, 3, 6, 12 or 24 h. Thirdly, the effect of IL-4 or IFNγ on IL-1β-induced TSLP secretion was evaluated by incubating the cells in 48-well plates with IL-4 (10 ng/ml, R&D systems) or IFN-γ (10 ng/ml, R&D systems) together with IL-1β (0.1 ng/ml) for 24 h. Finally, since IL-1β activates p38 mitogen-activated protein kinase (MAPK), p42/44 MAPK and stress-activated protein kinase (SAPK)/c-Jun kinase (JNK; Yoshino et al., 2004), the effect of inhibitors of these MAPKs on IL-1β-induced TSLP secretion was evaluated. For this purpose, we preincubated the cells in 48-well plates with SB202190 (10 μM), PD98059 (25 μM) and SP600125 (10 μM; inhibitors of p38 MAPK, p42/44 MAPK and SAPK/JNK, respectively, Calbiochem, La Jolla, CA, USA) for 1 h before the addition of IL-1β (1 ng/ml), then incubated the cells with IL-1β for 24 h. The time-course experiment was performed under condition with 5% FBS to keep the influence of culture conditions constant between the different time points. All the other cultures were performed under serum-free condition. After the treatments, the cells or the conditioned medium were collected and stored at -80°C until subsequent analysis. The characteristics of the study subjects used in the above experiments are shown in Supplementary data, Table S1.

Measurement of TSLP in serum and PF

Concentrations of TSLP in the serum and PF were measured using a specific ELISA kit (Duoset, R&D Systems) according to the manufacturer’s protocol. The absorbance was read at 450 and 550 nm with the Epoch Multi-Volume Spectrophotometer System (BioTek, Winowski, VT). The sensitivity of the assay was 7.81 pg/ml.

RNA extraction, reverse transcription and real-time quantitative PCR

We extracted the total RNA from ESCs using an RNeasy minikit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse transcribed in a 20-μl volume using a RT–PCR kit (Toyobo, Osaka, Japan). To assess TSLP mRNA expression, real-time quantitative PCR and data analysis were performed using Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). The expression of TSLP mRNA was normalized to RNA loading for each sample using human glyceraldehyde dehydrogenase (GAPDH) mRNA as an internal standard. TSLP primers (sense, 5′-GCCATGAAAACTAACGCTGC-3′; antisense, 5′-GCGGAAATTTTGAACGCTTGC-3′) and GAPDH primers (sense, 5′-ACCACGCTCCAGTGCAGTC-3′; antisense, 5′-TCCACACACCCTTGTGGTGTA-3′) were chosen to amplify a 152 and 425 bp fragment, respectively.

PCR conditions were as follows: for TSLP, 40 cycles at 95°C for 10 s, 60°C for 10 s, 72°C for 7 s; for GAPDH, 30 cycles at 95°C for 10 s, 64°C for 10 s, 72°C for 18 s. All PCR conditions were followed by melting curve analysis.

Immunohistochemistry

Endometrioma tissues were washed with phosphate-buffered saline (PBS), embedded in the OCT compound (Sakura, Tokyo, Japan), and snap frozen in liquid nitrogen. Sections were sliced at a 6-μm thickness. Sections were fixed with cold acetone for 10 min and then washed three times in PBS for 5 min. The sections were treated with 1% H₂O₂ for 20 min to eliminate endogenous peroxidase. After blocking with protein block, serum-free, ready-to-use (Dako), the sections were incubated with 1 μg/ml of anti-human TSLP antibody (R&D systems) and 1 μg/ml of sheep IgG (R&D systems) at 4°C overnight. The sections were washed with PBS twice and incubated with biotinylated anti-sheep IgG (Vector, Burlingame, CA, USA) for 30 min at room temperature. The sections were incubated with Vectastain elite ABC reagent (Vector) for 30 min at room temperature. Staining was detected with the Vector NovaRED substrate kit (Vector). All sections were counterstained with hematoxylin and evaluated under a light microscope.

Statistical analysis

TSLP concentrations in the culture media and TSLP mRNA levels in the cultured ESCs were analyzed by ANOVA followed by post hoc analysis. TSLP concentrations in the PF and the serum were analyzed by the Wilcoxon test. P < 0.05 was considered significant.

Results

IL-1β-induced increase in protein secretion and mRNA expression of TSLP in ESCs

IL-1β at doses of 0.01, 0.1, 1 and 10 ng/ml significantly increased TSLP secretion. The increase was stepwise from 0 to 0.1 ng/ml and then appeared to decrease slightly (Fig. 1A). The time-course study demonstrated that IL-1β increased the expression of TSLP mRNA by 8-fold 3 h after it was added. This was the highest level observed (Fig. 2A).

Effect of IL-4 and IFNγ on IL-1β-induced increase in TSLP secretion in ESCs

IL-4, a representative cytokine of the Th2 immune response, significantly enhanced the IL-1β-induced increase in TSLP secretion (Fig. 2A). In contrast, IFNγ, a representative cytokine of the Th1 immune response, significantly suppressed IL-1β-induced increase in TSLP secretion (Fig. 2B). Neither IL-4 nor IFNγ alone affected basal TSLP secretion.
Effect of MAPK inhibitors on IL-β-induced increase in TSLP secretion in ESCs

The inhibitors of p38 MAPK (SB202190), p42/44 MAPK (PD98059) and SAPK/JNK (SP600125) inhibited the IL-1β-induced increase in TSLP significantly (Fig. 3).

Expression of TSLP in endometrioma tissue

We detected obvious staining of TSLP in 3 out of 10 samples (Supplementary data, Table S1). The representative picture is shown in Fig. 4A and C. While the stromal cells were diffusely stained positive, the staining seemed intense near the epithelium. Although most of the epithelial cells were detached, the remaining epithelial cells were stained positive for TSLP. No staining was observed when normal sheep IgG (negative control) was used as a primary antibody (Fig. 4B and D).

Concentration of TSLP in serum and PF

The concentrations of TSLP in the serum from two patients without endometriosis, four patients with stage I/II and one patient with endometriosis are shown in Fig. 5A. The conditioned medium was collected and assayed for TSLP by a specific ELISA. Values are the mean ± SEM of four separate cultures. Different letters denote significant differences between groups (P < 0.05). The result is representative of four repeated experiments using samples from four different women.
stage III/IV endometriosis and in the PF from one, two and one patient(s), respectively, were below the limit of assay (7.81 pg/ml). TSLP concentrations below the limit of assay were assigned to the limit value in the analysis. The TSLP concentrations in the PF of women with stage III/IV endometriosis were significantly higher than those of women without endometriosis, whereas those of women with stage I/II endometriosis appeared to be equivalent to those of women without endometriosis (Fig. 5A). Similarly, the concentrations of TSLP in the serum of women with III/IV endometriosis were significantly higher than those of women without endometriosis and with stage I/II endometriosis. Meanwhile, the concentrations of TSLP in the serum of women without endometriosis were comparable to those of women with stage I/II endometriosis (Fig. 5B).

Discussion

In the present study, IL-1β increased mRNA expression and protein secretion of TSLP from ESCs. The highest level of TSLP mRNA expression observed was at 3 h; this pattern is similar to that reported in airway epithelial cells (Lee and Ziegler, 2007). IL-4 enhanced but IFNγ suppressed IL-1β-induced secretion of TSLP. Inhibitors of p38 MAPK, p42/44 MAPK and SAPK/JNK abrogated the IL-1β-induced increase in TSLP secretion. Immunoreactivity of TSLP was detected in the stromal area of endometrioma. TSLP concentrations in the PF and serum were both increased in women with stage III/IV endometriosis, while those of women with stage I/II endometriosis were similar to those of women without endometriosis.

IL-1β has been suggested to support the development of endometriosis through the production of various inflammatory molecules including IL-6, IL-8, MCP-1 and COX-2. The present finding raises a novel notion that IL-1β may promote the disease through the Th2 immune response stimulated by TSLP secreted by ESCs. In this context, it is intriguing to note our recent finding that IL-1β stimulates activin-A expression in ESCs (Yoshino et al., 2011), because activin-A functions as a Th2 cytokine to stimulate the differentiation of M2 macrophage (Ogawa et al., 2006). Thus, the secretion of both TSLP and activin-A from ESCs appears to mediate the effect of IL-1β to promote the disease via the Th2 immune response.

The enhancement of IL-1β-induced TSLP secretion with IL-4 and the suppression of that with IFNγ imply an exquisite mechanism to amplify the Th2 response in endometriosis. In view of increased expression of IL-4 and decreased expression of IFNγ in women with endometriosis (Ho et al., 1996; Hsu et al., 1997; Wu et al., 1998; Szyllo et al., 2003; Gmyrek et al., 2008), it is plausible that IL-1β-induced secretion of TSLP is augmented in women with the disease. In addition, on the assumption that TSLP-induced Th2 cell differentiation leads to a higher amount of IL-4, the higher amount of IL-4 further promotes the production of TSLP in the endometriotic tissue. Consequently, endometriosis-associated Th2 skewed status may contribute to the development of the disease in a perpetuating manner.

Meanwhile, another finding that IL-4 and IFNγ had no effect on TSLP secretion in the absence of IL-1β may indicate the essential role of IL-1β in the TSLP-mediated Th2 immune response in endometriosis. In contrast to the requirement of IL-1β for the IL-4-induced increase in TSLP secretion, IL-4 by itself increases eotaxin, a stimulant
of the Th2 immune response (Ouyang et al., 2010). Accordingly, IL-4 seems to promote the Th2 immune response in endometriosis in various manners.

The inhibitors of p38 MAPK, p42/44 MAPK and SAPK/JNK inhibited IL-1β-induced increase in TSLP secretion. The inhibitory effect of these inhibitors is also observed in IL-1β-induced secretion of IL-8 and CCL20 from ESCs (Yoshino et al., 2004; Hirata et al., 2010), both of which are suggested to stimulate inflammation in endometriosis. Accordingly, these MAPK inhibitors may hinder the progression of the disease by suppressing both inflammation and Th2 immune response. Actually, the efficacy of inhibitors of p42/44 MAPK and p38 MAPK to subside the disease has been demonstrated in mouse models of endometriosis (Yoshino et al., 2006; Ngô et al., 2010; Zhou et al., 2010). In contrast, protein kinase A inhibitor H89 and Janus kinase 3 inhibitor CP-690550, which are not expected to affect the pathways activated by IL-1β, did not affect the secretion of IL-1β-induced increase in TSLP secretion (Supplementary data, Fig. S1). To understand the precise mechanism, however, more detailed analysis of the signaling networks is needed in future studies.

The increase in TSLP levels in the PF and the serum of women with stage III/IV endometriosis endorses the notion that TSLP is involved in endometriosis. The immunohistochemical finding that TSLP was detected in stromal cells of endometrioma implies that TSLP increased in the PF and the serum may be derived from the lesion. This speculation is consistent with the finding that TSLP concentrations in the PF and the serum were comparable in women without endometriosis and with stage I/II endometriosis, in which the volume of the lesion is remarkably small compared with stage III/IV endometriosis.

TSLP increased gene expression and protein secretion of IL-6 and IL-8 in human airway smooth muscle cells (Shan et al., 2010), implying some inflammatory function of TSLP other than stimulating the Th2 immune response. However, we could not detect gene expression of TSLP receptor in ESCs and TSLP did not increase the gene expression of IL-6 and IL-8 in ESCs (data not shown). Therefore, we speculate that TSLP exerts mainly on immune cells as a driver of the Th2 immune response in endometriotic tissues.

In the current treatment of endometriosis, most therapies depend on the suppression of ovarian function. Therefore, therapies which maintain ovarian function are demanded. The blockade of TSLP is expected as a future therapy in some allergic diseases (Koyama et al., 2007; Zhang et al., 2011). Given that the Th2 immune response may exacerbate endometriosis, the blockade of TSLP might suppress the development of the disease, leading to a novel therapy without harm to ovarian function. Further studies are thus warranted to examine the effects of TSLP suppression in endometriosis.

This study has its limitation in interpreting the findings of immunohistochemistry. First, we did not compare TSLP expression in the endometrioma and the healthy ovary, although TSLP might be expressed in the healthy ovary. Second, we could detect obvious staining of TSLP only in 3 out of 10 samples. One possible reason is that the antibody used in the present study could detect only high levels of TSLP expression. Another reason may be that complex inflammatory reactions that regulate TSLP expression occur in a spatio-temporally heterogeneous manner in endometrioma tissues. This notion is in accord with a report that aromatase, an enzyme-stimulating endometriosis, is expressed in a spatially heterogeneous manner in endometrioma tissues (Yang et al., 2002).

In summary, the present study demonstrated that TSLP secretion from ESCs was up-regulated by IL-1β and that TSLP was increased in the serum and the PF of women with endometriosis. IL-1β-induced increase in TSLP secretion from ESCs may develop the disease through the Th2 immune response.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles

Y.O. was the principal investigator and takes primary responsibility for the paper. Y.U. and Y.O. contributed to the study design and manuscript writing. Y.U., G.I., M.T. and M.N. contributed to the execution of the experiments. All the authors contributed to the data analysis and critical discussion.

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Conflict of interest

None declared.

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