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Original Paper

IL-29 Enhances LPS/TLR4-Mediated Inflammation in Rheumatoid Arthritis

Donghua Xu[a] Shushan Yan[b] Huijuan Wang[c] Bingjie Gu[d] Keyi Sun[c] Xiaofan Yang[c] Bin Sun[e] Xiaodong Wang[a]

[a]Department of Rheumatology, the Affiliated Hospital of Weifang Medical University, Weifang, [b]Department of Surgical Oncology, the Eighty-first Hospital of People's Liberation Army, Nanjing, [c]Department of Microbiology and Immunology, Nanjing Medical University, Nanjing, [d]Department of Rheumatology and Immunology, Nanjing First Hospital, Nanjing Medical University, Nanjing, [e]Department of Nephrology, the First Affiliated Hospital, Nanjing Medical University, Nanjing, China

Key Words
Interleukin-29 • Nuclear factor-κB • Rheumatology arthritis • Toll-like receptor 4

Abstract
Background/Aims: Interleukin-29 (IL-29), a critical member of type III interferons (IFNs) family, has been implicated in protecting against viral infection and modulating autoimmune inflammation. Toll-like receptor 4 (TLR4) plays a crucial role in synovial inflammation and may contribute to the pathogenesis of rheumatology arthritis (RA). However, little is known about the modifying effect of IL-29 on TLR4-mediated inflammation in RA. We aim to investigate the potential association between IL-29 and TLR4 in RA. ***Methods:*** Peripheral blood mononuclear cells (PBMCs) and serum from 77 patients with RA and 70 controls were collected to determine levels of IL-29 and TLR4 mRNA by real-time polymerase chain reaction (PCR). Levels of IL-29 and TLR4 in synovial tissues and fluid from 25 RA patients and 24 controls were detected by enzyme-linked immunosorbent assay (ELISA) or western blot assay, respectively. RAW264.7 cells were stimulated by lipopolysaccharide (LPS) and/or IL-29. The production of inflammatory cytokines including IL-6, IL-8 as well as TNF-α and the activation of nuclear factor-κB (NF-κB) signaling were determined. ***Results:*** In comparison with controls, increased IL-29 was observed in PBMCs, synovial tissue, serum and synovial fluid of patients with RA. Besides, TLR4 was significantly elevated in PBMCs and synovium of RA patients. Moreover, IL-29 was positively associated with TLR4 in RA, suggested by Pearson's correlation analysis. When RAW264.7 cells were stimulated by LPS with or without IL-29 *in vitro*, IL-29 could enhance LPS-mediated TLR4 expression and the production of IL-6, IL-8 and TNF-α in RAW264.7 cells via the activation of NF-κB signaling. ***Conclusion:*** The present study suggests, for the first time, that IL-29 can aggravate LPS/TLR4-mediated inflammation in RA depending on NF-κB signaling activation.

D. Xu, S. Yan, H. Wang and B. Gu contributed equally contributed to this work.

Xiaodong Wang, M.D.
And Bin Sun, M.D.

Department of Rheumatology, the Affiliated Hospital of Weifang Medical University, Weifang 261000, Shandong Province (China); Department of Nephrology, the First Affiliated Hospital, Nanjing Medical University, Nanjing 210029, Jiangsu Province (China), E-Mail xiaodongw_doc@163.com, E-Mail sunbin@medmail.com.cn

Introduction

Type III interferons (IFNs) are comprised of three identified molecules, including interleukin-29 (IL-29), IL-28A and IL-28B, and belong to the superfamily of human class II cytokines [1, 2]. They are also known as the IFNλ family and engage through a common complex of IL-28RA and IL-10R2 [3]. IFNλ exhibits multiple immune regulatory activities, such as antivirus, antiproliferation, and antitumor properties. Accumulated evidence has suggested that IFNλ is involved in the regulation of T helper (Th)1/Th2 response and functions of macrophages, B cells and plasmacytoid dendritic cells [4-8]. IL-29 (IFNλ1) is the most active cytokine among all type III IFNs. A previous study has implicated that IL-29 was involved in renal disorder and arthritis progression of systemic lupus erythematosus (SLE) through regulating the production of several chemokines [9]. Interestingly, increased level of IL-29 has been observed in patients with rheumatology arthritis (RA) as compared with healthy controls [10]. In addition, IL-29 could aggravate synovial inflammation by enhancing pro-inflammatory cytokines production, such as IL-6 and IL-8 [10]. Nonetheless, whether and how IL-29 contributes to RA pathogenesis remains obscure to date.

Toll-like receptors (TLRs) and the signaling through TLR pathways have been implicated in the pathogenesis of autoimmune diseases, for instance, RA and SLE [11, 12], which may contribute to the understanding of role of infections in autoimmune disorders. TLR4 is one of the most extensively studied TLRs involved in autoimmune diseases. A number of studies have suggested that TLR4-mediated inflammation may play a critical role in the development of RA [13, 14]. Xu and the colleagues firstly reported that IL-29 might enhance TLR-mediated production of inflammatory mediators in synovial fibroblasts of RA patients [15]. Nonetheless, the findings were primarily based on a human rheumatoid fibroblast-like synoviocyte (MH7A cell line) *in vitro*. Moreover, the modifying effect of IL-29 on TLR4-mediated inflammation in RA and the underlying molecular mechanisms remain largely unknown. Apart from synovial fibroblasts, synovial macrophages are also predominately involved in the pathogenesis and progression of RA. Thus, we aim to investigate the potential role of IL-29 and TLR4 and their interaction in RA. RAW264.7 macrophages are also used to elucidate the association between IL-29 and TLR4 *in vitro*.

Material and Methods

Patients and controls

Patients with RA and controls were recruited randomly from the first affiliated hospital of Nanjing medical university between 2013 and 2015 and the affiliated hospital of Weifang medical university between 2014 and 2015. Controls were patients without RA and enrolled from the same hospitals for synovectomy or joint replacement surgery or amputations or other regular treatments after trauma. Peripheral blood mononuclear cells (PBMCs) and serum were obtained from blood samples of 77 patients with RA and 70 controls. Synovial tissues and synovial fluid were collected from 25 patients with RA and 24 controls among all participants. Informed consent for sample test was obtained. The study was approved by our Institutional Ethics Committee. Table 1 showed the clinical and laboratory characteristics of patients with RA and controls.

Cells culture

RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen Corp., Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin with 5% CO2 at 37 °C. RAW264.7 macrophages were exposed to LPS (Sigma, USA, 0.5ug/ml) and/or recombinant human IL-29 (R&D Systems, Minneapolis, MN, USA) at different concentrations (100, 10, 1ng/ml) for 2, 6, 12, or 24 hours.

Real-time polymerase chain reaction (PCR)

Total RNAs of PBMCs or synovial tissues were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed in triplicate assay according to the protocol of SYBR Green Mastermix

Table 1. Clinical and laboratory characteristics of RA patients and controls. ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; CRP, C-reactive protein

	RA patients (n=77)	Controls (n=70)
Age (year)	49.2 ± 19.0	47.8 ± 23.2
Gender (female/male)	65/12	61/9
Duration of disease (year)	6.1 ± 2.1	-
Score of disease activity in 28 joints	3.9 ± 1.1	-
ESR (mm/h)	37.6 ± 19.2	10.8 ± 3.1
RF (U/l)	76.4 ± 13.9	-
CRP (mg/l)	12.1± 5.2	-

Table 2. Primers for real-time PCR

Genes	Forward primers (5′ to 3′)	Reverse primers (5′ to 3′)
Mouse TLR4	AAGAGCCGGAAGGTTATTGT	AGCTTAGCAGCCATGTGTTC
Human TLR4	CCAGCCTCCTCAGAAACAGA	TCCCTCCAGCAGTGAAGAAG
Mouse IL-6	AGGAAGCGGTCCAGGTAGTT	CTCCCAAGTCCTCCATAGCAG
Mouse IL-8	CTAGGCATCTTCGTCCGTCC	CAGAAGCTTCATTGCCGGTG
Mouse TNF-α	TCTTGAGGCCACTTTCTGCT	ACTCATCCTCCACGTCCTTG
Mouse GAPDH	GCCTCGTCCCGTAGACAAAA	GATGGGCTTCCCGTTGATGA
Human GAPDH	AAGGAAATGAATGGGCAGCC	TAGGAAAAGCATCACCCGGA

kit (Takara, Tianjin, China) on the Step one plus machine (Applied Biosystems, Darmstadt, Germany). Primers for specific genes were shown in Table 2. All primers were designed by use of Primers 3.0 software.

Enzyme-linked immunosorbent assay (ELISA)

Levels of IL-29 in serum and synovial fluid samples were determined by ELISA according to the protocol of human IL-29 ELISA reagent kit from ADL (San Diego, CA, USA). IL-6 (R&D Systems, USA), IL-8 (Kemin Tech, China), and TNF-α (R&D Systems, USA) in culture supernatant were assayed by ELISA kits in accordance with the manufacturer's recommendations. The lowest detection limits for IL-29, IL-6, IL-8, and TNF-α were 0.2, 7.8, 31.25, and 31.2 pg/ml, respectively.

Flow cytometry analysis (FCA)

RAW264.7 cells were stimulated by LPS (0.5ug/ml) and/or IL-29 (100ng/ml) for 6, 12, and 24 hours. Cells were pelleted, resuspended and incubated with FITC-conjugated anti-mouse TLR4 (eBioscience Inc. San Diego, CA) or corresponding isotype control (eBioscience Inc. San Diego, CA) at 37°C for 1 h. After washing for three times, RAW264.7 cells were finally resuspended in PBS and analyzed on a FACS Calibur flow cytometer equipped with CellQuest software (BD Biosciences).

Western blot analysis

PBMCs, fresh synovial tissues, and RAW264.7 cells were lysed with RIPA buffer plus inhibitors of protease and phosphatase. Protein samples were quantified with the Bradford assay (Bio-Rad Laboratories, Hercules, CA), and then separated by SDS-PAGE at 30μg per well. All samples were transferred to a PVDF membrane, which was incubated with specific antibodies including TLR4 (Santa Cruz), p-IκBα (CST, USA), p-NF-κB/P65 (CST, USA), β-actin (CST, USA) and GAPDH (CST, USA) as described previously [16].

Immunofluorescence

RAW264.7 cells were stimulated with LPS (0.5ug/ml) with or without IL-29 (100ng/ml) for 2h, and the phosphorylation of NF-κB and its nuclear translocation were determined by use of confocal laser

scanning microscope after staining with the UltraCruzTM Mounting Medium (Santa Cruz) as previously described [16].

Statistical analysis

Results are presented as mean ± SEM. Independent-Samples T test or One-Way ANOVA was used for analysis. Pearson's correlation and linear regression analysis were used to determine the correlation between parameters. SPSS 16.0 and Graphpad 5.0 were adopted for calculation. All tests were two-tailed. P value less than 0.05 was considered to be statistically significant.

Results

Increased expression of IL-29 in RA

As shown in Fig. 1A, increased mRNA level of IL-29 was observed in PBMCs and synovial tissue of RA patients compared with controls. Similarly, the level of IL-29 protein was also elevated in serum and synovial fluid of RA patients (Fig. 1B). Taken together, the expression of IL-29 was significantly elevated in RA patients.

Increased expression of TLR4 in RA

In comparison with controls, TLR4 was significantly enhanced in PBMCs and synovium of RA patients at levels of both mRNA (Fig. 1C) and protein (Fig. 1D).

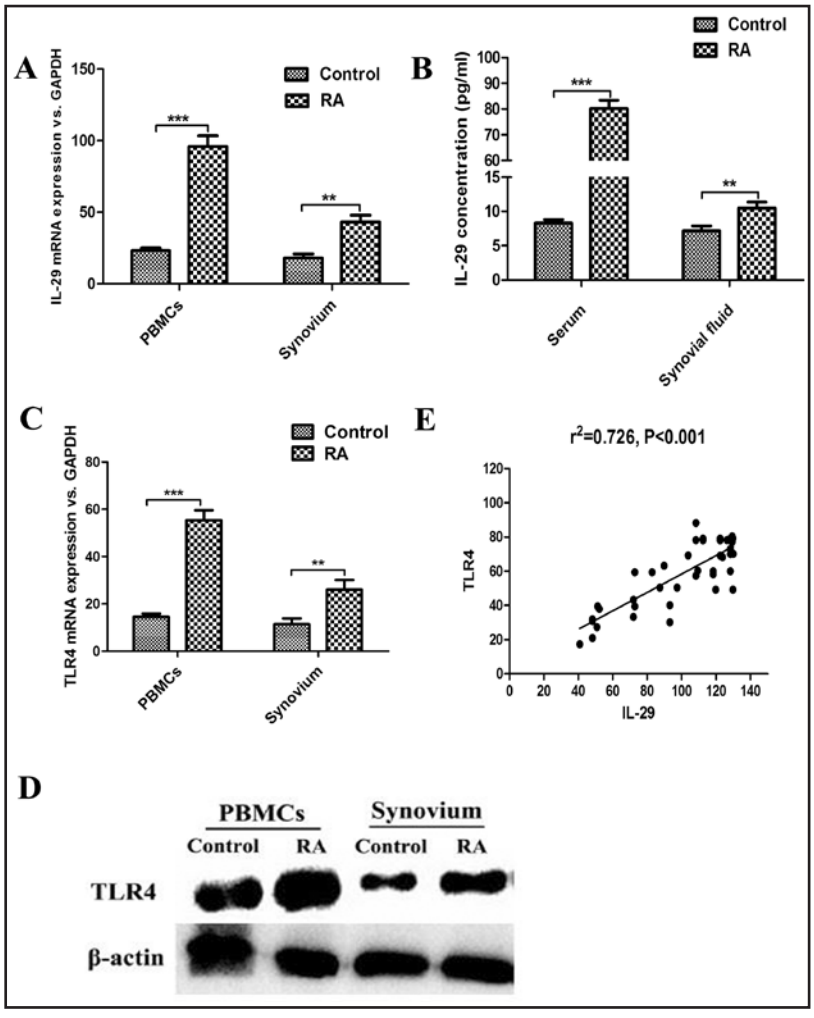

Fig. 1. Increased expression of IL-29 and TLR4 in RA. (A) Level of IL-29 mRNA in PBMCs (RA Patients/Controls: 77/70) and synovial tissues (RA Patients/Controls: 25/24) of patients with RA and controls. (B) Level of IL-29 protein in serum (RA Patients/Controls: 77/70) and synovial fluid (RA Patients/Controls: 25/24) of patients with RA and controls. (C) Level of TLR4 mRNA in PBMCs (RA Patients/Controls: 77/70) and synovial tissues (RA Patients/Controls: 25/24) of patients with RA and controls. (D) Level of TLR4 protein in PBMCs and synovial tissues of patients with RA and controls (RA Patients/Controls: 25/24). (E) Pearson's correlation analysis for the association between IL-29 and TLR4 in RA. (** $P < 0.01$, *** $P < 0.001$).

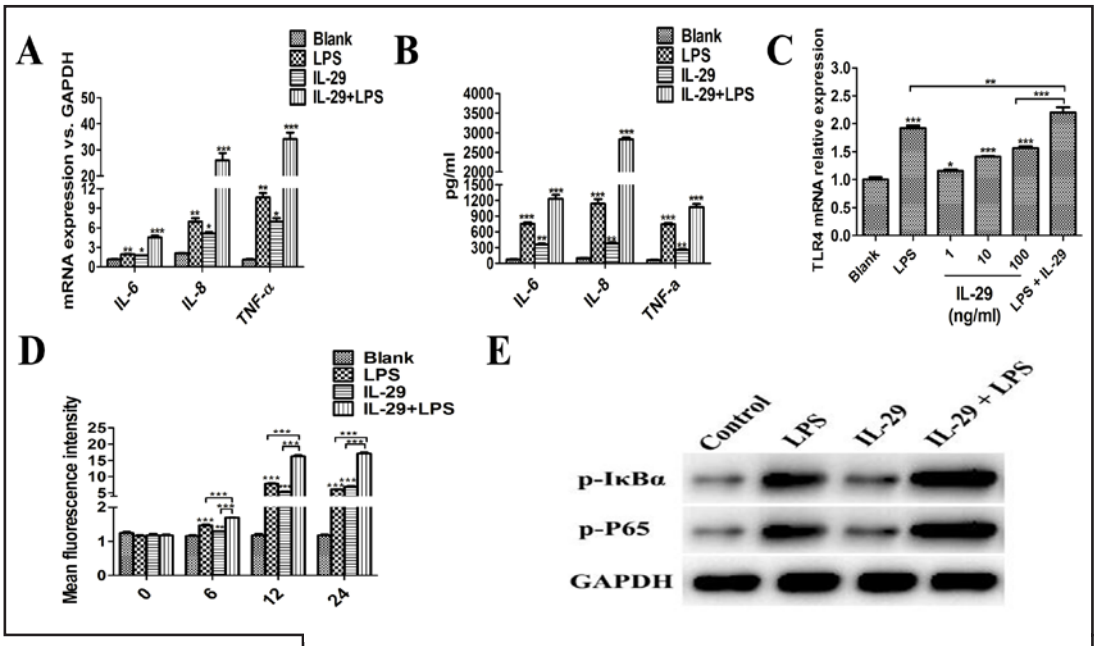

Fig. 2. IL-29 enhanced LPS-induced inflammatory cytokines production and NF-κB signaling activation in RAW264.7 cells. (A) Level of TNF-α, IL-6 and IL-8 mRNA in RAW264.7 cells (1×10^6 cells/well in 6-well plates, n=3). (B) Level of TNF-α, IL-6 and IL-8 protein in cultural supernatants of RAW264.7 cells (5×10^5 cells/well in 96-well plates, n=3). (C) Level of TLR4 mRNA in RAW264.7 cells (1×10^6 cells/well in 6-well plates, n=3). (D) The mean fluorescence intensity of TLR4 in RAW264.7 cells (5×10^5 cells/well in 24-well plates, n=3). (E) Activation of IκB-α and NF-κB/P65 in RAW264.7 cells (1×10^7 cells/well in cell culture plates, n=3). (F) Activation and nuclear translocation of NF-κB/P65 in RAW264.7 cells (X100, n=3). (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$).

Positive association between IL-29 and TLR4

We simultaneously determined the expression of IL-29 and TLR4 in PBMCs of 77 RA patients. The Pearson's correlation analysis showed that IL-29 was positively related to TLR4 in RA with an r^2 equal to 0.726 (Fig. 1E).

IL-29 elevated the expression of IL-6, IL-8, and TNF-α in RAW264.7 cells

The expression of IL-6, IL-8, and TNF-α was slightly elevated in IL-29-stimulated RAW264.7 macrophages (Fig. 2A and B). Besides, IL-29 could significantly enhance the expression of those inflammatory cytokines when combining with LPS (Fig. 2A and B).

IL-29 promoted LPS-induced TLR4 expression in RAW264.7 cells

IL-29 could independently up-regulate the expression of TLR4 in dose- and time-dependent manners (Fig. 2C and D). The level of TLR4 protein in RAW264.7 cells modestly increased when they were exposed to IL-29 (100ng/ml) for 6 hours, and considerably elevated at 12 hours (Fig. 2D). In addition, the expression of TLR4 was more significant in RAW264.7 cells stimulated by IL-29 plus LPS at different time points (Fig. 2C and D).

IL-29 enhanced LPS-induced NF-κB signaling activation in RAW264.7 cells

The levels of phosphorylated IκBα and NF-κB (P65) were less significant in IL-29-stimulated RAW264.7 cells, while IL-29 could remarkably enhance LPS-mediated IκBα and NF-κB/P65 phosphorylation activation in these cells (Fig. 2E). Similar to the results of western blot analysis, IL-29 could promote the LPS-induced nuclear translocation of phosphorylated NF-κB/P65 in RAW264.7 cells (Fig. 2F). Taken together, more significant activation of NF-κB signaling was observed in RAW264.7 cells stimulated by IL-29 and LPS.

Discussion

The present study showed that IL-29 and TLR4 significantly increased in PBMCs and synovium of patients with RA. Besides, IL-29 and TLR4 were positively associated with each other, suggesting IL-29 might confer modifying effect on TLR4-mediated inflammation and contribute to RA pathogenesis. When stimulating RAW264.7 cells by LPS and/or IL-29 *in vitro*, we found that IL-29 could promote LPS/TLR4 mediated inflammation by enhancing TLR4 expression and the production of inflammatory cytokines (IL-6, IL-8 and TNF-α). Additionally, the effect of IL-29 on LPS/TLR4 mediated inflammation in RA might be dependent on the activation of NF-κB signaling.

To the best of our knowledge, TLRs are involved in triggering innate immune responses and priming antigen-specific adaptive immunity [17-19]. TLR4 is a specific exogenous receptor for LPS, which plays a vital role in pathogen recognition and activation of the innate immune system [12, 20, 21]. It is most abundantly expressed on macrophages and mediates signaling transduction triggered by LPS through cooperating with MD-2 and CD14 [22]. TLR4-mediating immune response is not only necessary to invade pathogens, but also play a crucial role in maintaining homeostasis of commensal flora and in the response to dangerous signals [19, 23-25]. LPS can enhance TLR4 expression in macrophages and facilitate the activation of its downstream signaling pathways [26]. TLR4 can mediate synovial inflammation in RA [13, 24]. Macrophages and fibroblasts are the major cells in synovial tissue. TLR4 is expressed on the membrane of both cells. It has been demonstrated that IL-29 could up-regulate the expression of TLR4 in RA synovial fibroblasts and enhance inflammation in synovium [15]. Interestingly, TLR4 was demonstrated to be expressed at higher levels in moderately inflamed synovium compared with normal synovial tissues or synovium with severe inflammation in RA, which implicated that TLR4 signaling might lead to enhanced and sustained inflammation and contribute to RA pathogenesis [27]. In our study, the expression of TLR4 in PBMCs and synovial tissues of RA patients was significantly enhanced at levels of both mRNA and protein.

The increased expression of TLR4 in synovial tissues of RA patients might be attributed to elevated macrophages infiltrating in synovium. In addition, increased IL-29 level in synovium was another critical leading factor for the significant difference of TLR4 expression between RA patients and controls. However, due to relative small sample size, we failed to assess its association with the disease activity and stage of RA. Interestingly, positive

correlation between TLR4 and IL-29 was demonstrated in patients with RA. Furthermore, their interaction was further elucidated in one single cell type *in vitro*, which was accord with the findings *in vivo*. IL-29 might enhance LPS/TLR4-mediated inflammation in RAW264.7 cells depending on the activation of NF-κB signaling pathway.

Previous studies have implicated that type I IFNs could augment TLR4-mediated inflammatory response in RA [13]. Type III IFNs possess similar immune regulatory activities with type I IFNs. Xu et al. have found that IL-29 might enhances TLR-mediated IL-6 and IL-8 production in synovial fibroblasts [15], which suggested a critical role of IL-29 in RA development associated with infections. TLR4-mediated inflammation plays a critical role in the development and progression of a number of rheumatoid diseases, including RA [13, 15]. Accordingly, we hypothesize that IL-29 may modify the effect of TLR4-mediated inflammation in RA. Similar to the findings by Wang et al. [23], significantly elevated level of IL-29 was demonstrated in peripheral blood samples and synovial tissues from patients with RA. In addition, IL-29 could promote LPS-induced acute inflammatory response in RAW264.7 cells by promoting the secretion of inflammatory cytokines including IL-6, IL-8 as well as TNF-α. In addition to enhance pro-inflammatory cytokines production by IL-29 in macrophages, increased levels of TLR4 mRNA and protein were also observed in RAW264.7 cells. Those findings supported that infections induced abundant TLR4 in RA synovium, which was essential for the initiation of inflammatory response and the maintaining of chronic and persisting inflammation in local joints of patients with RA. Nonetheless, the primary synovial macrophages were not isolated and cultured *in vitro* for further investigation in this study. Furthermore, whether IL-29 interacts with TLR4 and contributes to RA development and progression *in vivo* warrants further elucidation.

In summary, our study firstly supports the hypothesis that IL-29 enhances inflammation in combination with TLR4 during RA pathogenesis. The positive association between IL-29 and TLR4 may be dependent on NF-κB signaling activation. Further studies are warranted to elucidate their potential interaction *in vivo*.

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Disclosure Statement

All authors have contributed adequately to this work and have no conflicts of interest to disclose.

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