Curcumin Alleviates Lipopolysaccharide (LPS)-Activated Neuroinflammation via Modulation of miR-199b-5p/IκB Kinase β (IKKβ)/Nuclear Factor Kappa B (NF-κB) Pathway in Microglia

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Background: Microglia reside in the spinal cord plays a key role in the onset, progression of post-spinal cord injury (SCI) neuroinflammation. Curcumin has been shown to exhibit diverse anti-inflammatory and anti-tumor activities. The aim of this study was to explore the effect of curcumin on the inflammatory response in lipopolysaccharide (LPS)-activated microglia and its mechanism.

Material/Methods: The expression levels of phosphorylated-p65 (p-p65), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IκB kinase β (IKKβ) were examined by western blot assay. MiR-199b-5p expression was detected by quantitative real-time polymerase chain reaction assay. The putative binding sites of miR-199b-5p in IKKβ 3’UTR were predicted by bioinformatics, and direct interaction between miR-199b-5p and IKKβ was verified by dual-luciferase reporter assay and RNA-immunoprecipitation assay.

Results: Curcumin significantly suppressed inflammatory response induced by LPS by inactivation of nuclear factor kappa B (NF-κB) in microglial cells, as reflected by the decreased levels of p-p65, as well as the pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS), TNF-α, and IL-1β. Moreover, curcumin increased the level of miR-199b-5p and decreased IKKβ expression in activated microglial cells. Knockdown of miR-199b-5p or overexpression of IKKβ reversed the inhibitory effect of curcumin on inflammatory response and NF-κB activation. MiR-199b-5p directly targeted IKKβ and suppressed its expression. Silencing of IKKβ abolished miR-199b-5p-stimulated inflammatory cytokines production and NF-κB activation.

Conclusions: Curcumin attenuated neuroinflammation induced by LPS through regulating miR-199b-5p/IKKβ/NF-κB axis in microglia.

MeSH Keywords: Curcumin • Inflammation Mediators • Microglia

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Background

Microglia, which are the resident macrophages of brain and spinal cord, serve as a prominent source of inflammatory mediators and play fundamental roles in central nervous system (CNS) disorders [1]. Overactivation of microglia under neuroinflammatory condition initiates an inflammatory cascade, leading to the excessive production of pro-inflammatory cytokines, including nitric oxide (NO), tumor necrosis factor (TNF-α), and interleukin (IL-1β) [2,3]. Inhibition of pro-inflammatory release in activated microglia may alleviate the severity of neuroinflammatory diseases.

Spinal cord injury (SCI) is one of the common CNS injuries caused by great physical and psychological trauma due to impaired sensory motor function [4]. SCI is usually caused by motor vehicle accidents, sports, natural disasters, and violence. The pathophysiologic processes of SCI involve primary and secondary mechanisms of injury [5]. Primary injury mainly refers to the destruction of spinal cord structure and function, which is considered to be irreversible. Following a few minutes to several weeks after a primary injury, a secondary injury can lead to an inflammatory response by activation of resident microglia and macrophages [6]. Therefore, attention should be focused on the secondary injury caused by activated microglia.

Curcumin, chemically known as 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a bioactive polyphenol that exerts a variety of pharmacologic effects, such as anticarcinogenic, anti-inflammatory, anti-oxidant, and anti-neuroinflammatory properties [7,8]. A previous study by Lin et al. showed that curcumin hampered the apoptosis of primary cultured astrocytes derived from SCI rats and inhibited neuron loss by downregulating glial fibrillary acidic protein (GFAP) expression [9]. Cemil [10] and Sahin et al. [11] stated that curcumin provided neuroprotective effects by exhibited an anti-oxidant action after SCI. Moreover, activation of Nrf2 by curcumin induced the blockade of nuclear factor-kappa B (NF-κB) pathway, leading to the decrease of inflammatory cytokines secretion in the injured spinal cord [12]. However, the molecular basis of curcumin is poor defined in LPS-induced inflammatory response in microglia.

MicroRNAs (miRNAs) are a type of non-protein coding RNA with larger than 18–22 nucleotides (nt) that negatively regulated gene expression at post-transcriptional level through binding with the 3’-untranslated region (3’-UTR) of target mRNA. Multiple researches have demonstrated that miRNAs can be involved in the initiation and development of various pathological processes, such as inflammation, cell survival, and tumor formation. Recently, abnormal versions of miRNAs have been shown to be associated with the pathological response after SCI [13]. For instance, high miR-126 expression favors angiogenesis and attenuates inflammation after SCI in rats by negative regulation of its targets SPRED1, PIK3R2, and VCAM1 [14]. Lentiviral delivery of miR-133b contributes to the recovery of motor function in mice with SCI [15]. Additionally, miR-34a is reported to be immediately downregulated after SCI, and dysregulation of this miRNA is associated with glial cells apoptosis and inflammatory response via altering the expression of Notch1, Csf1r, and PDGFRα [16]. As for miR-199b, it has been reported to be expressed at low level in acute SCI [17]. However, the function and underlying mechanisms of miR-199b-5p in the progression of SCI remain largely unknown.

IκB kinase β (IK-β, encoded by Ikbkb) is a critical coordinator of inflammatory responses by activation of NF-κB [18]. NF-κB is thought to play a crucial role in the regulation of cell survival genes and modulate the expression of pro-inflammatory enzymes and cytokines, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 [19]. In addition, previous study showed that IκB kinase β (IKKβ)/NF-κB signaling pathway was activated as well as the levels of pro-inflammatory cytokines TNF-α and IL-1β were increased in the spinal cord of the rat contusion epicenter [20]. In spite of these findings, the relationships among curcumin, miR-199b-5p, and IKKβ/NF-κB signaling pathway remain poorly understood.

In the present study, our research aimed to identify miRNAs that could be regulated by curcumin in activated microglia; and miR-199b-5p was identified to be upregulated in curcumin-treated microglia. Furthermore, the relationship between miR-199b-5p and IKKβ, an activator of NF-κB pathway, was further explored, and their functions on the curcumin-mediated anti-inflammatory effect in activated microglia was also investigated.

Material and Methods

Cell culture and treatment

BV2 microglial cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (HyClone, Logan, UT, USA) at 37°C with 5% CO2. To activate the microglia, cells were treated with 100 ng/mL lipopolysaccharide (LPS, Solarbio, Beijing, China) for 24 hours prior to this study.

Cell transfection

MiR-199b-5p inhibitor (anti-miR-199b-5p), miR-199b-5p mimics (miR-199b-5p), siRNA targeting silIKKβ, and relative controls
were obtained from Thermo Fisher Scientific (Waltham, MA, USA). IKKβ expressing plasmid (IKKβ) was synthesized by Thermo Fisher Scientific through inserting the full-length sequences of IKKβ into pcDNA3.1 vector. Activated microglial cells were treated with designated concentrations of curcumin (Klamar, Shanghai, China) and/or transfected with anti-miR-199b-5p, IKKβ, miR-199b-5p, siIKKβ, or relative control using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Forty-eight hours thereafter, cells were harvested for subsequent research.

Western blot

For western blot analysis, total protein was extracted from activated microglial cells using RIPA lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and protein concentration of each sample was determined by bicinchoninic acid (BCA) assay. After suffering from 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were subsequently blocked with 5% skimmed milk and incubated with primary antibodies (Abcam, Cambridge, MA, USA) against p-p65, total p65, iNOS, TNF-α, IL-1β, IKKβ, and GAPDH (as an internal control) overnight at 4°C. After washed with TBST buffer, the membranes were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam) for 1.5 hours at 37°C. Protein blots were exposed using enhanced chemiluminescent reagent (ECL, Millipore), followed by the determination of protein expression using Image Lab software (Bio-Rad, Hercules, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

For quantitative real-time polymerase chain reaction (qRT-PCR) analysis, total RNA was extracted from microglial cells using mirVana™ miRNA isolation Kit (Invitrogen) according to the manufacturer’s instructions, followed by the examination of RNA purity by a spectrophotometer. 10 ng of RNA from each sample was reverse-transcribed into complementary DNA (cDNA) using TaqMan® miRNA reverse transcription kit (Thermo Fisher Scientific), and qRT-PCR was conducted using SYBR Green PCR Master Mix (Thermo Fisher Scientific) and specific primers for miR-199b-5p (Ribobio, Guangzhou, China). The relative expression of miR-199b-5p was calculated using 2^ΔΔCt method with U6 snRNA as a housekeeping gene. Primer sequences were listed as below: miR-199b-5p: 5'-CAGCCCGAGCCGCGTCTTCGTT-3' (forward) and 5'-AGGGAGGCGCGAGCCGAGG-3' (reverse); U6: 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-AACGCTCCTTCCCTCACCA-3' (reverse).

Dual-luciferase reporter

Identification of the putative miR-199b-5p target was performed using TargetScan (http://www.targetscan.org/). Partial 3'UTR fragments of IKKβ containing the predicted miR-199b-5p binding sites was cloned into psiCHECK™-2 vector (Promega, Madison, WI, USA), and generated wild-type IKKβ-3'UTR plasmid (IKKβ-wt). Mutated IKKβ-3'UTR plasmid (IKKβ-mut) was conducted by using QuikChange II XL Site-directed Mutagenesis Kit (Agilent-Stmiceagene, Houston, TX, USA). For luciferase activity assay, BV2 microglial cells were co-transfected with IKKβ-wt or IKKβ-mut and NC or miR-199b-5p. Forty-eight hours thereafter, the luciferase activity was detected by dual luciferase reporter assay kit (Promega).

RNA-immunoprecipitation (RIP)

Ago2 protein is the core catalytic element of RNA-induced silencing complex (RISC) assembly by binding to miRNA. To determine the possible interaction between miR-199b-5p and IKKβ, RIP assay was carried out using RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) and anti-Ago2 antibody. In short, BV2 microglial cells were transfected with NC or miR-199b-5p for 48 hours, then cell extracts were incubated with the protein A/G magnetic beads conjugated with anti-Ago2 or anti-IgG antibody (Abcam). The enrichment level of IKKβ in immunoprecipitant complex was examined by RT-qPCR assay.

Statistical analysis

The data were analyzed using GraphPad Prism 7 (GraphPad Inc., La Jolla, CA, USA). Student’s t-test was used for comparisons between 2 groups. One-way analysis of variance (ANOVA) was used for multiple groups comparisons. P<0.05 was regarded as statistically significant.

Results

Curcumin attenuated the inflammatory response caused by LPS in microglia via inactivation of NF-κB

As already known, LPS-activated microglia can be used as a cell model for spinal cord injury (SCI) study [20]. To investigate the effect of curcumin on the inflammatory response in activated microglia, we employed LPS and different concentrations of curcumin (0 µM, 2 µM, 4 µM, and 8 µM) to treat BV2 microglial cells. The results of western blot analysis showed that LPS exposure significantly increased the enrichment of pro-inflammatory enzyme and cytokines (iNOS, TNF-α and IL-1β) (Figure 1A, 1B). The expression of pro-inflammatory enzyme and cytokines was significantly decreased by curcumin treatment (Figure 1A, 1B). The inflammatory response was significantly attenuated by curcumin in a dose-dependent manner.
the activation of NF-κB pathway [21]. However, introduction of curcumin dose dependently suppressed the expression of iNOS, TNF-α, IL-1β, and p-p65, which was initially stimulated by LPS (Figures 1A–1E).

Curcumin overturned the effects of LPS on miR-199b-5p and IKKβ expression in microglia

A previous study has shown that miR-199b could be involved in the progression of SCI by regulation of IKKβ/NF-κB in LPS-activated microglia [17]. Therefore, we attempted to investigate whether curcumin had the ability to mediate the expression of miR-199b and IKKβ in activated microglia. It could be seen that miR-199b-5p was downregulated and IKKβ was upregulated in microglia after LPS stimulation (Figure 2A, 2B), but curcumin reversed the effects of LPS on miR-199b-5p and IKKβ expression in a dose dependent manner.

**MiR-199b-5p knockdown abolished curcumin-inhibited inflammation and NF-κB activation in LPS-treated microglia**

In the present study, we performed a rescue experiment to explore whether miR-199b-5p was required for curcumin-mediated inhibitory effect on inflammatory response. Activated BV2 cells were transfected with anti-NC or anti-miR-199b-5p before treatment of 8 µM curcumin. The results of qRT-PCR showed that transfection of anti-miR-199b-5p effectively attenuated miR-199b-5p expression, which was initially stimulated by curcumin (Figure 3A), indicating the high transfection efficiency of anti-miR-199b-5p. Functionally, the inhibitory effects of curcumin on p-p65, iNOS, TNF-α, and IL-1β expression were abrogated following miR-199b-5p knockdown (Figure 3B–3F). These data suggested that curcumin attenuated neuroinflammation and NF-κB activation induced by LPS in BV2 cells via upregulating miR-199b-5p.
Figure 2. Effect of curcumin on miR-199b-5p and IKKβ expression. (A, B) LPS-induced BV2 cells were treated with different concentrations of curcumin, with untreated group as a blank control. qRT-PCR assay was performed to detect the expression of miR-199b-5p (A). Western blot assay was conducted to examine the level of IKKβ (B). * P<0.05. LPS – lipopolysaccharide; qRT-PCR – quantitative real-time polymerase chain reaction.

Figure 3. Effect of miR-199b-5p on curcumin-mediated pro-inflammatory cytokine production. (A–F) LPS-activated BV2 cells pretreated with 8 µM curcumin were transfected with anti-NC or anti-miR-199b-5p, with untreated group as a control. The relative expression of miR-199b-5p was measured by qRT-PCR assay (A). The protein levels of p-p65, total-p65, iNOS, TNF-α, and IL-1β was examined by western blot assay (B–F). * P<0.05. LPS – lipopolysaccharide; qRT-PCR – quantitative real-time polymerase chain reaction; p-p65 – phosphorylated-p65; iNOS – inducible nitric oxide synthase; COX-2 – cyclooxygenase-2; TNF-α – tumor necrosis factor-α; (IL)-1β – interleukin-1β.
Overexpression of IKKβ reversed the inhibitory effect of curcumin on inflammatory response by activation of NF-κB in LPS-induced microglia

To further investigate the role of IKKβ in curcumin-mediated inflammatory response in activated microglia, BV2 cells were transfected with vector or IKKβ overexpressing plasmid before treatment of 8 µM curcumin. Results revealed that transfection of IKKβ overexpressing plasmid effectively enhanced IKKβ expression, which was initially inhibited by curcumin (Figure 4A). As a major modulator of NF-κB pathway, IκB kinase β (IKKβ) is required for NF-κB activation [22]. Here, we observed that forced expression of IKKβ reversed curcumin-mediated p-p65 expression, as well as pro-inflammatory enzyme and cytokines (iNOS, TNF-α, and IL-1β) levels (Figure 4B–4F). Altogether, these data indicated that IKKβ reversed curcumin-mediated inflammatory response by activating NF-κB in LPS-induced BV2 cells.

Figure 4. Effect of IKKβ on curcumin-mediated proinflammatory cytokine production. (A–F) LPS-activated BV2 cells pretreated with 8 µM curcumin were transfected with vector or IKKβ, with untreated group as a control. The protein levels of IKKβ, p-p65, total-p65, iNOS, TNF-α, and IL-1β was examined by western blot assay. * P<0.05. LPS – lipopolysaccharide; p-p65 – phosphorylated-p65; iNOS – inducible nitric oxide synthase; TNF-α – tumor necrosis factor-α; IL-1β – interleukin-1β.
IKKβ was directly targeted by miR-199b-5p

To explore the molecular mechanism by which miR-199b-5p and IKKβ exerted their regulatory effect on the inflammatory response of activated microglia, TargetScan software was applied to find out the potential targets of miR-199b-5p. According to the prediction, IKKβ was identified as a candidate target of miR-199b-5p (Figure 5A). Afterwards, dual-luciferase reporter assay and RIP assay were performed to confirm the direct binding between miR-199-5p and IKKβ. As a result, forced expression of miR-199-5p significantly impaired the luciferase activity of IKKβ-wt reporter in BV2 cells. However, no obvious change was observed on the luciferase activity of IKKβ-mut reporter (Figure 5B). The results of RIP assay revealed that IKKβ could be highly enriched by anti-Ago2 antibody, but anti-IgG failed to show any efficacy of enrichment (Figure 5C). Moreover, overexpression of miR-199b-5p increased the enrichment level of IKKβ in Ago2 immunoprecipitation complex in BV2 cells compared with miR-NC group (Figure 5C). Next, we further analyzed the protein level of IKKβ by western blot in activated BV2 cells transfected with NC, miR-199b-5p, anti-NC, or anti-miR-199b-5p. As shown in Figure 5D, miR-199b-5p addition dramatically reduced the protein level of IKKβ, but knockdown of miR-199b-5p increased IKKβ level in BV2 cells. These findings indicated that miR-199b-5p directly targeted IKKβ and negatively regulated its expression.

Knockdown of miR-199b-5p induced curcumin-alleviated inflammatory response through IKKβ/NF-κB in activated microglia

Our previous studies have confirmed that miR-199b-5p negatively regulated IKKβ expression in curcumin-treated BV2 cells. Thus, we wondered whether miR-199b-5p participated in the modulation of curcumin-inhibited inflammatory response by IKKβ/NF-κB pathway. As shown in Figure 6A, transfection of silIKKβ weakened anti-miR-199b-5p-induced IKKβ expression. Moreover, inhibition of miR-199b-5p by anti-miR-199b-5p...
**Figure 6.** Effect of IKKβ knockdown on anti-miR-199b-5p-mediated pro-inflammatory cytokines production. (A–F) LPS-activated BV2 cells pretreated with 8 µM curcumin were transfected with anti-NC, anti-miR-199b-5p, anti-miR-199b-5p+scramble, or anti-miR-199b-5p+siIKKβ. The protein levels of IKKβ, p-p65, total-p65, iNOS, TNF-α, and IL-1β was examined by western blot assay. * P<0.05. LPS – lipopolysaccharide; p-p65 – phosphorylated-p65; iNOS – inducible nitric oxide synthase; TNF-α – tumor necrosis factor-α; IL-1β – interleukin-1β.
enhanced the abundance levels of p-p65, iNOS, TNF-α, and IL-1β initially suppressed by curcumin in activated BV2 cells. However, knockdown of IKKβ abolished the promotive effect anti-miR-199b-5p on p-p65, TNF-α, and IL-1β expression (Figures 6B–6F). Altogether, our data suggested that curcumin attenuated the neuroinflammation partly through modulation of miR-199b-5p/IKKβ/NF-κB pathway in activated microglia.

Discussion

SCI-induced damages are composed of primary and secondary mechanical injuries, in which microglial cells are reported to be a major regulator in secondary injury. In the early stage of SCI, microglial cells function as a “sweeper” to decompose and devour diseased neurons, which is beneficial to maintain the homeostasis of cell microenvironment. However, continuing activation of microglial cells induces the release of a plenty of cytokines involving in the oxidative stress and inflammatory response, which ultimately leads to the poor recovery of neurological function after SCI [23].

Curcumin has been identified as a potential anti-inflammatory agent [24]. For instance, Aggarwal et al. pointed out that curcumin exerted its anti-inflammatory activity by inhibiting the production of a variety of different molecules associated with inflammation [25]. Jobin et al. showed that curcumin promoted the neuroprotective effect affected with ischemia by suppressing an inflammatory reaction modulated by the TLR4/p38/MAPK pathway [26]. Also, curcumin resulted in the blockade of lipo-polysaccharide (LPS)-activated IκBα/ NF-kB pathway in BV2 microglia, exerting anti-inflammatory and neuroprotective effects [27]. Similarly, curcumin efficiently attenuated the production of pro-inflammatory mediators nitric oxide (NO) and prostaglandin E2 (PGE2), as well as the release of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and TNF-α in LPS-induced microglia [28]. In the present study, we aimed to investigate the molecular basis of curcumin in the inflammatory response of activated microglia. In line with the previous report [16], our study initially confirmed that curcumin attenuated the inflammatory response caused by LPS in microglia via inactivation of NF-κB, as evidenced by the decreased levels of iNOS, TNF-α, IL-1β, and p-p65. These findings showed that anti-inflammatory properties of curcumin might be useful for treating LPS-mediated SCI and inflammatory disease.

As mentioned in previous reports, curcumin has been shown to exert its anti-inflammatory function by regulating miRNA expression. For example, curcumin impedes the release of LPS-induced cytokines TNF-α and IL-6 may partly via downregulation of miR-155 in macrophages and mice [29]. Moreover, curcumin can participate in the regulation of neurological diseases via targeting miRNAs by inactivation of inflammatory signaling pathways [30]. As mentioned by Zaky et al., co-treatment of curcumin and valproic alleviated LPS-stimulated neuroinflammation in rat brain cortex by effectively suppressing the expression of let-7, ultimately augmenting neuroprotection and promoting brain recovery [31]. Pogue et al. stated that curcumin negatively regulated miR-125b and miR-146a through blockade of NF-κB pathway in metal-sulfate-stressed human astroglial cells and IL-1ß-stressed human neuronal-glial cells [32,33].

MiR-199b-5p has been well documented as a tumor suppressor in various human cancers such as live cancer [34], breast cancer [35], and renal cell carcinoma [36]. A recent report also demonstrated that reduced expression of miR-199b facilitated the SCI through regulation of IKKβ/NF-κB signaling pathway in activating microglial cells [17]. Also, by targeting miR-199b/IKKβ:NF-κB pathway, long noncoding RNA MALAT1 enhanced the inflammatory response of microglia following SCI [20]. IKKβ is a key catalytic subunit of IKK complex that composed of a kinase domain, a leucine zipper, and a helix-loop-helix. Elevated IKKβ phosphorylates κB proteins and triggers the activation of NF-κB, which is known as a major inflammatory initiator [37]. Mounting evidences have demonstrated that IKKβ/NF-κB signaling pathway participate in the regulation of pro-inflammatory effect of activated microglia after SCI by inducing the release of inflammatory cytokines [17], suggesting that inhibition of IKKβ/NF-κB pathway may be a potential therapeutic target for secondary injury after SCI. In view of the involvement of miR-199b-5p and IKKβ/NF-κB in the inflammatory response of microglia, we suppose that curcumin may attenuate the inflammation induced by LPS in microglia partly through regulating miR-199b-5p/IKKβ/NF-κB axis.

Based on the previous study, we analyzed the possible mechanism of how curcumin was involved in immunoregulation inactivated microglia. We found that treatment with curcumin potently suppressed miR-199b-5p expression and enhanced IKKβ expression in LPS-stimulated microglia in a dose-dependent manner. Exogenous knockdown of miR-199b or overexpression of IKKβ reversed the inhibitory effects of curcumin on iNOS, TNF-α, IL-1β, and p-p65 expression. Furthermore, IKKβ was identified as a target of miR-199b-5p. Inhibition of miR-199b-5p contributed to the inflammatory response in curcumin-treated microglia, which was further abrogated by IKKβ depletion. Collectively, these findings would provide a possible new strategy for the treatment of SCI and other inflammatory disorders in the future.

Conclusions

Therefore, we concluded that curcumin alleviated neuroinflammation induced by LPS in microglia via modulation of miR-199b-5p/IKKβ/NF-κB pathway. And, this finding may provide a new insight into the treatment of SCI patients.
Conflicts of interest

None.

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