Clinical, Cosmetic and Investigational Dermatology

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ORIGINAL RESEARCH

Effect of Chloroquine on Type 2 Inflammatory Response in MC903-Induced Atopic Dermatitis Mice

Mingjing Wei*, Huixue Yang*, Zhengchao Shao, Haoyue Wan, Yiheng Wang, Wenqi Chen

Department of Dermatology, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, 210000, People's Republic of China

*These authors contributed equally to this work

Correspondence: Wenqi Chen, Department of dermatology, Nanjing First Hospital, Nanjing Medical University, Changle Road 68, Nanjing, Jiangsu, 210000, People's Republic of China, Tel +8615261008432, Email WenqiChen0809@163.com

Introduction: Atopic dermatitis (AD) is a chronic, non-infectious inflammatory dermatosis. Chloroquine (CQ) has long been proven to possess anti-inflammatory properties.

Objective: This paper aims to investigate the impact of CQ on type 2 inflammatory response in MC903-induced AD mice.

Methods: An AD mouse model was established via MC903 induction. After CQ treatment, AD mice were intraperitoneally injected with polyinosinic: polycyclic acid [poly (I:C)] or Nigericin. Dermatitis severity was scored, and the thickness of the left ear was measured. The pathological changes in mouse skin tissues were observed by H&E staining. The number of mast cells was counted via TB staining. The content of peripheral blood T-helper 2 (Th2) cells and levels of immunoglobulin E (IgE), thymic stromal-derived lymphopoietin (TSLP), interleukin (IL)-4, IL-13, interferon (IFN)-γ, IL-1β, and IL-18 were assessed by flow cytometry and ELISA. The levels of toll-like receptor 3 (TLR3), NLRP3, ASC, and cleaved caspase-1 proteins in skin tissues were determined by Western blot.

Results: CQ treatment abated dermatitis severity and left ear thickness in AD mice, alleviated skin damage, reduced mast cell number, diminished IgE, TSLP, IL-4, and IL-13 levels, and peripheral blood Th2 cell content, with no significant changes in IFN-γ level. CQ alleviated type 2 inflammatory response in AD mice by inhibiting the activation of TLR3. CQ suppressed NLRP3 inflammasome activation. Activating TLR3/NLRP3 annulled CQ-mediated alleviation on type 2 inflammatory response in AD mice.

Conclusion: CQ alleviated type 2 inflammatory response in AD mice by inhibiting TLR3 activation and NLRP3 inflammasome activation.

Keywords: atopic dermatitis, chloroquine, toll-like receptor 3, NLRP3 inflammasome, type 2 inflammation, MC903

Introduction

Atopic dermatitis (AD) is a common chronic, non-infectious skin disease that arises from the intricate interplay of immunological, genetic, and environmental elements.[1] As a typical type 2 inflammatory disease, AD is characterized by elevated levels of serum immunoglobulin E (IgE), intense itch, recurrent eczematous lesions, epidermal hyperplasia, the generation of inflammatory mediators, and the infiltration of T-cells and dendritic cells (DCs).[2–4] For decades, the incidence rate of AD has been rising steadily,[5] influencing up to 10% of grown-ups,[6] which seriously interferes with the daily activities of the patients and greatly reduces their quality of life.[7] AD may present with distinct clinical phenotypes, leading to challenges in diagnosis.[6] Many countries have a dearth of approved medications for atopic dermatitis. Therefore, it is of great significance to deeply understand the mechanism of AD and search for effective methods for mitigating AD.

The excessive type 2 inflammatory response in AD is mainly driven by type 2 helper T cells (Th2) and group 2 innate lymphoid cells (ILC2s).[8,9] MC903 (calcipotriol) is an active vitamin D analogue,[10] which can induce AD-like type 2 skin inflammation in mice by up-regulating thymic stromal-derived lymphopoietin (TSLP) and is widely used to establish AD animal models.[11] Research has shown that TSLP serves as an early promoter of AD, originating from several cellular sources like mast cells and keratinocytes.[11,12] It has the capability to generate type 2 cytokines, including interleukin (IL)-4 and IL-13, which can activate mast cells, enhance IgE expression, and ultimately induce skin inflammation

Received: 14 September 2023
Accepted: 31 March 2024
Published: 14 May 2024

resembling AD.[13,14] Toll-like receptors (TLRs) are a group of receptors crucial for innate immune signaling and inflammatory response triggering, closely linked to the onset and progression of AD.[15] An existing study has found that using the TLR3 agonist polyinosinic:polycytidylic acid [poly (I:C)] to induce TLR3 activation can exacerbate AD-like symptoms in mice induced by MC903.[16] Notably, activation of TLR3, a member of the TLR family, can induce the production and release of TSLP, further exacerbating Th2 inflammatory response.[17] However, the study of TLR3 in AD is still controversial. According to reports, inflammasomes are multi-protein complexes assembled by intracellular NOD-like receptors (NLRs),[18] which participate in type 2 inflammatory responses in AD,[19,20] especially the nucleotide-binding oligomerization domain-like receptor 3 (NLRP3) inflammasomes. Generally, the NLRP3 inflammasomes are activated through the TLR/NF-κB signaling, further up-regulating caspase-1 and pro-inflammatory factors IL-1β and IL-18.[21] What's more, multiple studies have shown that TLR3 can mediate the activation of the NLPR3 inflammasomes.[22–25] In AD-like skin lesions in 2.4-dinitrochlorobenzene-treated fat-1 mice, the NLRP3 inflammasomes are activated, with ASC, NLRP3, IL-1β, and mature caspase-1 mediating pyroptosis injury.[26] Accordingly, repression of the NLRP3 inflammasomes may be an attractive treatment method for AD.

Chloroquine (CQ) is not only used to treat malaria but is also the most commonly used drug to alleviate acute and chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, and Sjogren's syndrome.[27–29] CQ can hinder the activation and differentiation of several subtypes of helper T cells and B cells, as well as the production of diverse inflammatory mediators by DCs, monocytes/macrophages, and polymorphonuclear neutrophils (PMNs).[30] CQ can reduce TLR ligand-driven HIV-1 infection-related immune activation via TLR antagonist property.[31] Accumulating evidence has revealed that CQ can suppress TLR3 expression.[32,33] What's more, one of the potential anti-inflammatory mechanisms of CQ is to weaken the activity of the NLRP3 inflammasomes.[34] Also, the latest research has shown that CQ suppresses NLRP3 inflammasome activation and abates renal fibrosis in mice with hyperuricemic nephropathy.[35] However, there are currently few reports on whether CQ relieves AD by suppressing TLR3 activation and NLRP3 inflammasome activation. The purpose of this study is to investigate the effect of CQ on the MC903-induced type 2 inflammatory response in AD mice, providing some theoretical basis for clarifying the pathogenesis of AD and new ideas for its treatment.

Materials and Methods

Ethics Statement

The experiments were authorized by the Institutional Animal Ethics Committee of Nanjing Hospital Affiliated to Nanjing Medical University (project license number DWSY-23022326). All animal manipulations and experimental techniques were conducted in accordance with the standards set forth by the committee. All the laboratory procedures were used to minimize the pain of mice. All experiments in this study followed the "3R" principles of animal welfare guidelines, namely replacement, reduction, and refinement.

Experimental Animals

Male BALB/c mice of the SPF (pathogen-free) grade (N = 30, 6 weeks old, weighing around 24 g) bought from VT Lihua (Beijing, China) were raised under a clean condition. The 30 mice were evenly housed in 6 cages with common feed and unpolluted water, constant temperature and humidity in a 12-h light and dark cycle for a 14-day adaptive feeding.

Establishment of MC903-Induced AD Model

An AD mouse model was established by topically utilizing a low-calcemic analogue calcipotriol of vitamin D3, MC903 (Sigma-Aldrich, St. Louis, MO, USA), as per a reported protocol.[36] In short, MC903 (2 nmol, 20 μL) dissolved in 95% ethanol was applied topically to the dorsal side of the left ear. On the 7th day, an MC903-induced AD model was successfully established. Subsequently, MC903 was still administered to mice in the AD group, the AD + CQ group, the AD + CQ + Nigericin group, and the AD + CQ + poly (I:C) group.[37] The Control group was given an equal amount of 95% ethanol.

Animal Grouping and Treatment

Following the random number table method, mice were divided into the following 5 groups (6 mice/group): the Control group (mice were given an equal amount of 95% ethanol after shaving), the AD group (mice were induced AD with MC903),[37] the AD + CQ group (AD mice were intraperitoneally injected with 5 μg/g CQ once a day for 5 consecutive days[38]), the AD + CQ + poly (I:C) group (after CQ treatment, the AD mice were intraperitoneally injected with 5 μg/g bodyweight poly (I:C), once a day for 2 consecutive days),[16] and the AD + CQ + Nigericin group (after CQ treatment, AD mice were injected intraperitoneally with 5 mg/kg Nigericin once a day for 2 consecutive days).[39] CQ medication interventions began on the 8th day, with the AD and Control groups injected with equal amounts of normal saline. Among these, MC903 was purchased from LEO Pharma A/S (Ballerup, Denmark). CQ (HY-17589), poly (I:C) (HY-107202, TLR3 activator), and Nigericin (HY-127019, NLRP3 activator) were all purchased from MedChemExpress (Shanghai, China).

On the 15th day, mice were anesthetized by intraperitoneal injection of 0.3% pentobarbital sodium (50 mg/kg), followed by 0.1 mL of peripheral blood collection from the tail vein, partially for flow cytometry detection, and partially separated to obtain serum for ELISA detection. After dermatitis scoring and left ear thickness measuring, the mice were euthanized by intraperitoneal injection of 3% pentobarbital sodium (100 mg/kg), with the left ear skin tissues collected, partially prepared into tissue sections for histological staining, and partially prepared into tissue homogenate for Western blot detection. The diagram of the treatments is shown in Supplementary Figure 1.

Dermatitis Score and Ear Thickness

The dermatitis score was evaluated.[40] The scores ranging from 0 (none), 1 (mild), 2 (moderate), or 3 (severe) were tested for the 5 symptoms (lichenification, erythema/edema, excoriation, erosion, and dryness), with the total score quantified as the sum for the 5 symptoms of all the individual scores (maximum score of 15 points). On days 7, 10, 12, and 15, left ear thickness was measured using a micrometer (Mitutoyo, Kanagawa, Japan).

Histopathological Studies

The mouse left ear was fixed with 4% paraformaldehyde. After paraffin embedding, 5 μm skin sections were subjected to hematoxylin and eosin (H&E) staining for observation of pathological changes in skin tissues. The sections were also stained with toluidine blue (TB) for mast cell counting. Mast cells were counted using a light microscope (× 200 magnification) from five randomly chosen fields of view.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of serum IgE (PI476, Beyotime, Shanghai, China), TSLP (ab155461, Abcam, Cambridge, UK), Th2 cytokines [IL-4 (PI612, Beyotime), IL-13 (PI539, Beyotime)], IFN-γ (PI507, Beyotime), and inflammatory factors [IL-1β (PI301, Beyotime), IL-18 (PI553, Beyotime)] in mice were determined by ELISA. All operations were strictly carried out in accordance with the instructions. The data were collected using a microplate reader (Bio-Rad 680, Bio-Rad, Hercules, CA, USA).

Flow Cytometry

The content of Th2 (CD3+CD4+CD193+) in mouse peripheral blood was measured using flow cytometry (Cytoflex, Beckman Coulter, CA, USA). Firstly, peripheral blood was collected in eppendorf tubes, added with 10 × red blood cell Lysis Buffer (420,301, Biolegend, San Diego, CA, USA), and lysed at room temperature for 10–12 min to remove red blood cells. After sample centrifugation, the supernatant was removed, and the samples were resuspended with phosphate-buffered saline (PBS). The 1 mL PBS-resuspended cells were added with 2 μL Cell Activation Cocktail (423,303, Biolegend), mixed well for stimulation at 37°C for 4 h, followed by centrifugation at 350 g for 5 min, resuspension with 100 μL PBS, and a 30-min incubation with 5 μL/tube Purified anti-mouse CD3 Antibody (100,201, Biolegend) and 5 μL/tube Purified anti-mouse CD4 Antibody (100,401, Biolegend) in the dark at 4°C. After that, cells were subjected to a 5-min centrifugation at 350 g, with the supernatant discarded, rinses with PBS, another 5-min

centrifugation at 350 g, with the supernatant discarded, and a 20-min incubation with 0.5 mL/tube Fixation Buffer (420,801, Biolegend) at room temperature in the dark. Subsequently, cells were centrifuged at 350 g for 5 min, with the supernatant removed, resuspended with 1 × Permeabilization Wash Buffer (1 mL/tube; 421,002, Biolegend), and centrifuged at 400 g for 5 min, with the supernatant removed. After two repetitions, cells were resuspended with 100 μL Permeabilization Wash Buffer working solution, labelled and incubated with 5 μL/tube Purified anti-mouse CD193 (CCR3) Antibody (144,502, Biolegend) in the dark for 30 min, followed by a centrifugation at 400 g for 5 min, with the supernatant discarded, 2 rinses with 1 mL PBS, with the supernatant discarded, and a resuspension with 500 μL PBS. Finally, the content of Th2 (CD3+CD4+CD193+) cells was analyzed on a flow cytometer.

Western Blot

The homogenate of mouse ear skin tissues was lysed using protein lysis solution (R0278, Sigma-Aldrich), with the protein content quantitatively analyzed using a bicinchoninic acid protein assay kit (Beyotime). The protein was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto the polyvinylidene fluoride membranes, which were then blocked for 1 h in Tris-buffered saline with Tween-20 (TBST; 20 mM Tris, 137 mM NaCl, 0.1% Tween-20) containing 5% skim milk. Then, the membranes were added with primary antibodies anti-TLR3 (1:1000, ab62566, Abcam), anti-NLRP3 (1:1000, ab263899, Abcam), ASC (1:1000, ab307560, Abcam), cleaved caspase-1 (1:1000, GTX133447, GeneTex, CA, USA), and β-actin (1:1000, ab8227, Abcam) and incubated overnight at 4°C. After washing in TBST, the samples were incubated with Goat Anti-Rabbit IgG H&L (HRP) (1:2000, ab6721, Abcam) at room temperature for 2 h, with β-actin serving as the internal reference. Protein bands were detected using an enhanced chemiluminescence kit (ECL Plus, Life Technology, MA, USA), and grayscale analysis was performed using Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

GraphPad Prism 8.01 (GraphPad Software Inc., San Diego, CA, USA) was utilized for data analysis and plotting. The measurement data were presented in the form of mean ± standard deviation. Independent sample *t*-test was applied for comparisons between two groups; one-way analysis of variance (ANOVA) analysis was employed for comparisons among groups, with Tukey's test used afterwards. Mouse body weight and left ear thickness were analyzed using two-way ANOVA. Tukey's multiple comparison test or Šídák's multiple comparison test was used for the post hoc test. *P* was obtained from a bilateral test. A value of $P < 0.05$ was regarded as statistically significant.

Results

CQ Alleviated MC903-Induced Type 2 Inflammatory Response in AD Mice

The severity of ear skin lesions in each group of mice is shown in Figure 1A. Compared with the Control group, the dermatitis score severity and left ear thickness in the AD group were evidently increased, while those of the CQ treatment group were apparently mitigated versus the AD group (Figure 1A–C, all $P < 0.05$). As reflected by H&E and TB staining, there was no obvious damage to the left ear skin of the Control group, while the AD group exhibited hyperkeratosis, acanthosis, erosion, and infiltration of mast cells in the skin of the left ear, but CQ treatment prominently alleviated skin damage in AD mice and reduced epidermal thickness and number of mast cells (Figure 1D and E, all $P < 0.05$). ELISA manifested that serum TSLP, IL-4, IL-13, and IgE levels in the AD group were substantially higher than those in the Control group (Figure 1F), while they were measurably diminished after CQ treatment (all $P < 0.05$); and there was no significant change in the serum IFN-γ level among the three groups ($P > 0.05$). In addition, flow cytometry demonstrated a significant increase in peripheral blood Th2 cell content in the AD group and a salient reduction in Th2 cell content in peripheral blood after CQ treatment (Figure 1G, all $P < 0.05$). The above results indicated that CQ alleviated the MC903-induced type 2 inflammatory response in AD mice.

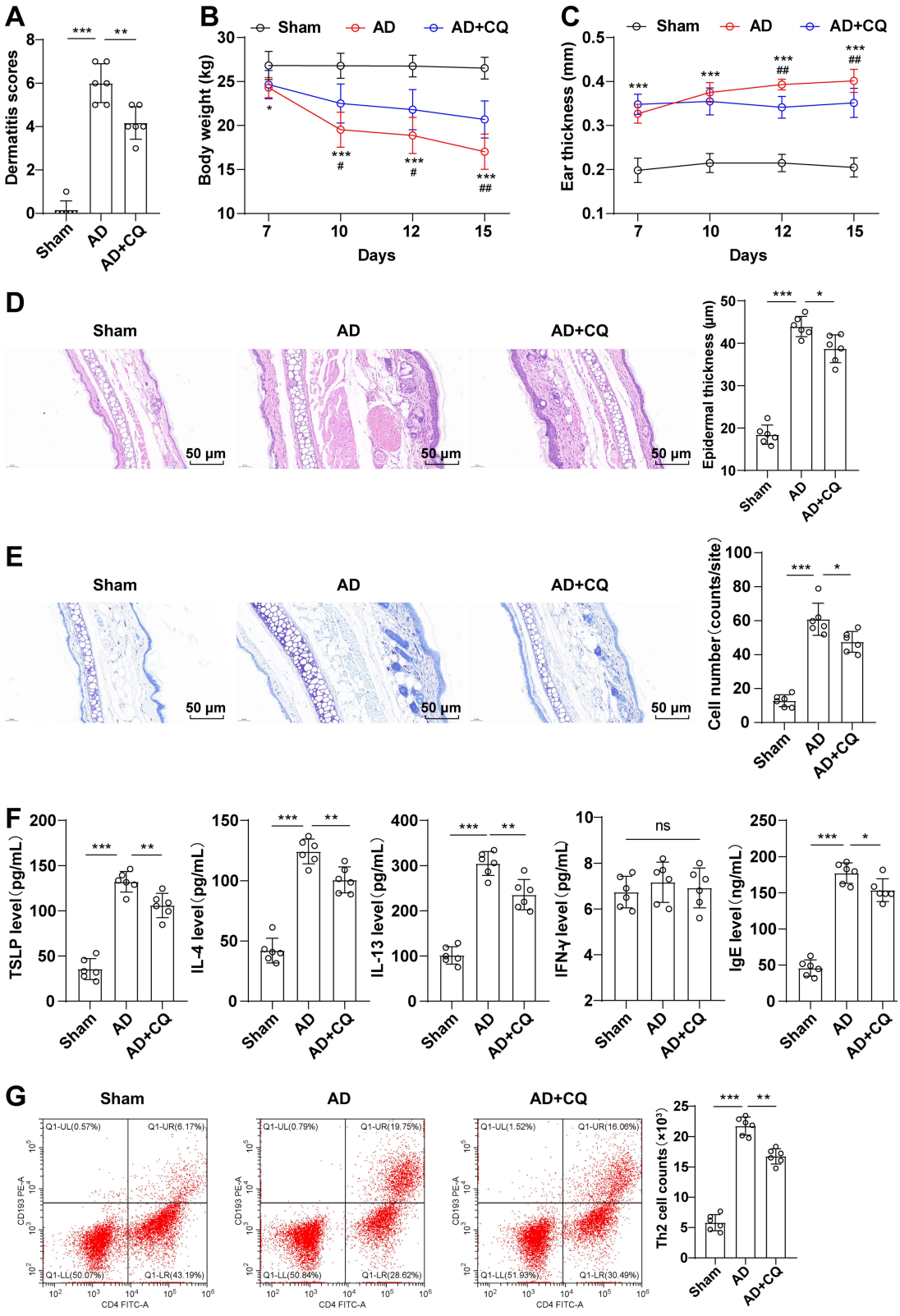

Figure 1 CQ relieved MC903-induced type 2 inflammatory response in AD mice. (**A**) The severity of ear skin lesions; (**B**) dermatitis severity score; (**C**) left ear thickness; (**D**) H&E staining; (**E**) TB staining; (**F**) ELISA measurements of serum TSLP, IL-4, IL-13, IFN-γ, and IgE levels; (**G**) flow cytometry to determine the content of peripheral blood Th2 cells (CD3+CD4+CD193+). N = 6. Data were represented as mean ± standard deviation, one-way ANOVA was for data comparisons in panels A/D-G, with Tukey's test serving for post hoc testing. ns represented $P > 0.05$, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Two-way ANOVA was adopted in panels B-C, and Tukey's multiple comparisons test was implemented for post hoc test.*Represented comparisons with the Control group, *$P < 0.05$, ***$P < 0.001$, # represented comparisons with the AD group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

CQ Alleviated Type 2 Inflammatory Response in AD Mice by Curbing TLR3 Activation

To further investigate whether CQ alleviated type 2 inflammatory response in AD mice by inhibiting TLR3 activation, 5 μg/g bodyweight poly (I:C) (TLR3 activator) was injected intraperitoneally into AD mice after CQ treatment. Western blot results uncovered that the TLR3 protein level in the left ear skin tissues of AD mice was conspicuously elevated relative to the Control group, while after CQ treatment, the level was significantly diminished relative to the AD group. However, the TLR3 protein level in the left ear skin tissues of the AD + CQ + poly (I:C) group was observably raised (Figure 2A, all $P < 0.05$). Subsequently, further detection on the changes in type 2 inflammatory response in mice in the AD + CQ group and the AD + CQ + poly (I:C) group elicited that activating TLR3 partially reversed the alleviative effect of CQ on type 2 inflammatory response in AD mice (Figure 2B–H, all $P < 0.05$), and there was no significant difference in the serum IFN-γ level ($P > 0.05$). These results were indicative of the fact that CQ attenuated type 2 inflammatory response in AD mice by inhibiting the activation of TLR3.

CQ Weakened the Activation of the NLRP3 Inflammasomes

On top of the previous results, we speculated that CQ might suppress TLR3 activation to repress NLRP3 inflammasome activation. Western blot results revealed that in contrast to the Control group, NLRP3, ASC, and cleaved caspase-1 protein levels in the left ear skin tissues of the AD group conspicuously rose, while in contrast to the AD group, these levels of the AD + CQ group memorably dropped (Figure 3A, all $P < 0.01$). Moreover, as reflected by ELISA, serum IL-1β and IL-18 levels in AD mice were considerably up-regulated relative to the controls, while the levels in the AD + CQ mice were distinctly down-regulated versus the AD mice (Figure 3B, all $P < 0.05$). The results suggested that CQ limited the activation of the NLRP3 inflammasomes.

Activation of NLRP3 Partly Annulled CQ-Mediated Attenuating Effect on Type 2 Inflammatory Response in AD Mice

To further validate the role of the NLPR3 inflammasomes in alleviating type 2 inflammatory response in AD mice, 5 mg/kg Nigericin (NLPR3 inflammasome agonist) was injected intraperitoneally into AD mice following CQ treatment. NLRP3, ASC, and cleaved caspase-1 protein levels and serum IL-1β and IL-18 levels in the left ear skin tissues of the AD + CQ + Nigericin group mice were markedly facilitated in contrast to the AD + CQ group mice (Figures 4A and G, all $P < 0.05$). Relative to the AD + CQ group, dermatitis severity and left ear thickness in the AD + CQ + Nigericin group were obviously increased, with salient increases in epidermal thickness and mast cell number, as well as elevated serum TSLP, IL-4, IL-13, and IgE levels, and peripheral blood Th2 cell content (Figures 4B–H, all $P < 0.05$). These results prompted that activating NLRP3 partially abrogated CQ-mediated attenuation on type 2 inflammatory response in AD mice.

Discussion

AD is the most common chronic inflammatory skin disease across the globe, causing extensive burden on adults and children and also having impacts on the lives of family members and patient caregivers.[41] Roughly 33% of children and 50% of adults diagnosed with AD exhibit symptoms indicative of a moderate-to-severe illness.[42] In certain cases, patients may find that phototherapy and topical treatment are insufficient for achieving effective disease control, necessitating systemic therapy.[43] Currently, systemic immunomodulatory medicines employed for AD treatment encompass a range of older medications such as cyclosporine, methotrexate, azathioprine, and mycophenolate, as well as the biologic compound known as dupilumab,[44,45] of which dupilumab is clinically effective.[46–48] Evidence has suggested CQ as a therapy for AD,[49] but its efficiency and mechanism in AD remain unclear. Consequently, our findings highlighted that CQ weakened the activation of the TLR3 and NLRP3 inflammasomes, thus reducing type 2 inflammatory responses in AD mice.

The characteristics of AD include itchy and recurrent eczematous skin injury, raised serum levels of IgE, skin barrier defects, epidermal thickening, and infiltration of inflammatory cells such as mast cells and eosinophils.[50,51] AD is mainly linked to skin barrier dysfunction and IgE elevation due to decreased filaggrin expression.[52] From the results of existing

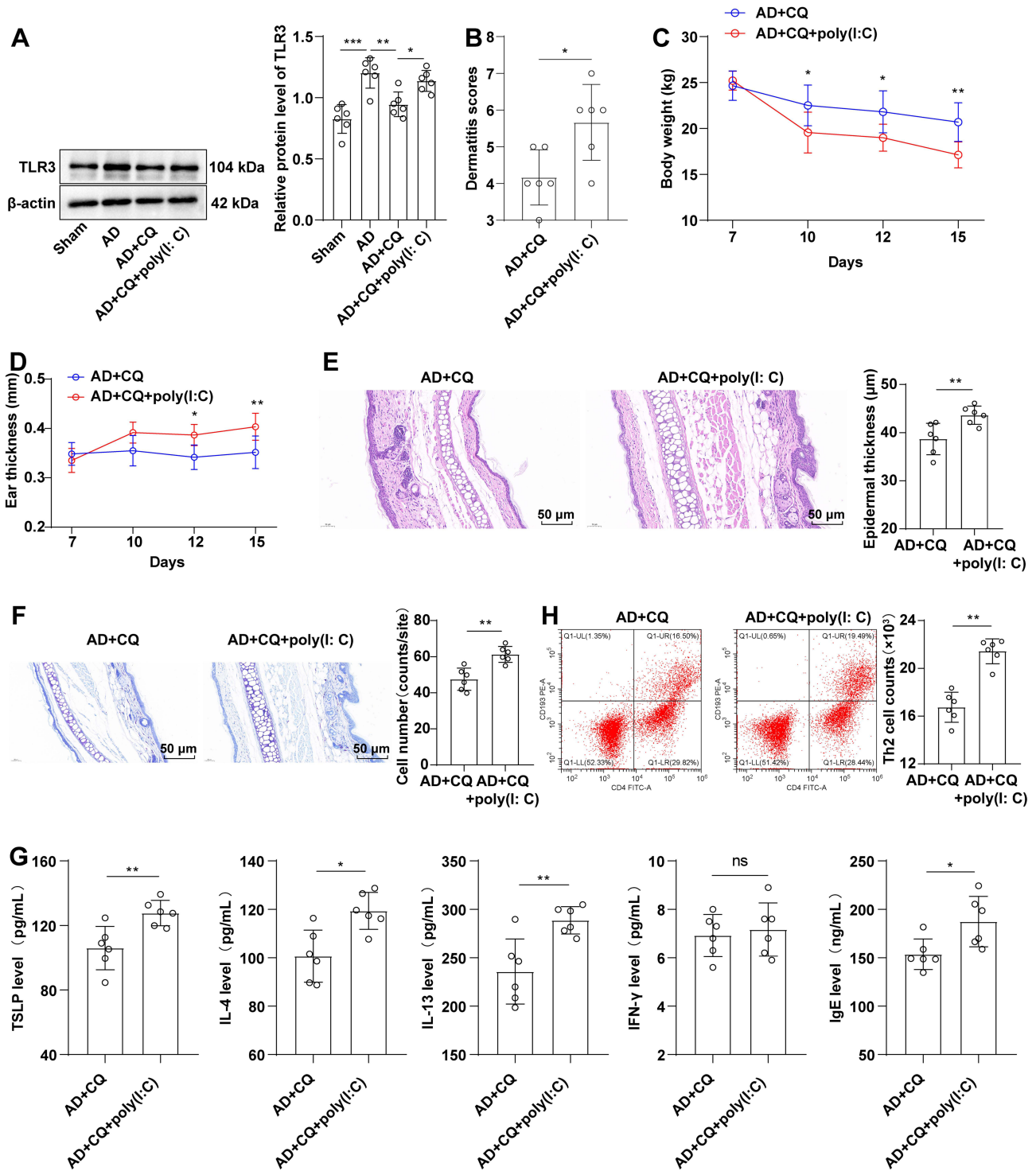

Figure 2 CQ abated type 2 inflammatory response in AD mice by inactivating TLR3. (**A**) Western blot detection of TLR3 protein expression; (**B**) the severity of ear skin lesions; (**C**) dermatitis severity score; (**D**) left ear thickness; (**E**) H&E staining; (**F**) TB staining; (**G**) ELISA detections of serum TSLP, IL-4, IL-13, IFN-γ, and IgE levels; (**H**) flow cytometry to assess the content of Th2 cells (CD3+CD4+CD193+). N = 6. Data were represented as mean ± standard deviation. Data in panel A were tested by one-way ANOVA, followed by post hoc testing using Tukey's test. Two-way ANOVA was used for panels (**C** and **D**), and Šídák's multiple comparisons test was used for post hoc test. Data in panels B/E-H were examined by independent sample *t*-test. ns represented $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

research, it has been manifested that Th2 cell-mediated pruritus, skin barrier dysfunction, and immunity induce a vicious cycle of AD, and on the other hand, IL-4, IL-13, and TSLP occupy a vital position for immune reactions mediated by Th2 cells.[53] Moreover, dramatic responses to mirtazapine and CQ for recalcitrant AD have been reported in a previous

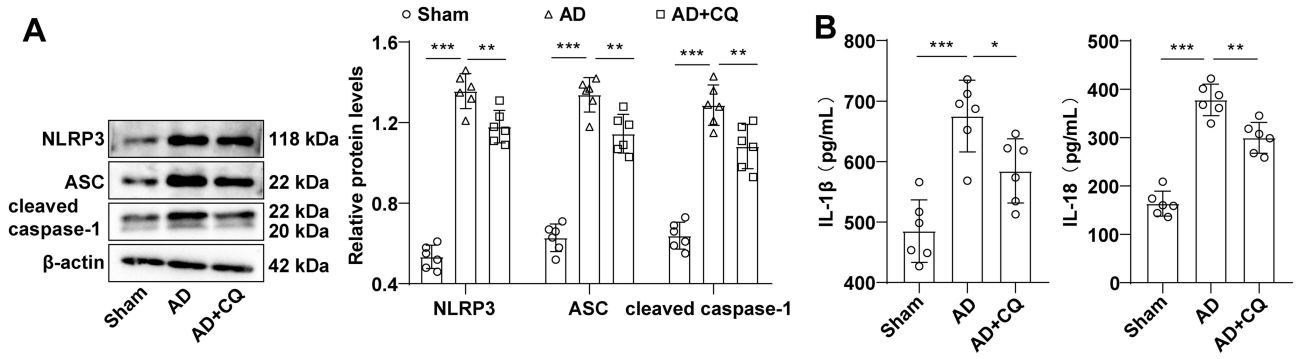

Figure 3 CQ suppressed the activation of the NLRP3 inflammasomes. (**A**) Western blot to measure the expression patterns of NLRP3, ASC and cleaved caspase-1 proteins; (**B**) ELISA detection of serum IL-1β and IL-18 levels in mice. N = 6. Data were represented as mean ± standard deviation. One-way ANOVA was used for inter group data comparisons, followed by Tukey's test, where *Represented $P < 0.05$, **Represented $P < 0.01$, and ***Represented $P < 0.001$.

study.[54] Based on these, we also uncovered increased left ear thickness and dermatitis score, erosion, acanthosis, hyperkeratosis, and infiltration of mast cells in the skin of the left ear, as well as raised content of peripheral blood Th2 cells and levels of serum TSLP, IL-4, IL-13, and IgE in AD mice, while CQ treatment led to opposite trends. Consistently, an existing study has also come across the anti-inflammatory property of CQ via regulating TSLP levels in an in vivo PMA-irritated ear edema model and an in vitro phorbol myristate acetate + A23187-activated human mast cell (HMC-1) model, suggesting that CQ treatment diminishes TSLP mRNA expression in activated HMC-1 cells and ear tissues and reduces ear thickness in ear edema.[12] To conclude, CQ retarded type 2 inflammatory responses induced by MC903 in AD mice.

The activation of TLR3 leads to the production and release of TSLP, thereby further aggravating Th2 inflammatory responses.[17] We treated AD mice with TLR3 activator on top of CQ treatment and disclosed that poly (I:C) treatment up-regulated CQ treatment-reduced TLR3 protein level and CQ-abated type 2 inflammatory responses in AD mice were consequently aggravated. Intriguingly, in another prior investigation, it is believed that TLR3 is more crucial in regulating type 1 immunity via cytokines like IFN-γ, potentially contributing to the balance of type 2 cytokines.[15] Nevertheless, there was no significant difference in the serum IFN-γ level among the treatment groups in our findings. Likewise, CQ limits the TLR3/IFN-β signaling in cultured normal human mesangial cells, exerting effect on protecting against renal damage in lupus nephritis.[55] Endosomal TLR3 suppression by CQ dose-dependently represses TLR3 agonists-induced TSLP generation in Th2-like inflammation.[56] Altogether, CQ reduced the activation of TLR3 to suppress type 2 inflammatory responses in AD mice.

The NLRP3 inflammasomes are high molecular weight protein complexes that have been associated with various inflammatory and allergic diseases in human, especially AD.[57] Inflammasomes are triggered by the activation of NLRP3 and cause caspase-1 activation, which in turn stimulates the release and maturation of cytokines including IL-18 and IL-1β, as well as the initiation, modulation and amplification of various immune responses, with the NLRP3 inflammasomes identified as vital modulator of contact hypersensitivity, suggesting a new therapeutic method for allergic disease treatment, such as AD.[58] As reported, AD patients exhibited dramatically higher NLRP3 mRNA expression in the lesions than healthy controls.[59] It was also reported that AD patients showed lower NLRP3 expression in epidermal cells than healthy controls, which was ascribed to the inhibition caused by heightened levels of type 1 cytokines, whereas NLRP3 was up-regulated by IFN-γ.[19] Our results manifested that the levels of cleaved caspase-1, ASC, and NLRP3 proteins in the left ear tissues of AD mice were elevated, and the levels of IL-18 and IL-1β in serum were increased, while CQ treatment decreased these protein levels. Consistent with the vast majority of recent research reports, NLRP3 expression is up-regulated in AD, and repressing NLRP3 activation can mitigate AD.[58,60–64] A previous study has also indicated that one of the possible anti-inflammatory mechanisms of CQ is repression of the NLRP3 inflammasome activity.[34] The antimalarial CQ reduces LPS-induced activation of the NLRP3 inflammasomes, thereby conferring protection against murine endotoxic shock.[65] Taken together, CQ repressed the activation of the NLRP3 inflammasomes. To further substantiate this conclusion, we treated AD mice with NLPR3 inflammasome activator on the basis of CQ treatment, and discovered increased mast cell number, dermatitis

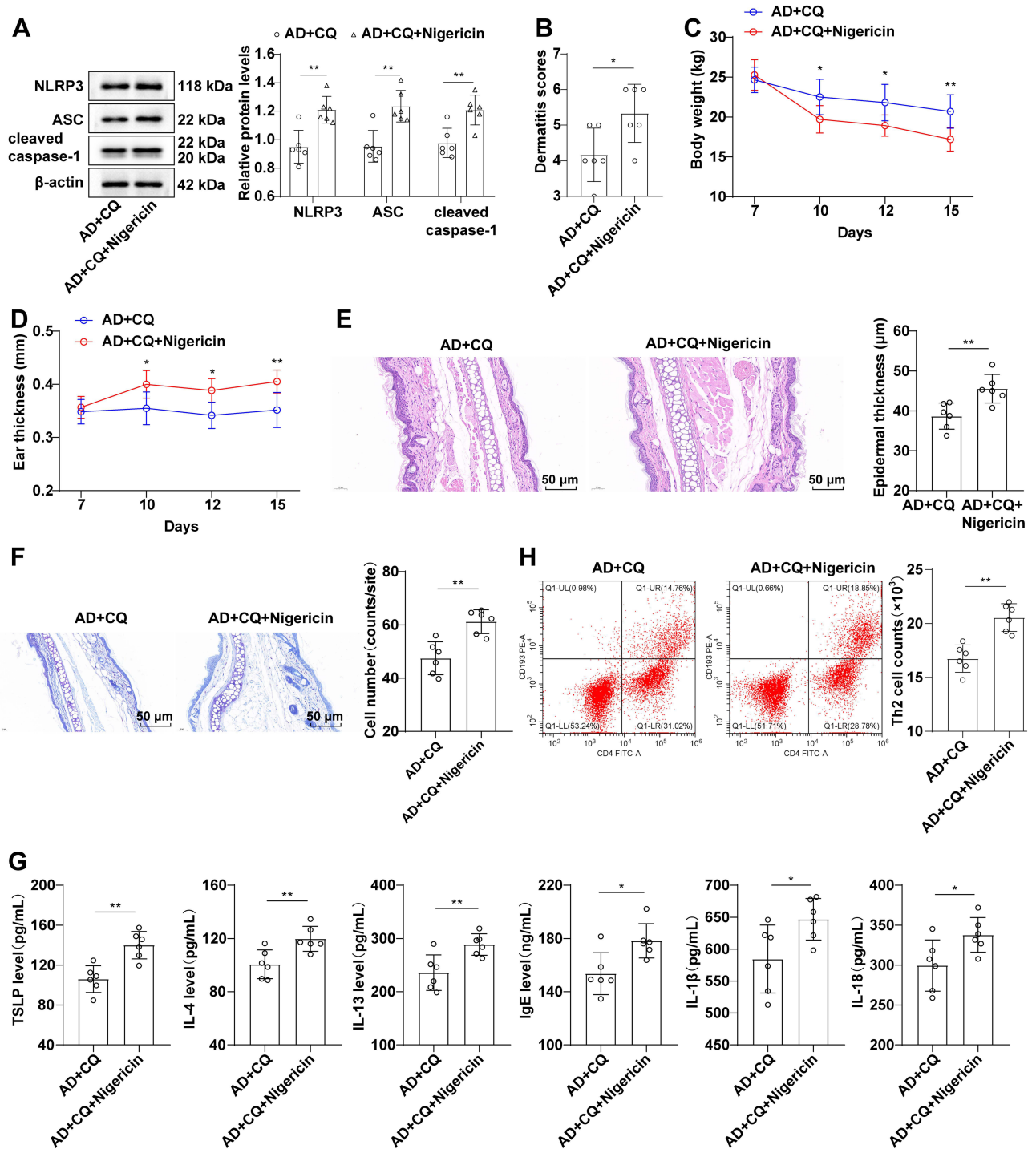

Figure 4 Activation of NLRP3 partially averted the alleviating effect of CQ on type 2 inflammatory response in AD mice. (**A**) Western blot to test the expression levels of NLRP3, ASC and cleaved caspase-1 proteins; (**B**) the severity of ear skin lesions; (**C**) dermatitis severity score; (**D**) left ear thickness; (**E**) H&E staining; (**F**) TB staining; (**G**) ELISA detection of serum TSLP, IL-4, IL-13, IgE, IL-1β and IL-18 levels; (**H**) flow cytometry to assess the content of Th2 cells (CD3+CD4+CD193+). N = 6. Data were represented as mean ± standard deviation, and independent sample *t*-test was employed for comparisons in panels A-B/E-H. Two-way ANOVA was used in panels C-D, and Šídák's multiple comparisons test was conducted for post hoc test. **P* < 0.05, ***P* < 0.01.

severity, epidermal thickness and left ear thickness, facilitated content of peripheral blood Th2 cells and serum levels of TSLP, IL-4, IL-13, and IgE, which suggested for the first time that activating NLRP3 partly diminished CQ-mediated attenuation on AD mouse type 2 inflammatory responses.

Strengths and Limitations

This study supported that CQ inactivated TLR3 to limit NLRP3 inflammasome activation, thereby alleviating type 2 inflammatory responses in AD mice, which provided some theoretical basis for clarifying AD pathogenesis and new ideas for its treatment. Nevertheless, we only used MC903-induced male AD mouse model to explore the therapeutic effect of CQ on AD mice, without considering the influence of sex-based differences in mice on the results of the study, and did not involve human samples or human cells. Meanwhile, CQ is a known pruritogen,[66] and the mechanism in mice is well established.[67] Without measuring pruritus, the application value of CQ in the treatment of AD remains to be further explored. What's more, the optimal dosage of CQ for treating AD mice was not studied in-depth. These above-mentioned problems will be further improved in the follow-up study.

Conclusion

CQ relieved the type 2 inflammatory response in AD mice by curbing TLR3 activation and NLRP3 inflammasome activation.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement

The experiments were authorized by the Institutional Animal Ethics Committee of Nanjing Hospital Affiliated to Nanjing Medical University (project license number DWSY-23022326). All animal manipulations and experimental techniques were conducted in accordance with the standards set forth by the committee. All the laboratory procedures were used to minimize the pain of mice. All experiments in this study followed the "3R" principles of animal welfare guidelines, namely replacement, reduction, and refinement.

Author Contributions

All authors have made significant contributions to conception, study design, execution, data acquisition, analysis, and interpretation. All authors participated in the drafting or writing of the manuscript and substantially revised or critically reviewed the article. All authors review and agree on all versions of the article before submission, during revision, acceptance of the final version for publication, and any significant changes introduced at the proofing stage. All authors have agreed on the journal to which the article will be submitted. All authors agree to take responsibility and be accountable for the contents of the article. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Declaration

The work described has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Funding

This work was partially supported by grants from Nanjing Health Science and Technology Development Special Fund (No. YKK22121). All authors ensure that the information in the grant section is accurate and that the grant number is correct.

Disclosure

The authors have no conflicts of interest to declare.

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