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Original Research Article

Curcumin alleviates asthma in rat by targeting Foxp3

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Abstract

Purpose: *To examine the mitigating influence of curcumin on asthma in children, and the involvement of Foxp3 in the process.*
Methods: *A rat model of asthma was successfully induced using ovalbumin (OVA). Twenty-four (24) rats were assigned to 3 cohorts: model, curcumin and control groups. At the cellular level, sorted CD4+ T cells were differentiated into regulatory T (Treg) cells and divided into 4 groups: Treg, Treg + curcumin, Treg + STAT5-IN-1 (STAT5 inhibitor) and Treg + curcumin + STAT5-IN-1 groups.*
Results: *Curcumin significantly inhibited OVA-induced increases in eosinophils and lymphocytes and reduced airway pathological changes, relative to model rats ($p < 0.05$). There were marked increases in TGF-β and IL-10 contents in bronchoalveolar lavage fluid (BALF), relative to model rats. Furthermore, curcumin up-regulated p-STAT5 and Foxp3 in the lung tissues of rats, when compared with model group ($p < 0.05$). In cell experiments, curcumin significantly increased p-STAT5, Foxp3 and IL-10 levels, and enhanced the transformation of CD4+ T cells to more Treg cells ($p < 0.05$).*
Conclusion: *Curcumin may affect the differentiation of Treg cells, as well as alleviate asthma in rats by targeting Foxp3 through STAT5 regulation. This research has identified the likely beneficial role of curcumin as therapeutic agent for pediatric asthma, but this has to be clinically validated.*

Keywords: *Curcumin, Foxp3, STAT5, Treg, Asthma in children*

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INTRODUCTION

Asthma is a chronic airway inflammatory disease that manifests primarily as acute bronchial asthma, a frequently-occurring disease in children. Under numerous exogenous and endogenous environmental stimuli, the airways undergo excessive constriction which is accompanied by enhanced airway responsiveness and increased mucus secretion [1]. Bronchial asthma occurs in infancy and adulthood, but in infants, bronchial asthma develops throughout the entire developmental period of the patients. With continuous deterioration of the environment, especially poor air quality, the incidence of bronchial asthma in children has been on the increase from year to year. Unfortunately, there is still no specific drug for the treatment of pediatric asthma, and the pathogenesis of the disease is still unclear.

Asthma is a chronic inflammatory disease of the airways which involves a variety of inflammatory cells such as T cells, eosinophils, and macrophages. Regulatory T (Treg) cells act as a negative feedback regulation mechanism for

activation of the T cells, and they modulate the response of T cells within an appropriate range without causing damage to the body. Treg cells are divided into $CD4^{+}CD25+$Treg, Tr1, Th3, NKT, and others (according to phenotype, secreted cytokines and regulatory mechanisms), among which the most important is the CD4+CD25+Treg [2].

Foxp3 is a grasp regulator of the biological roles of Treg cells. It regulates immunity in asthma by enhancing the production and development of Treg cells. Moreover, Foxp3 modulates Treg cell immunosuppressive function and coordinates lymphocyte immune responses to pathogens [3]. Foxp3 is special to Treg cells, being a characteristic index of these cells. Foxp3 is activated through the STAT5 signal route. Moreover, STAT5 performs a crucial part in Treg cell function, and it is activated by the binding of IL-2 to the IL-2R receptor on CD4+CD25+ Treg cells. On activation, STAT5 becomes attached to the non-coding sequence 2 of the conserved area of Foxp3 enhancer [4].

Curcumin extracted from turmeric is a polyphenol which has antioxidant, anti-inflammatory, antibacterial, antiviral and other biological activities [5]. Research has shown that curcumin exerts a regulatory impact on cancer by targeting signaling pathways involved in cancer cell proliferation, implying that this polyphenolic compound may be used, alone or in combination with other drugs, as an effective cancer therapy [6].

In recent studies, curcumin has been shown to exert regulatory effects on human immune responses, and it may act as an effective drug for treating inflammatory diseases [7]. However, not much is known about the effect of curcumin on asthma and the associated mechanisms.

EXPERIMENTAL

Animals

Male Sprague-Dawley (SD) rats (n = 24) aged 3 weeks, with mean weight of 150 ± 10 g, were provided by Zhejiang Laboratory Animals Center (Hangzhou, China). The rats were maintained in a clean-grade environment with 40 - 70 % humidity at room temperature, and the animals had free access to de-ovalbumin (de-OVA) and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee (approval no. ZJCL A-IACUC-20040157), and the International Guidelines 2.0 were followed in conducting the study [8].

Grouping and establishment of rat model of asthma

The SD rats were randomly assigned to three groups: asthma model, curcumin, and control groups, each with eight rats. Rats in the asthma model and the curcumin groups were sensitized *via* intraperitoneal injection of 2 mL of sensitization solution (100 mg OVA + 100 mg $Al(OH)_3$) on days 1 and 7, while rats in control group were injected 2 mL of saline. From day 15 to 28, the asthma model group and the curcumin group rats were stimulated *via* aerosol inhalation of 5 % OVA solution in glass containers daily for 30 min. The control rats received saline aerosol inhalation. The rats were administered OVA every other day from day 30 to day 44, each time for 30 min, in a total of 8 times. One hour before atomization, curcumin solution was injected intraperitoneally into rats in curcumin cohort at a dose of 0.10 g/kg. The rats in asthma model group were not subjected to intraperitoneal injection of curcumin, while rats in the control group were intraperitoneally injected 2 mL saline.

Bronchoalveolar lavage fluid (BALF) collection and cell sorting

Saline (3 mL) was injected 3 times into the left lung lavage from tracheal intubation. The recovery rate of BALF was about 90 %, and the BALF was pooled in a centrifuge tube. After centrifugation, the pellet was smeared and stained with Wright stain, and the cells were counted and sorted under an oil microscope.

Hematoxylin and eosin (H&E) staining

Rat lung tissues from the three groups were fixed with 4 % formaldehyde, dehydrated, embedded and sectioned. Finally, H&E staining was done on the lung sections, and the tissues were examined microscopically.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-10 and TGF-β in BALF supernatants were determined with ELISA (JingMei Biotech, China) kits, in line with the kit protocol. Optical density was read at 450 nm in a plate reader, and the protein levels were calculated.

Western blot assay

Total protein was extracted from the collected tissues of rats using RIPA buffer. The proteins were subjected to electrophoresis using SDS-PAGE and transferred to PVDF membranes. Then, the membranes were incubated overnight

at 4 ^{o}C with the primary antibodies anti-Foxp3, anti-p-STAT5, and anti-STAT5, with anti-GAPDH (Abcam, UK) as the internal reference. All antibodies were diluted 1:1000. Membrane incubation with 2^{o} immunoglobulin IgG-HRP (Abcam, UK; 1:5000 dilution) was done for 1 h under laboratory conditions. The GEL-PRO gel imaging system was used for analysis of relative protein expression levels.

Isolation of rat CD4+ T lymphocytes and differentiation of Treg cells

The rats were euthanized using cervical dislocation, and the spleens were aseptically removed using laparotomy. The mononuclear cells were routinely isolated. After obtaining the splenic mononuclear cell suspension, the CD4+ T cells were sorted with immunomagnetic bead cell sorting kit [9]. The α-T cell receptor (α-TCR) stimulators (anti-CD3+and anti-CD28, 5 µg/mL) and the cytokines ATRA, IL-2 and TGF-β are required for CD4+ T cells to be transformed into Treg cells. Cell maintenance was done in RPMI-1640 tainted with 10 % FBS, streptomycin and penicillin. The cells were grouped as follows: Treg, Treg + curcumin (5 µM), Treg + STAT5-IN-1 (STAT5 inhibitor, 47 µM), and Treg + curcumin + STAT5-IN-1 groups.

Statistical analysis

The SPSS 12.0 software was used to analyze the data obtained, while two groups were compared with *t*-test. The significance of differences was assumed at $p < 0.05$.

RESULTS

Effect of curcumin on cell classification in BALF of young rats

The leukocytes in the BALF of control group rats were mainly monocytes, with few lymphocytes, neutrophils and eosinophils. The populations of lymphocytes and eosinophils in rats in the asthma model group were significantly elevated, relative to control rats. However, the populations of eosinophils and lymphocytes were markedly less in rats in the curcumin group than in asthma model rats (Figure 1 A - D).

Impact of curcumin on airway histopathology in young rats

Rats in the control group had neat airway mucosa epithelium and regular lumen. There was no inflammatory cell infiltration under the mucosa or around the vascular wall (Figure 2 A). In the model group, a large quantity of inflammatory cells, mainly eosinophils and lymphocytes, infiltrated and clustered around the airway mucus and vascular wall in rats. The airway wall and smooth muscles were thickened, and there were increases in number of mucosal epithelial folds. The lumen was narrow and partially blocked with mucus plugs (Figure 2 B). The results revealed that the airway pathological symptoms in the curcumin group of rats were extensively lower than those in the asthma model, suggesting that curcumin may mitigate airway remodeling in asthma (Figure 2 C).

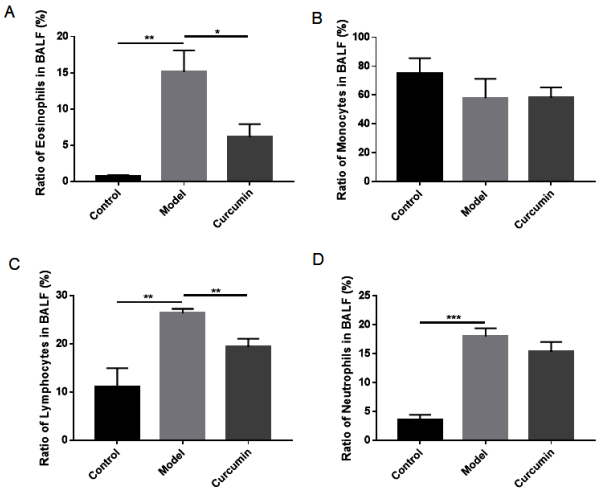

Figure 1: Effect of curcumin on cell classification in BALF of young rats. (A) % of eosinophils, (B) % of monocytes, (C) % of lymphocytes, (D) % of neutrophils. *$P < 0.05$; **$p < 0.01$; ***$p < 0.001$

Effect of curcumin on the levels of TGF-β and IL-10 in BALF

Compared with the control group, the levels of TGF-β and IL-10 in the BALF in model group rats were markedly increased, while TGF-β and IL-10 content in BALF was markedly greater in curcumin-treated rats than in the asthma model ($p < 0.05$; Figure 3 A and B).

Effect of curcumin on the expression of STAT5 and Foxp3

The STAT5 and Foxp3 expressions in right pulmonary tissues of rats were determined using Western blotting experiment. As shown in Figure 4, p-STAT5 and Foxp3 proteins in the asthma model group were up-regulated, when compared with those in control rats. However, these protein levels were markedly increased in curcumin rats, relative to asthma model rats ($p < 0.05$; Figure 4).

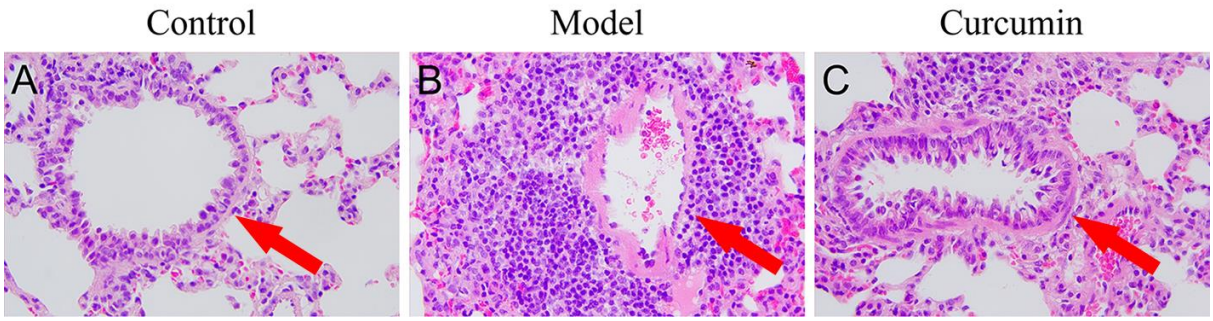

Figure 2: Effect of curcumin on the airway histopathology of young rats. (A) H&E staining of the airway of a rat in the control group, (B) H&E staining of the airway of a rat in the model group, (C) H&E staining of the airway of a rat in the curcumin group

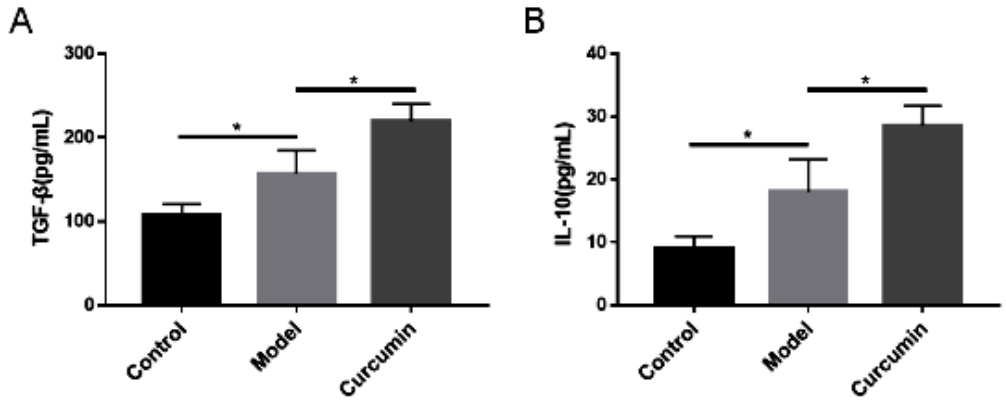

Figure 3: Effect of curcumin on TGF-β and IL-10 in BALF. (A) TGF-β in BALF; (B) IL-10 in BALF. *P < 0.05 vs control

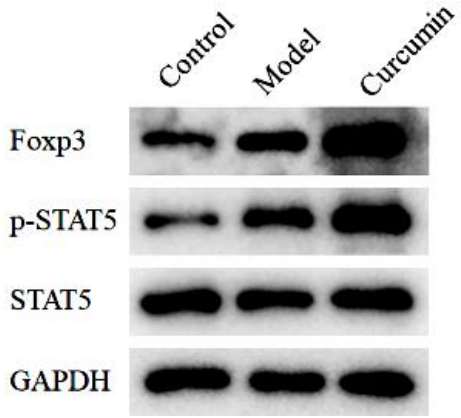

Figure 4: Effect of curcumin on the expressions of STAT5 and Foxp3

Effect of curcumin on the differentiation of Treg cells

In order to further find out whether curcumin affects Treg cell differentiation by targeting Foxp3 through STAT5 regulation, CD4+ T cells obtained in this study were subjected to Treg cell differentiation. When curcumin was added to the differentiated microenvironment, the expressions of p-STAT5 and Foxp3 were significantly increased, and IL-10 expression was also shown to be increased. More differentiated Treg cells were observed. However, when STAT5-IN-1 was added to the microenvironment for directed differentiation, p-STAT5 and Foxp3 were significantly down-regulated, while IL-10 expression was decreased, and the number of differentiated Treg cells was reduced. Furthermore, when both STAT5-IN-1 and curcumin were added to the differentiated microenvironment, the expressions of p-STAT5 and Foxp3 were not clearly up-regulated, when compared with Treg+STAT5-IN-1, and IL-10 expression was also not significantly increased. There were no more differentiated Treg cells (Figure 5 A and B).

DISCUSSION

Presently, asthma symptoms in children are alleviated only by long-time use of glucocorticoids. However, it is widely acknowledged that high doses and long-term use of hormones cause local and systemic side effects. It is of great importance to understand the pathogenesis of asthma in children and to identify drugs that interfere with the asthma progression in this group.

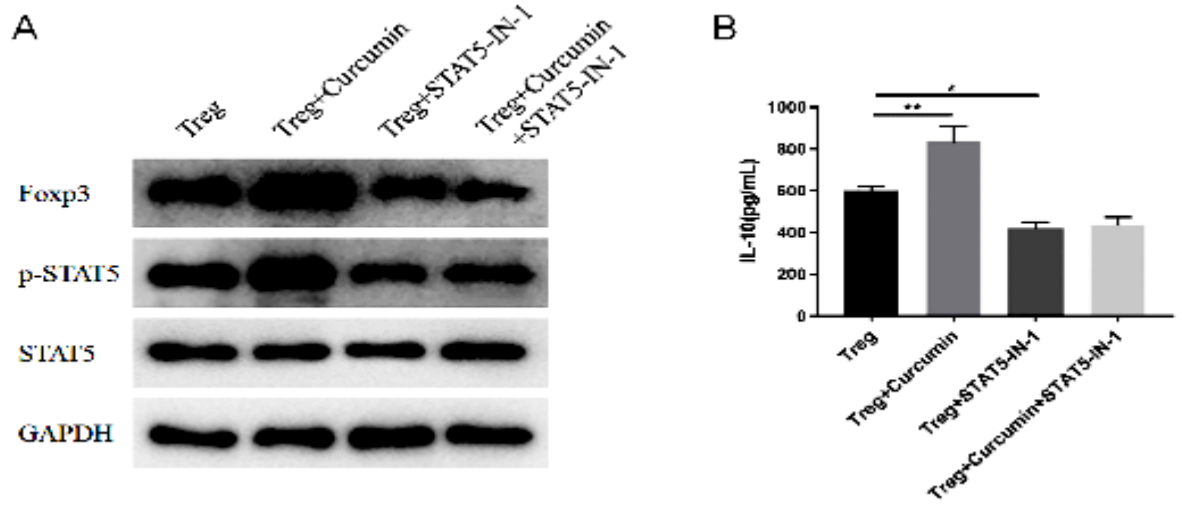

Figure 5: Curcumin affected the differentiation of Treg cells by regulating STAT5/ Foxp3. A: Effect of curcumin on STAT5 and Foxp3 contents in the microenvironment of directed differentiation; B: effect of curcumin on the secretion of IL-10 in the microenvironment of directed differentiation. *$P < 0.05$; **$p < 0.01$, vs Treg

Bronchial asthma is a chronic airway condition due to inflammation, with various effector cells such as eosinophils, lymphocytes, neutrophils and airway epithelial cells. However, the key effector cells of asthma airway inflammation are eosinophils. Indeed, eosinophil accumulation around the bronchus is a prominent feature of airway inflammation in asthma [10]. The main manifestations of airway inflammation in asthma are a series of pathological changes such as infiltration of inflammatory cells dominated by eosinophils, proliferation and hypertrophy of the airway wall and smooth muscles, damage and shedding of the airway epithelium, edema of airway mucosa, and sub-mucosal gland hyperplasia. This research showed that the proportions of lymphocytes and eosinophils were markedly greater in BALF of asthma rats than in control rats. This confirmed the critical roles of these cells in the pathological process of bronchial asthma. The results from pathological examination of the lung tissues showed significantly increased damage to the bronchial wall and infiltration of inflammatory cells around the tube wall. Therefore, curcumin significantly inhibited airway inflammation in rats with asthma.

The CD4+T cells have the potential to differentiate into Th1, Th2, Th17 and CD4+CD25+Treg under the influence of different cytokines and environmental conditions. The most effective mechanism in peripheral immune tolerance in the body is active Treg cell-mediated inhibition. Treg cells secrete the inhibitory cytokines IL-10 and TGF-β1. The cytokine IL-10 activates inflammatory responses by stimulating the production of Th2, thereby causing eosinophil infiltration [11]. Moreover, TGF-β acts on inflammatory cells as a pro-inflammatory cytokine, thereby exacerbating the pathogenesis of asthma by recruiting leukocytes into the airways and bronchi, resulting in induction of the differentiation of CD4+T cells into effector Th17 cells [12]. In this study, the levels of TGF-β and IL-10 in BALF were clearly higher in model rats than in control rats. Interleukin 10 (IL-10) and TGF-β1 secreted by Treg cells are immunosuppressive agents. Studies have revealed that curcumin attenuates pulmonary inflammatory injury during cecal ligation and puncture (CLP)-induced severe pulmonary damage. A study showed that after curcumin treatment, the levels of TGF-β and IL-10 in BALF were increased [13]. This is in agreement with the results of the present study. The concentrations of TGF-β and IL-10 in BALF were markedly greater in curcumin-treated rats than in model rats, implying that curcumin has potential to alleviate asthma-induced airway inflammation.

A dysfunction in Foxp3 may lead to the occurrence of autoimmune and inflammatory diseases. Recent studies revealed that abnormal expression of Foxp3 is strongly associated with the progression of asthma in children. Based on studies on asthma in young mice, it has been shown that the occurrence of asthma was suppressed in transgenic T cells by up-regulating Foxp3 expression [14]. Moreover, the expressions of Foxp3 and p-STAT5 are abnormally up-regulated in a mouse model of sepsis and in a rat model of chronic osteomyelitis, when compared with normal controls [3]. In this study, abnormal up-regulations of Foxp3 and p-STAT5 were also observed in the rat lung tissues of the asthma

model group, when compared to the control rats. The development and differentiation of CD4+CD25+Treg are regulated by the transcription factor Foxp3 which is modulated by the activation of STAT5. Previous studies have found that, relative to the peripheral blood eosinophils of patients with asthma, there was significant decrease in IL-5 family-induced STAT5 phosphorylation in airway eosinophils, with the airway eosinophils showing capacity to preferentially reduce STAT5 signal transduction [15]. Continuous activation of STAT5 enhances Foxp3 production and levels Treg cells. It is known that CD4+CD25+Treg is regulated by activating STAT5 through up-regulation of the expression of Foxp3 [16]. Murawski *et al* report the expression of STAT5 was blocked by the JAK inhibitor (AG-490), resulting in significant decrease in Foxp3 level in Treg cells [17]. In this study, curcumin increased the Foxp3 and p-STAT5 expressions, indicating that it may slow down the progression of asthma in rats by promoting the activation and phosphorylation of STAT5. This study found that the protein expressions of Foxp3 and p-STAT5 increased after adding curcumin to the microenvironment of directed differentiation of CD4+T cells. Moreover, cytokine IL-10 level secreted by Treg cell was increased. Following addition of the STAT5 inhibitor, the contents of Foxp3, p-STAT5 and IL-10 were decreased. Thus, it was inferred that curcumin enhanced the transformation of CD4+ into Treg, and delayed asthma progression.

Curcumin has been shown to produce various biological effects with no obvious adverse effects. For example, it exerts anti-inflammatory and immune-regulatory effects [5]. Studies have shown that curcumin regulates multiple signaling pathways and corresponding signaling molecules: it down-regulates COX-2, TNF-α, IL-1β and cyclin D1, and inhibits the NF-kB signal route [18]. In this study, curcumin inhibited eosinophil recruitment and mucus overproduction, and attenuated airway inflammation in rats with OVA-induced asthma. Therefore, curcumin may regulate the STAT5-Foxp3 signal route, affect transformation of CD4+ T into Treg, and effectively modulate levels of TGF-β and IL-10. These findings indicate the potential benefits of curcumin as a therapeutic drug for asthma in children.

CONCLUSION

Under allergen stimulation, the expression of STAT5 in CD4+ T cells is down-regulated, which in turn led to the inhibition of expression of its downstream factor Foxp3, thereby aggravating pediatric asthma. However, curcumin upregulates Foxp3 expression by upregulating STAT5 expression in CD4+ T cells; it enhances the differentiation of CD4+ T cells to more Treg cells, thereby ultimately alleviating rat asthma. However, clinical applicability of the results should be investigated in pediatric asthma.

DECLARATIONS

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Funding

None provided.

Ethical approval

All experimental procedures were approved by the Institutional Animal Care and Use Committee (approval no. ZJCL A-IACUC-20040157).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Meiling Sheng designed the study, performed formal analysis, and prepared a draft of the manuscript. Ruju Xu participated in formal analysis and analyzed the data. Xiangying Wang and Yanyan Zhu analyzed the data and validated the results, while Chunli Zhang reviewed the draft of the manuscript and made substantial revision to the draft. All authors read and approved the manuscript for publication.

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