Catalpol Inhibits Tregs-to-Th17 Cell Transdifferentiation by Up-Regulating Let-7g-5p to Reduce STAT3 Protein Levels

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Purpose: Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease, and Th17 cells are key factors in the pathogenesis of human inflammatory conditions, such as RA. Catalpol (CAT), a component in Rehmanniae Radix (RR), has been found to regulate human immunity. However, the effects of CAT on Th17 cell differentiation and improvement of RA are not clear.

Materials and Methods: Collagen-induced arthritis (CIA) mice were constructed to detect the effects of CAT on arthritis and Th17 cells. The effect of CAT on Th17 differentiation was evaluated with let-7g-5p transfection experiments. Flow cytometry was used to detect the proportion of Th17 cells after CAT treatment. Levels of interleukin-17 and RORγt were assessed by qRT-PCR and enzyme-linked immunosorbent assay. The expression of signal transducer and activator of transcription 3 (STAT3) was determined by qRT-PCR and Western blot.

Results: We found that the proportion of Th17 cells was negatively associated with let-7g-5p expression in CIA mice. In in vitro experiments, CAT suppressed traditional differentiation of Th17 cells. Simultaneously, CAT significantly decreased Tregs-to-Th17 cells transdifferentiation. Our results demonstrated that CAT inhibited Tregs-to-Th17 cells transdifferentiation by up-regulating let-7g-5p and that the suppressive effect of CAT on traditional differentiation of Th17 cells is not related with let-7-5p.

Conclusion: Our data indicate that CAT may be a potential modulator of Tregs-to-Th17 cells transdifferentiation by up-regulating let-7g-5p to reduce the expression of STAT3. These results provide new directions for research into RA treatment.

Key Words: Collagen-induced arthritis, CAT, Th17 cell differentiation, microRNA let-7g-5p, STAT3

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease involving inflammation and synovial hyperplasia.1,2 Frequently, RA results in severe destruction of cartilage and disability.3 Rehmanniae Radix (RR) is a traditional Chinese medicine found across Henan, Hebei, Shanxi, and many other provinces in China. Catalpol (CAT) is a component isolated from RR, and many researches have reported that CAT shows distinct immunoregulatory, antitumor, and antimicrobial activities.4-7

A key factor in the pathogenesis of RA, interleukin (IL)-17-producing helper T cells (Th17) can produce a large number of inflammatory cytokines such as IL-6 and TGF-β, a process referred to as traditional differentiation.8-11 Furthermore, research has shown that T regulatory cells (Tregs) have the ability to transdifferentiate into Th17 cells in the presence of inflammatory signals.12,13 Micro-RNAs (miRNAs) are a class of tiny non-coding RNA
molecules that regulate gene expression at the post-transcriptional level. There is increasing evidence indicating that miRNAs play an important role in many inflammatory and auto-immune diseases, including RA. Furthermore, studies have suggested a strong association for miRNAs in Th17 cells differentiation. Let-7 is a member of the miRNA family that regulates target genes involved in cell proliferation, differentiation, and apoptosis. According to our previous report, Th17 cells differentiation could be inhibited by up-regulating let-7g-5p.

In this study, we aimed to investigate the relationship between CAT and Th17 cells in collagen-induced arthritis (CIA) mice. We found that joint injury and inflammatory responses were inhibited after CAT treatment in CIA mice. Also, we discovered that the proportion of Th17 cells was negatively associated with let-7g-5p expression in CIA mice. In in vitro experiments, CAT suppressed traditional differentiation of Th17 cells. Simultaneously, CAT significantly decreased transdifferentiation of Tregs-to-Th17 cells. Overall, we demonstrated that CAT inhibits Tregs-to-Th17 cell transdifferentiation by up-regulating let-7g-5p and that the suppressive effect of CAT on traditional differentiation of Th17 cells is not related with let-7-5p. Moreover, our results indicate that CAT inhibits Tregs-to-Th17 cell transdifferentiation via up-regulating let-7g-5p to reduce the expression of signal transducer and activator of transcription 3 (STAT3), a potential target mechanism for treating RA.

MATERIALS AND METHODS

Animals
C57BL/6 mice were purchased from the Qinglongshan Laboratory Animal Center [Nanjing, P.R. China; animal license number: SCXK (Su) 2017-0001]. DBA/1 mice were also purchased from the Qinglongshan Laboratory Animal Center [Nanjing, China, animal license number: SCXK (Su) 2015-0001]. They were fed standard mouse feed and water without specific pathogens at the Experimental Animal Center of Nanjing University of traditional Chinese medicine under artificial illumination for 12 hours at a room temperature of 23±2°C and relative humidity of 50%–60%. All animal experiments were conducted in accordance with the principles for the use of Animals and the guidelines for the Care and use of Experimental Animals.

In vivo experiments

In vivo experiment protocol
After 7 days of accommodation, DBA/1 mice were randomly divided into three groups (n=6 per group) as follows: control, CIA, and CAT. All mice, except for the control mice, were used for CIA induction. Briefly, on day 0, mice were injected intradermally at the base of the tail with 100 μL of type II bovine collagen (2 mg/mL, Chondrex, Inc., Washington, DC, USA) emulsified in 0.05 M acetic acid and an equal volume of complete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO, USA). On day 21, mice were given a booster immunization with 100 μL of type II bovine collagen (2 mg/mL) emulsified in equal volumes of incomplete Freund’s adjuvant (IFA, Sigma-Aldrich). Control mice were injected with normal saline intracutaneous in the tail root on day 0 and day 21, respectively. Arthritic scores were monitored every 4 days after secondary immunization.

On the booster immunization day, mice in the CAT group were treated with CAT (30 mg/kg) via gavage administration daily for 4 weeks. The dose of CAT (30 mg/kg) was based on the long-term experience of our lab: at this dose, arthritis was effectively relieved, and no toxic side effects were observed in mice (not published). Mice in the control and model groups received the same volume of saline by gavage administration daily for 4 weeks. Mice were sacrificed on day 44, and peripheral blood, spleens, and ankle joints were obtained for further studies.

Separation of cells from peripheral blood and spleens in CIA mice
We collected blood samples in heparin tubes. To obtain peripheral blood mononuclear cells (PBMCs), samples were isolated within 1 h by Ficoll density gradient centrifugation (TBD sciences, LTS1092). In addition, to separate the red blood cells, we harvested the spleens of mice, which were mashed and washed with phosphate buffer saline (PBS) (Boster Biological Technology Co., Ltd, Fremont, CA, USA). Splenocytes were attained by ACK lysis buffer (KeyGEN BioTECH, Nanjing, China) to remove the red blood cells and filtered through a 70-μm cell strainer (BD Biosciences, San Diego, CA, USA).

Histologic analysis
The ankle joints of mice were fixed with 4% paraformaldehyde, decalciﬁed with decalciﬁed solution, embedded in paraﬁn, sectioned, and stained with hematoxylin-eosin. Pathological changes in the joints were observed under light microscope. Histology scores were assessed as previously described.

In vitro experiments

Cell culture
The cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum under 5% CO2 at 37°C.

Dose
CAT was purchased from Yuanye (Shanghai, P.R. China). At 20 μg/mL, CAT inhibited the differentiation of Th17 cells but did not elicit significant toxic side effects on cell viability (not published).

Separation of naive CD4+ T cells from spleens in C57BL/6 mice
Naive CD4+ T cells were separated from the spleens of C57BL/6 mice with a mouse naive CD4+ T cell isolation kit (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer’s in-
Differentiation and transfection assay

Let-7g-5p mimics, inhibitors, and negative control RNA (miR-mimic control and miR-inhibitor control) were purchased from GenePharma (Shanghai, P.R. China) to achieve the overexpression or knockdown of let-7g-5p. The oligonucleotides were mixed with siRNA-Mate (GenePharma) following the manufacturer's instructions and then added to Tregs under stimulation by 2 μg/mL of plate-bound anti-CD3 (BD Pharmingen, San Diego, CA, USA) and 2 μg/mL of anti-CD28 antibody (BD Pharmingen).

Detection of Th17 by flow cytometry

In order to determine the different phenotypes of cell differentiation, flow cytometry was used. PBMCs and splenocytes from CIA mice, as well as cultured Th17 cells in vitro, were collected. Cells were then washed twice with PBS and activated with leukocyte activation cocktail (BD Pharmingen) for 4 h. After staining of Th17 cells with anti-CD4-FITC (BD Pharmingen) and anti-IL-17A-PE (BD Pharmingen) for 30 min, intracellular cytokines were analyzed by flow cytometry (Beckman FC-500, Beckman Coulter, Inc., Brea, CA, USA).

Cytokines detection by enzyme-linked immunosorbent assay

The plasma of mice in each group was collected, and the concentrations of IL-2 (Catalog #JEB-12265), IL-6 (Catalog #JEB-12267), IL-10 (Catalog #JEB-12260), IL-17 (Catalog #JEB-12836), IL-21 (Catalog #JEB-12999), and IL-23 (Catalog #JEB-12650) were determined. Also, the supernatants of cell cultures were collected, and the concentration of IL-17 (Catalog #EK117HS) was determined.

Real-time quantitative polymerase chain reaction analysis

Total RNA samples were isolated by TRIzol reagent (Ambion, Austint, TX, USA) according to the manufacturer’s protocol. Reverse transcription of total RNA into cDNA was conducted using miRNA 1st Strand cDNA Synthesis Kits (Vazyme, Nanjing, P.R. China), and cDNA amplification was carried out with HiScript II Q RT SuperMix for qPCR (Vazyme). Real-time PCR was performed using ChamQ Universal SYBR qPCR Master Mix.

Table 1. Primers Used for Quantitative Real-Time PCR

| Gene name | Primers sequence 5’-3’ |
|-----------|----------------------|
| GAPDH     | F: AGTCGGTGTGAACGGATTG | R: TGTAGACCATGTAGTTGAGTC |
| RORγt     | F: GCCCACACCTCACAATGGA | R: AGTAGGCCCACTACCTGCT |
| IL-17     | F: GGAGTGGACGCTCGGAAAGA | R: GGAGCGGTTCTGGAATTCAC |
| Let-7g-5p | F: GGGTGAGGTAGTAGTTTGT | R: CAGTGCGTGTCGTGGAGT |

Fig. 1. CAT prevents the progression of arthritis in CIA mice. (A) Histopathological characteristics of mice joints. (B) Gross images of the hind limb of mice. (C) Arthritis scores of mice. (D) Histopathologic scores of CIA mice. (E) IL-2 levels in the plasma of mice. (F) IL-6 levels in the plasma of mice. (G) IL-21 levels in the plasma of mice. (H) IL-23 levels in the plasma of mice. Values are presented as a mean±SEM (n=6). *p<0.5, **p<0.01, ***p<0.001 compared with the model group. CAT, catalpol; CIA, collagen-induced arthritis; IL, interleukin.
Mix and miRNA Universal SYBR qPCR Master Mix (Vazyme) on an ABI 7500 thermocycler (Applied Biosystems Life Technologies, Foster City, CA, USA). Analyses were performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as invariant control (Table 1).

**Western blot**

Cells were lysed in a lysis buffer, and the protein content of the lysates was determined with bicinchoninic assay. Equal amounts of protein per sample were separated by 10% SDS-PAGE and subsequently transferred to PVDF membranes. The membranes were blocked for 2 h with 5% skimmed milk and then incubated with primary antibodies overnight at 4°C. The membranes then were incubated with secondary antibodies (1:5000 diluted) for 2 h. The blots were visualized with electrochemiluminescence detection kits. Western blot analyses were performed using the quantitative software Image Lab 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative protein expression of the target band was calculated by grayscale scanning (S): relative protein expression=S target protein/S internal reference.

**Statistical analysis**

Statistical significance was determined using GraphPad Prism Software (Version 8 for Windows; GraphPad Prism, San Diego, CA, USA). The data are presented as a mean±SEM, and 95% confidence intervals (CIs) were calculated. *p values of 0.05 or less were considered to be statistically significant.

**RESULTS**

CAT attenuates arthritis and inflammatory response in CIA mice

To verify the therapeutic effect of CAT on CIA, swelling of the hind paw and histopathological lesions in the joints of mice were investigated. Compared with normal control mice, CIA mice exhibited massive joint destruction and severe paw swelling (Fig. 1A, B, and C). In contrast, CAT (30 mg/kg/day) treatment had significant effects on the severity of joints inflammation. After CAT treatment, the observed joint destruction, clinical scores, and paw swelling significantly decreased (Fig. 1A, B, and C). Also, we quantified histopathological results, and we found that CIA mice after CAT treatment had less in-

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Fig. 2. The effects of CAT on CD4+IL-17+ Th17 cells and the expression of let-7g-5p in CIA mice. (A) The percentage of CD4+IL-17+ Th17 cells in PBMCs of CIA mice. (B) The percentage of CD4+IL-17+ Th17 cells in splenocytes from CIA mice. (C) Proportion of CD4+IL-17+ Th17 cells in PBMCs from CIA mice. (D) Proportion of CD4+IL-17+ Th17 cells in spleens from CIA mice. (E) IL-17 levels in plasma from CIA mice were measured by ELISA. (F) The mRNA levels of RORγt in splenic CD4+ T cells were detected by qPCR. (G) The mRNA levels of let-7g-5p in splenic CD4+ T cells were detected by qPCR. Values are presented as a mean±SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 compared with the model group. CAT, catalpol; CIA, collagen-induced arthritis; ELISA, enzyme-linked immunosorbsent assay; qPCR, quantitative polymerase chain reaction; PBMC, peripheral blood mononuclear cells; IL, interleukin.
flammmation and arthrosis than the model group (Fig. 1D). Additionally, CAT significantly decreased the levels of pro-inflammatory cytokines, including IL-2, IL-6, IL-21, and IL-23 (Fig. 1E-H). Taken together, these results indicated that CAT inhibits joint injury and inflammatory responses in CIA mice.

CAT decreases the ratio of CD4+ IL-17+ Th17 cells, but up-regulates let-7g-5p in CIA mice

PBMCs and splenocyte isolated from peripheral blood and the spleens of CIA mice were analyzed by flow cytometry to evaluate the proportion of CD4+IL-17+ Th17 cells in all mice. Compared with control mice, CIA mice showed markedly higher percentages of CD4+IL-17+ T cells (Fig. 2A-D). As expected, the ratio of CD4+IL-17+ T cells was markedly decreased by CAT treatment. Subsequently, we investigated the effects of CAT on Th17-related cytokines in plasma (Fig. 2E). The levels of IL-17 in CIA mice were higher than those in control mice. After CAT treatment, the levels of IL-17 decreased. RORγt is a transcription factor required for the differentiation and function of Th17 cells, and let-7g-5p has been shown to inhibit the differentiation of Th17 cells in our previous study.20 To determine the effect of CAT on RORγt and let-7g-5p expression, we evaluated their mRNA levels in CD4+T cells from the peripheral blood and spleen in DBA/1 mice. As shown in Fig. 2F and G, mRNA levels of RORγt markedly increased in CIA mice, and CAT treatment significantly inhibited the expression of RORγt mRNA. Meanwhile, CAT treatment elicited a dramatic increase in let-7g-5p mRNA, which was significantly inhibited in CIA mice. These results indicated that CAT might negatively regulate Th17 cell differentiation in CIA mice.

CAT suppresses the differentiation of Th17 cells in vitro

CAT treatment decreased the proportions of CD4+IL-17+ Th17 cells in CIA mice (Figs. 1 and 2). As the effect of CAT (20 μg/mL) on the differentiation of Th17 cells has been shown in previous research, we examined whether CAT could inhibit traditional differentiation of Th17 cells and Tregs-to-Th17 cell transdifferentiation in vitro. Not surprisingly, treatment with CAT (20 μg/mL) resulted in a significant reduction in the proportion of CD4+IL-17+ Th17 cells under traditional differentiation of Th17 cells (Fig. 3A and B). Similarly, CAT also reduced the proportion of CD4+IL-17+ Th17 cells in Tregs-to-Th17 cell transdifferentiation (Fig. 3C and D). Additionally, we evaluated the levels of IL-17 (Fig. 3C and G) and mRNA levels of the Th17 transcription factor RORγt (Fig. 3D and H) and found that CAT significantly inhibited IL-17 levels and the expression of RORγt mRNA. Taken together, CAT was deemed to suppress the traditional differentiation of Th17 cells and Tregs-to-Th17 cell transdifferentiation.

CAT suppresses Tregs-to-Th17 cell transdifferentiation by up-regulating let-7g-5p

In addition to suppression of the differentiation of Th17 cells in CIA mice, treatment with CAT also significantly increased let-
7g-5p expression, which has been found to inhibit Th17 cell differentiation in our previous research. To this end, we examined the expression of let-7g-5p in traditional differentiation of Th17 cells and Tregs-to-Th17 cell transdifferentiation to determine whether the effect of CAT is relevant to let-7g-5p. Interestingly, upon incubation with CAT, there was no significant change in the expression of let-7g-5p during traditional differentiation of Th17 cells (Fig. 4A). In contrast, CAT significantly increased the expression of let-7g-5p in Tregs-to-Th17 cell transdifferentiation (Fig. 4B). These findings demonstrated that the suppression of CAT on Tregs-to-Th17 cell transdifferentiation might be associated with let-7g-5p.

To verify the potential effect of let-7g-5p on Tregs-to-Th17 cell transdifferentiation, let-7g-5p mimics, inhibitors, and negative control RNAs were transfected into Treg cells and then cultured in the condition of Th17-polarization. A significant reduction in the proportion of CD4+IL-17+ Th17 cells was observed in the let-7g-5p mimics group relative to that of control mimics (Fig. 5A and B). Furthermore, IL-17 levels in culture supernatant, as well as the expression of IL-17 and RORγt mRNA, were notably repressed after let-7g-5p mimics transfection (Fig. 5C–E). In contrast, the proportion of CD4+IL-17+ Th17 cells, expression of RORγt mRNA, and IL-17 levels were elevated after let-7g-5p inhibitor transfection (Fig. 5). These findings indicated that let-7g-5p is capable of inhibiting Tregs-to-Th17 cell transdifferentiation.

The observed inhibition of let-7g-5p on Tregs-to-Th17 cell transdifferentiation spurred us to determine whether CAT suppresses Tregs-to-Th17 cell transdifferentiation by up-regulating let-7g-5p. Compared with let-7g-5p mimics, CAT treatment elicited a significant decrease in the proportion of CD4+IL-17+ Th17 cells (Fig. 6A and B). In addition, incubation with CAT resulted in further reductions in IL-17 protein levels and mRNA levels of IL-17 and RORγt (Fig. 6D, E, and F). Compared with the let-7g-5p inhibitor group, CAT decreased the proportion of CD4+IL-17+ Th17 cells, protein levels of IL-17 and mRNA levels of IL-17 and RORγt (Fig. 6A and B). These findings demonstrated that CAT could suppress Tregs-to-Th17 cell transdifferentiation by up-regulating let-7g-5p in vitro.

**CAT suppresses Tregs-to-Th17 cell transdifferentiation in relation to STAT3**

The STAT3 signaling pathway is a potential therapeutic target for RA. STAT3 plays a critical role in the differentiation and proliferation of Th17 cells. To investigate the mechanism by which CAT influences Tregs-to-Th17 cell transdifferentiation, the pro-
tein levels of STAT3 were analyzed. The protein and mRNA levels of STAT3 were notably repressed by let-7g-5p mimics treatment (Fig. 7A, C, and E). In contrast, let-7g-5p inhibitor treatment resulted in a significant increase in the protein and mRNA levels of STAT3. These results suggested a possible effect for let-7g-5p on the expression of STAT3. Subsequently, the effects of CAT on the expression of STAT3 were investigated. After CAT treatment, compared with the control mimics group and the control inhibitor group, the protein and mRNA levels of STAT3 were decreased significantly. Together, the results suggested that CAT could up-regulate let-7g-5p to decrease the expression of STAT3.

**Fig. 6.** CAT suppresses Tregs-to-Th17 cell transdifferentiation by up-regulating let-7g-5p in vitro. (A) Representative dot plots of CD4+IL-17+ Th17 cells in vitro. (B) Proportion of CD4+IL-17+ Th17 cells after let-7g-5p mimics transfection. (C) Proportion of CD4+IL-17+ Th17 cells after let-7g-5p inhibitor transfection. (D) IL-17 levels in culture supernatants after let-7g-5p mimics transfection were measured by ELISA. (E) mRNA levels of RORγt after let-7g-5p mimics transfection were detected by qPCR. (F) mRNA levels of IL-17 after let-7g-5p mimics transfection were detected by qPCR. (G) IL-17 levels in culture supernatants after let-7g-5p inhibitor transfection were measured by ELISA. (H) mRNA levels of RORγt after let-7g-5p inhibitor transfection were detected by qPCR. (I) mRNA levels of IL-17 after let-7g-5p inhibitor transfection were detected by qPCR. Values are presented as a mean±SEM (n=3). *p<0.5, **p<0.01, ***p<0.001 compared with the model group. CAT, catalpol; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction.
DISCUSSION

Expanding research has shown that Th17 cells are potent inducers of RA and that IL-17, which is secreted by Th17, increases during RA activity, causing cartilage destruction and bone erosion by activating synovial cells and stimulating the production of pro-inflammatory cytokines, such as TNF-α and IL-1.22,23 Our previous report indicated that decreased expression of let-7g-5p is associated with increases in Th17 cells.18 However, the mechanism of how let-7g-5p represses the differentiation of Th17 cells is still not known.

Emerging evidence has indicated that traditional Chinese medicine has a positive effect on RA.24-26 In this study, we investigated the effect of CAT from traditional Chinese medicine on Th17 cells differentiation. We first investigated the effect of CAT on murine CIA in CIA mice and found that Th17 cells and levels of the inflammatory cytokines IL-2, IL-6, IL-21, and IL-23 were markedly decreased after CAT treatment, suggesting that CAT might negatively regulate Th17 cell differentiation. Additionally, let-7g-5p mRNA was found to be negatively related with the proportion of Th17 cells after CAT treatment. We then investigated percentages of Th17 cells after CAT treatment in vitro experiments. As expected, results from flow cytometry, enzyme-linked immunosorbent assay, and qPCR demonstrated that both traditional differentiation of Th17 cells and Tregs-to-Th17 cell transdifferentiation were inhibited by CAT. However, treatment with CAT did not alter let-7g-5p mRNA during traditional differentiation of Th17 cells. On the contrary, mRNA levels
of let-7g-5p were elevated by CAT in Tregs-to-Th17 cell transdifferentiation. These data indicated that the effect of CAT on traditional differentiation of Th17 cells has nothing with let-7g-5p, whereas the effect of CAT on Tregs-to-Th17 cell transdifferentiation might be related with let-7g-5p.

The STAT3 signaling pathway, which plays a critical role in early Th17 cell development, is a downstream target in autoimmune diseases, such as RA.27-30 Recently, STAT3 has been shown to play a role in regulating Th17 cell function and differentiation.31,32 Inhibition of STAT3 expression has been found to inhibit Th17 cell differentiation.35,36 To explore further mechanisms of CAT inhibition of Tregs-to-Th17 cell transdifferentiation, we detected the expression of STAT3 by qRT-PCR and Western blot. We found that STAT3 was inhibited after transfection with let-7g-5p mimics, and the inhibition of STAT3 was more significant after CAT treatment. Taken together, these results suggested that CAT inhibits Tregs-to-Th17 cell transdifferentiation by up-regulating let-7g-5p to inhibit the expression of STAT3.

In conclusion, CAT appears to play an inhibiting role in the differentiation of Th17 cells and CIA symptoms. Additionally, CAT could inhibit Tregs-to-Th17 cell transdifferentiation by up-regulating let-7g-5p to reduce the expression of STAT3. However, the mechanism of how CAT inhibits traditional differentiation of Th17 cells needs to be explored further in the future. These data suggest the possible use of CAT in novel therapeutic regimens for treating autoimmune diseases such as RA.

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AUTHOR CONTRIBUTIONS
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