CircEpc1 Promotes Ricin Toxin-Induced Inflammation Via Activation of NF-κB And MAPK Signaling Pathways By Sponging miR-5114

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Research

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Abstract

**Background:** Increasing studies have concentrated on investigating circular RNAs (circRNAs) as pivotal regulators in the progression of numerous diseases and biological processes and abundant evidence shows that circRNAs are participated in the regulation of innate immune responses. Several studies showed that Ricin Toxin (RT) could induce inflammatory injury. There was no research on the particular functions and underlying mechanisms of circRNAs in RT-induced inflammation.

**Results:** In this study, RNA sequencing performed on RT-treated and normal RAW264.7 macrophage cells was used to investigated the differentially expressed circRNAs. Based on the dataset, the expression of circEpc1 (mmu_circ_0000842) was identified higher in RT-treated cells. Moreover, gain-and-loss function assays showed that circEpc1 function as a promoter in RT-induced inflammation in vivo and in vitro. Mechanistically, circEpc1 acted as a miR-5114 sponge to relieve the suppressive effect of miR-5114 on its target NOD2 and thereby activating NF-κB and MAPK signaling pathways.

**Conclusions:** Our results illuminated a link between RT-induced inflammation and the circEpc1 regulatory loop and provided novel insight into the functions of circRNA in innate immune, which may emerge as a potential target in immunotherapy to control the RT-induced inflammatory injury.

**Background**

Ricin toxin (RT) is a natural plant toxin derived from the seeds of the castor plant (*Ricinus communis*), which is widely cultivated and processed worldwide [1]. Because of the extreme toxicity of RT, incidents of human and animal poisoning or death due to contact or accidental ingestion of RT have been reported continually [2]. Considering the mechanism of RT cytotoxic, it was identified as type II ribosome-inactivating protein (RIP) [3]. Moreover, the damage of the 28S rRNA by RT triggers a specific kinase-activated pathway and induces inflammation [4]. Based on our previous work, RT-treated macrophages release various types of cellular factors, including IL-6, TNF-α, IFN-β, and other cytokines [5, 6]. When these cellular events occur, an intracellular signaling cascade is activated through various pattern-recognition receptors (PRRs) spoke on macrophages. One of the most critical PRRs involved in inflammation is nucleotide-binding oligomerization domain 2 (NOD2) which recognizes bacterial cell walls [7, 8]. Activation of NOD2 results in activation of multiple signaling pathways, such as NF-κB and MAPK pathways, and ultimately leads to types of inflammation responses.

CircRNAs, a novel class of non-coding RNAs, as a type of regulatory RNAs have attracted extensive research interest. Structurally, circRNAs are characterized by closed continuous loop structures with neither 5'-3' polarity nor a polyadenylated tail [9]. The majorities of circRNAs are abundant, highly conserved across species, and tissue or developmental-stage specific [10, 11]. Compared with their linear counterparts, circRNAs exhibit higher stability due to the loop structure being resistant to RNase R in vivo [12].
Currently, with the rapid development of high-throughput sequencing techniques and bioinformatics, RNomics has gradually become a focus of attention. Thus, accumulated knowledge of characteristics and functions of circRNAs is beginning to be understood. Studies have shown that circRNAs play essential roles in human diseases, including atherosclerotic vascular disease [13], neurological disorders [14], cardiovascular disease [15], and cancer [16, 17]. Emerging evidence has shown that circRNAs have been identified as competing endogenous RNAs (ceRNAs) that function as miRNA sponges via complementary base paring [18]. For instance, the circRNA ciRS-7 inhibits miRNAs in murine tissue by sponging miR-7 [19]. Additionally, besides the ceRNA mechanism, circRNAs can also interact with RNA-binding proteins (RBP) to regulate target gene expressions and some of them can encode functional proteins [20–22]. These studies strongly confirm that circRNAs play a fundamental role in a variety of cellular processes. Our previous study have confirmed that RT can cause inflammation in RAW264.7 macrophage cells [23]. Researches have showed that circRNAs plays an important role in the occurrence and progression of many diseases. However, the biological effects and underlying mechanisms of circRNAs in RT-induced inflammation have not been explored comprehensively.

In this study, we investigate the expression profiling of circRNAs in RT-treated and normal RAW264.7 macrophage cells by RNA-Seq. We found a significant upregulated circRNA, mmu_circ_0000842, designated as circEpc1, is initially identified which may promote inflammation. Mechanistically, circEpc1 functions as miRNA sponge to regulate the expression of NOD2 by competitive binding to miR-5114, leading to the activation of NF-κB and MAPK pathways. Collectively, our results revealed that the circEpc1/miR-5114/NOD2 axis has a considerable role in RT-induced inflammation, identifying circEpc1 as a potential biomarker and therapeutic target for RT-induced inflammatory injury.

Results

Differential expression of circRNAs in RT-treated RAW264.7 macrophage cells

To identify circRNAs that are crucial to RT-induced inflammation, RNA-seq was performed for RT-treated RAW264.7 macrophage cells and matched normal RAW264.7 macrophage cells. As shown in Fig. 1A, a total of 4273 dysregulated circRNAs were identified in RT-treated RAW264.7 cells, of which 1634 circRNAs were upregulated and 2639 circRNAs were downregulated. In addition, we found that 17 circRNAs were significantly upregulated, and 6 circRNAs were significantly downregulated in RT-treated RAW264.7 cells (filtered by fold change ≥ 2 and P < 0.05) (Fig. 1B, C).

Among these circRNAs, we focused on the top 8 significantly dysregulated circRNAs to verify their abundance in RT-treated cells based on their fold change. Interestingly, a circRNA (circRNA ID in circbase: mmu_circ_0000842, http://www.circbase.org/, termed circEpc1 in the remainder of the article), which was derived from the Epc1 gene locus and most highly upregulated circRNA attracted our attention (Fig. 1D). To further investigate the expression level of circEpc1 according to the RNA-seq data, we detected higher circEpc1 expression in RT-treated RAW264.7 cells via qRT-PCR, which was consistent with the RNA-seq
data (Fig. 1E). Taken together, these results suggested that circEpc1 as an upregulated circRNA participate in RT-induced RAW264.7 macrophage inflammation.

**Identification of the circular structure of circEpc1**

CircEpc1 arises from the Epc1 gene, which is located at chromosome 18 and consists of the head-to-tail splicing of exon 3 and exon 8 (6448902–6455334). Sanger sequencing was performed to validate its back-splicing using the RT-PCR product of circEpc1. The sequence is consistent with circBase database annotation (http://www.circbase.org/) (Fig. 2A). To further confirm the circular form of circEpc1, we designed divergent and convergent primers to amplify the circular and linear forms of Epc1, respectively. RT-PCR was performed to detected the expression level of circular and linear forms of Epc1 with or without RNase R supplementation in the reverse-transcribed RNA (cDNA) and genomic DNA (gDNA) of RAW264.7 cells. Results showed that divergent primers could amplify products from cDNA but not from gDNA (Fig. 2B). Moreover, analysis for stability of circEpc1 and linear Epc1 in RAW264.7 cells treated with Actinomycin D, an inhibitor of transcription, showed that circEpc1 was more stable than linear Epc1 (Fig. 2C). In addition, the nuclear-cytoplasmic fractionation results revealed that circEpc1 was primarily located in the cytoplasm rather than nuclear (Fig. 2D). These results further confirm the characteristics of circEpc1 as a circRNA and imply that its function may be benefited from the biological stability.

**CircEpc1 acts as a facilitator in RT-induced inflammation of RAW264.7 cells**

Next, we examined the expression of circEpc1 in RT-treated and normal RAW264.7 cells via qRT-PCR. The expression of circEpc1 was significantly increased in RT-treated cells (Fig. 3A). Meanwhile, based on our previous work, ELISA assays showed that the secretion of TNF-α and IL-6 in the supernatant of RT-treated cells was significantly higher than that of the control group [6]. Collectively, these results suggested that circEpc1 may be involved in RT-induced inflammation.

To investigate the role of circEpc1 in RT-induced inflammation, two small hairpin RNAs (sh-circEpc1#1 and sh-circEpc1#2) were constructed to target the unique back-splicing junction of circEpc1. The shRNAs significantly decreased circEpc1 expression without decreasing the linear Epc1 mRNA level. In addition, RAW264.7 cells were also transfected with the circEpc1 lentivirus vector to overexpress circEpc1. The efficiency and specificity of circEpc1 knockdown and overexpression in RAW264.7 cells verified by qRT-PCR (Fig. 3B, C). However, after knockdown of circEpc1, the secretion of TNF-α and IL-6 was significantly decreased. On the contrary, the overexpression of circEpc1 showed the opposite result. (Fig. 3D). Taken together, these data suggest that circEpc1 played as a facilitator in RT-induced inflammation.

**MiR-5114 is sponged by circEpc1 and plays a negative role in RT-induced inflammation**

Extensive studies have reported that circRNAs mostly function as miRNAs sponge to regulate downstream genes [24, 25]. Therefore, taking the sequence of circEpc1 as a bait, we constructed a circEpc1-miRNAs-NOD2 network and performed a cross-analysis using two miRNA target prediction
online databases (TargetScan and miRanda). This network included 3 candidate miRNAs (miR-1930-3p, miR-5114, and miR-719), containing common binding sites for the circEpc1 and NOD2 (Fig. 4A). Based on our previous work, RNA-Seq technology was used to perform an analysis of the miRNA profiles of RT-treated RAW264.7 macrophage cells. Interestingly, we found that the expression of miR-5114 was significantly downregulated and the same results were obtained by qRT-PCR assays in RT-treated RAW264.7 cells (Fig. 4B), suggesting that there may be a regulatory relationship between circEpc1 and miR-5114.

To confirm miR-5114 could be regulated by circEpc1, a dual-luciferase reporter assay was used to determine the direct binding between circEpc1 and miR-5114 based on their complementary sequences. We constructed luciferase reporters containing wild type and mutated putative binding sites of circEpc1 transcripts (Fig. 4C). Then, we co-transfected a miR-5114 mimic with the reporter gene into HEK293T cells. Results of luciferase reporter assays showed that the luciferase activities of circEpc1 wild type reporter were significantly reduced when transfected with miR-5114 mimics compared with control reporter or mutated luciferase reporter (Fig. 4D). Thus, the direct interaction between circEpc1 and miR-5114 was confirmed. Furthermore, FISH analysis was performed in RT-treated RAW264.7 cells, and we found that miR-5114 was co-localized with circEpc1 in the cytoplasm, which further verification the interaction between circEpc1 and miR-5114 (Fig. 4E).

To study the function of miR-5114, miR-5114 mimics was separately transfected into RAW264.7 cells and its effect on the expression of miR-5114 was detected (Fig. 4F). Moreover, we found that, miR-5114 mimics significantly suppressed the TNF-a and IL-6 secretion, and this effect could be reversed after transfection with circEpc1 overexpressing lentivirus vector (Fig. 4G). In conclusion, these results revealed that miR-5114 played a negative role in RT-induced inflammation and circEpc1 serves as a sponge of miR-5114.

CircEpc1 facilitates RT-induced inflammation by upregulating the expression of NOD2 and activating the NF-κB and MAPK signaling pathways

With the aim to detect the detailed mechanism of circEpc1 in RT-induced inflammation, we focused on a gene involved in inflammation responses to get some key clues. Increasing evidences demonstrate that NOD2, an intracellular PRR, plays an important role in innate immune regulation. Once activated, NOD2 oligomerizes and interacts with the serine/threonine kinase receptor-interacting protein 2 (RIP2) though the CARD domains, resulting in the activation of NF-κB and MAPK signaling pathways [26–28]. Here, we investigated whether there is interaction between circEpc1, miR-5114 and NOD2.

Online bioinformatics predictions (TargetScan) indicated that miR-5114 possesses binding sites that are potentially complementary to NOD2 (Fig. 5A). Intriguingly, NOD2 and RIP2 were both significantly increased in RT-treated RAW264.7 cells at mRNA and protein levels according to the results of qRT-PCR
and Western blot assays (Fig. 5B, C), suggesting that NOD2 expression was positively correlated with the circEpc1 expression level in RT-induced inflammation. According to the NOD-Like Receptor signaling pathway shown in Fig. 5D, NOD2 mediates the expression of inflammatory cytokines by activating downstream NF-κB and MAPK signaling pathways. Thus, we confirmed this signal transduction mechanism in RT-treated RAW264.7 cells (Fig. 5E, F). Furthermore, the regulatory function of circEpc1 was also confirmed in lentivirus-constructed cell lines (Fig. 5G, H). Sum up the above results, CircEpc1 facilitates RT-induced inflammation by upregulating the expression of NOD2 and activating the NF-κB and MAPK signaling pathways.

**CircEpc1 promotes RT-induced inflammation in vivo**

To evaluate the contribution of circEpc1 to RT-induced inflammation in vivo, the following animal experiment was conducted. Mice were challenged with RT by means of liquid aerosol lung delivery, and the effects of challenge dose and time on the secretion of TNF-α and IL-6 in BALF of mice were evaluated via ELISA (Fig. 6A). Based on the results, treatment with RT (2 μg/mL) for 24 h was took as the condition of the follow-up animal experiment. To further investigate the effects of circEpc1 in vivo, gain-and-loss function assays were performed through AAV transfection system. Mice were randomly divided into the following three groups: NC, OE-circEpc1, and sh-circEpc1. The transfection effect was detected by ex vivo fluorescence imaging system and qRT-PCR after transfection 3 weeks (Fig. 6B, C). Meanwhile, overexpression of circEpc1 significantly suppressed the expression of miR-5114 (Fig. 6D). In the case of exposed to RT (2 μg/mL), the protein level of NOD2 and RIP2 were significantly upregulated in OE-circEpc1 group, but showed the opposite trend in sh-circEpc1 group (Fig. 6E). Furthermore, to investigate the potential pro-inflammatory function of circEpc1, H&E staining was used to detect inflammatory injury in lung tissue (Fig. 6F). Following RT exposure, the alveolar wall was thickened, there was extensive neutrophil infiltration, and a large number of inflammatory cell foci appeared around the blood vessels. It was further showed that circEpc1 could significantly promote the secretion of TNF-α and IL-6 (Fig. 6G). These results suggested that circEpc1 functions as a promoter in RT-induced inflammation in mice.

**Discussion**

Currently, due to the supreme cytotoxicity of RT, it is still a great challenge to deal with the severe inflammatory injury caused by inhaled RT. However, although significant improvements have been made in diagnosis and treatment strategies, there is no effective and approved treatment for RT inhalation poisoning, so new and effective treatments for this injury are needed. Growing evidence indicates that circRNAs, as novel noncoding RNAs, play important roles in multiple inflammatory responses and may be involved in the pleiotropic modulation of cellular functions [29, 30]. Thus, it is essential to analyze the differentially expressed circRNAs in RT-treated RAW264.7 macrophage cells and elucidate its basic mechanism in RT-induced inflammation.

In the present study, we profiled circRNA expression in RT-treated and normal RAW264.7 macrophage cells by RNA-Seq, resulting in the identification of 23 significantly differentially expressed circRNAs, including 17 upregulated and 6 downregulated circRNAs. We identified circEpc1 as a significantly
upregulated circRNA in RT-treated RAW264.7 cells and RT-injured mice. Gain-and-loss function assays suggested that the circEpc1 promotes RT-induced inflammation through circEpc1/miR-5114/NOD2 axis (Fig. 7).

Our circRNA high-throughput sequencing and qRT-PCR results showed that circEpc1 is significantly overexpressed in RT-treated RAW264.7 cells compared with the normal RAW264.7 cells, and it was the first time to scan circRNAs which play a promoting role in RT-induced inflammation. To elucidate the detailed mechanism underlying circEpc1 function as a facilitator in RT-induced inflammation, we constructed cell lines with circEpc1 overexpression and knockdown using lentiviral vectors. CircEpc1 overexpression significantly promotes the secretion of TNF-α and IL-6 in RAW264.7 cells, indicating a pro-inflammatory effect of circEpc1 in RT-induced inflammatory injury and its potential value as a biomarker for the diagnosis of RT poisoning.

Although it is not clear how circRNAs regulate the progression of inflammation, studies have shown that they may function as miRNA sponges to regulate the expression of target genes via the miRNA response element [31]. It has been reported that CircBbs9 can promote PM2.5-induced lung inflammation by activating NLRP3 via sponge miR-30e-5p [32]. In addition, circGLIS2 abnormally activates NF-κB signaling pathway through miR-671 sponge mechanism in colitis cells [33]. Moreover, circRNA, which is widely located in the cytoplasm, often acts as a miRNA sponge [34]. We confirmed that circEpc1 was mainly located in the cytoplasm by nucleocytoplasmic separation experiments. Furthermore, bioinformatics analysis showed that there were potential binding sites between circEpc1 and miR-5114. Our results showed that circEpc1 contains the binding site of miR-5114, and it could bind to miR-5114 in RT-treated RAW264.7 cells as verified by FISH and Dual-Luciferase reporter assays. In addition, we also constructed cell lines with miR-5114 overexpression using lentiviral vectors. Compared with the control cells, the secretion of TNF-α and IL-6 in miR-5114 overexpression cells was significantly downregulated. On the contrary, when co-transfected with miR-5114 overexpression and circEpc1 overexpression lentivirus vectors, this trend was reversed. Therefore, we concluded that miR-5114 is sponged by circEpc1 and plays a negative role in RT-induced inflammation.

To further explore the regulatory mechanism of circRNA as ceRNA, we identified a target gene NOD2 which can bind to miR-5114 through TargetScan. NOD2 belongs to the intracellular pathogen recognition receptors (PRRs) family, which can sense bacterial peptidoglycan (PGN) and muramyl dipeptide (MDP) [35]. Furthermore, NOD2 regulates multiple signaling pathways involved in a variety of cellular responses, including inflammatory responses via activation of NF-κB, MAPK, and type I IFNs, and autophagy [36]. Recent studies suggest that inhibition of NOD2 signaling pathway may be beneficial to the development of inflammatory disorders [37, 38]. Therefore, we thought that RT-induced RAW264.7 cells inflammation may due to NF-κB and MAPK activation downstream of NOD2 signaling pathway. Studies have shown that RIP2 is the essential adaptor kinase in NOD2 signaling pathway, and formation of the NOD2-RIP2 multiprotein complex after ligand recognition drives NF-κB/MAPK activation [39, 40]. In this study, we found that the expressions of NOD2 and RIP2 were significantly upregulated in RT-treated RAW264.7 cells via qRT-PCR and WB analysis. Furthermore, the phosphorylation of IκB-α, p38, ERK, and JNK in RAW264.7
cells were increased following RT treatment. Studies on the regulatory mechanism of ceRNA confirmed that circEpc1 upregulated the expression of NOD2 and RIP2. Compared with the control cells, the phosphorylation of IκB-α, p38, ERK, and JNK in circEpc1 overexpression cells was significantly increased, while miRNA reversed these results. Above all, circEpc1 functions as a ceRNA to up-regulate the expression of NOD2 by competitive binding to miRNA-5114, leading to the progression of RT-induced inflammation via NF-κB and MAPK signaling pathways.

Presently, there are relatively few reports about the role of circRNAs in innate immunity and our study provided a novel insight into the role of circRNA in RT-induced inflammation. For this study, only the function of circEpc1 as a miRNA sponge has been explored. However, besides this mechanism, circRNAs can interact with RNA-binding protein to regulate gene expressions and encode functional proteins, indicating there may be other potential mechanisms for the role of circEpc1 in RT-induced inflammation and further research is needed.

Conclusions

In summary, our findings reveals that circEpc1 competitively binds miR-5114 to abolish the suppressive effect of miR-5114 on NOD2, then promotes RT-induced inflammation via NF-κB and MAPK signaling pathways. The data provides a link between circRNAs, NOD2 signaling pathway and innate immunity. Based on this mechanism, it is believed that the circEpc1/miR-5114/NOD2 axis has great potential as a new biomarker and new therapeutic target for RT-induced inflammation in the future.

Methods

Cell line and culture

Mouse mononuclear macrophage cell line RAW264.7 was purchased from the American Type Culture Collection (ATCC, VA, USA). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂ and maintained in RPMI-1640 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, UT, USA), 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen, CA, USA).

circRNA library construction and sequencing

RAW264.7 cells were prepared as described previously [6]. RT (20 ng/mL) was used to treat cells for 8 h. Total RNA was extracted using TRIzol reagent (Takara, Tokyo, Japan) following the manufacturer’s instructions. The total RNA quantity and purity were analysis of Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number >7.0. Approximately 10 ug of total RNA representing a specific adipose type was used to deplete ribosomal RNA according to the manuscript of the Epicentre Ribo-Zero Gold Kit (Illumina, San Diego, USA). Following purification, the poly(A)- or poly(A)+ RNA fractions is fragmented into small pieces using divalent cations under elevated temperature. Then the cleaved RNA fragments were reverse-transcribed to create the final cDNA library in accordance with the protocol for the RNA-Seq sample preparation kit (Illumina, San Diego, USA), the average insert size for the
paired-end libraries was 300 bp (±50 bp). And then we performed the paired-end sequencing on an Illumina Hiseq4000 at the (LC Sciences, USA) following the vendor’s recommended protocol. CircRNA expressions from different samples or groups were calculated by scripts in house. Only the comparisons with P < 0.05 were regarded as showing differential expression.

**RNA extraction, reverse transcription, and qRT-PCR analysis**

Total RNA was extracted from cultured cells and lung tissue using TRIzol reagent (Takara, Tokyo, Japan) according to the manufacturer's instructions. gDNA was extracted using Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). Reverse transcription was performed using Oligo (dT) primer for mRNA into cDNA with M-MLV Reverse Transcriptase, RNase H- (Takara, Tokyo, Japan). A Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) was used to verify the existence of circRNAs. The qRT-PCR was conducted using Applied Biosystems QuantStudio 3 Real-Time PCR Systems (Thermo Fisher Scientific, MA, USA) with SYBR Green PCR Master Mix (Takara, Tokyo, Japan). The expression levels of miRNA were determined using an All-in-One miRNA qPCR Kit (GeneCopoeia, MD, USA). β-actin was measured as an endogenous control for mRNA and circRNA, and U6 was used as a control for miRNA. The relative fold-change in expression with respect to a control sample was calculated by the $2^{-\Delta\Delta Ct}$ method. Relative primers are shown in Table S1.

**RNase R treatment**

2 μg of total RNA was incubated with or without 5 U/μg RNase R at 37°C for 30 min in the buffer provided with the kit (Epicentre Technologies, Wisconsin, USA), and subsequently purified by RNeasy MinElute Cleaning Kit (Qiagen), then analyzed via RT-PCR.

**Nucleocytoplasmic separation and RNA isolation**

PARIS Kit (Invitrogen, CA, USA) was used to separately isolate nuclear and cytoplasmic RNA from cultured cells, according to the manufacturer's protocol.

**Actinomycin D assay**

RAW264.7 cells were exposed to 2 μg/ml actinomycin D (Sigma Aldrich, St. Louis, MO, USA) at indicated time point. Then the cells were harvested, and total RNA was extracted. The stability of circEpc1 and Epc1 mRNA was analyzed using qRT-PCR.

**Lentivirus vector constructs and cell transfection**

For in vitro studies, the lentivirus vectors were constructed using the pHBLV-CMV-Circ-MCS-EF1-ZsGreen-T2A-PURO vector purchased from Hanbio Biotechnology (Shanghai, China), and further confirmation was obtained by Sanger Sequencing. The circEpc1 overexpressing lentivirus vector, two shRNAs (sh-circEpc1#1 and sh-circEpc1#2) targeting circEpc1, and miR-5114 mimics were also designed and synthesized by HanBio. Cell transfection was performed as we described previously [6]. Then, 5 μg/mL of
Puromycin (MedChemExpress, NJ, USA) was used to select the stably infected cells. The transfection efficiency was determined by qRT-PCR.

**Dual-Luciferase reporter assay**

Luciferase assays were performed using the DLR Assay System (Promega, WI, USA) according to the manufacturer's instructions. Briefly, the HEK293T cells were co-transfected by Lipofectamine 3000 (Invitrogen, CA, USA) with miR-5114 mimics or nonsense control (NC) mimics (Sequence: 5′-AGAACGUCGAAGGCAGAGGUCA-3′) and luciferase reporter constructs containing the wild-type or mutant 3′-UTR of circEpc1. The mimics and luciferase reporter constructs were purchased from Hanbio (Shanghai, China). The cells were lysed 24 h after transfection, then Renilla luciferase (RLuc) and Firefly luciferase (Fluc) activities were measured on a Synergy 2 luminometer (BioTek, USA). Rluc signals were normalized to the intraplasmid Fluc transfection control.

**Fluorescence in situ hybridization (FISH)**

For FISH, the RT-treated RAW264.7 cells were incubated using specific probes of circEpc1 and miR-5114 according to user manual of RNA FISH Kit (GenePharma, Shanghai, China). The cells and fluorescence-labeled probes were hybridized in a hybridization buffer and hybridized overnight at 37°C. The next day, after stringent washing with SSC buffer, the nucleus were counterstained with DAPI. Images were acquired using a LSM-780 confocal laser scanning microscope (Carl Zeiss, Germany) and digitized with a software program Zen Light Edition.

**Animal experiments**

Male BALB/c mice aged 6-8 weeks, weighting 20-25 g, were purchased from the Liaoning Changsheng biotechnology co., Ltd (Benxi, China). To explore the regulatory function of circEpc1 in vivo, the adeno-associated virus (AAV) circEpc1 overexpressing, shRNA targeting circEpc1, and negative control (NC) were constructed and packaged by HanBio. The mice were randomly divided into three groups (NC, circEpc1-overexpression, and sh-circEpc1) with eight mice in each group. One week after the mice have acclimated to the environment, a total of 50 µL solution containing above virus or RT was slowly injected into trachea by means of liquid aerosol lung delivery. All mice were sacrificed three weeks after the injection, and the lungs were cut out for histopathological analysis.

**Ex vivo fluorescence imaging**

In order to detect the transfection effect of AAVs in vivo, we carried out Ex vivo tissue fluorescence imaging experiment. Then, under anesthesia, three weeks after AAVs transfection, the imaging system was used for fluorescence imaging of lung tissue in mice. Maestro software was used to remove the mouse background fluorescence.

**Histopathological analysis**
Histopathological analysis was performed according to the manufacturer's protocol. After injection, the mouse lungs were cut out and immersed in 4% paraformaldehyde overnight, embedded in paraffin and then sections 4 μm thick were cut. The sections were dehydrated in xylene and ethanol successively. Hematoxylin-eosin (H&E) staining was performed to evaluate the morphological variation in lung and inflammatory cell infiltration. Sections were microscopically examined using Nikon (Eclipse 80i; Tokyo, Japan), and the images were acquired on the imaging system (digital sight DS-FI2, Nikon, Japan).

**Western blot analysis**

Protein of RT-treated RAW264.7 cells and lung tissue were homogenized in RIPA lysis and extraction buffer (Thermo Fisher Scientific, MA, USA) supplemented with PMSF. A BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) was used to quantify the concentration of protein samples in the cell lysates. Then, the proteins were separated by 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Following blocking the PVDF membranes with 5% nonfat milk (BD Biosciences, Franklin Lakes, NJ, USA) in 0.1% Tween-20 TBST buffer at room temperature for 1 h, the membranes incubated with antibodies included NOD2 (1:2000, proteintech, 66710), RIP2 (1:1000, Abcam, ab8428), p65 (1:2000, proteintech, 66535), IκB-α (1:4000, Abcam, ab32518), P-IκB-α (1:1000, CST, 28595), p38 (1:2500, BD Biosciences, 612280), p-p38 (1:5000, BD Biosciences, 612168), ERK (1:4000, BD Biosciences, 610030), p-ERK (1:1000, BD Biosciences, 612358), JNK (1:250, BD Biosciences, 612540), p-JNK (1:250, BD Biosciences, 610627) overnight at 4°C. β-actin (1:10000, proteintech, 66009) was used as the internal protein loading control. After being washed 5 times in TBST for 50 min, membranes were incubated with HRP-labelled secondary antibody at room temperature for 1 h. Finally, immunodetection was performed using an enhanced chemiluminescence (ECL) detection system with Chemiluminescence HRP Substrate (GE Healthcare, Buckinghamshire, UK).

**Cytokine assays**

After RT treated, mice lungs were irrigated three times with 1.0 mL PBS. The collected bronchoalveolar lavage fluid (BALF) was immediately centrifuged to separate the cells and supernatant. Measurement of cytokine in the supernatants of BALF and RT-treated RAW264.7 cells was carried out using Mouse TNF-α ELISA kit (Dakewe, Shenzhen, China) and Mouse IL-6 ELISA kit (Biolegend, San Diego, CA, USA) according to the manufacturer’s instructions.

**Statistics**

We performed our experiments in triplicate, and the results are presented as mean ± standard deviation of the mean. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA), and consisted of analysis of variance followed by Student’s t-test when comparing two experimental groups. P < 0.05 was considered statistically significant, P < 0.01 and P < 0.001 were considered indicative of highly significant difference.

**Declarations**
Authors’ contributions

M.D., X.Z., and H.Y.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing. N.X. and W.L.: conception and design, supervision of the study, and final approval of manuscript. Y.W., Y.C., C.S., and J.Z.: design, collection and/or assembly of data, data analysis and interpretation, and statistical analysis; N.Z., K.Y., G.S., and G.Z.: collection and/or assembly of data. All authors read and commented on the article. The authors read and approved the final manuscript.

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Availability of data

This RNA-seq data are deposited in the NCBI Gene Expression Omnibus (GEO) datasets under the accession number GSE175619 (https://www.ncbi.nlm.nih.gov/geo/).

Ethics approval and consent to participate

All animal experiments were performed with the approval from the Institutional Animal Care and Use Committee of Changchun Veterinary Research Institute. Consent to participate is not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Audi J, Belson M, Patel M, Schier J, Osterloh J. Ricin poisoning: a comprehensive review. JAMA. 2005;294(18):2342–51. http://doi.org/10.1001/jama.294.18.2342.

2. Assiri AS. Ricin poisoning causing death after ingestion of herbal medicine. Ann Saudi Med. 2012;32(3):315–7. http://doi.org/10.5144/0256-4947.2012.315.
3. Endo Y, Mitsui K, Motizuki M, Tsurugi K. The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28 S ribosomal RNA caused by the toxins. J Biol Chem. 1987;262(12):5908–12.

4. Sowa-Rogozinska N, Sominka H, Nowakowska-Golacka J, Sandvig K, Slominska-Wojewodzka M. Intracellular Transport and Cytotoxicity of the Protein Toxin Ricin. Toxins (Basel). 2019;11(6):http://doi.org/10.3390/toxins11060350.

5. Liao P, Li Y, Li H, Liu W. Organellar proteome analyses of ricin toxin-treated HeLa cells. Toxicol Ind Health. 2016;32(7):1166–78. http://doi.org/10.1177/0748233714549066.

6. Dong M, Yu H, Wang Y, Sun C, Chang Y, Yin Q, Zhao G, Xu N, Liu W. Critical role of toll-like receptor 4 (TLR4) in ricin toxin-induced inflammatory responses in macrophages. Toxicol Lett. 2020;321:54–60. http://doi.org/10.1016/j.toxlet.2019.12.021.

7. Chen G, Shaw MH, Kim YG, Nunez G. NOD-like receptors: role in innate immunity and inflammatory disease. Annu Rev Pathol. 2009;4:365–98. http://doi.org/10.1146/annurev.pathol.4.110807.092239.

8. Gresnigt MS, Cunha C, Jaeger M, Goncalves SM, Malireddi RKS, Ammerdorffer A, Lubbers R, Oosting M, Rasid O, Jouvin G, et al. Genetic deficiency of NOD2 confers resistance to invasive aspergillosis. Nat Commun. 2018;9(1):2636. http://doi.org/10.1038/s41467-018-04912-3.

9. Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. Nat Biotechnol. 2014;32(5):453–61. http://doi.org/10.1038/nbt.2890.

10. Rybak-Wolf A, Stottmeister C, Glazar P, Jens M, Pino N, Giusti S, Hanan M, Behm M, Bartok O, Ashwal-Fluss R, et al. Circular RNAs in the Mammalian Brain Are Highly Abundant, Conserved, and Dynamically Expressed. Mol Cell. 2015;58(5):870–85. http://doi.org/10.1016/j.molcel.2015.03.027.

11. Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO. Cell-type specific features of circular RNA expression. PLoS Genet. 2013;9(9):e1003777. http://doi.org/10.1371/journal.pgen.1003777.

12. Li Y, Zheng Q, Bao C, Li S, Guo W, Zhao J, Chen D, Gu J, He X, Huang S. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. Cell Res. 2015;25(8):981–4. http://doi.org/10.1038/cr.2015.82.

13. Holdt LM, Stahringer A, Sass K, Pichler G, Kulak NA, Wilfert W, Kohlmaier A, Herbst A, Northoff BH, Nicolaou A, et al. Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. Nat Commun. 2016;7:12429. http://doi.org/10.1038/ncomms12429.

14. Lukiw WJ. Circular RNA (circRNA) in Alzheimer's disease (AD). Front Genet. 2013;4:307. http://doi.org/10.3389/fgen.2013.00307.

15. Lei K, Bai H, Wei Z, Xie C, Wang J, Li J, Chen Q. The mechanism and function of circular RNAs in human diseases. Exp Cell Res. 2018;368(2):147–58. http://doi.org/10.1016/j.yexcr.2018.05.002.

16. Guarnerio J, Bezzi M, Jeong JC, Paffenholz SV, Berry K, Naldini MM, Lo-Coco F, Tay Y, Beck AH, Pandolfi PP. Oncogenic Role of Fusion-circRNAs Derived from Cancer-Associated Chromosomal Translocations. Cell. 2016;166(4):1055–6. http://doi.org/10.1016/j.cell.2016.07.035.

17. Guarnerio J, Bezzi M, Jeong JC, Paffenholz SV, Berry K, Naldini MM, Lo-Coco F, Tay Y, Beck AH, Pandolfi PP. Oncogenic Role of Fusion-circRNAs Derived from Cancer-Associated Chromosomal
Translocations. Cell. 2016;165(2):289–302. http://doi.org/10.1016/j.cell.2016.03.020.

18. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. Nature. 2013;495(7441):384–8. http://doi.org/10.1038/nature11993.

19. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature. 2013;495(7441):333–8. http://doi.org/10.1038/nature11928.

20. Du WW, Yang W, Liu E, Yang Z, Dhaliwal P, Yang BB. Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. Nucleic Acids Res. 2016;44(6):2846–58. http://doi.org/10.1093/nar/gkw027.

21. Yang Y, Gao X, Zhang M, Yan S, Sun C, Xiao F, Huang N, Yang X, Zhao K, Zhou H, et al. Novel Role of FBXW7 Circular RNA in Repressing Glioma Tumorigenesis. J Natl Cancer Inst. 2018;110(3):http://doi.org/10.1093/jnci/djx166.

22. Legnini I, Di Timoteo G, Rossi F, Morlando M, Briganti F, Sthandier O, Fatica A, Santini T, Andronache A, Wade M, et al. Circ-ZNF609 Is a Circular RNA that Can Be Translated and Functions in Myogenesis. Mol Cell. 2017;66(1):22–37 e29. http://doi.org/10.1016/j.molcel.2017.02.017.

23. Xu N, Yuan H, Liu W, Li S, Liu Y, Wan J, Li X, Zhang R, Chang Y. Activation of RAW264.7 mouse macrophage cells in vitro through treatment with recombinant ricin toxin-binding subunit B: involvement of protein tyrosine, NF-kappaB and JAK-STAT kinase signaling pathways. Int J Mol Med. 2013;32(3):729–35. http://doi.org/10.3892/ijmm.2013.1426.

24. Yu J, Xu QG, Wang ZG, Yang Y, Zhang L, Ma JZ, Sun SH, Yang F, Zhou WP. Circular RNA cSMARCA5 inhibits growth and metastasis in hepatocellular carcinoma. J Hepatol. 2018;68(6):1214–27. http://doi.org/10.1016/j.jhep.2018.01.012.

25. Han D, Li J, Wang H, Su X, Hou J, Gu Y, Qian C, Lin Y, Liu X, Huang M, et al. Circular RNA circMTO1 acts as the sponge of microRNA-9 to suppress hepatocellular carcinoma progression. Hepatology. 2017;66(4):1151–64. http://doi.org/10.1002/hep.29270.

26. Fridh V, Rittinger K. The tandem CARDs of NOD2: intramolecular interactions and recognition of RIP2. PLoS One. 2012;7(3):e34375. http://doi.org/10.1371/journal.pone.0034375.

27. Tao M, Scacheri PC, Marinis JM, Harhaj EW, Matesic LE, Abbott DW. ITCH K63-ubiquitimates the NOD2 binding protein, RIP2, to influence inflammatory signaling pathways. Curr Biol. 2009;19(15):1255–63. http://doi.org/10.1016/j.cub.2009.06.038.

28. Hasegawa M, Fujimoto Y, Lucas PC, Nakano H, Fukase K, Nunez G, Inohara N. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. EMBO J. 2008;27(2):373–83. http://doi.org/10.1038/sj.emboj.7601962.

29. Li X, Jia Y, Nan A, Zhang N, Zhou H, Chen L, Pan X, Qiu M, Zhu J, Zhang H, et al. CircRNA104250 and IncRNAuc001.dgp.1 promote the PM2.5-induced inflammatory response by co-targeting miR-3607-5p in BEAS-2B cells. Environ Pollut. 2020;258:113749. http://doi.org/10.1016/j.envpol.2019.113749.
30. Li Y, Yin Z, Fan J, Zhang S, Yang W. The roles of exosomal miRNAs and IncRNAs in lung diseases. Signal Transduct Target Ther. 2019;4:47. http://doi.org/10.1038/s41392-019-0080-7.

31. Chen J, Liu G, Wu Y, Ma J, Wu H, Xie Z, Chen S, Yang Y, Wang S, Shen P, et al. CircMYO10 promotes osteosarcoma progression by regulating miR-370-3p/RUVBL1 axis to enhance the transcriptional activity of beta-catenin/LEF1 complex via effects on chromatin remodeling. Mol Cancer. 2019;18(1):150. http://doi.org/10.1186/s12943-019-1076-1.

32. Li M, Hua Q, Shao Y, Zeng H, Liu Y, Diao Q, Zhang H, Qiu M, Zhu J, Li X, et al. Circular RNA circBbs9 promotes PM2.5-induced lung inflammation in mice via NLRP3 inflammasome activation. Environ Int. 2020;143:105976. http://doi.org/10.1016/j.envint.2020.105976.

33. Chen J, Yang X, Liu R, Wen C, Wang H, Huang L, Li W, Zhu Z, Zhu Y, Liu H. Circular RNA GLIS2 promotes colorectal cancer cell motility via activation of the NF-kappaB pathway. Cell Death Dis. 2020;11(9):788. http://doi.org/10.1038/s41419-020-02989-7.

34. Su H, Tao T, Yang Z, Kang X, Zhang X, Kang D, Wu S, Li C. Circular RNA cTFRC acts as the sponge of MicroRNA-107 to promote bladder carcinoma progression. Mol Cancer. 2019;18(1):27. http://doi.org/10.1186/s12943-019-0951-0.

35. Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, Sansonetti PJ, Mengin-Lecreulx D. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J Biol Chem. 2003;278(43):41702–8. http://doi.org/10.1074/jbc.M307198200.

36. Trindade BC, Chen GY. NOD1 and NOD2 in inflammatory and infectious diseases. Immunol Rev. 2020;297(1):139–61. http://doi.org/10.1111/imr.12902.

37. Cavallari JF, Fullerton MD, Duggan BM, Foley KP, Denou E, Smith BK, Desjardins EM, Henriksbo BD, Kim KJ, Tuinema BR, et al. Muramyl Dipeptide-Based Postbiotics Mitigate Obesity-Induced Insulin Resistance via IRF4. Cell Metab. 2017;25(5):1063–74 e1063. http://doi.org/10.1016/j.cmet.2017.03.021.

38. Miller MH, Shehat MG, Alcedo KP, Spinel LP, Soulakova J, Tigno-Aranjuez JT. Frontline Science: RIP2 promotes house dust mite-induced allergic airway inflammation. J Leukoc Biol. 2018;104(3):447–59. http://doi.org/10.1002/JLB.4HI0118-017RR.

39. Chin AI, Dempsey PW, Bruhn K, Miller JF, Xu Y, Cheng G. Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. Nature. 2002;416(6877):190–4. http://doi.org/10.1038/416190a.

40. Park JH, Kim YG, McDonald C, Kanneganti TD, Hasegawa M, Body-Malapel M, Inohara N, Nunez G. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. J Immunol. 2007;178(4):2380–6. http://doi.org/10.4049/jimmunol.178.4.2380.

Figures
Figure 1

Identification of circRNAs expression profile. a Volcano plots were constructed using fold change values and P-values. The red dot in the plot represents the significantly different expression (fold change ≥ 2, P<0.05) of circRNAs showing statistical significance. b, c Hierarchical clustering analysis of circRNAs that are significantly differentially expressed in control and RT-treated RAW264.7 cells, including 17 upregulated and 6 downregulated circRNAs; each group contained three biological repeats (fold
change $\geq 2$, $P < 0.05$). The expression levels are presented in different colors indicating expression levels above and below the median expression level across all samples. d ID number and alias of mmu_circ_0000842 from the circBase database. e Expression levels of top8 significantly differentially expressed circRNAs were determined by qRT-PCR.

Figure 2
Characterization of circEpc1 in RT-treated RAW264.7 cells. a Genomic loci of circEpc1 gene. circEpc1 is produced at the Epc1 gene (ID: 13831) locus containing exons 3–8. The back-splice junction of circEpc1 was identified by Sanger sequencing. b RT-PCR analysis for circEpc1 and its linear isoform Epc1 in cDNA and gDNA in the presence or absence of RNase R supplementation from RAW264.7 cells using divergent and convergent primers. c qRT-PCR for the abundance of circEpc1 and Epc1 in RAW264.7 cells treated with Actinomycin D at the indicated time point. c ID number and alias of mmu_circ_0000842 from the circBase database. d Levels of circEpc1 in the nuclear and cytoplasmic fractions of RAW264.7 cells. Data represent the mean ± SD from three representative experiments. (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 3

Characterization of circEpc1 in RT-treated RAW264.7 cells. a Expression level of circEpc1 in control and RT-treated RAW264.7 cells. b Two shRNAs were designed to silence circEpc1 by targeting the spliced junction of circEpc1, it revealed that the shRNAs could significantly downregulate the expression level of circEpc1 but had no effect on that of linear Epc1. c Expression level of circEpc1 in RAW264.7 cells after transduction with over-expressing circEpc1 lentivirus. d ELISA assays were performed to determine the role of circEpc1 in RT-induced inflammation. Data represent the mean ± SD from three representative experiments. (*P < 0.05, **P < 0.01, ***P < 0.001).
**Figure 4**

CircEpc1 serves as a miRNA sponge of miR-5114. a The schematic flowchart shows the pipelines of miRNAs which could bond to circEpc1 and NOD2 3'UTR via online bioinformatic network. b Expression level of miR-5114 in control and RT-treated RAW264.7 cells. c, d Dual-luciferase reporter activity of circEpc1 in HEK-293T cells co-transfected with miR-5114 mimics or mimics NC. e MiR-5114 co-localized with circEpc1 in the cytoplasm by FISH analysis, scale bar = 100 μm. f Expression level of miR-5114 in...
RAW264.7 cells after transduction with miR-5114 mimics lentivirus. g TNF-α and IL-6 secretion in RT-treated RAW264.7 cells transfected with miR-5114 mimics alone or co-transfected with circEpc1. Data represent the mean ± SD from three representative experiments. (*P < 0.05, **P < 0.01, ***P<0.001).

Figure 5

CircEpc1 facilitates RT-induced inflammation by relieving repression of miR-5114 for NOD2 expression in RAW264.7 cells. a The binding sites of miR-5114 and NOD2 were predicted by TargetScan database. b
Expression level of NOD2 and RIP2 in control and RT-treated RAW264.7 cells. c The protein expression level of NOD2 and RIP2 in control and RT-treated RAW264.7 cells. d NOD-Like Receptor signaling pathway. e, f Western blot assays were used to evaluate the activation of MAPK and NF-κB signaling pathways in RAW264.7 cells induced by RT. g, h Western blot assays were used to evaluate the effects of circEpc1 and miR-5114 on NOD2, RIP2, MAPK and NF-κB signaling pathways in RT-induced inflammation. The intensity of bands were scanned and measured by Image J software, and were summarized as bar graphs. Data represent the mean ± SD from three representative experiments. (*P < 0.05, **P < 0.01, ***P<0.001).
Figure 6

CircEpc1 promotes RT-induced inflammation in vivo. a Effects of RT concentration and exposure time on TNF-α and IL-6 secretion in mice. b Ex vivo fluorescence imaging of lung tissue in control and AAVs transfected mice groups. c, d qRT-PCR assays were used to determine the expression of circEpc1 and miR-5114 in AAVs transfected mice. e Western Blot assays were used to determine the protein expression level of NOD2 and RIP2 in AAVs transfected mice. f The image (H&E staining) of mouse lung
morphological changes and inflammatory cell infiltration in control and RT exposed groups of AAVs transfection model mice (scale bar: 100 μm). g CircEpc1 promotes the secretion of TNF and IL-6 in mice exposed to RT. Data represent the mean ± SD from three representative experiments. (*P < 0.05, **P < 0.01, ***P<0.001).

Figure 7

The mechanism of circEpc1 promotes RT-induced inflammation through the circEpc1/miR-5114/NOD2 axis.

Supplementary Files
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