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Curcumin alleviates Aflatoxin B1-triggered chicken liver necroptosis by targeting the LOC769044/miR-1679/STAT1 axis

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ABSTRACT Aflatoxin B1 (**AFB1**) is an unavoidable environmental toxin. The accumulation of AFB1 and its metabolites in the liver poses a threat to both human and animal health. Curcumin exhibits anti-oxidative, anti-tumor, and anti-inflammatory properties. There is no report on the mechanism regarding how curcumin relived liver necroptosis in chickens induced by AFB1 based on the regulatory network of ceRNA. To explore this, we performed transmission electron microscopy and sequenced lncRNA and mRNA in chicken livers treated with AFB1 and/or curcumin for 28 d *in vivo*. We observed substantial alterations in the lncRNA and mRNA expression profiles within the chicken liver, indicating that curcumin can mitigate AFB1-induced necroptosis both *in vivo* and *in vitro*. Further analysis, including the establishment of an lncRNA-miRNA-mRNA network and the utilization of a dual luciferase reporter assay, revealed that LOC769044 acts as a competing endogenous RNA (ceRNA) for miR-1679. In addition, STAT1 was identified as a direct target of miR-1679. Modulating miR-1679 levels through overexpression, and silencing LOC769044 and STAT1, effectively reversed the necroptotic effects induced by AFB1, a reversal that was also observed with curcumin supplementation. In conclusion, our data demonstrate that curcumin alleviates AFB1-induced liver necroptosis through the LOC769044/miR-1679/STAT1 signaling axis. This study suggests that LOC769044 may serve as a novel therapeutic target for managing AFB1-mediated liver toxicity.

Key words: Aflatoxin B1, curcumin, chicken liver, ceRNA, necroptosis

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INTRODUCTION

Aflatoxins is synthesized by *Aspergillus parasticus* and widely existed in moldy peanuts, soybeans and et al (Rushing and Selim, 2019; Williams, et al., 2004). Aflatoxin B1 (**AFB1**) belongs to a group 1 cancerogenic substances and serves as a pollution indicator in food monitoring. Observational data from 2018 to 2020 indicate a notably high incidence of AFB1 contamination, affecting 81.9 to 100% of feedstuffs and complete feeds sampled across different regions in China (Zhao, et al., 2021). Feed contaminated with AFB1 can cause organ damage in poultry, decrease production performance and feed utilization, induce immunosuppression, increase morbidity and mortality, and affect meat quality (Li, et al., 2023). AFB1 can also harm human health throughout the food chain "from farm to fork" (Liu, et al., 2023). It has been reported that AFB1 can leads to immune toxicity, genetic toxicity, nephrotoxicity, and particularly hepatotoxicity (Frangiamone, et al., 2024).

Necroptosis is a unique type of cell death and is crucial in certain acute and chronic liver injuries, making it an appropriate target for liver injury treatment. Necroptosis has its own unique regulatory targets and signaling pathways, among which TNFR1 (tumor necrosis factor receptor 1a)-mediated RIPK1 (receptor interacting

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serine/threonine kinase 1)-RIPK3 (receptor interacting serine/threonine kinase 3)-MLKL (mixed lineage kinase domain-like) is the classic signal transduction pathway (Xie et al., 2013). Activated MLKL can lead to membrane disintegration which is a key step necessary to initiate cell necroptosis (Cai et al., 2014; Hildebrand et al., 2014). It has been proved that Cd triggered chicken liver necroptosis through activating RIPK1/RIPK3/MLKL expression and MAPK (mitogen-activated protein kinases)/NF-κB (nuclear factor kappa B)pathway (Liu et al., 2022). At present, the literatures about AFB1-induced liver damage now focuses on apoptosis (Wang et al., 2019), inflammation (Zhang et al., 2019), and oxidative stress (Wu et al., 2022a). Our team previously preliminarily found that AFB1 induces necroptosis through TLR4 (toll-like receptor 4)/RIPK pathway (Li et al., 2022). Hence, blocking the necroptosis signaling pathway is a new potential approach for AFB1-induced hepatotoxicity in future. With the in-depth study of necroptosis, more and more genes involved in the regulation of liver necroptosis have been discovered and identified. Our liver transcriptome data showed that STAT1 (signal transduction and transcriptional activator) is significantly expressed after AFB1 exposure, thus we speculate that STAT1 plays an important role in AFB1-induced liver toxicity.

The lncRNAs refer to a group of single-stranded RNAs that are longer than 200 nt and are incapable of encoding proteins (Zhang et al., 2018). LncRNAs were originally thought to be "gene transcription noise". Small non-coding RNAs with a length of 20 to 25 nt are called miRNAs. LncRNAs and miRNAs have a role in nearly every biological function, such as cellular proliferation, differentiation, cell death, organ development (Bandiera et al., 2015) and pathological processes (Yan et al., 2018). A new mechanism "ceRNA (competitive endogenous RNAs)" proposes that lncRNAs serve as a miRNA sponge by binding sites, relieving miRNA suppression on target genes and enhancing target genes expression level (Salmena et al., 2011). The mechanism is crucial in regulating the emergence and progression of liver diseases. LncRNA MEG3 can serve as ceRNA through targeting miR-34a/Nrf2 (nuclear factor, erythroid 2 like 2) to modulate HIRI (hepatic ischemia reperfusion injury) (Huang et al., 2018). AFB1 can also affect lncRNAs transcripts to impair porcine alveolar macrophage proliferation (Chao et al., 2022) and rats that developed hepatocellular carcinoma (Shi et al., 2016). Our earlier research discovered that AFB1 might harm chicken liver while altering the lncRNA expression profiles, and ceRNA network exists in this process (Li et al., 2021). Nevertheless, the involvement of ceRNA in AFB1-induced liver injury even necroptosis remains further exploration.

Curcumin is a low cost, accessible and low toxic phenolic chemical extracted from *Curcuma longa L.* Numerous studies have shown its significant biological effects such as anti-oxidative stress, anti-inflammation, anti-tumor, protection of mitochondrial function and signaling pathways (Benzer, et al., 2018; Wang, et al., 2018). Curcumin is a potential hepatoprotective medicine due to its specific beneficial effects on hepatocytes in hepatic injury, hepatitis, and even liver cancer (Antonio et al., 2019). It can exhibit hepatoprotective effect through antioxidant, modulate enzyme activity, suppress the expression of NF-κB and activity of TLR receptor and regulate pro-inflammatory cytokines (Boozari et al., 2019; Patel et al., 2019). Lu reported that curcumin can also dose-dependently ameliorate alcohol-induced Nrf2 expression inhibition and mice hepatocyte necroptosis (Lu et al., 2016).

In this study, based on established models of liver injury caused by AFB1 exposure and curcumin intervention model *in vivo* and *in vitro*, morphological changes, lncRNAs and mRNAs expression profiles, enzymes activities, levels of necroptosis and inflammatory signaling pathways, establish and verification of lncRNA-miRNA-mRNA network were analyzed. This prospective investigation was meant to evaluate the manner of cell death generated by AFB1 exposure and the roles of lncRNA as ceRNA, uncover the mechanism whereby curcumin alleviates AFB1-induced liver injury and discover novel strategies for AFB1 hepatotoxicity. It also has important scientific significance to promote the development of the chicken industry and guarantee the safety of chicken source food and human health.

METHODS AND MATERIALS

Animals and Treatment

Northeast Agricultural University Laboratory Animal Ethics Committee approved all procedures. Thirty-two 1-day-old broilers were assigned randomly to four groups: control group treated with a normal diet, AFB1 group treated with 1 mg/kg AFB1, AFB1 + curcumin group treated with 1 mg/kg AFB1 and 300 mg/kg curcumin, and curcumin group treated with 300 mg/kg curcumin as previous conducted. All groups had ad libitum access to feed and clean water. The broilers were euthanized on the 28th d.

Electron Microscopy

After the broilers were sacrificed, the livers were quickly harvested and cut into a size of 1 mm^3. The liver tissue was carefully washed by PBS and fixed with glutaraldehyde solution and osmic acid. Then the samples were dehydrated with graded ethanol and acetone at 4 °C. The prepared tissues were finally embedded in araldite, and cut into thicknesses of 50−70 nm slices. The transmission electron microscope (HITACHI, Japan) was used for observation of the liver cells alterations.

RNA-seq and Data Analysis

LncRNA and mRNA sequences were performed by Sangon Biotech (Shanghai, China) to reveal the transcription level changes of broilers liver. Briefly, RNA extraction and quality identification were conducted by

Sangon Biotech (Shanghai, China). An Illumina HiSeq 2000 with 150-bp paired-end was used for generating the library. The raw data for lncRNA and mRNA were submitted to the NCBI Gene Expression Omnibus (GSE148014). Differentially expressed lncRNAs and mRNAs (DELs and DEMs) were identified by using the DEGseq algorithm. Heatmaps of differential gene profiles were drawn by Sangerbox (Shen et al., 2022).

The lncRNA-miRNA-mRNA network was established. Briefly, RegRNA 2.0 and miRDB databases were used to predict the miRNAs that could bind to differentially expressed LncRNAs. The predicted miRNA results obtained from the 2 databases were intersected. The RNAhybrid software was applied to calculate the binding site's minimum free energy (**mfe**), and miRNAs with mfe <-20 kcal/mol were identified as the final target gene. miRDB database was used to predict the mRNA that could bind to miRNAs. The intersections of the database prediction results and differentially expressed mRNAs were identified as the final target mRNAs. According to the above predicted results, RAWGraghs 2.0 beta was used to draw the lncRNA-miRNA-mRNA network.

LMH Cells Culture

LMH cells (donated by Prof. Xu) were maintained in DMEM medium. LMH cells are the first chicken hepatoma cell line to be established. This cell line was derived from a hepatocellular carcinoma cell. LMH cells provide some potential advantages over primary cultures: continuous long-term culture, cell type homogeneity, and a virtually endless supply. Thus, LMH cells are more suitable for long-term studies and stable transfections (Gabis, et al., 1996). Cells were cultured with 5% CO_2 humidified atmosphere at 37 °C. Before cell treatment, LMH cells were transplanted into 6-well plates for culture until 70 to 80% density. The cell medium was changed into a fresh medium every 48 h.

Cell Viability Analysis

Cell viability assay was conducted as previously described (Wu et al., 2022b). Briefly, LMH cells were grown in DMEM medium treated with different AFB1 concentrations (0, 2.5, 5, 10, 20, 40, 80 μM), curcumin (0, 2.5, 5, 10, 20, 40 μM), or nec-1 (0, 5, 10, 20, 40, 50 μM) for 12 h in 96-well plates. After treatment, the cell viability of LMH cells was assessed by CCK8 (Abmole, USA) kits according to the manufacturer's instruction. The optical density (**OD**) was measured at 450 nm. The following formula was utilized for calculating cell viability:

$$\text{cell viability} = \frac{\text{A(treatment)} - \text{A(blank)}}{\text{A(control)} - \text{A(blank)}} \times 100\%$$

Cell Transfection

GenePharma (Shanghai, China) designed and manufactured LOC769044 siRNA, STAT1 siRNA and miR-1679 mimics. LOC769044 siRNA, STAT1 siRNA and miR-1679 mimics sequences were shown in Table S1. For LMH cells transfection in 6-well plates, LMH cells were transfected with LOC769044 siRNA (50 nM) and STAT1 siRNA (50 nM) using GP-transfect-Mate (Shanghai, China) and transfected miR-1679 mimics (50 nM) using Lipofectamine 3000 (Invitrogen, CA, USA) in Opti-MEM medium. After 6 h transfection at 37°C, 5% CO_2 atmosphere, LMH cells were incubated with 20 μM AFB1, 20 μM curcumin and 10 μM necrostatin-1 (nec-1) for 12 h. LMH cells were collected immediately for the following experiments.

Double Luciferase Reporter Assays

The 180 bp of LOC769044/STAT1 segment containing miR-1679 binding site and corresponding mutated sequences were designed and synthesized by GenePharma (Shanghai, China). Recombinant wild-type LOC769044/STAT1 and mutant-type LOC769044/STAT1 plasmids were manufactured using pmirGLO vector (supplied by VectorBuilder) and named pMIR-LOC769044-WT/MUT and pMIR-STAT1-WT/MUT. Transient cotransfection was conducted in LMH cells with wild-type (pMIR-STAT1-WT or pMIR-LOC769044-WT) or mutant-type (pMIR-STAT1-MUT or pMIR-LOC769044-MUT) and miR-1679 mimics NC or miR-1679 mimics using lipofectamine 3,000. Following 48 h transfection, we used Dual-Lumi Kit (Beyotime, Shanghai, China) to determine the luciferase activities.

Annexin V-FITC/PI Staining Assay

The percentages of live cells, death cells and necrotic cells were assessed by Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). After treating LMH cells with AFB1, curcumin or nec-1 for 12 h in 24-well plates, Annexin V-FITC and propidium iodide (**PI**) staining were performed. The final images were observed under fluorescence microscope (Nikon, Japan). The necrotic cells show both green and red fluorescence.

Biochemical Analysis and ATPase Activity

The activities of alanine aminotransferase (**ALT**) and aspartate aminotransferase (**AST**), as well as ATPase activities including Ca^{2+}-ATPase, Mg^{2+}-ATPase, Ca^{2+} Mg^{2+}-ATPase, and $Na^{+}K^{+}$-ATPase activities were assessed using commercial kits (Jiancheng Bio, Nanjing, China) following the user guide.

ELISA for Proinflammatory Cytokines and ROS

To assess the generation of proinflammatory cytokines and reactive oxygen species (**ROS**) in curcumin and/or AFB1 treated LMH cells, interleukin 6 (**IL-6**), interleukin-1β (**IL-1β**), tumor Necrosis Factor α (**TNF-α**), inducible nitric oxide synthase (**iNOS**) and ROS

level were assayed by commercial ELISA kits (Meimian, Jiangsu, China) following the user guide.

Separation of Nuclear-Cytoplasmic Fractions and qPCR Analysis

Nuclear and cytoplasmic fractions were extracted from liver tissue using the Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek, Canada) following the instructions.

For lncRNA and mRNA qPCR analysis, BioRT cDNA First Strand Synthesis Kit (Bioer, Hangzhou, China) was used for mRNA cDNA synthesis. LncRNA cDNA was synthesized by lnRcute lncRNA First-Strand cDNA Kit (Tiangen, Beijing, China). The lncRNAs and mRNAs specific primers were shown in Table S2 and S3. GAPDH was considered as an internal reference.

For miRNA qPCR analysis, miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) was used for miRNA cDNA synthesis and the stem-loop primers and qPCR specific primers for miRNAs were designed using miRNA Design V1.01 (Vazyme, China) and shown in Table S4 and S5. The gga-5s-rRNA was selected as an internal reference. QPCR for lncRNAs, mRNAs and miRNAs were carried out on LightCyler 96 system (Roche, Switzerland).

Western Blotting Analysis

Liver tissue and LMH cells protein were extracted by RIPA and quantified by BCA kit (Beyotime, China). SDS-PAGE electrophoresis was used to separate the protein samples, after which the target protein bands were excised and transferred to PVDF membranes. The target membranes were blocked with 5% skim milk for 2 h, then exposed to primary antibodies for overnight incubation at 4°C. The membranes were finally incubated with secondary antibodies at room temperature for 1 h. Blots signal detection was conducted using ECL luminescence reagent (Absin, Shanghai, China) and relative protein level quantification was performed using Image J.

Statistical Analysis

All tests were carried out in triplicate. The ONE-WAY ANOVA approach was utilized for data analysis and the SPSS 26.0 was employed. Mean ± SD was used to present data. Significant statistically was defined as $P < 0.05$. Graghpad Prism 8.0 was used to draw figures. The graphical abstract was made by Figdraw.

RESULTS

Curcumin Alleviates AFB1-Induced Liver Necroptosis

To investigate curcumin's protective effects against liver injury in broilers caused by AFB1, the ultrastructural alterations of liver cells were observed using an electron microscope. There was intact liver cell ultrastructure in the control and curcumin group, liver cells have integral membranes, uniformly distributed chromatin, and abundant mitochondria with clear mitochondrial cristae (Figure 1A). In AFB1 exposure caused obvious necrotic characteristics in liver cells including abnormal nuclear morphology, disrupted nuclear membrane, swelling mitochondria, absence of mitochondrial cristae and vacuolar degeneration in cytoplasm. Whereas liver cells in AFB1 + cur group showed restored ultrastructure of nuclear membrane and mitochondria. The necroptosis signals including RIPK1, RIPK3, and MLKL were activated at the molecular level (Figures 1D−1G) in AFB1-exposed broiler liver. Our previous studies also have shown that AFB1 can cause liver pathological damage, accompanied by significant changes in liver injury and biochemical parameters (ALT, AST, alkaline phosphatase (**ALP**) and γ-glutamyl transpeptidase (**γ-GT**) activities, catalase (**CAT**) and superoxide dismutase (**SOD**) activities, glutathione (**GSH**) contents and malondialdehyde (**MDA**) levels), furthermore, curcumin obviously alleviates these serious alterations induced by AFB1 (Li, et al., 2021; Li, et al., 2022), showing a good protective effect.

Effect of Curcumin and AFB1 on lncRNAs and mRNAs Expression Profiles in Broiler Livers

To better understand the mechanisms by which curcumin mitigates AFB1-induced liver damage in broilers, we performed RNA-seq and analysis of DELs and DEMs profiles (Figures 1B and 1C). The data showed that 717 mRNAs and 34 lncRNAs were significantly changed overlapped in AFB1 and AFB1 + cur group. We constructed the network of lncRNA-miRNA-mRNA for DELs and DEMs. Ten known lncRNAs of 34 total DELs were selected and the intersection of miRDB and RegRNA 2.0 database predicting outcomes calculated by RNAhybrid (minimum free energy, mfe ≤ −20) were final target binding miRNAs. miRDB was used to predict target binding mRNAs, the intersection of miRDB predicted results and differentially expressed mRNAs were taken as the final results. The network of lncRNA-miRNA-mRNA was shown in Figure S1.

LOC769044 Functions as a ceRNA for miR-1679

According to the ceRNA mechanism, highly expressed lncRNAs in cytoplasm may competitively bind with miRNAs to indirectly regulate downstream target genes, and thus modulate cellular functions. Ten lncRNAs in the top 20 differentially expressed lncRNAs were selected for intracellular localization through isolated nuclear-cytoplasmic fractions and qPCR. The result showed (Figures 2A and 2C) that LOC769044 was mainly localized in cytoplasm and significantly increased in AFB1-exposed broiler livers and decreased in

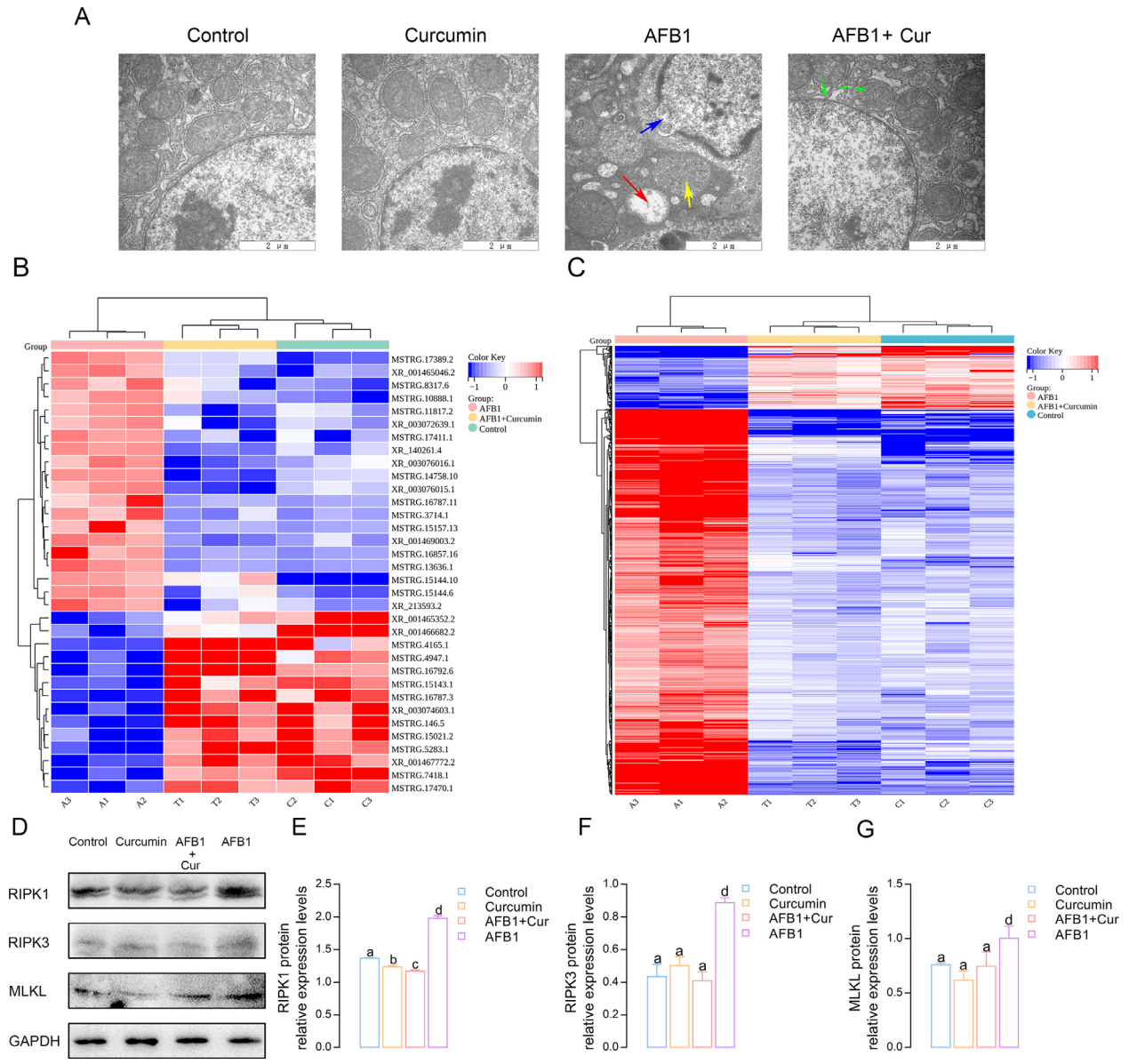

Figure 1. Curcumin alleviates AFB1-induced necroptosis in broiler liver. (A) Ultrastructural alternations observation of broiler liver cells. Bar = 2 μm. Blue arrow indicates disrupted nuclear membrane, yellow arrow indicates swelling mitochondria and absence of mitochondrial cristae, red arrow indicates vacuolar degeneration in cytoplasm, and green arrows indicates normal mitochondria cristae and intact nuclear membrane. (B–C) Heatmap of lncRNAs (B) and mRNAs (C) differently expressed in broiler liver (n = 3). (D–G) Relative protein expression of necroptosis genes in broiler liver (n = 8). Significant differences are indicated by different letters on the graph ($P < 0.05$).

AFB1 + cur group. As shown in Figure 2B, the network of lncRNA-miRNA-mRNA for LOC769044 was constructed, we found that miR-1679, miR-1700 and miR-1567 had potential binding sites with LOC769044. QPCR results (Figure 2D) showed that miR-1679 was down-regulated in AFB1 group, miR-1700 and miR-1567 were up-regulated in AFB1 group, while these changes were reversed in AFB1 + cur group. Thus, we speculated that LOC769044, as a molecular sponge, may competitively bind with miR-1679 (the potential binding site was shown in Figure 3H). In double luciferase reporter assays, we found that LMH cells co-transfected with miR-1679 mimics and LOC769044 WT plasmid showed a significant decrease in luciferase activity (Figure 2E). Whereas, there were no changes in luciferase activity in LMH cells co-transfected with miR-1679 mimics and the LOC769044 MUT plasmid, indicating that LOC769044 can interact directly with miR-1679 at the binding site. Furthermore, si-LOC769044 transfection strongly increased miR-1679 expression level (Figure 2F), suggesting LOC769044 had a negative regulatory effect with miR-1679.

STAT1 is a Downstream Target of miR-1679

Based on the miRDB database, we found that the 3′ UTR of STAT1 contains the binding site for miR-1679 (Figure 3H). AFB1 exposure remarkably up-regulated STAT1 mRNA and protein expression levels in broiler liver, and the activation of STAT1 was inhibited by curcumin supplementation (Figures 3A–3C). STAT1 expression level was shown to be positively related to LOC769044 and negatively to miR-1679. We

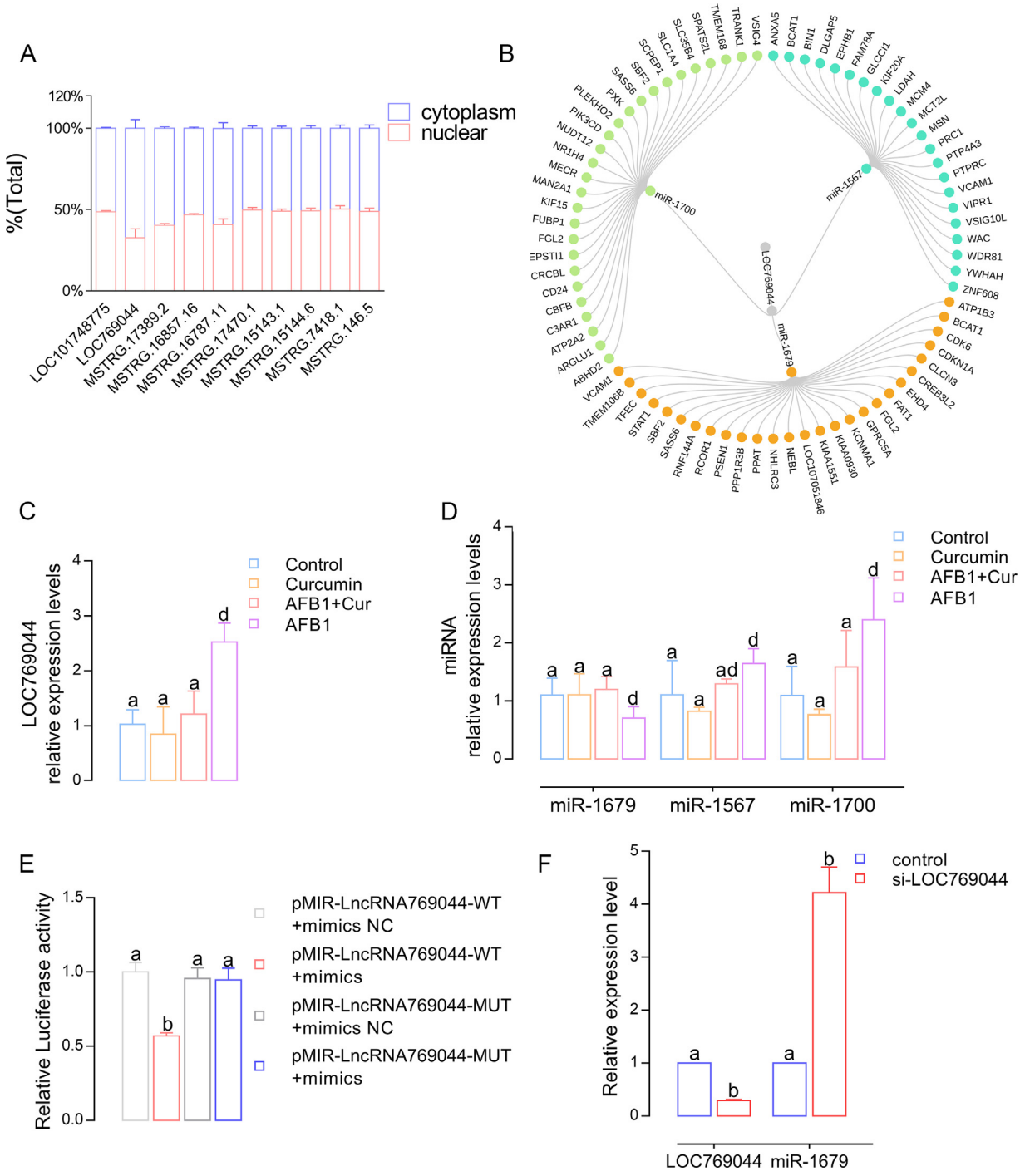

Figure 2. LOC769044 acts as a molecular sponge for miR-1679. (A) Intracellular localization (cytoplasm/nuclear) of differentially expressed LncRNAs (n = 24). (B) LOC769044-miRNA-mRNA network. (C) AFB1 exposure increased the relative expression of LOC769044, and curcumin decreased LOC769044 expression level in broiler liver (n = 8). (D) The relative expression of miR-1679, miR-1700 and miR-1567 in broiler liver (n = 8). (E) Co-transfection with WT/MUT LOC769044 luciferase reporter plasmids and miR-1679 mimics or miR-1679 mimics NC in LMH cells (n = 3). (F) LOC769044 knockdown increased the expression of miR-1679 in LMH cells. Before qPCR analysis, LMH cells were transfected with si-LOC769044 for 6 h (n = 3). Significant differences are indicated by different letters on the graph ($P < 0.05$).

constructed the WT/MUT STAT1 luciferase reporter plasmids and co-transfected with miR-1679 mimics or mimics NC. The double luciferase reporter analysis results (Figure 3D) showed that miR-1679 can bind to STAT1 in potential site. Over-expressed miR-1679 could suppress STAT1 mRNA and protein expression levels obviously (Figures 3E−3G). These results indicate LOC769044 may act as ceRNA of miR-1679 by targeting binding with STAT1. The potential binding sites of LOC769044-miR-1679-STAT1 were shown in Figure 3H.

Curcumin Alleviates AFB1-Induced Necroptosis and Inflammation in LMH Cells

We established AFB1, curcumin and nec-1 exposure model in vitro. As shown in Figure 4A, cell viability

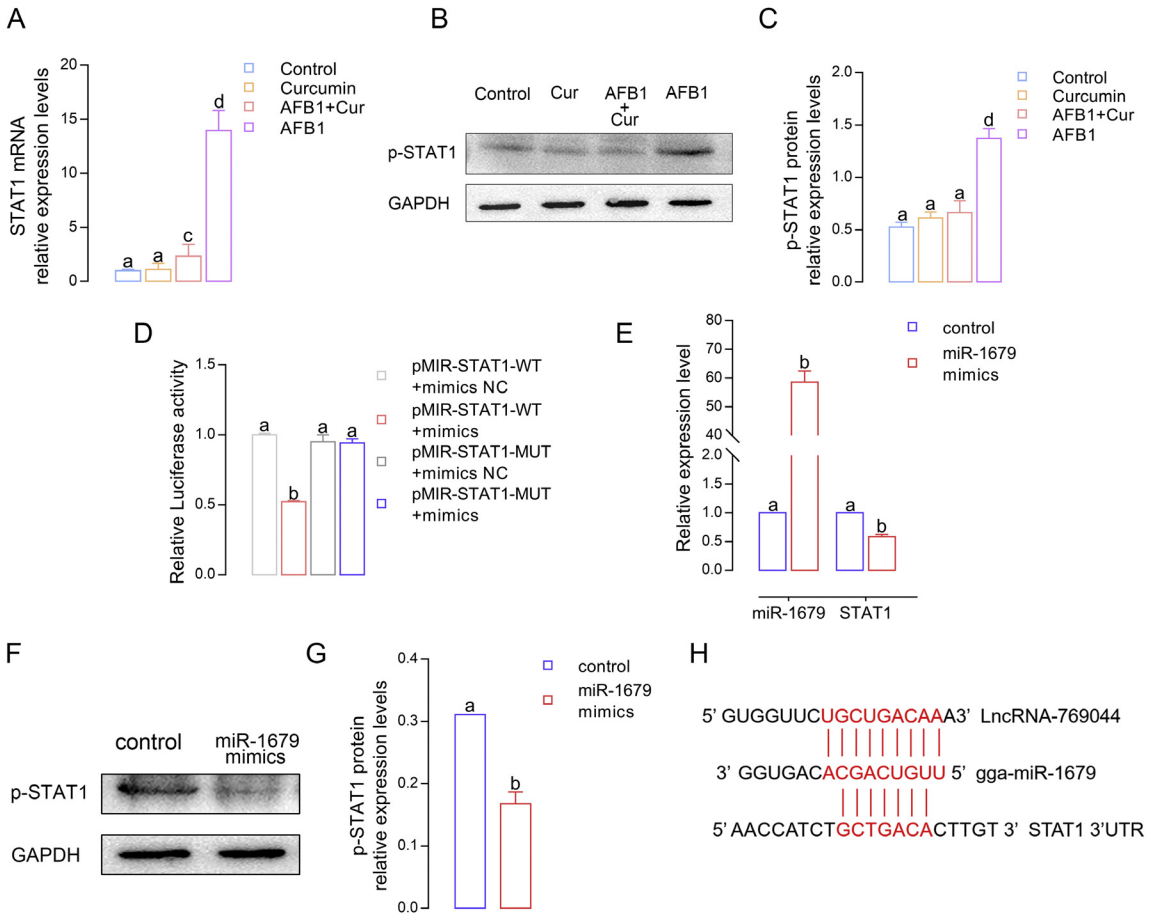

Figure 3. STAT1 is a downstream target of mi-1679. (A−C) The mRNA and protein expressions of STAT1 were upregulated after AFB1 exposure in broiler liver (n = 24). (D) Cotransfection with WT/MUT STAT1 luciferase reporter vectors and miR-1679 mimics or miR-1679 mimics NC in LMH cells (n = 3). (E−G) Overexpression of miR-1679 decreased the mRNA and protein expressions of STAT1 in LMH cells (n = 3). Before qPCR and western blotting analysis, LMH cells were transfected with miR-1679 mimics for 6 h. (H) Potential binding sites of LOC769044-miR-1679-STAT1. Significant differences are indicated by different letters on the graph ($P < 0.05$).

decreased in a dose-independent manner when exposed to both AFB1 and curcumin. In detail, the high-dose (20 μM) AFB1 and 20 μM curcumin significantly decreased the LMH activity. Meanwhile, 2.5 to 10 μM curcumin could effectively alleviate AFB1-induced cell viability reduction. While the nec-1 presents nontoxic to LMH cells. To determine the adverse effects of AFB1 exposure to LMH cells and the application of curcumin to possibly reduce the AFB1-induced cytotoxicity, 20 μM AFB1, 10 μM curcumin or 10 μM nec-1 was supplemented into the culture medium.

Necroptosis often accompanies inflammatory response, oxidative stress and energy metabolism disturbance (Chi et al., 2019). Thus, ATPases activities and inflammatory response were determined. The ATPases activities were significantly decreased, while AST and ALT levels were increased after AFB1 exposure (Figures 4B and 4C). Obviously, curcumin and nec-1 reversed the changes of ATPases activities and ALT, and AST levels induced by AFB1. AFB1 treatment strongly up-regulated the mRNA and protein levels of inflammatory genes (Figures 4F and 4G) as well as the contents of proinflammatory cytokines and ROS (Figures 4D and 4E). Conversely, curcumin supplementation as well as nec-1 revealed protective effects on AFB1-induced inflammation in LMH cells by inhibiting the levels of inflammatory genes and proteins as well as cytokines contents.

We further analyzed the necrotic cells by Annexin V-FITC staining. The increased number of necrotic cells observed from AFB1 group can be remarkably reduced by curcumin supplementation and nec-1 treatment (Figure 5A). AFB1 led to a strong activation of necroptosis signal including the transcriptional and protein levels of RIPK1, RIPK3 and MLKL, whereas curcumin and nec-1 protect LMH cells from AFB1-induced toxicity by greatly suppressing the activation of necroptosis pathway (Figures 5D and 5F).

The LOC769044/miR-1679/STAT1 Axis Participates in Necroptosis in LMH Cells

As shown in Figures 5B, 5C, and 5E, AFB1 exposure increased LOC769044 and STAT1 expression and decreased miR-1679 level. While curcumin and nec-1 supplementation strongly reversed these expression levels.

Subsequently, to investigate the role of LOC769044 in necroptosis, we transfected si-LOC769044 into LMH cells. As shown in Figure 6A, transfection with si-

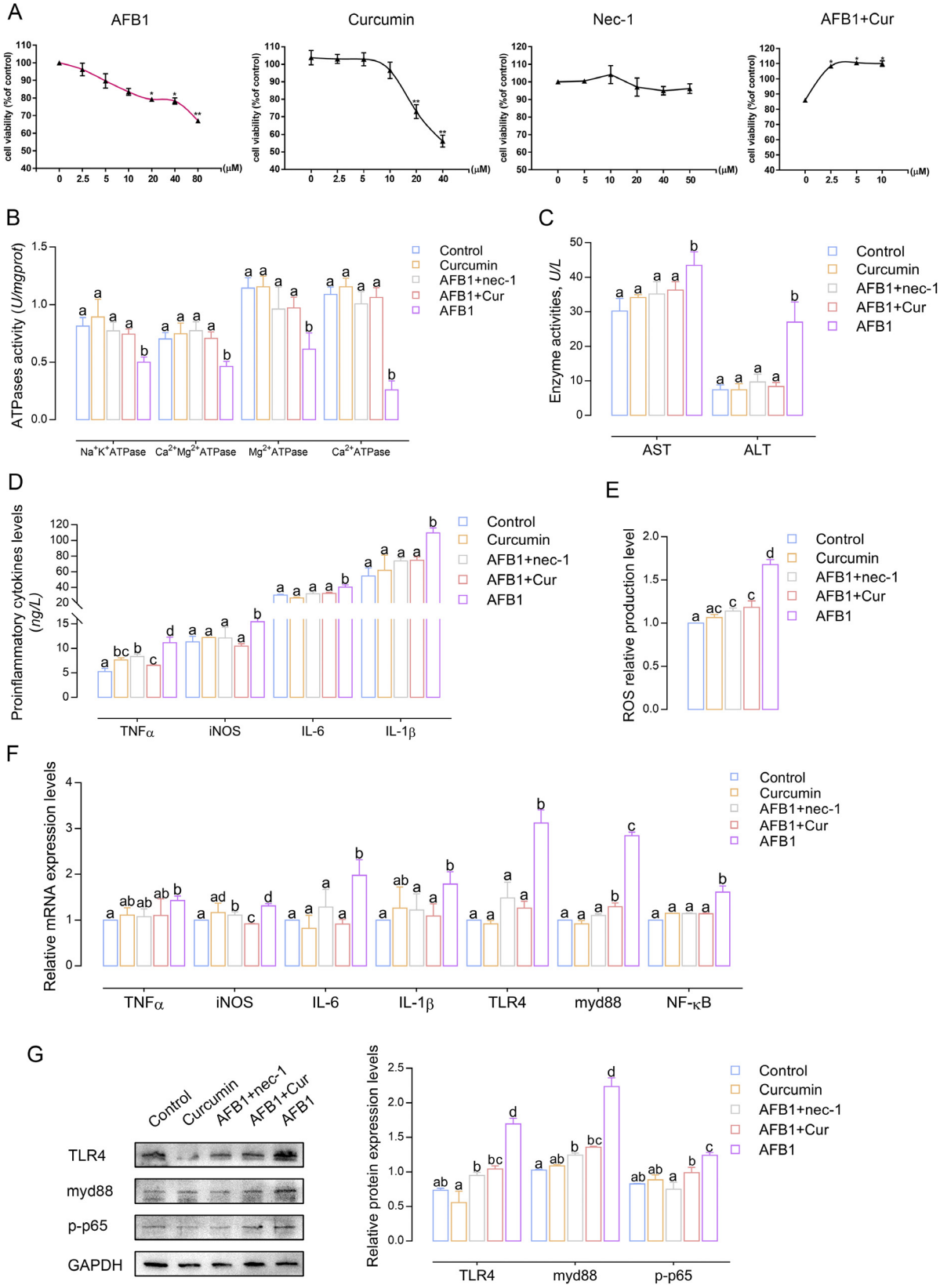

Figure 4. Curcumin alleviates AFB1-induced inflammation in LMH cells. (A) Effects of AFB1 (0−80 μM), curcumin (0−40 μM) and nec-1 (0−50 μM) exposure for 12 h on LMH cells viability. (B−E) Effects of AFB1, curcumin and nec-1 on ATPases, AST and ALT activities, ROS levels and proinflammatory cytokines contents of LMH cells. (F−G) Effects of AFB1, curcumin and nec-1 on mRNA and protein levels of inflammatory genes of LMH cells. Significant differences are indicated by different letters on the graph ($P < 0.05$).

LOC769044 successfully reduced the AFB1-induced production of LOC769044, and the number of necrotic cells decreased obviously (Figure S2 A). Compared to AFB1 group, after transfection of si-LOC769044, the miR-1679 expression was significantly increased, S6TAT1 mRNA and protein levels were down-regulated, and necroptosis

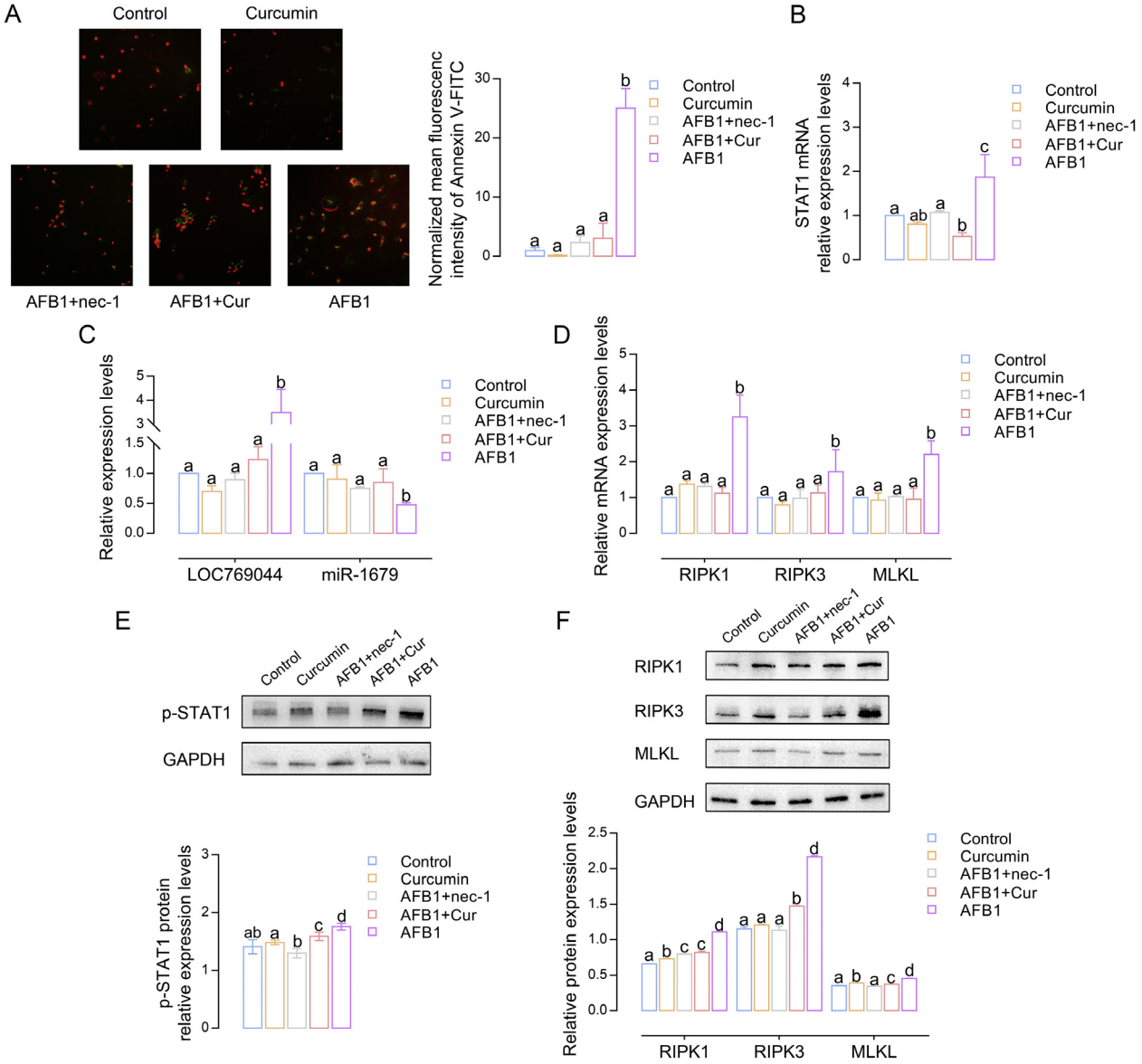

Figure 5. Curcumin alleviates AFB1-induced necroptosis in LMH cells. (A) Effects of AFB1, curcumin and nec-1 exposure on LMH cells death. (B−C, E) LOC769044, miR-1679 and STAT1 relative expression levels in LMH cells. (D, F) The relative mRNA and protein levels of necroptosis genes of LMH cells. Significant differences are indicated by different letters on the graph ($P < 0.05$).

genes mRNA and protein levels were reduced, these results were accordance with curcumin supplementation (Figures 6B−6K). It is suggested that LOC769044 silencing can block the necroptosis signaling pathway and LOC769044 is one of the effective targets of curcumin alleviating AFB1-induced necroptosis. Transfection of miR-1679 mimics increased the level of miR-1679 in LMH cells (Figure 7A). MiR-1679 over-expression in AFB1 group leads to a significant reduction in necrotic cells number (Figure S2 B) and suppressed the STAT1, RIPK1, RIPK3 and MLKL expression level (Figure 7B −7J) compared to AFB1 alone treated LMH cells. Moreover, miR-1679 over-expression has the same protective effects as curcumin supplementation against necroptosis caused by AFB1 exposure.

To further explore the regulation of necroptosis signaling pathway by STAT1, we silenced STAT1 in LMH cells by using STAT1 siRNA (Figure 8A). Necroptosis signals including RIPK1, RIPK3, and MLKL transcriptional and protein levels in AFB1 + si-STAT1 and AFB1 + Cur + si-STAT1 group (Figures 8B−8I), were dramatically reduced in comparison to the AFB1 group, as well as the rate of necrotic cells (Figure S2 C). These results represent that silencing LOC769044 and STAT1 and overexpression of miR-1679 could effectively reverse AFB1-induced LMH cells necroptosis, suggesting that inhibition of LOC769044 results in the level of miR-1679, which enhances the suppression effects of miR-1679 on STAT1, alleviating LMH cells necroptosis.

DISCUSSION

AFB1 is a highly carcinogenic substance which exists widely in mouldy diet. Low-dose AFB1 exposure leads to chronic liver disease even hepatocellular carcinoma in human and animals (Rushing and Selim, 2019). Long non-coding RNA has been proved in recent years to

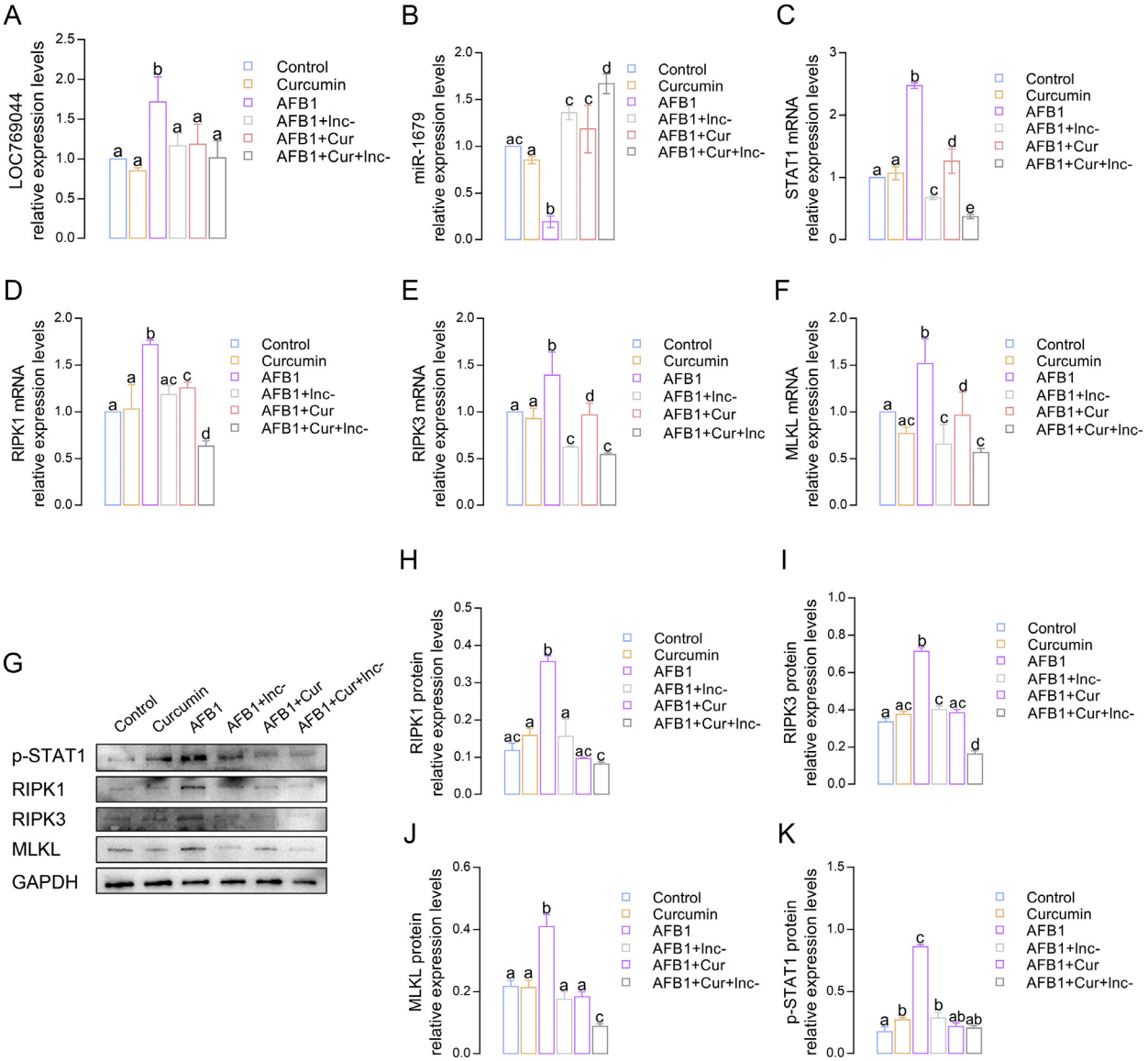

Figure 6. Effects of LOC769044 on LMH cells necroptosis. Before qPCR and western blotting analysis, LMH cells were transfected with LOC769044 siRNA for 6 h, then treated with AFB1, curcumin or nec-1 for 12 h. (A−F) The relative mRNA levels of LOC769044, miR-1679, STAT1 and necroptosis signaling in LMH cells. (G−K) The relative protein levels of STAT1 and necroptosis signaling proteins in LMH cells. Significant differences are indicated by different letters on the graph ($P < 0.05$).

have an vital function in liver disease by modulating cell proliferation, apoptosis, and inflammation (Schueller, et al., 2018). Curcumin, a natural plant extract, has several pharmacological qualities such as antioxidant and anti-inflammatory activities (Ailioaie and Litscher, 2020). Yet, the mechanism by which lncRNAs participate in AFB1-induced hepatocyte necroptosis and curcumin protective effects are uncertain. We found that AFB1 exposure induced necroptosis and led to differential expression of lncRNAs and mRNAs, and LOC769044 works as a ceRNA for miR-1679 to affect the downstream STAT1. Further investigation showed that AFB1 exposure results in necroptosis via LOC769044/miR-1679/STAT1 axis and curcumin significantly improved AFB1-induced liver necroptosis.

Whether the liver injury is acute or chronic and induced by pathogenic agents such as AFB1, the mechanism is hepatocyte death. Necroptosis is a unique kind of cell death. Necroptosis is a form of programmed cell death that, like apoptosis, is regulated by specific genes, necroptosis cells exhibit necrotic morphological traits such as cell and organelle expansion, mitochondrial dysfunction and massive cellular components overflow (Li et al., 2022). Meanwhile, necroptosis often accompanies with oxidative stress and inflammation (Chi et al., 2019). A previous study demonstrated that hexafluoropropylene oxide trimer acid exposure triggers necroptosis and inflammation through the Wnt/β-catenin/NF-κB axis in the liver (Zhang et al., 2023). While LPS can lead to chicken liver necroptosis via the miR-155/TRAF3/MAPK axis (Zhirong et al., 2021). Curcumin can protect against podocyte necroptosis induced by high glucose via suppression of ROS level and RIPK3 pathway (Chung et al., 2022). Curcumin has also been shown to ameliorate hepatocyte necroptosis dose-dependently and reverse alcohol-induced inhibition of Nrf2

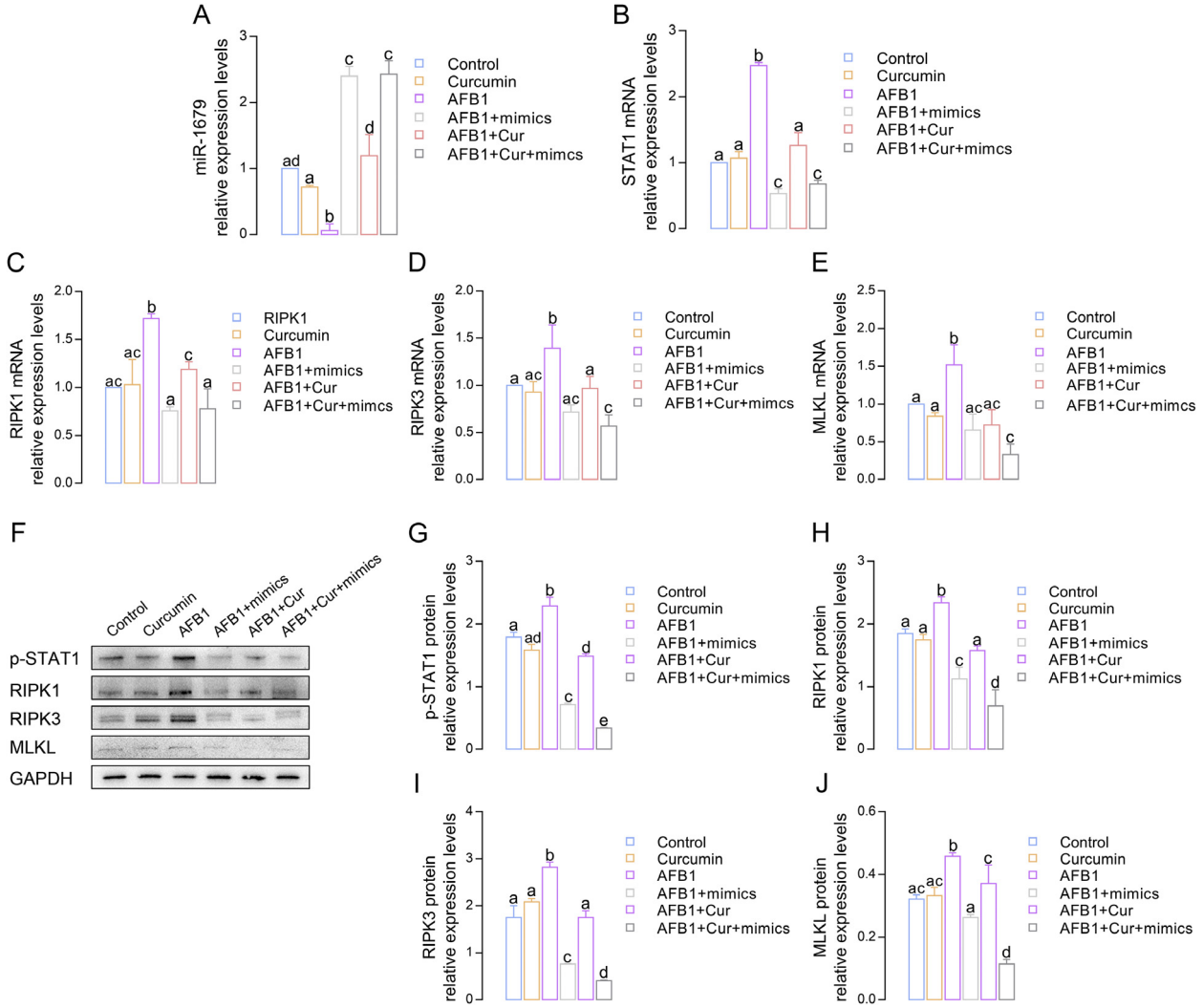

Figure 7. Effects of miR-1679 on LMH cells necroptosis. Before qPCR and western blotting analysis, LMH cells were transfected with miR-1679 mimics for 6 h, then treated with AFB1, curcumin or nec-1 for 12 h. (A−E) The relative mRNA levels of miR-1679, STAT1 and necroptosis signaling in LMH cells. (F−J) The relative protein levels of STAT1 and necroptosis signaling proteins in LMH cells. Significant differences are indicated by different letters on the graph ($P < 0.05$).

expression in mice (Lu et al., 2016). There are few studies about AFB1-induced liver necroptosis and the protective effects of curcumin. We demonstrated that AFB1 can lead to necroptosis both *in vivo* and *in vitro* (chicken liver (Li et al., 2022) and LMH cells), which is specifically showed as ultrastructural damages, decreased ATPases activity, increased ALT and AST enzymes activity, elevated ROS levels, and obvious activation of necroptosis and inflammatory pathways. It has been reported that necrostatins are a group of compounds named for their capability to prevent necroptosis, among which nec-1 has been used to study the contribution of necroptosis and target RIP1 kinase activity in a wide range of pathological cell death events (Cao and Mu, 2021). In this study, nec-1 was selected as positive control for necroptosis therapy to present the protective effect of curcumin on AFB1. The results showed that curcumin and nec-1 (necroptosis inhibitor) can significantly antagonize AFB1-induced necroptosis and inflammatory changes of hepatocytes.

With advances in high-throughput sequencing technology, transcriptomics has been increasingly utilized for the discovery of new biomarkers. Transcriptomics quickly and sensitively identify changes in molecular expression levels in early toxic mechanisms and provide an approach for further toxicity mechanism exploration of exogenous toxins (Waters and Fostel, 2004; McBurney et al., 2009; Lu et al., 2013). A significant body of research in recent years has found that aberrant expression of lncRNAs is closely associated with liver disease (Takahashi, et al., 2014). LncRNAs such as MALAT1 (Jiang and Li, 2015), lncHULC (Xiong, et al., 2017) and HOTAIR (Zhang, et al., 2016) were involved in multiple pathways and regulate a variety of cellular physiological processes in liver cancer. We found that compared with AFB1 groups, there are 717 mRNAs and 34 lncRNAs were differentially expressed in AFB1 + Cur group. LncRNA can act as ceRNA in cytoplasm, competitively interacting with miRNA to exhibit its role in biological processes (Salmena, et al., 2011; Tay, et al., 2014). LncRNA NRF acts as a ceRNA for miR-873 and reduces its regulation of RIPK1/RIPK3, promoting necroptosis in myocardial cells (Wang, et al., 2016). We screen cytoplasmic expressed LOC769044 as key lncRNA and

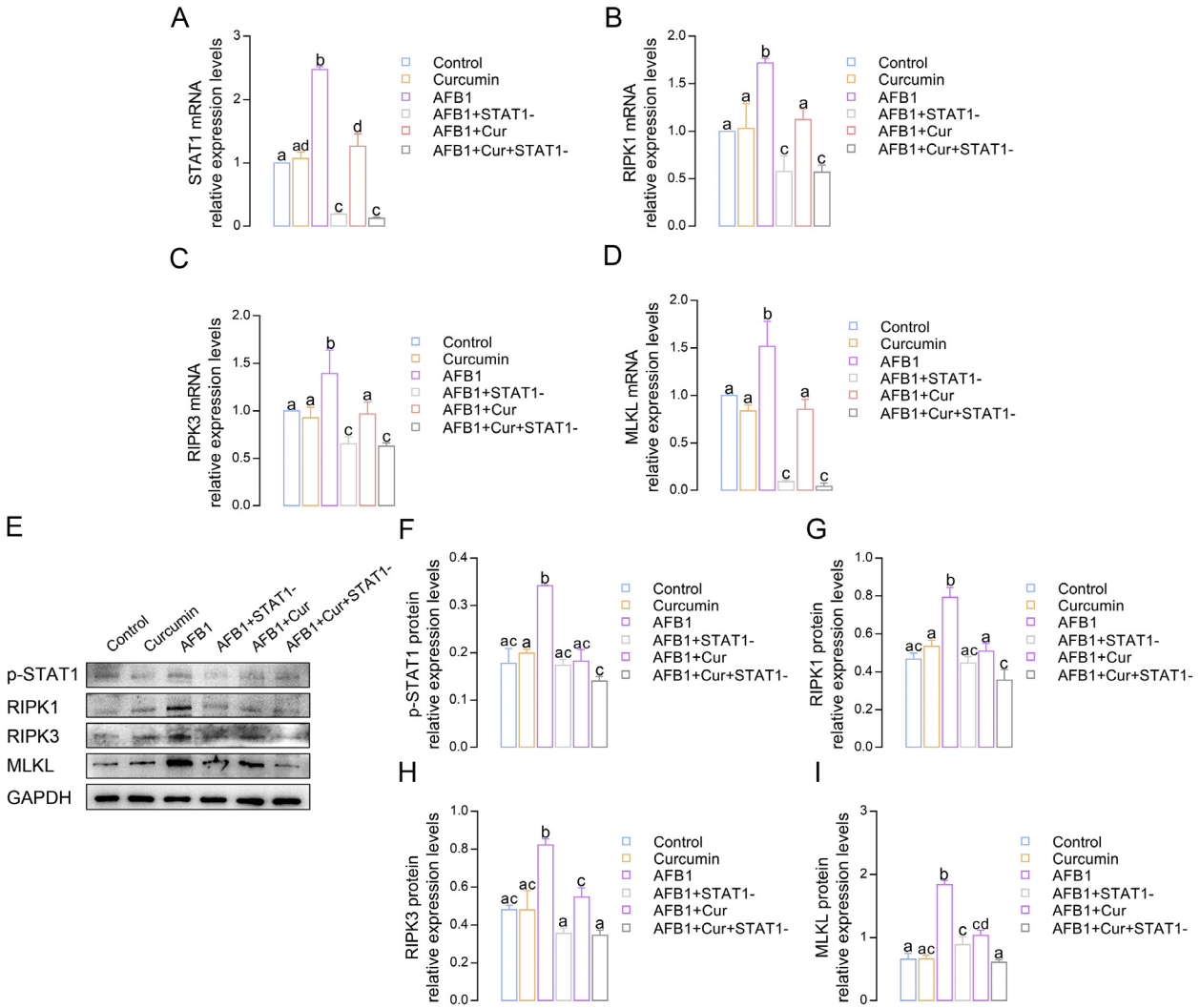

Figure 8. Effects of STAT1 on LMH cells necroptosis. Before qPCR and western blotting analysis, LMH cells were transfected with STAT1 siRNA for 6 h, then treated with AFB1, curcumin or nec-1 for 12 h. (A–D) The relative mRNA levels of STAT1 and necroptosis signaling in LMH cells. (E–I) The relative protein levels of STAT1 and necroptosis signaling proteins in LMH cells. Significant differences are indicated by different letters on the graph ($P < 0.05$).

directly binding to miR-1679 through nuclear-cytoplasmic localization, network analysis and double luciferase reporter gene system analysis. In addition, LOC769044 showed a negative regulation correlation with expression of miR-1679. LOC769044 silence inhibits its LMH cells necroptosis by decreasing RIPK1, RIPK3 and MLKL expression levels. These suggest that LOC769044 functions as a competing molecular sponge for miR-1679 in AFB1-induced hepatocytes necroptosis.

Previous studies have reported that miRNA regulates multiple genes in necroptosis pathway. Selenium deficiency increases miR-16-5p expression level, then aggravate necroptosis induced LPS in chicken tracheal epithelial cells (Wang et al., 2020). Methionine-selenium can inhibit the miR-155/TRAF3/MAPK signaling pathway to antagonize the LPS-induced necroptosis in chicken liver (Zhirong et al., 2021). In this study, bioinformatics, network analysis and double luciferase reporter gene system analysis results revealed that miR-1679 and STAT1 had direct binding sites with negative regulatory correlation in expression levels. These indicate that STAT1 was the downstream target gene of miR-1679. STAT1, a member of the signal transducer and activator of transcription (STAT) family, plays a role in modulating various cellular biological processes, including differentiation, proliferation, and immune response. The pro-inflammatory cytokine IFN-γ (interferon-γ) can activate STAT1 in hepatocytes, activated STAT1 dimerizes and transports into the nucleus to induce transcription of MLKL and other genes involved in necroptosis and inflammation, exacerbating liver injury (Günther et al., 2016). Besides, it has been demonstrated that LPS can induce the continuous and significant expression of STAT1, STAT2, and IRF9 (interferon regulatory factor 9), resulting in macrophage necroptosis and inflammation. STAT1 knockdown of macrophage showed that RIPK3 phosphorylation level was significantly reduced, which antagonized the occurrence of necroptosis (McComb et al., 2014). These findings revealed that STAT1 is crucial in the modulation of necroptosis and inflammation.

MiR-1679 over-expression or STAT1 knockdown were carried out in LMH cells to confirm the function of miR-1679 and STAT1 in curcumin alleviating necroptosis

caused by AFB1 in hepatocytes, miR-1679 over-expression or STAT1 knockdown decreased the up-regulation of STAT1 and necroptosis genes RIPK1, RIPK3, and MLKL induced by AFB1, which are equivalent to the effects of LOC769044 silencing and curcumin intervention. These findings suggest that LOC769044/miR-1679 triggers AFB1-induced necroptosis via targeting STAT1 and curcumin can attenuate AFB1-induced necroptosis by targeting LOC769044/miR-1679/STAT1 axis.

CONCLUSIONS

Taken together, our findings indicate that LOC769044/miR-1679 triggers AFB1-induced chicken liver necroptosis by targeting STAT1 and curcumin protects against AFB1-induced necroptosis by targeting LOC769044/miR-1679/STAT1 axis. This work provides evidence that LOC769044 may be a new marker of AFB1-induced necroptosis and important theoretical and technical support for the identification of potential drug, as well as for elaborating and understanding the curcumin's detoxifying mechanism against liver toxicity caused by AFB1.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103883.

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