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Microcystin-RR promote lipid accumulation through CD36 mediated signal pathway and fatty acid uptake in HepG2 cells

Wenjuan Jia [a,b,1], Lin Zhong [a,b,1], Qingmiao Ren [c], Da Teng [a,b], Lei Gong [b], Haibin Dong [b], Jun Li [a,b], Chunxiao Wang [b], Yong-Xing He [d,**], Jun Yang [a,b,*]

[a] School of Basic Medical Sciences, Qingdao University, Qingdao, 266071, China
[b] Department of Cardiology, Yantai Yuhuangding Hospital, Qingdao University, Yantai, 264000, China
[c] The Precision Medicine Laboratory, The First Hospital of Lanzhou University, Lanzhou, 730000, China
[d] Ministry of Education Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou, 730000, China

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ABSTRACT

Microcystins (MC)-RR is a significant analogue of MC-LR, which has been identified as a hepatotoxin capable of influencing lipid metabolism and promoting the progression of liver-related metabolic diseases. However, the toxicity and biological function of MC-RR are still not well understood. In this study, the toxic effects and its role in lipid metabolism of MC-RR were investigated in hepatoblastoma cells (HepG2cells). The results demonstrated that MC-RR dose-dependently reduced cell viability and induced apoptosis. Additionally, even at low concentrations, MC-RR promoted lipid accumulation through up-regulating levels of triglyceride, total cholesterol, phosphatidylcholines and phosphatidylethaolamine in HepG2 cells, with no impact on cell viability. Proteomics and transcriptomics analysis further revealed significant alterations in the protein and gene expression profiles in HepG2 cells treated with MC-RR. Bioinformatic analysis, along with subsequent validation, indicated the upregulation of CD36 and activation of the AMPK and PI3K/AKT/mTOR in response to MC-RR exposure. Finally, knockdown of CD36 markedly ameliorated MC-RR-induced lipid accumulation in HepG2 cells. These findings collectively suggest that MC-RR promotes lipid accumulation in HepG2 cells through CD36-mediated signal pathway and fatty acid uptake. Our findings provide new insights into the hepatotoxic mechanism of MC-RR.

1. Introduction

The frequently occurring cyanobacterial blooms in eutrophic waterbodies worldwide has become a serious global public health concern in recent years. These cyanobacterial blooms are harmful partly due to the production of a variety of microcystins (MCs), which are mainly produced by *Microcystis aeruginosa* and associated with mortality and illness in both animals and humans (Huisman et al., 2018; Rastogi et al., 2014). MCs are a group of cyclic heptapeptide substances with the general structure of cyclo-(D-Ala^1 -X^2 -D-Masp^3 -Z^4 -Adda^5 -D-γ-Glu^6 -Mdha^7) in which X and Z are variable amino acids(Bouaïcha et al., 2019). Until now, more than 279 variants of MCs have been identified (Du, 2019). Among them, MC-LR and MC-RR are among the most abundant and share significant structural similarity. The maximum recommended concentration of MCs in drinking water by the World Health Organization (WHO) is 1 μg/L(Pham and Utsumi, 2018). However, the concentration of MCs ranges from 19 to 141 μg/L in water bodies experiencing worldwide algal blooms(Funari and Testai, 2008). In Chinese freshwater bodies, the maximum total MCs concentrations occurred in Lake Dianchi (23.06 μg/L)(Wei et al., 2022; Zhang et al., 2024), and the most common variants detected are MC-LR and MC-RR (Huo et al., 2021).

MC-LR is extensively studied and has been identified as a hepatotoxin, exerting potent inhibition on the eukaryotic protein phosphatase families PP1 and PP2A(Schreidah et al., 2020; Zhang et al., 2022). Multiple studies have demonstrated that both long-term and short-term exposures to MC-LR can induce apoptosis and oxidative DNA damage, impair vital immune responses, and elevate the risk of cancers in mice(Krishnan et al., 2020; Lone et al., 2016; Zegura et al., 2003). In addition, MC-LR can also alter cellular metabolism, promote lipid

* Corresponding author. Department of Cardiology, Yantai Yuhuangding Hospital, Qingdao University, Yantai, 264000, China.
** Corresponding author.
E-mail addresses: jiawj15@163.com (W. Jia), heyx@lzu.edu.cn (Y.-X. He), yangjyhd@163.com (J. Yang).
[1] Wenjuan Jia and Lin Zhong contributed equally to this study.

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accumulation and exacerbate nonalcoholic fatty liver disease (NAFLD) in mice or fish (Albadrani et al., 2019; Ma et al., 2017a). Moreover, it has been found to contribute to liver lipid metabolism disorders in high-fat diet-induced obese mice by upregulating lipid synthesis and inhibiting fatty acid β-oxidation(Chu, 2022). MC-RR also has been found to be associated with apoptosis induction, toxicity, and liver proteome alterations (Rai et al., 2018), its specific role in cellular metabolism and the underlying mechanism of remains unclear.

Lipid metabolism is a critical liver function, and dysregulation of lipid metabolism has been implicated in various human diseases, including NAFLD, diabetes and cardiovascular diseases(Yi et al., 2023). NAFLD affects approximately 25 % of the adult population and is commonly recognized as the hepatic manifestation of metabolic syndrome as it can develop to steatohepatitis (NASH), fibrosis, cirrhosis, and even liver cancer(Kasper et al., 2021; Michelotti et al., 2013). In a healthy state, there is a balance between fatty acids uptake, fatty acids synthesis, and fatty acid disposal in the liver(Hodson and Gunn, 2019). However, exposure to environmental stimuli or the onset of disease can disrupt the balance of fatty acids metabolism, leading to dysregulated lipid metabolism, which creates a lipotoxic environment and facilitates the progression of NAFLD(Geng et al., 2021). Environmental contaminants, such as microcystins, have been identified as concrete environmental risk factors associated with NAFLD, potentially influencing fatty acid uptake and influencing the development of NAFLD (Dolce and Della Torre, 2023; Zheng et al., 2021). Studies have shown that early exposure to MC-LR leads to an adolescent NAFLD phenotype in mice(Al-Badrani et al., 2020), and individuals with long-term environmental exposure to MCs may be be more susceptible to NAFLD(Zhao et al., 2020). However, the impact of MC-RR on lipid metabolism remains relatively limited.

In present study, we have successfully identified the minimal concentration of MC-RR capable of enhancing lipid accumulation in HepG2 cells, while preserving cell viability. Through a comprehensive approach integrating metabolic and proteomic analyses, our investigation unveiled that exposure to MC-RR resulted in a significant augmentation of cellular triglycerides (TG), total cholesterol (T-CHO), phosphatidylethanolamine (PE), and phosphatidylcholine (PC), thus driving the observed lipid accumulation. Furthermore, we have elucidated that MC-RR likely exerts its influence on lipid accumulation through a complex mechanism that involves the uptake of lipids mediated by CD36 and the modulation of cellular metabolism through the AMP-activated protein kinase (AMPK) and the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway.

2. Materials and methods

2.1. Reagent

Microcystin-RR (MC-RR) was purchased from Qingdao IniKem Bio-PharmaTech Co., Ltd (Qingdao, China) and dissolved in double-distilled water (200 mg/ml). The stock solution was diluted using complete medium in order to achieve the appropriate concentration which ranged from 0.5 to 2 mg/ml. Oleic acid (OA) and palmitic acid (PA) were acquired from Sigma-Aldrich (Saint Louis, MO). A solution containing a mixture of free fatty acids (20 mM oleic acid + 10 mM palmitic acid) was prepared as described previously(Li et al., 2021). In brief, 20 mM OA and 10 mM PA were dissolved in 0.1 mol/L NaOH solution at 70 °C and isometrically mixed with 20 % FA-free BSA solution. The mixed free FA stock solution (10 mM OA, 5 mM PA and 10 % BSA) was diluted 50-fold with the complete medium immediately before use.

2.2. Cell culture and treatment

The HepG2 (human hepatocellular carcinoma) cells were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 % (w/v) penicillin and streptomycin (Sangon, Shanghai, China) at a temperature of 37 °C in a 5 % CO_2 environment.

The cell-culture dish was treated with 100 μg/ml poly-D-lysine (Sangon, Shanghai, China). MC-RR and mixed free FA solution were added depending on experimental requirement.

2.3. shRNA, transfection and stable cell lines construction

Small hairpin (sh)RNA targeting human CD36 and negative control shRNA (shNC) were cloned into miRZip™ shRNA Expression Lentivector (Systembio, Shanghai, China). The target sequence of CD36 was 5′-CACTATCAGTTGGAACAGA-3′(sh1) and 5′-CCGACGTTAATCTGAAAGGA-3′ (sh2), respectively. The establishment of stable HepG2 cell lines with CD36 knockdown was achieved using lentivirus infection. The lentivirus particles were generated by HEK 293T cells transfected with packaging plasmid psPAX2, envelope plasmid pMD2.G, and target plasmids. The plasmids harboring shNC and shCD36 were introduced into HEK 293T cells by transfection using ExFect Transfection Reagent (Vazyme, China) in accordance with the instructions provided by the manufacturer. Following a 48–72 hours incubation period in the presence of lentivirus, a concentration of 2 μg/mL puromycin was introduced to facilitate the identification of cells exhibiting positive characteristics. The media containing puromycin was replenished at 48 hours intervals.

2.4. Cell viability (CCK-8 assay)

The effects of MC-RR on cell viability were determined by the Cell Counting Kit-8 (CCK8) assay. HepG2 cells were seeded into 96-well plates. The medium was then substituted with complete medium containing MC-RR (0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 μM) after cell adherence, respectively. Cells were incubated for further 24 hours, then 10 μl CCK8 reagent (Vazyme, China) was added into each well, following with and culture another 2hr at 37 °C. Finally, optical density value (OD450) was measured and cell viability was calculated followed the formula with three independent biological replicates experiments:

$$\text{Cell viability} = [(OD450_{treatment}\text{-}OD450_{blank})/[(OD450_{control}\text{-}OD450_{blank})] \text{ x100 \%}$$

2.5. Flow cytometry analysis

To evaluate apoptosis, cellular samples were exposed to staining with FITC and propidium iodide (PI) using the Annexin V-FITC/PI Apoptosis Detection Kit (Yeason, China) in accordance with the provided instructions.

For measurement of lipid accumulation, HepG2 cells were stained with Bodipy 493/503 or Nile red (Medchemexpress, China) according to the manufacturer's recommended procedure and subjected to flow cytometry using MoFlo XDP (Beckman Coulter Inc, USA). Both the measurement of apoptosis and lipid accumulation were performed three independent biological replicates experiments.

2.6. Bodipy 493/503 and oil red O staining

HepG2 cells were fixed and subjected to staining with either bodipy 493/503 or Oil Red O solution (Oil Red O staining kit, Solarbio, China), following the instructions provided by the manufacturer, respectively. Stained cells were visualized on microscope and representative images were shown in each figure.

2.7. Determination of intracellular total triglyceride and cholesterol content

The intracellular levels of total triglyceride (TG) and cholesterol (T-CHO) were measured and standardized to the total intracellular protein concentration using a TG or T-CHO assay kit (Nanjing Jiancheng

Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol with three independent biological replicates experiments.

2.8. Metabolomics analysis

Cells were collected and subjected to quality control (QC), then the QC samples were prepared and dissolved in 50 % acetonitrile. Each sample underwent filtration with a disposable 0.22 μm cellulose acetate and subjected to analysis using a mass spectrometry instrument. The metabolomics profiling was conducted by employing a UPLC-ESI-Q-Orbitrap-MS system (UHPLC, Shimadzu Nexera X2 LC-30AD, Japan), in conjunction with the Q-Exactive Plus instrument manufactured by Thermo Scientific in the United States. The acquired mass spectrometry (MS) data underwent processing using MS-DIAL software to perform peak alignment, retention time correction, and peak area extraction. The identification of metabolites was conducted by accuracy mass (mass tolerance <10 ppm) and MS/MS data (mass tolerance <0.02Da). These data were compared against various databases including HMDB, mass bank, and other publicly available databases, as well as our own metabolite standard library. The extracted-ion features were filtered to retain only variables that had a minimum of 50 % nonzero measurement values in at least one group.

2.9. Proteomics analysis

Protein was extracted from the cell sample and the supernatant was collected and subjected to quantification. Then the protein (200 μg for each sample) was digested with FASP method(Wiśniewski et al., 2009). Briefly, dithiothreitol (DTT) and iodoacetamide (IAA) were introduced into the UA buffer to inhibit the activity of reduced cysteine. The protein suspension was subjected to digestion with trypsin overnight at 37 °C and subsequent collecting and desalting using a C18 StageTip in preparation for LC-MS analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were conducted using a Q Exactive Plus mass spectrometer, which was connected with an Easy 1200 nLC system from Thermo Fisher Scientific. The obtained raw files were searched against uniprot-Homo sapiens (Human)[9606]-207393-20221227.fasta (207393 total entries, downloaded 2022.12.27) using Proteome Discoverer software version 2.4.

2.10. Western Blotting analysis

The cells were rinsed with ice-cold phosphate-buffered saline (PBS) and then disrupted in radioimmune precipitation assay buffer. This buffer consisted of 150 mM sodium chloride (NaCl), 50 mM Tris–hydrochloride (Tris–HCl), 1 % Nonidet P-40, and 0.1 % sodium dodecyl sulfate (SDS). Additionally, a protease inhibitor combination from Roche (Indianapolis, USA) was included in the solution. Protein concentrations were measured using the BCA assay kit (Yeason, China). An equivalent quantity of protein was subjected to SDS-PAGE, thereafter transferred onto nitrocellulose membranes, and subsequently probed with the following primary antibodies: anti-CD36 (1:1000, Immunoway, China); anti-AMPK/p-AMPK (1:1000, Abclonal, China), anti-PI3K/p-PI3K (1:1000, Abclonal, China), anti-AKT/p-AKT(1:1000, Abclonal, China); anti-mTOR/p-mTOR (1:1000, Santa Cruz Biotechnology, USA) and anti-β-actin (1:3000, Proteintech, China). All of Western Blotting analysis were conducted in three independent biological replicates experiments.

2.11. Bioinformatic analysis

Metabolome, transcriptome and proteome data was analyzed with R (version:4.0.3), R packages and DAVID (https://david.ncifcrf.gov/) and the analysis results are presented using Graphpad Prism 8.0.

2.12. Statistical analysis

Statistical analysis was performed using two-tailed unpaired Student's *t*-test with Graphpad Prism 8.0. Statistical significance is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001. The density of the bands was quantified by ImageJ (Rawak Software, Inc.).

3. Results

3.1. MC-RR reduced cell viability and caused apoptosis in a dose-dependent manner

As in vitro alternatives to primary human hepatocytes, HepG2 cells are most commonly used in drug metabolism and hepatotoxicity studies. To examine the impact of MC-RR on HepG2 cells (Fig. 1A) on HepG2 cells, MC-RR was introduced into the cell culture, followed by a CCK8 assay to evaluate cell viability. As shown in Fig. 1B, MC-RR inhibited viability after concentrations greater than 0.75 μM. Flow cytometry analysis (FACS) with Annexin V-FITC/PI double staining also confirmed that treatment with MC-RR led to cell apoptosis in a dose-dependent manner (Fig. 1C). MC-RR did not induce obvious apoptosis within the dose ranging from 0 to 0.5 μM, indicating that low concentration of MC-RR did not affect cell viability.

3.2. Low concentration of MC-RR aggravated lipid accumulation in HepG2 cells

Recent studies suggested that MC-LR aggravates liver lipid metabolism disorders. To study whether MC-RR treatment also affected lipid metabolism, HepG2 cells were cultured in low fatty acid (FA) conditions (0.2 mM Oleic acid and 0.1 mM palmitic acid) and treated with MC-RR for 24 hours. Quantitative analysis using FACS revealed a dose-dependent increase in lipid accumulation upon exposure to MC-RR. Notably, even at low concentrations, MC-RR was found to promote lipid accumulation (Fig. 2A). Bodipy 493/503 and Oil red O staining confirmed that MC-RR facilitated lipid accumulation in HepG2, respectively (Fig. 2B). Exposure to MC-RR also increased total intracellular triglyceride (TG, Fig. 2C) and cholesterol (T-CHO, Fig. 2D). In summary, low concentration of MC-RR could facilitate lipid accumulation in HepG2 cells without affecting cell viability.

Since MC-LR has been found to promote lipid accumulation through regulating serum choline in rat livers(Zhang et al., 2016), non-targeted metabolome analyses were performed to gain further insights into the alteration of metabolites after MC-RR exposure. Clear separations between two groups were observed in the principal component analysis (Fig. 3A) and orthogonal partial least squares-discriminant analysis (OPLS-DA, Fig. 3B) both before and after MC-RR treatment. The average expression values of metabolites in MC-RR and control group (foldchange>2, p value < 0.05) indicated differential expression of lipid and lipid-like molecules (Fig. 3C). Exposure to MC-RR disrupted the level of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), such as PE (16:0/20:4 (5Z,8Z,11Z,14Z)), PC (18:1 (9Z)/14:0, PE (18:1 (11Z)/20:4 (5Z,8Z,11Z,14Z)), PC (15:0/20:4), PC (O-16:0/18:1) and PC (O-16:0/20:5) (Fig. 3D). In addition, exposure to MC-RR resulted in an upregulation of the relative abundance of both PE and PC (Fig. 3E). These findings indicated that exposure to MC-RR increased cellular TG, T-CHO, PE and PC, ultimately lead to lipid accumulation in HepG2 cells.

3.3. Identification of differentially expressed proteins and related pathways in MC-RR exposure cells

To further interrogate the function of MC-RR in the regulation of lipid metabolism, HepG2 cells were cultured and then subjected to RNA sequencing and proteomic analysis (Figure S1 and Fig. 4). Proteomic analysis identified a total of 5791 unique proteins (Fig. 4B). PCA analysis revealed that the proteome profiles of samples in the MC-RR group were

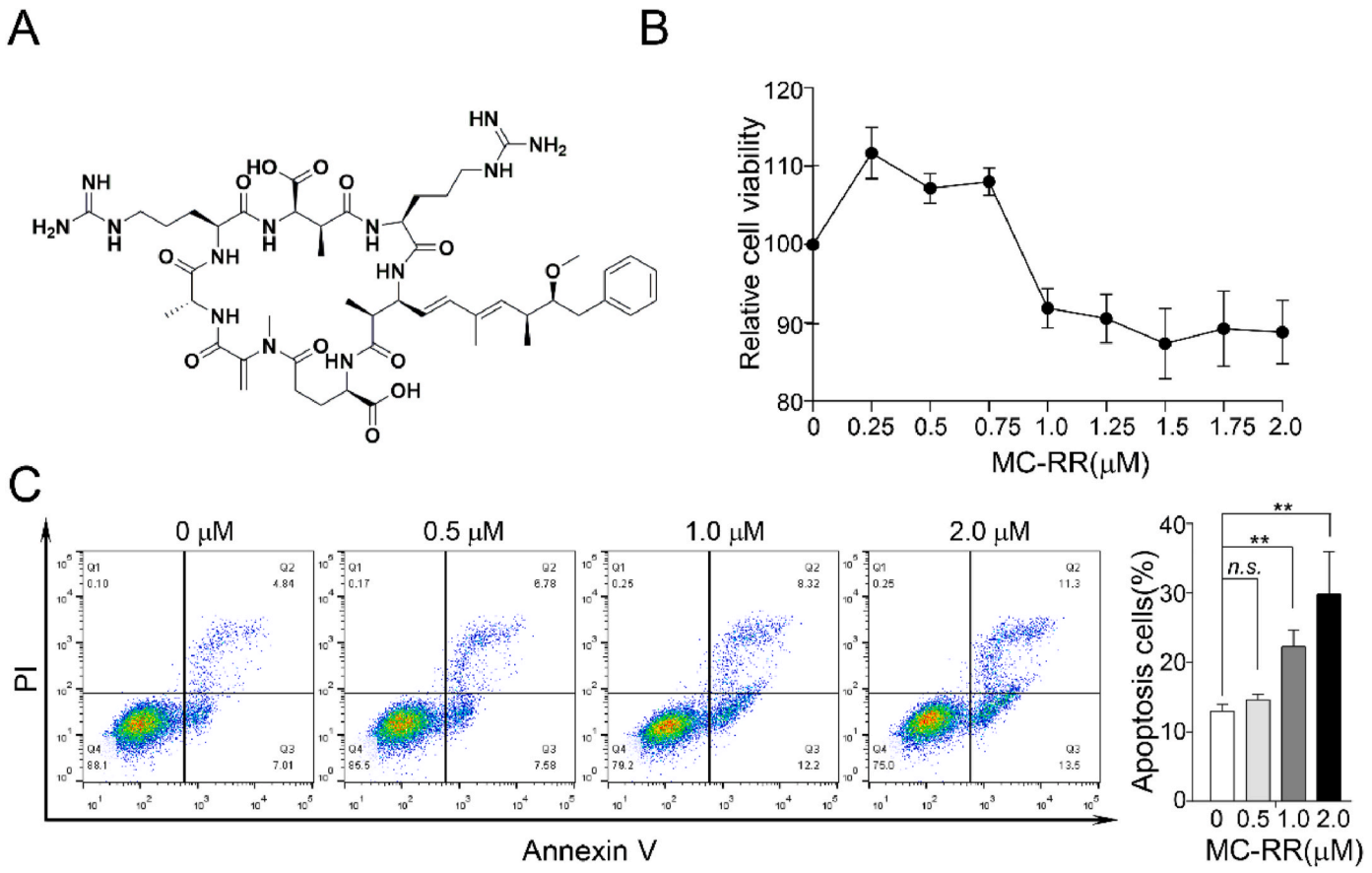

Fig. 1. MC-RR decreased cell viability and induced cell apoptosis in human hepatoma HepG2 cells. (A) Chemical structures of MC-RR. (B–C). Cell viability (B) and cell apoptosis (C) were determined after HepG2 cells were exposed to different doses of MC-RR for 24 hours. The data are presented as mean ± SD. *:P < 0.05; **:P < 0.01.

separated from those in the control group (Fig. 4C). Based on a fold-change of 1.5 and p-value less than 0.05 between the two groups, we identified 207 significantly differentially expressed proteins in the MC-RR group compared with the control group. Notably, the scavenger receptor SR-B3(CD36), which mediates the transport of fatty acid, was more highly upregulated (Fig. 4D). Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene ontology (GO) analysis were performed to gain insights into their biological functions. This bioinformatics enrichment analysis showed that these differentially expressed proteins contribute to various signaling pathways associated with lipid metabolism, such as AMPK signaling pathway, PI3K-Akt signaling pathway, insulin resistance and Hippo pathway signaling pathway (Fig. 4E–F). Intriguingly, RNA sequencing analysis showed the enrichment of the processing of phagosome, inflammatory and immune-related pathways (Fig. S1), the integrated analysis of metabolic and proteomic showed that steroid biosynthesis and ATP binding cassette transporter (ABC) transporters were affected (Fig. S2) after MC-RR treatment. Those multi-omics analysis data further support the association between MC-RR and lipid metabolism.

3.4. MC-RR up-regulated CD36 and activated AKT/PI3K/mTOR pathway

In order to further explore the molecular mechanism whereby MC-RR affects lipid metabolism in HepG2 cells, we then validated the proteomic data by QRT-PCR and western blot. As shown in Fig. 4A and Fig. S2, dose dependent increase of CD36 were observed at protein and mRNA levels after MC-RR treatment. Additionally, the expression of AMPK and p-AMPK were assessed and quantitatively analyzed. We observed a significant decrease of AMPK by adding FA in medium. Exposure to MC-RR reduced AMPK expression even further while remarkably elevate expression of p-AMPK (Fig. 4A). On the other hand, the relative expression of p-PI3K, p-Akt, and p-mTOR proteins were found to increase significantly in the MC-RR group. Notably, transcriptional level of AMPK, PI3K, AKT and mTOR were all up-regulated after MC-RR treatment (Fig. S2). These results suggested that not only AMPK signaling pathway but also PI3K/Akt/mTOR signaling pathway was involved in MC-RR regulated lipid metabolism.

3.5. Knockdown of CD36 meliorated MC-RR induced lipid accumulation in HepG2 cells

To investigate role of CD36 in lipid metabolism regulated by MC-RR, we knocked down the endogenous expression of CD36 by CD36-targeting short hairpin RNA (shRNA) in HepG2 cells. Knockdown efficiency was confirmed at RNA level and protein level (Fig. 6A and B), the results indicated that CD36 was more significantly suppressed by sh2. The control and knockdown cells with sh2 were then subjected to FACS analysis with Nile red lipid visualization method to detect lipid content in cells exposed to 0, 0.5, 2 μM MC-RR, respectively. As shown in Fig. 6C, the FACS results indicated that the silencing of CD36 significantly decreased up-regulated lipid content induced by MC-RR, confirming that CD36 contributes to MC-RR mediated lipid accumulation in HepG2 cells.

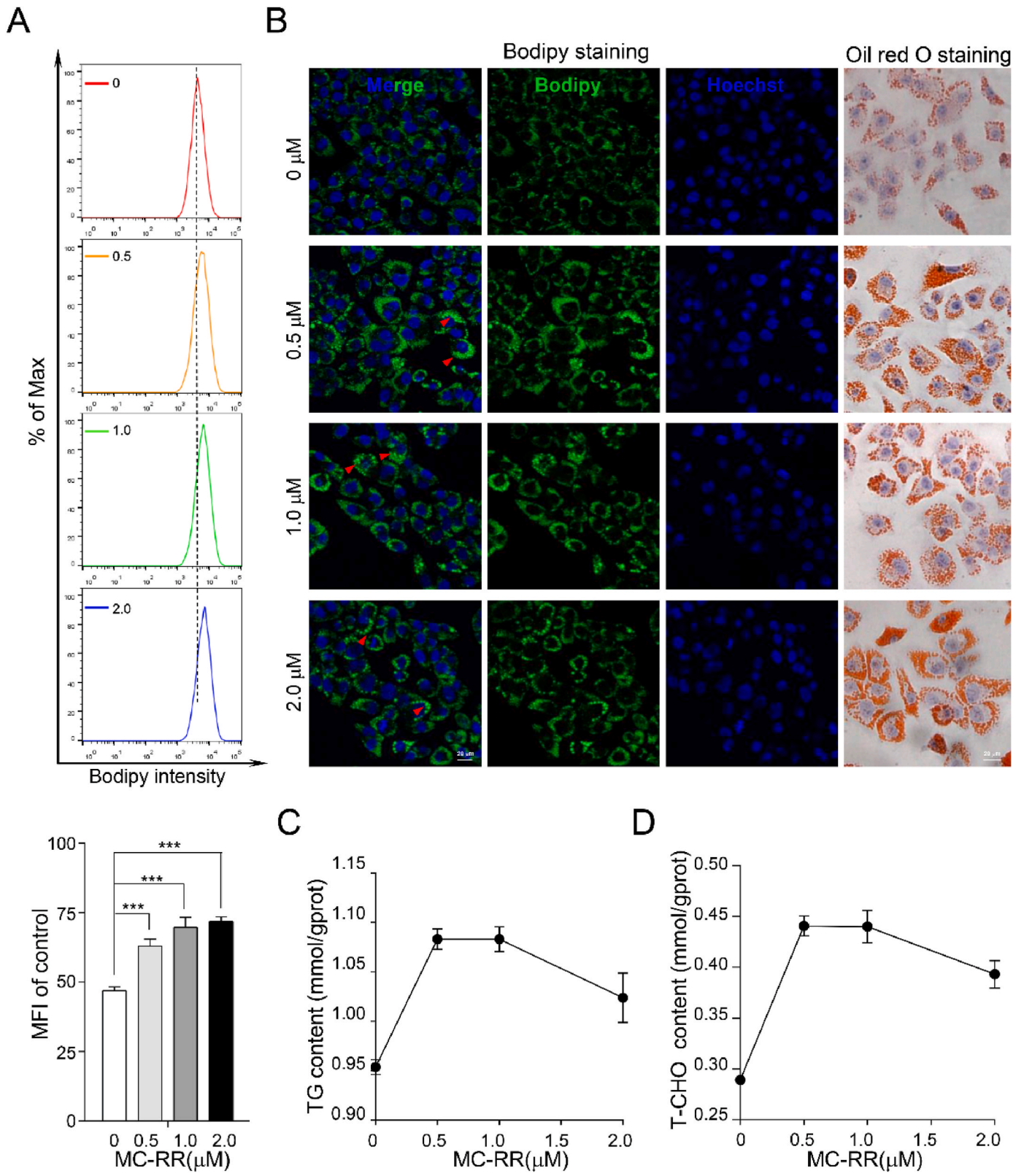

Fig. 2. MC-RR facilitated lipid accumulation in HepG2 cells. (A–D) HepG2 cells were treated with FA and different doses of MC-RR for 24 hours, respectively, and then lipid accumulation was detected through (A–D) flow cytometer with Bodipy 493/503 (A), Bodipy 493/503 staining and Oil Red O staining (B), total triglyceride test (C) and total cholesterol test (D).

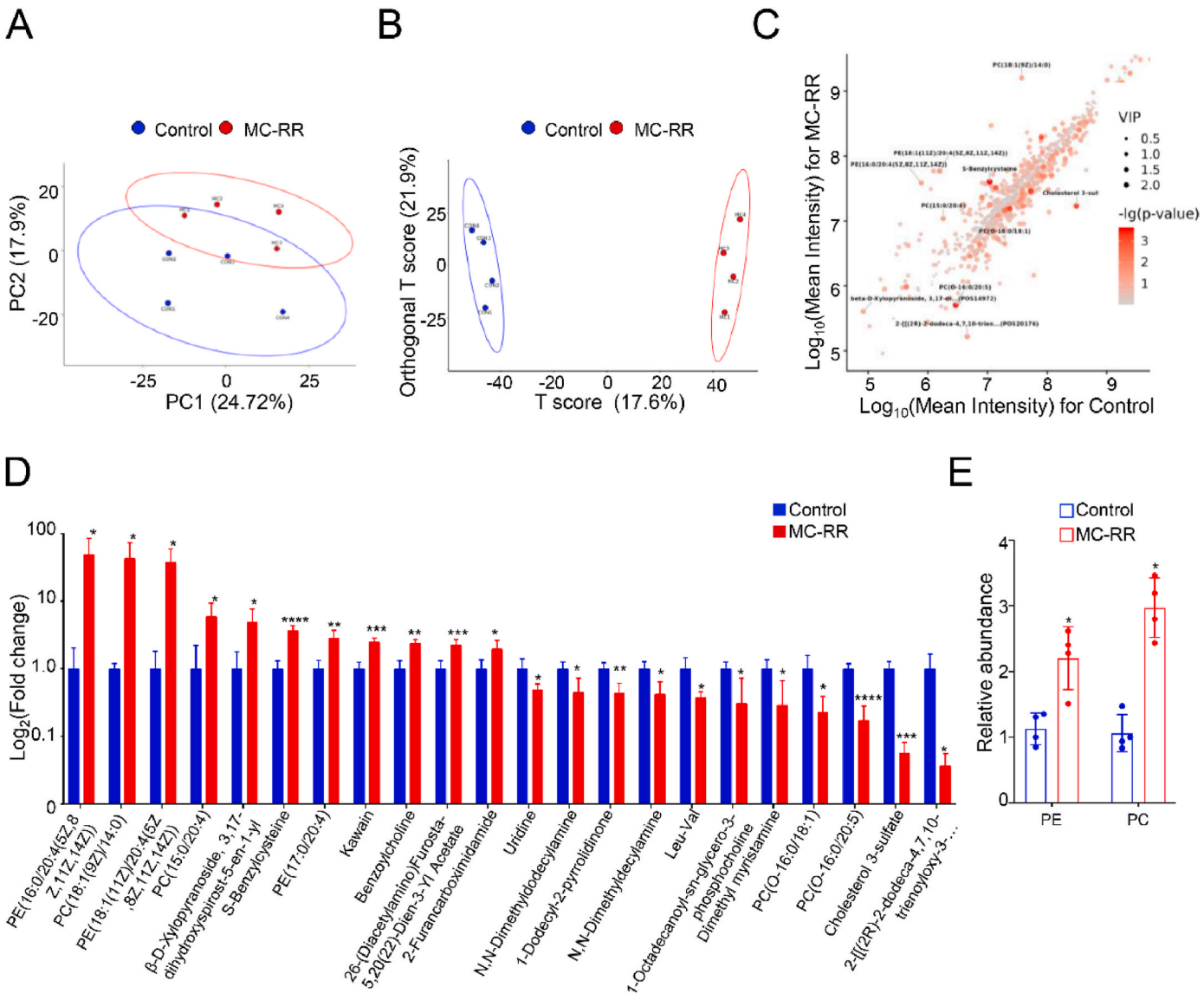

Fig. 3. Comprehensive analysis of untargeted metabolomics of MC-RR treated HepG2 cells and controls. (A–B) PCA (A) and OPLS-DA plots of metabolomics data. (C) Compare scatter plots of average expression values for MC-RR. vs. Control. (D) Changes of identified metabolites (Fold change >2, p value < 0.05) in response to MC-RR exposure. (E) Relative abundance of total PE and PC in MC-RR treated and control group. The data are presented as mean ± SD. *:P < 0.05; **:P < 0.01; ***:P < 0.001; ****:P < 0.0001.

4. Discussion

Up to now, more than 279 variants of MCs have been identified. Among them, MC-RR is second most toxic. In the present study, we found that MC-RR reduced cell viability and caused apoptosis in a dose-dependent manner, as well as facilitated lipid accumulation through regulating CD36/AMPK and activating PI3K/AKT/mTOR signaling pathway.

MCs, which are the most widely distributed cyanotoxins, have the potential to be absorbed by the human body through the digestive tract, respiratory tract and skin contact, leading to detrimental effects on to liver, gonad, brain, kidney, spleen and resulting in multi-organ toxicity (Ma et al., 2021).MCs are typical hepatotoxin and could induce hepatic injury and exacerbate NAFLD(Albadrani et al., 2017; Zhang et al., 2022). MC-LR has been reported to affect HepG2 cell physiology at the concentration of 0.5 μM(Zhang et al., 2013). Ma et al. discovered that exposure to 10 and 50 μM MC-LR for 24 hours induced cytotoxicity, altered microRNA expression profiling, and influenced signaling pathways including MAPK, biosynthesis of secondary metabolites, and pyrimidine and purine metabolism(Ma et al., 2017b). Meanwhile, a low concentration of MC-LR (e.g., 0.5 μM) was found to have no effect on cell growth and survival(Ma et al., 2016). However, under hypoxic conditions, such as at a concentration of 0.1 μM, MC-LR decreased cell viability (Zhang et al., 2013). Interestingly, we observed that the cytotoxicity of MC-RR on HepG2 cells is dependent on its concentration. At low concentrations (less than 0.75 μM), MC-RR failed to induce cytotoxicity or show any significant difference in apoptosis compared to the control group after 24 hours of treatment. In contrast, at high concentration (more than 1.0 μM), MC-RR significantly reduced viability and induced apoptosis (Fig. 1B and C). A previous study indicated that the administration of MC-RR to mice at a concentration of 200 μg/kg caused toxicity and alterations in the liver proteome (Rai et al., 2018), our study further clarified the toxic concentration of MC-RR in HepG2 cells. Notably, the transcriptome analysis revealed enrichment of inflammatory and immune-related pathways (Fig. S1), suggesting that low concentration of MC-RR may have transcriptional effects on cells that may not immediately reflected in aspects such as cell activity and apoptosis.

MCs have been reported to interfere with lipid metabolism in liver

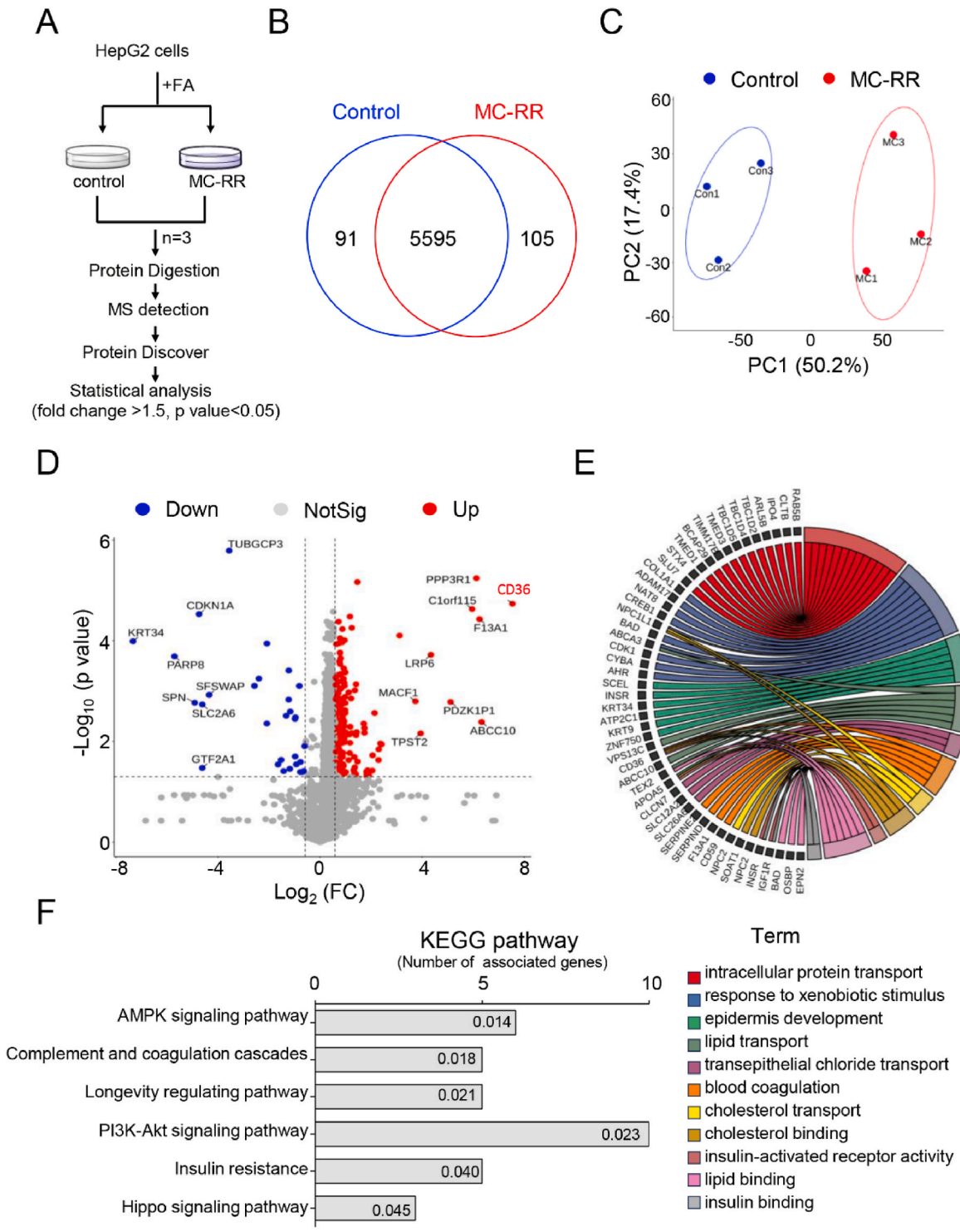

Fig. 4. The proteomic response to low concentration of MC-RR treatment. (A) Experimental strategy to characterize proteomic changes. (B) The principal component analysis (PCA) chart of MC-RR group and control group. (C) Venn diagram of detected proteins in two groups. (D) Volcano graph of the differential protein distribution. (E–F) Signaling pathways involved by altered expression of proteins in MC-RR group with KEGG (E) and GO analysis (F).

tissue and cells. Zhang et al. indicated that chronic exposure to MC-LR induces abnormal lipid metabolism via endoplasmic reticulum stress in male zebrafish(Zhang, 2020). In mice, MC-LR causes serum metabonomic alterations and hepatic lipid vacuoles accumulation(Chu, 2022; Zhang et al., 2016). MC-LR can altered cellular metabolism of HepG2 cells and induced lipid peroxidation(Ma et al., 2017a). In addition, it has been reported that both MC-LR and MC-RR can perturb cholesterol synthesis, fatty acids and lipid oxidation in zebrafish(Pavagadhi et al., 2013). In present study, we have provided additional evidence supporting MC-RR as a promoter of lipid accumulation in HepG2 cells. Notably, we observed that low concentrations of MC-RR (0.5 μM) did not adversely affect cell viability or induce apoptosis (Fig. 1B and C), but they accelerated lipid accumulation and increased cellular TG and T-CHO (Fig. 2), both which up-regulation promote liver lipid accumulation and contribute to NAFLD progression (Kerr and Davidson, 2012). We also discovered that exposure to MC-RR disrupts lipid homeostasis,

including PE and PC, as indicated by metabolomics analysis (Fig. 3). Given that the highest concentration of MCs can reach up to 141 μg/L in water bodies and MC-RR is one of the commonly found MC variants (Funari and Testai, 2008), our study suggests that low concentrations of MC-RR in the environment may not significantly impair cell activity but can potentially influence cellular metabolism. Therefore, it is important to pay attention to the potential link between exposure to MCs in polluted waters and the increased incidence of certain metabolic diseases in the surrounding area.

TG and CHO are the main constituents of cellular lipid droplets (Gluchowski et al., 2017), and their accumulation is considered a key characteristics of NAFLD(Kerr and Davidson, 2012). The relative abundance of PC and PE plays a crucial role in the surface dynamics of lipid droplets in liver cells(Guo et al., 2008). Alterations in the absolute concentrations of PC and PE, as well as changes in the molar ratio between PC and PE, have been reported to contribute to the development of NAFLD, impair liver regeneration, or exacerbate alcoholic fatty liver disease(Kharbanda et al., 2007; Li et al., 2006; Ling et al., 2012). Exposure to MC-RR increases the levels of TG, T-CHO, PE and PC (Fig. 2C–D, Fig. 3E), suggesting that MC-RR may accelerate the formation of lipid droplets during lipid metabolism.

PC and PE typically contains saturated fatty acyl chains or polyunsaturated FAs in their side chains(Gibellini and Smith, 2010). CD36 is of utmost importance in facilitating the uptake of FAs and contributes under excessive fat supply to lipid accumulation and metabolic dysfunction(Pepino et al., 2014). Exposure to MC-RR increased expression of CD36 in HepG2 cells (Fig. 3C–F; Fig. 4A and Fig. S2), indicating MC-RR may accelerate lipid accumulation through CD36 mediated FA uptake. We also observed that the addition of FA to the medium reduced the expression of AMPK, while exposure to MC-RR up-regulated expression of phosphorylated AMPK (p-AMPK) (Fig. 4A), indicating the activation of the AMPK pathway by MC-RR. The AMPK pathway plays a crucial role in regulating energy homeostasis and metabolic processes, and its activity can be suppressed by excessive nutrient levels (Coughlan et al., 2013). Dysregulation of AMPK signaling may impair the ability to adjust FA oxidation to FA availability, ultimately resulting in lipid accumulation and insulin resistance in skeletal muscle(Ruderman et al., 2013; Samovski et al., 2015). Energy stresses activates AMPK, which subsequently upregulates nutrient uptake and catabolism(Hardie et al., 2016). Intriguingly, CD36 was found to regulate lipophagy in

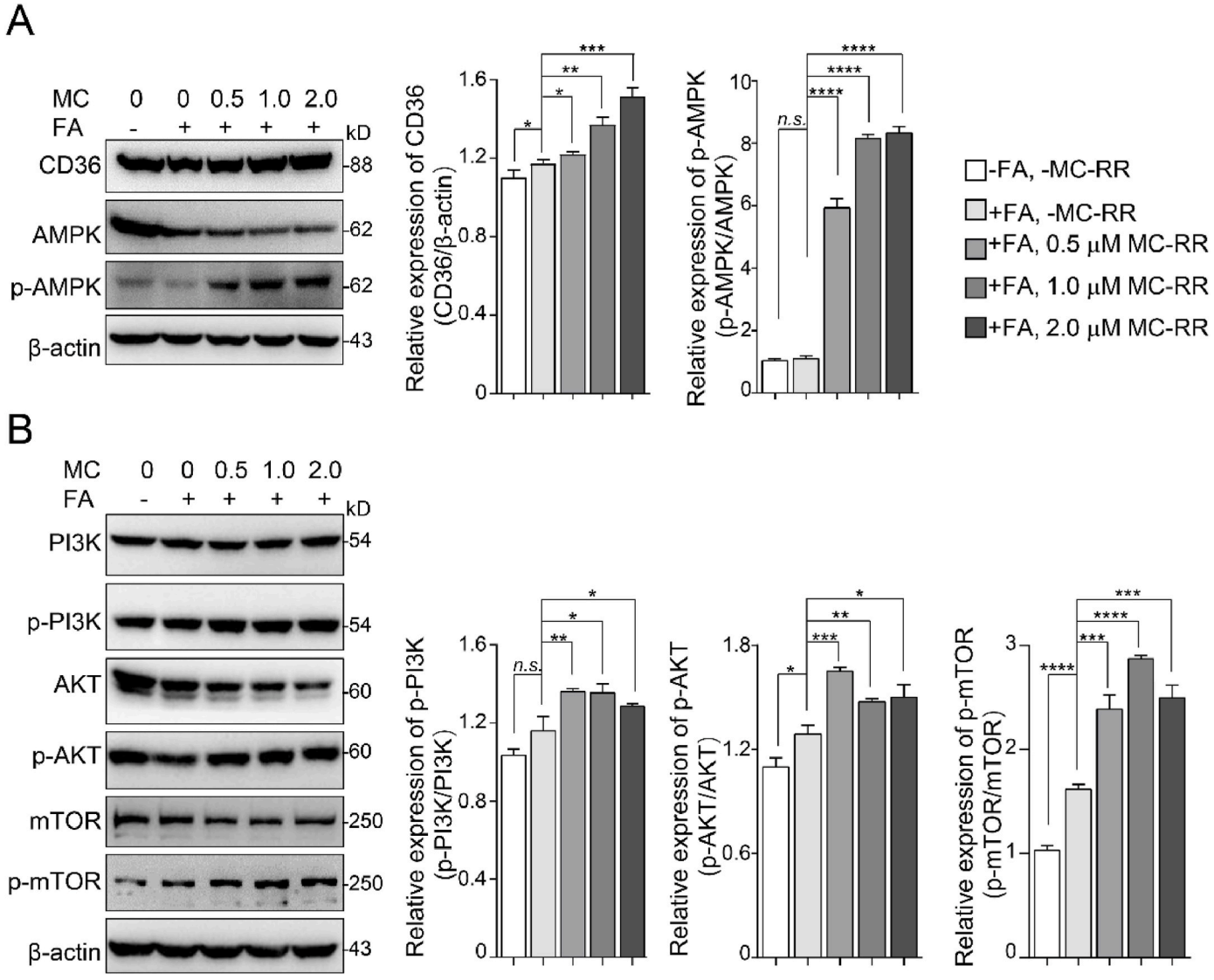

Fig. 5. MC-RR up-regulated CD36 and activated PI3K/AKT/mTOR signaling pathway. Lysates of HepG2 cells exposed to different dose of MC-RR were immunoprecipitated with (A) lipid metabolism related CD36, AMPK, p-AMPK, and (B) proteins involved in PI3K/AKT/mTOR pathway (PI3K, p-PI3K, AKT, p-AKT, mTOR and p-mTOR). Data are expressed as the phosphorylation levels of individual proteins were normalized to the total abundance of the respective proteins and CD36 was normalized with β-actin. The data are presented as mean ± SD. *:P < 0.05; **:P < 0.01; ***:P < 0.001; ****:P < 0.0001.

hepatocytes through an AMPK-dependent pathway(Li et al., 2019). AMPK is significantly activated when phosphorylated at Thr172, and the activated AMPK further enhances recruitment of CD36(Hao et al., 2020; Samovski et al., 2015). On the other hand, activated AMPK in hepatocytes also upregulates CD36 expression, resulting in uptake of fatty acids, hepatocellular lipid accumulation, and the development of fatty liver(Choi et al., 2017). Alternatively, CD36-mediated FA uptake also regulates the PI3K/AKT/mTOR signaling pathway, which plays a crucial role in maintaining cellular FA homeostasis(Samovski et al., 2015).We detected that the relative expressions of phosphorylated PI3K (p-PI3K), phosphorylated Akt (p-Akt), and phosphorylated mTOR (p-mTOR) were upregulated after treatment with MC-RR, indicating activation of the PI3K/Akt/mTOR signaling pathway after exposure to MC-RR (Fig. 4B). AKT modulates the mTORC1-S6K1 pathway, which in turn regulates lipid metabolism through mTORC1 and SREBP(Huang et al., 2018). mTORC1 is involved in the regulation of PC synthesis, thus influencing hepatic lipid homeostasis(Quinn et al., 2017). CD36 has been shown to mediate the activation of the PI3K/AKT/mTOR signaling pathway, and promote the progression of hepatocellular carcinoma (HCC) (Luo et al., 2021). Interestingly, MC-LR has been reported to enhance lipid accumulation by inducing unsaturated fatty acid biosynthesis through the PI3K/AKT/mTOR signaling pathway(Chu, 2022). In summary, exposure to MC-RR may upregulate CD36 expression and activated AMPK and PI3K/AKT/mTOR (Figs. 4 and 5), thereby promoting lipid accumulation in HepG2 cells.

CD36 has been found to mediate the activities of ABC transporters in liver and macrophages(Ulug and Nergiz-Unal, 2021), which is associated with cholesterol synthesis and homeostasis (Chatuphonprasert et al., 2018). ABC transporter also plays critical roles in lipid transport across the cytomembrane(Neumann et al., 2017). Interestingly, the

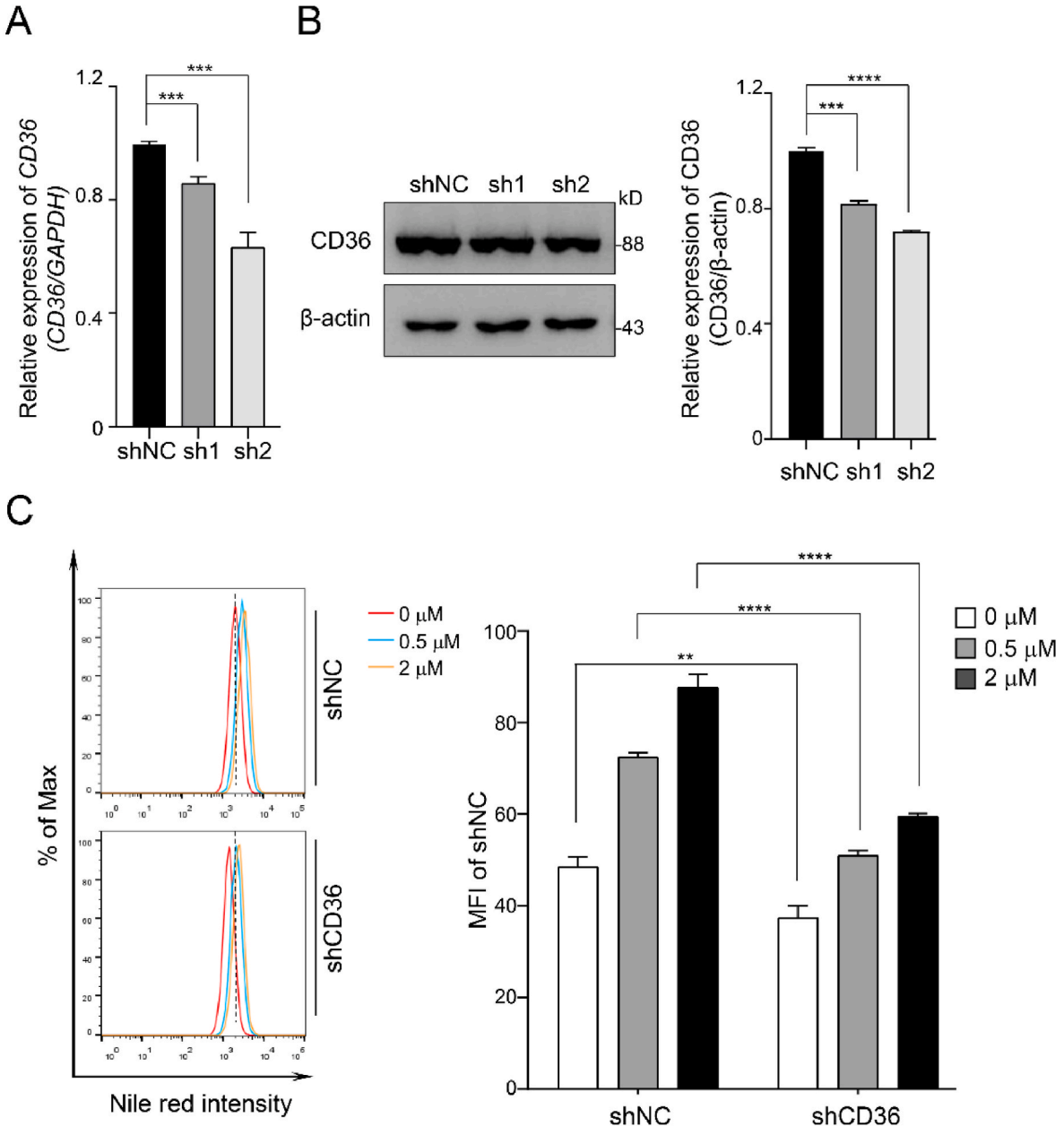

Fig. 6. Knockdown of CD36 in HepG2 cells suppressed lipid accumulation induced by MC-RR. (A–B) qPCR (A) and western blot analyses (B) of CD36 expression in control (shNC) and shRNA-CD36 (sh1 and sh2) cells. (C) CD36 knockdown cells with sh2 (shCD36) and control cells (shNC) were incubated with FA and 0, 0.5, 2 μM MC-RR, respectively, and then subjected to FACS analyses with Nile red. The data are presented as mean ± SD. *:P < 0.05; **:P < 0.01; ***:P < 0.001; ****:P < 0.0001.

results of multi-omics integrated analysis indicate that exposure to MC-RR affected steroid biosynthesis and ABC transporters (Fig. S2). Suppression of CD36 attenuates hepatic lipid accumulation, while increased expression of CD36 in the liver leads to lipid accumulation and significantly influences the progression of NAFLD(Miquilena-Colina et al., 2011; Zhang et al., 2018). In our study, we observed that the deficiency of CD36 decreased lipid accumulation induced by MC-RR (Fig. 6). Taken together, our finding suggests that MC-RR may accelerate the lipid droplets formation through CD36 mediated FA uptake, lipid synthesis, and CD36 related signaling pathway.

5. Conclusions

This study demonstrated that low concentration of MC-RR can induce an increase in the levels of TG, T-CHO, PC and PE and finally lead to lipid accumulation in HepG2 cells. Furtherly, it was determined that MC-RR may promote lipid accumulation through CD36-AMPK and CD36-PI3K/AKT/mTOR mediated FA uptake and lipid metabolism. These findings provide new insights into the mechanisms underlying the effect of MC-RR on hepatic cell lipid metabolism and further confirm the promoting effect of MCs in the development and progression of NAFLD. Furthermore, the study also suggests that both high and low concentrations of MCs in polluted waters should be taken into consideration, since low concentration of MC-RR may not affect cell viability, but still exert transcriptional effects and promote lipid accumulation.

Environmental Implication

The production of a variety of microcystins by *Microcystis aeruginosa* is the main cause of frequently occurring cyanobacterial blooms, which have become a serious global public health concern. Microcystins have been found to act as hepatotoxins, potently inhibiting the eukaryotic protein phosphatase families PP1 and PP2A, and can induce apoptosis and increase risk of cancers. In this study, we determined the minimum concentration of the toxic microcystins-RR and elucidated its potential impact on lipid accumulation through a multifaceted mechanism. These findings shed new light on the mechanisms underlying microcystin-RR's effect on lipid metabolism in hepatic cells.

CRediT authorship contribution statement

Wenjuan Jia: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Lin Zhong:** Validation, Project administration. **Qingmiao Ren:** Software, Methodology. **Da Teng:** Methodology. **Lei Gong:** Resources. **Haibin Dong:** Formal analysis, Data curation. **Jun Li:** Data curation. **Chunxiao Wang:** Methodology. **Yong-Xing He:** Writing – review & editing, Conceptualization. **Jun Yang:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.118402.

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