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Microcystin-leucine arginine mediates apoptosis and engulfment of Leydig cell by testicular macrophages resulting in reduced serum testosterone levels

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ABSTRACT

Microcystin-leucine arginine (MC-LR) causes decline of serum testosterone levels resulting in impaired spermatogenesis; however, the underlying molecular mechanisms are not fully understood. In this study, we aimed to investigate the effects of MC-LR exposure on the number of Leydig cells (LCs) in testis. Following chronic low dose exposure to MC-LR, the number of LCs was markedly decreased while macrophages were significantly increased. Then, we established a co-culture system to study the interaction between macrophages and LCs in the presence of MC-LR. No significant apoptosis of LCs cultured alone was observed after MC-LR (< 5 000 nM) treatment; however, apoptosis was robustly increased when LCs were co-cultured with macrophages in the presence of MC-LR. Further studies identified that MC-LR could stimulate macrophage to produce TNF-α, and secreted TNF-α induced LC apoptosis by binding to the tumor necrosis factor receptor 1 (TNFR1) on the LCs and thus activating reactive oxygen species (ROS)-p38MAPK signaling pathway. Furthermore, we also examined increased expression of Axl receptor and growth arrest-specific 6 (Gas6) in macrophages after MC-LR treatment. GAS6 mediates phagocytosis of apoptotic LCs by binding to the Axl receptor on macrophages and phosphatidylserine (PtdSer) on apoptotic LCs. Together, these results suggested that reduced serum testosterone levels may be associated with decrease of LCs as a result of LC apoptosis and phagocytosis by immune cells in MC-LR-treated mice.

1. Introduction

Recently, an increasing number of lakes and rivers are facing the threat of microcystins (MCs) as a result of increased proliferation of freshwater cyanobacteria (Adamovsky et al., 2015; Chen et al., 2017a; Merel et al., 2013; Wood, 2016). Thus aquatic organisms suffer severe toxic effects due to their chronic exposure to toxins (Ilfergane and Henkel, 2017; Kim et al., 2017; Paulino et al., 2017). Moreover, MCs also pose a substantial health hazard to humans owning to its enrichment in aquatic creatures, and thus the World Health Organization (WHO) set an upper limit of 1 μg/L MCs in freshwater (Chen et al., 2009; Li et al., 2011). Alarmingly, the concentrations of MCs in some natural water bodies are much higher in various countries (Backer et al., 2010; Song et al., 2007). Up to date, more than 100 structural analogues of MCs have been identified, among of which MC-leucine arginine (MC-LR) is the most common and toxic variant (Blom and Juttner, 2005). Up to date, MC-LR has been identified to be able to bring about colorectal cancer, renal impairment, liver cancer, diabetes, and reproductive system toxicity (Chen et al., 2009; Chen et al., 2016a; Li et al., 2011; Lin et al., 2016; Lone et al., 2015; Zheng et al., 2017; Zhou et al., 2002).

Previously, we have identified that MC-LR exposure caused decline of serum testosterone levels resulting in impaired spermatogenesis (Chen et al., 2011; Li et al., 2008). However, previous *in vitro* studies found that MC-LR cannot enter into Leydig cells (LC) or inhibit testosterone synthesis (Wang et al., 2013). Moreover, MC-LR treatment causes testicular inflammation, with significant enrichment of immune cells and proinflammatory cytokines including TNF-α, IL-6, MCP-1, and CXCL10 in testis (Chen et al., 2017b; Chen et al., 2016b). In aging CYP19-aromatase transgenic male mice (AROM$^+$ mice), macrophages were also observed to be significantly enriched in testes, and macrophages could engulf surrounding LCs resulting in decline of LCs (Yu et al., 2014). In MC-LR-treated mice, whether infiltrated macrophages also take part in engulfing LCs deserves to be explored.

Apoptotic cells are engulfed and digested by professional phagocytes (macrophages and dendritic cells) or by neighboring

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nonprofessional phagocytes to maintain homeostasis in animals (Seitz et al., 2007). With the help of phosphatidylserine (PtdSer) exposed on the cell surface that acts as an "eat me" signal (Savill and Fadok, 2000; Toda et al., 2015), apoptotic cells could be efficiently cleared by phagocytes. Moreover, Protein S, milk fat globule-EGF factor 8 (MFG-E8) and growth arrest-specific 6 (Gas6) were reported to mediate phagocytosis of apoptotic cells by surrounding macrophages by functioning as bridges that bind to PtdSer on apoptotic cells and tyrosine kinase receptors on macrophages including Tyro3, Axl, and MerTK (TAM receptors) (Seitz et al., 2007; Toda et al., 2015).

In this study, we demonstrated chronic low-dose exposure to MC-LR cause decline of LCs and significant enrichment of macrophages in testes. Moreover, MC-LR stimulates macrophages to express and secrete TNF-α, which could induce LC apoptosis by binding to the tumor necrosis factor receptor 1 (TNFR1) on the cell membrane of LCs and then activating ROS-p38MAPK signaling pathway. MC-LR also induced upregulated expression of Axl and GAS6 in macrophage; GAS6 mediates phagocytosis of apoptotic LCs by macrophages by serving as a bridging molecule that binds to the tyrosine kinase receptor Axl on macrophages and PtdSer on apoptotic LCs.

2. Materials and methods

2.1. Main reagents and cell culture

MC-LR with a purity ≥ 95% was obtained from Alexis Biochemical (Lausen, Switzerland). MC-LR (1 mg) was dissolved in 100 μL DMSO and diluted to 1 mL with DMEM-F12 to prepare the stock solution (1 mM). TNF-α was purchased from PeproTech (Rocky Hill, NJ). The p38 MAPK inhibitor SB203580 was purchased from Medchemexpress (Monmouth Junction, NJ). The antibodies used in this study are listed in Supplementary Table S1.

The TM3 cells sharing many properties of primary LCs have been extensively used as a surrogate for primary LCs in the literature (Guo et al., 2017a; Ilfergane and Henkel, 2017). TM3 cells were cultured in DMEM/F12 media supplemented 10% FBS, 100 U/ml penicillin, and 100 μg/mL streptomycin. The RAW264.7 cells were cultured in DMEM media containing 4.5 g/L D-glucose. To establish the co-culture system, TM3 cells (5×10^5) were cultured in the lower compartment of a 6-well transwell system, whereas RAW264.7 cells (2.5×10^5, 5×10^5, 10×10^5) were seeded on a 0.4 μm Transwell insert (Millipore). Moreover, MC-LR was added into the culture media to a final concentration of 500 nM. After co-culture treatment, we collected the LCs and detected the ratio of apoptotic cells by flow cytometry.

2.2. Animals and treatment

Male specific pathogen-free (SPF) BALB/c mice aged six weeks were given drinking water containing 1 μg/L, 10 μg/L, 20 μg/L, or 30 μg/L MC-LR for 180 consecutive days. Control mice were provided with only the blank water. Moreover, we have performed experiments to demonstrate that mice consume a similar amount of water containing even the highest dose of MC-LR (30 μg/L). Following exposure to MC-LR, mice were sacrificed by CO_2 asphyxiation and testes were obtained for subsequent analyses of flow cytometry, histopathology, q-PCR, and western blotting. All procedures carried out on animals were approved by the Animal Care and Use

Committee of Nanjing University under the animal protocol number SYXK (Su) 2009-0017.

2.3. Flow cytometry

To determine the effects of MC-LR exposure on macrophage polarization, RAW264.7 cells were stimulated with MC-LR (500 nM) for 24 h. Target cells were harvested and stained with primary antibody targeting CD86, and then flow cytometry assay was performed on a FACS CaliburTM flow cytometer (BD Biosciences, San Jose, CA). In this study, RAW264.7 cells treated with LPS (100 ng/mL) were used as a positive control. Additionally, flow cytometric analyses were also used to examine macrophages (F4/80-positive cells) enrichment in testes of MC-LR-treated mice. The specific protocols were performed as previously reported (Chen et al., 2016b). Annexin V-FITC/PI staining was used to identify apoptotic cells according to the manufacturer's instructions (Vazyme, Nanjing, China).

2.4. Quantitative real-time PCR (qRT-PCR) assay

Total RNA was isolated from each sample using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). HiScript Q RT SuperMix for qPCR kit (Vazyme, Nanjing, China) was used for reverse transcription polymerase chain reaction, and then qRT-PCR assay was conducted with SYBR Green I mix (Takara, Dalian, China) on an ABI ViiA 7 Q-PCR System (Applied Biosystems, Waltham, MA). The relative mRNA levels of target genes were measured using *GAPDH* mRNA as an internal control. The primer sets used in this study were listed in Supplementary Table S2.

2.5. Western blotting, coimmunoprecipitation (Co-IP) and Elisa analyses

Proteins were separated on a 10% SDS-PAGE gel and then electrophoretically transferred onto polyvinylidene fluoride (PVDF) membrane. Next, these plots were incubated overnight at 4 °C with rabbit anti-p38MAPK, rabbit anti-p-ERK, rabbit anti-JNK, rabbit anti-active caspase-3, rabbit anti-TNF-α, mouse anti-TNFR1, and mouse anti-GAPDH. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti- mouse IgG (Boster, Wuhan, China) were used as secondary antibodies. Co-IP analysis was performed as previously described (Chen et al., 2018). Briefly, anti-TNFR1 antibody was used as the precipitating antibody to first isolate TNFR1 from LC lysates by immunoprecipitation, followed by immunoblotting using antibody against TNF-α. TNF-α and GAS6 levels in cell supernatant and testis homogenate were measured by ELISA kits (R&D systems, Minneapolis, MN), and all protocols were performed according to the manufacturer's instructions.

2.6. Immunohistochemical analyses

The tissue sections were treated with 3% H_2O_2 for 10 min and blocked with 3% BSA for 20 min at 37 °C, followed by incubation with rat anti-F4/80 or mouse anti-3βHSD at 4 °C overnight. The target proteins were incubated with HRP-conjugated secondary antibodies at 37 °C for 1 h, and then the specific binding was examined using diaminobenzidine (DAB) method according to the manufacturer's instructions (Zhongshan Biotechnology, Beijing, China). For each section, seven images were captured at 200× magnification under a light microscope. After counting the F4/80 or 3βHSD-positive cells in each image, the average of five images, excluding the minimum and maximum values, was calculated; five sections per mice were taken for statistical analysis.

2.7. Immunofluorescent staining

Immunofluorescence analyses of testicular tissues were performed as previously described (Chen et al., 2018). Rat anti-F4/80, rabbit anti-active caspase-3, mouse anti-3 beta-hydroxysteroid dehydrogenase (3βHSD), rabbit anti-TNFR1, rabbit anti-GAS6, and rabbit-Axl were used as primary antibodies. Alexa Fluor 488-conjugated donkey anti-rat IgG (Invitrogen), Alexa Fluor 488-conjugated donkey anti-mouse IgG (Invitrogen), or Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) was used as secondary antibodies. Nuclei were stained with DAPI (Sigma, St. Louis, MO). The images were captured using a confocal fluorescence microscope (Olympus, Tokyo, Japan).