In vivo Studies on the Immunotoxic Effects of Microcystins on Rabbit

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ABSTRACT: Microcystins (MCs) are the toxic molecules produced by common cyanobacterium in freshwater blooms. Their toxicities raise severe health issues in livestock and human beings. In current study, the immunotoxic effects of MC-LR were investigated in rabbit through evaluating the dynamics of white blood cell (WBC) numbers and cytokine production such as interleukin-3 (IL-3), IL-4, IL-6, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α). MCs at the high dose (50 μg MC-LReq kg^{-1}) significantly induced increase in the WBC number but decrease in the Th1 (IFN-γ, TNF-α) and Th2 (IL-3, IL-4, IL-6) production. In the low dose group(12.5 μg MC-LReq kg^{-1}), the number of WBC and the production of IFN-γ, IFN-α, IL-4, IL-3, and IL-6 increased gradually in first 12 h, reach the peaks at 12 h, and dropped after 24 h. Significantly positive correlations were found between the cytokines production of IL-4 and IL-6, IFN-γ and IFN-α, or IL-4 and IFN-γ. In conclusion, MC-LR is able to disturb the rabbit immune system and there exists time-dose response relationship in the MC-LR-eliciting perturbation, which probably give a better insight into investigating the immunotoxicity mechanisms of MCs in vivo. © 2010 Wiley Periodicals, Inc. Environ Toxicol 27: 83–89, 2012.

Keywords: microcystin-LR (MC-LR); immunotoxicity; rabbit; cytokine; WBC

INTRODUCTION

Cyanobacterial blooms in eutrophic fresh surface water occur worldwide with increasing frequency (Paerl et al., 2001; Falconer and Humpage, 2005). Microcystins (MCs), secondary metabolites of toxic cyanobacteria, are considered to be one of the most dangerous groups (Codd, 1995; Dawson, 1998). MCs are produced by various species within the genera Microcystis, Anabaena, Oscillatoria, Nodularia, Nostoc, Cylindrospermopsis, and Umezakia (Keshavanath et al., 1994). So far, more than 70 variants of MCs have been reported (Fastner et al., 2002). Of the known MCs, MC-LR, -RR, and -YR are three variants, is most commonly found and frequently examined in an aquatic system.

Some reports of animal or human illness on the globe have been linked to the presence of MCs in drinking water (Carmichael, 1994; Codd et al., 1999; Carmichael et al., 2001; Falconer and Humpage, 2005). Recently, epidemiological studies have indicated that one of the risk factors for high occurrence of primary hepatocellular carcinoma is to drink MCs-contaminating water (Falconer and Humpage, 2005). Moreover, the most severe health case attributed to direct exposure to MCs has been emphasized by deaths of hemodialysis patients in Brazil in 1996 (Pouria et al., 1998). It was also reported that the incidence of primary liver cancers (PLC) in

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certain areas of China was related to the presence of MCs in drinking water (Yu 1995; Ueno et al., 1996) In a recent study by Chen et al. (2009), MC were identified for the first time in the serum of a chronically exposed human population together with indication of hepatocellular damage. MCs can cause over-phosphorylation of liver enzymes, liver necrosis, and lethal intrahepatic bleeding (Duy et al., 2000; Eriksson et al., 1990; Falconer and Yeung, 1992), and even death in only a few hours through disruption of hepatic architecture, a consequence of massive intrahepatic hemorrhage. Chronic uptake of MCs results in general hepatocyte degeneration via necrosis, progressive fibrosis, and even tumorigenesis (Dabholkar and Carmichael, 1987; Miura et al., 1989). MC-induced damage is not restricted to liver, however, as was supported by evidence of kidney impairment, gastrointestinal disorder, immune intruders, and MC-related embryotoxicity (Beasley et al., 1989; Zhang et al., 2008).

Currently the exact mechanisms of MCs toxicity are not fully understood, but it is suggested that their immunomodulatory reactivities may play a significant role in the pathogenesis (Cooper et al., 2001; Vitale et al., 2002). Earlier studies have shown that excessive amounts of MCs are especially harmful to immune cells, leading to cell damage or death by oxidizing the membrane lipids, protein carbohydrates, and nucleic acids (De la Fuente and Victor, 2000). Cyanobacterial toxins are able to enhance the early adherence of human peripheral polymorphonuclear leukocytes (Hernandez et al., 2000). Yea et al. (2000) have demonstrated that MCs and nodularin could down-regulate splenocyte functions in mice and suppress the IL-2 and IL-4 expression (Yea et al., 2000). It was also observed that the lethal effect of MCs on livestock and human being also significantly depended on stimulation of immune system (Pahan et al., 1998; Yea et al., 2001). MCs have a dose-dependent clastogenic effect on human lymphocytes connected with chromosomal breakage (Repavich et al., 1990). Mankiewicz et al. observed that MCs from cyanobacterial blooms collected from a Polish reservoir caused apoptosis effect on rat hepatocytes and human lymphocytes (Mankiewicz et al., 2001).

Cytokines are important markers to indicate the nature of immune disturbance that is inflicted by MCs immunotoxicity in many animal models. Cytokines play important roles in regulation of inflammatory responses (Adams and Tepperman, 2001; Murata et al., 1995), tumor immunity (Ohe et al., 2001) and susceptibility to allergy (Cyr and Denburg, 2001; Larche, 2001). Because of potent immunoregulatory activities of cytokines, their production changes could be relevant to the pathogenesis of many diseases. Although MC compounds are found to regulate the production of IL-1, TNF-γ, and inducible nitric oxide synthase in macrophages (Nakano et al., 1989; Pahan et al., 1998), their effects on the immune system including lymphocytes are still unclear. Moreover, few studies have been conducted to examine the immunotoxic effects of MC-LR in vivo. This study was to investigate the acute toxicity of MC-LR through evaluation of leukocyte and cytokine dymanics in rabbit, giving us better understanding of the in vivo immunotoxicity of MCs.

Abbreviations

MC-LR	microcystin-LR
MCs	microcystins
WBC	white blood cells
IL	interleukin
IFN-γ	interferon-γ
TNF-α	tumor necrosis factor-α

MATERIALS AND METHODS

Toxin

Cyanobacterial material used in this experiment was collected from surface blooms of Lake Dianchi, Yunnan in China. According to microscopic examinations, cyanobacterial material refers to phytoplankton cells from the surface of water in which the predominant species belong to Cyanophyta, and MCs was the predominant species. Freeze-dried crude algae were extracted as described previously (Park et al., 1998). Quantitative analysis of MC was performed using a reverse-phase high-performance liquid chromatography (HPLC, LC-20A, Shimadzu Corporation, Kyoto, Japan). MC concentration was determined by comparing the peak areas of the samples with those of the standard controls available (MC-LR and MC-RR, Wako Pure Chemical Industries, Japan). The MC content was 1.41 mg g^{-1} dry weight (DW), among which MC-RR′-LR′ and -YR were 0.84′0.50 and 0.07 mg g^{-1} DW, respectively.

Rabbit

Healthy rabbits weighing about 2000 $\pm$ 250 g were purchased from a local warren in Wuhan, China. Animals were given pathogen-free water and food. Feeding was terminated two days before initiation of the experiment, and water but no food was supplied throughout the experiment.

Experimental Protocol

A total of 12 rabbits were randomly divided into three groups of four each. Two MC-LR exposure doses, 50 μg kg^{-1} and 12.5 μg kg^{-1}, were chosen in our study according to our previous experiments (Zhao et al., 2008), and MC-free saline solution was used as the control. Rabbits were injected intraperitoneally (i.p.). Sera were collected from the hearts of rabbits with 50 μg kg^{-1}MC-LR 0, 1 and 3 h post treatment and rabbits with12.5 μg kg^{-1}MC-LR at 0, 1, 3, 12, 24, 48, and 168 h post treatment.

White Blood Cells Separation

Blood samples were taken by caudal puncture with heparinized syringes. Blood was centrifuged at 3000 $\times$ g for

15 min at 4°C and plasma was stored at −70°C until analysis. White Blood Cells (WBCs) (104 cells/μL) were determined by hemocytometer method (Stevens, 1997). WBC number was measured in triplicates.

ELISA for Cytokine Production

The harvested sera of the control and MC-LR treated rabbits at each time point were used to measure levels of IFN-γ, TNF-α, IL-4, IL-3, and IL-6 by using ELISA kits (Rapidbio Lab, CA). The sensitivity of the kits is: from 5 to 1000 pg/mL for IL-3 and IL-6, from 5 to 600 pg/mL for IL-4, from 5 to 500 pg/mL for IFN-γ, from 5 to 100 pg/mL for TNF-α, respectively.

The assays were carried out according to the manufacturer's instructions and calibrated with cytokine standards supplied with the kits. Breifly, ninety-six-well plates were coated with 50 μL of 5 μg/mL rat antirabbit IFN-γ (TNF-α, IL-4, IL-3, or IL-6) per well in PBS overnight. The plates were then washed three times with PBS and blocked with PBS for 2 h at 37°C. Totally, 25 μL of standard control (positive and negative) and samples were added into each well. After 2-h incubation at 37°C in 5% CO2, 25 μL of biotinylated antirabbit IFN-γ (TNF-α, IL-4, IL-3, or IL-6) mAb diluted by 1:500 were added after thorough washing and then incubated for 2 h at room temperature. After washing, 25 μL of the avidin-horseradish peroxidase were added and incubated for another 1 h at room temperature. Following five wash with PBS, color was developed by adding 3, 3′, 5, 5′-tetramethyl benzidine (TMB) substrate. Finally, 50 μL of the stop solution were added and the optical density was measured at 450 nm using ELISA reader (Spectramax 250, Molecular Devices, CA). Cytokine concentration was assessed in triplicates.

Statistical Analysis

All experiments were examined in duplicates, and every sample was measured in triplicates. All data were presented as the mean ± SD. Data were analyzed by repeated measures analysis of variance (ANOVA) and Dunnett's post test using STATISTICA software package (Version 6.0, Statsoft, Inc.). Differences were measured against control values and considered to be statistically significant at $P \leq 0.05$.

RESULTS

Change of WBC Number

In the high-dose group (50 μg kg^{-1}), 2–3 rabbits died 3 h post exposure with MC-LR. (Fig. 1). The number of WBC showed a significant increase ($P < 0.01$) 1 h postinjection, but tended to significantly increase 3 h postinjection. Compared with the control, there was still an upward trend 3 h postinjection. In the low-dose group (12.5 μg kg^{-1}), the number of WBC began to increase after 1h post injection, and the fastest increase appeared 3 h post injection and reached a peak 12 h after exposure, and significant decline was observed 24 h post injection. The WBC number dropped to the lowest 168 h post injection, even lower than the control group.

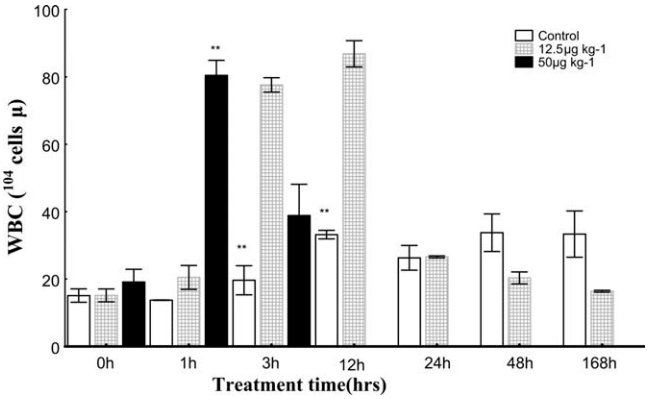

Fig. 1. The effect of microcystin-LR on White blood cells production by rabbit blood plasma in vivo. Data are presented as mean values ± S.E. The number of measurements performed in each group was three. The significant levels observed are $^{**}P < 0.01$ in comparison to control group values. These results are representative of three independent experiments.

Cytokine Production

Treatment of rabbits with MC-LR at a dose of 50 μg kg^{-1} resulted in an obvious decrease trend of IFN-γ, TNF-α, IL-4, IL-3, and IL-6 after 1 h and 3 h in comparison to the control (Figs. 2–6), whereas treatment with 12.5 μg kg^{-1} MC-LR resulted in a gradual increase of IFN-γ, TNF-α, IL-4, IL-3, and IL-6 production after 1 and 3 h in comparison to the control group (Figs. 2–6). All the cytokine production in the low dose group showed an obvious increase and reached a peak 12 h post exposure (Figs. 2–6), especially for the IL-3 production which was markedly enhanced after 12 h (Fig. 3). And all the cytokine production in the low dose group began to decrease after 24 h compared with the control (Figs. 2–6).

Correlation Between Cytokine Production

Correlation between the cytokine production of IL-4 and IL-6, IFN-γ and IFN-α, IL-4, and IFN-γ in the MC-LR-treated rabbits were analyzed. Significantly positive correlation was found between Th2-type cytokines IL-4 and IL-6 ($r = 0.95$, $P < 0.01$) (Fig. 7), between Th1-type cytokines IFN-γ and IFN-α ($r = 0.70$, $P < 0.01$) (Fig. 8), as well as

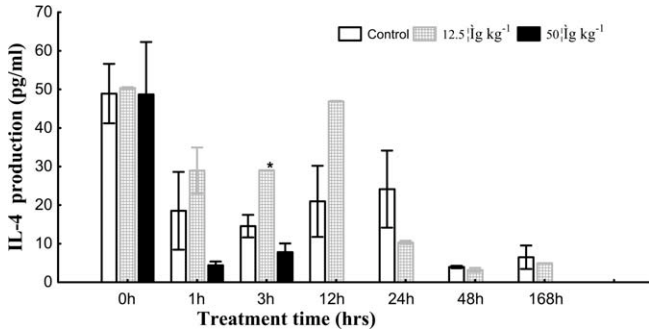

Fig. 2. The effect of microcystin-LR on IL-4 (pg/mL) production by rabbit blood plasma in vivo. Data are presented as mean values ± S.E. The number of measurements performed in each group was three. The significance levels observed are *$P < 0.05$ in comparison to control group values.Values smaller than 0.05 were considered statistically significant. These results are representative of three independent experiments.

between Th2-type cytokine IL-4 and Th1-type cytokine IFN-γ ($r = 0.87$, $P < 0.01$) (Fig. 9).

DISCUSSION

WBCs are a main mediator of immune system functions, defending the body against both infectious disease and foreign materials (Abbas et al., 1994). Changes of WBC number in the peripheral blood may reflect immune response. In our current study, although the number of WBC in the low-dose group increased during a short period, it began to

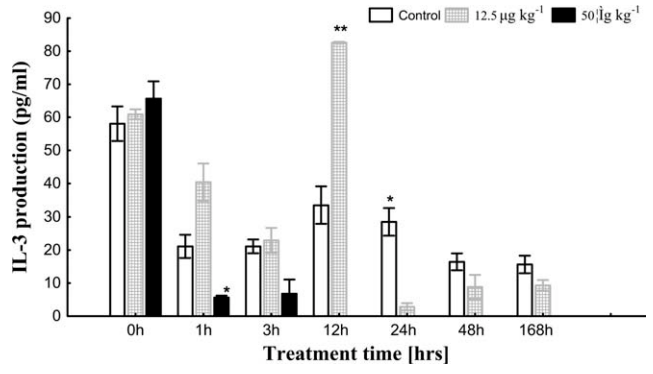

Fig. 3. The effect of microcystin-LR on IL-3 (pg/mL) production by rabbit blood serum in vivo. Data are presented as mean values ± S.E. The number of measurements performed in each group was three. An asterisk denotes a response that is significantly different from the control group as determined by Dunnett's two-tailed t test ($P < 0.05$), and two asterisks denotes a response that is extremely significantly different from the control group ($P < 0.01$). These results are representative of three independent experiments.

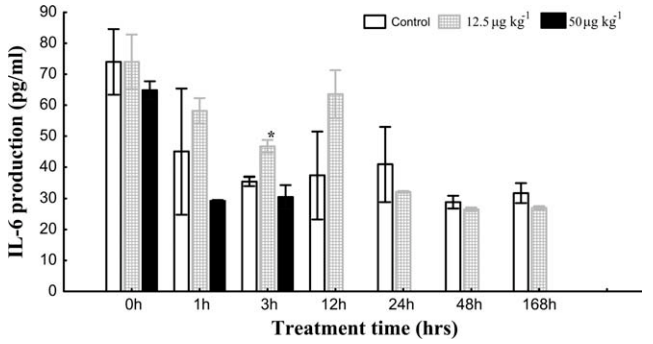

Fig. 4. The effect of microcystin-LR on IL-6 (pg/mL) production by rabbit blood serum in vivo. Data are presented as mean values ± S.E. The number of measurements performed in each group was three. The significance levels observed are *$P < 0.05$ in comparison to control group values. These results are representative of three independent experiments.

decline sharply afterwards, suggesting that MC-LR induced obviously immune toxicity to leukocyte system and subsequently resulted in the reduction of cellular immune functions. Some researchers have showed the effects of MCs on the function of mononuclear phagocyte system in mammalian (Pahan et al., 1998). Therefore, it could be deduced that MC-LR could interfere with the normal function of immune system through affecting the immune cells.

Regulation of immune system is largely dependent upon mediators known as cytokines. In the immune system, various cytokines comprise a complex network to maintain the immune balance, and if this balance is destroyed, the self-regulating functions will decline, inducing various diseases (Romagnani, 1991; Sandmand et al., 2002). Our results

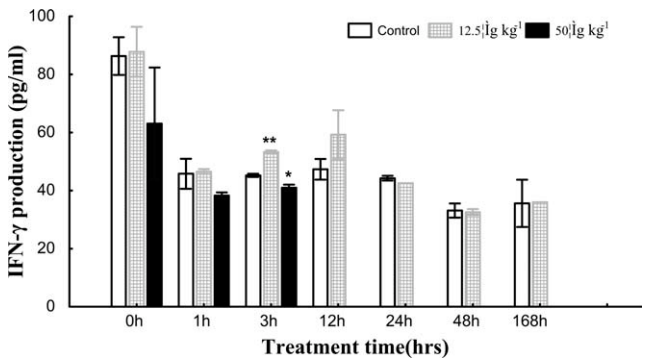

Fig. 5. The effect of microcystin-LR on IFN-γ (pg/mL) production by rabbit blood serum in vivo. Data are presented as mean values ± S.E. The number of measurements performed in each group was three. * Indicates significant differences at $P < 0.05$ between MC-treated groups and the control group, and the ** indicates extremely significant differences at $P < 0.01$. These results are representative of three independent experiments.

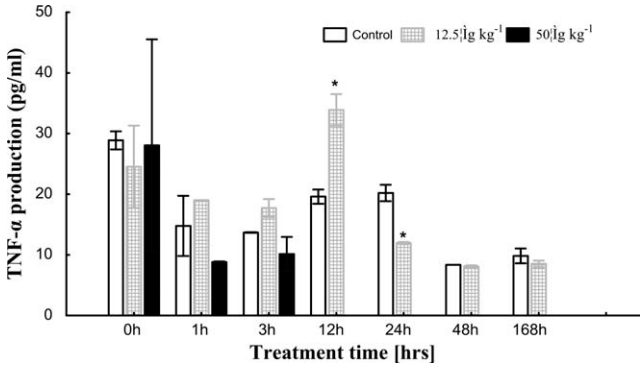

Fig. 6. The effect of microcystin-LR on TNF-α (pg/mL) production by rabbit blood serum in vivo. Data are presented as mean values ± S.E. The number of measurements performed in each group was three. The significance levels observed are $^*P < 0.05$ in comparison to control group values. These results are representative of three independent experiments.

show that, in the low dose group, the cytokines IFN-γ, IFN-α, IL-4, IL-3, and IL-6 gradually increased in first 12 h, reached the peak at 12 h, and then began to decrease after 24 h. In the high-dose group, the majority was dead after 3 h, and showed a distinct decrease trend of IFN-γ, TNF-α, IL-4, IL-3, and IL-6 expression. It could be deduced that the cytokine production may rapidly decline with increases of both MC-LR dose and exposure time.

In this study, there were significantly positive correlations between the cytokine production of IL-4 and IL-6, IFN-γ and IFN-α, IL-4 and IFN-γ. IL-3, IL-4, and IL-6 are produced by Th2 cells, whereas IFN-γ and IFN-α are secreted by Th1 cells (Cooper et al., 1994; Gajewski et al., 1990). The balance between Th1 and Th2 type cytokines is implicated in the regulation of many immune responses and is thought to be crucial for the outcome of several diseases (Sandmand et al., 2002). As for the results of the low dose group, we speculate that the increase of Th1 and Th2 cytokines at 12 h postinjection may be responsible for the irritative effects of MC-LR in rabbit and the decrease of Th1 and Th2 cytokines at 24 h postinjection may be potentially the consequence of the immunotoxicity effects of MC-LR. The reason for the decreased cytokine production after treatment with the high dose of MC-LR might be just the immunotoxicity effects of MC-LR. For the exact mechanism of cytokine production, the effect of the MC-LR immunotoxicity on the cytokines, in particular to unearth its effect on the molecular level of cytokines that regulate the Th1, Th2, and innate immunity, should be investigated further, and information on this level will give a better insight into providing preventive measures that are necessary in reducing the lethal consequences of the MC-LR immunotoxicity.

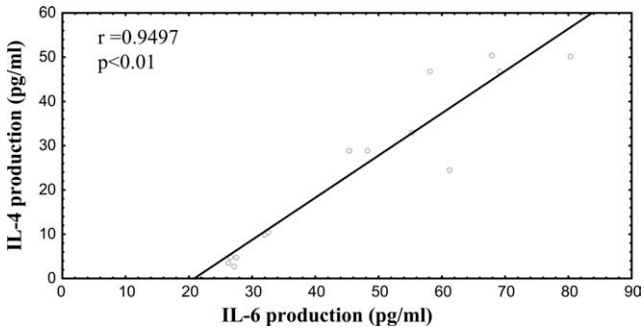

Fig. 7. Correlation between IL-4 and IL-6 production by rabbit blood serum. For the analysis of correlation the results of all concentrations were pooled. All experiments were examined in duplicates, and every sample were measured in triplicates.

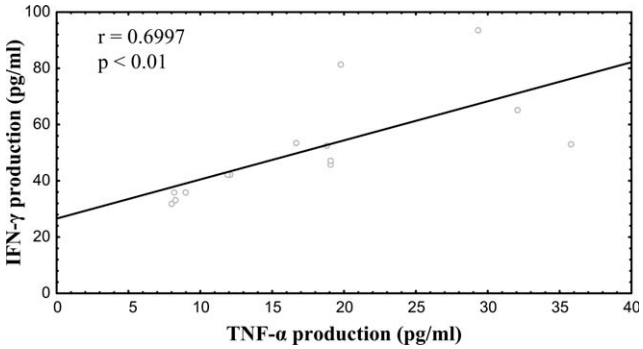

Fig. 8. Correlation between IFN-γ and TNF-α production by rabbit blood serum. For the analysis of correlation the results of all concentrations were pooled. All experiments were examined in duplicates, and every sample were measured in triplicates.

There are several reports investigating the immunosuppression effects of MC-LR on some cytokines in vitro. The immunomodulating mechanism of MC-LR on some cytokines in rat had been studied at the protein level (Rocha

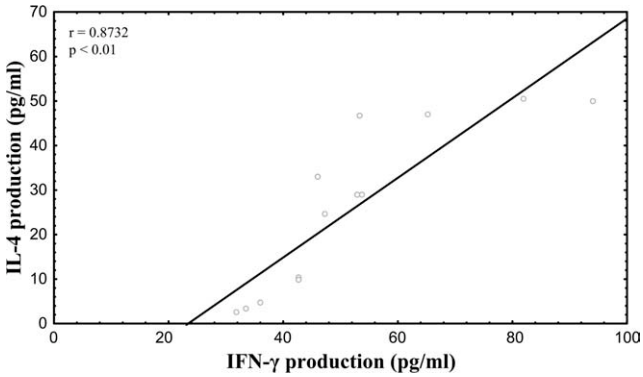

Fig. 9. Correlation between IL-4 and IFN-γ production by rabbit blood serum. For the analysis of correlation the results of all concentrations were pooled. All experiments were examined in duplicates, and every sample were measured in triplicates.

et al., 2000). Chen et al. (2004) has observed the significant repressive effects of MC-LR on cytokines IL-1, TNF-α, GM-CSF, IFN-γ at the mRNA level. Currently, our results illustrate that MC-LR is able to disturb the rabbit immune system and there is time-dose response relationship in the MC-LR-induced perturbation, which clearly indicates the comprehensive effects on Th1, Th2 cells, and probably accessory cells like macrophages and natural killer (NK) cells. Our results are not consistent with previous findings the dynamics of cytokins production (Rocha et al., 2000; Chen et al., 2004), probably due to the different experimental model used. Since our data are unable to furnish us with information on how MC-LR modulates the immune system at the cellular and molecular levels, more studies are required to elucidate the detailed immunotoxic mechanism of MC-LR in vivo.

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