00FC-99 ALLEVIATES LUPUS NEPHRITIS IN MRL/LPR LUPUS MICE BY INHIBITING THE ACTIVATION OF MAPK PATHWAY

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ABSTRACT

Purpose: *To investigate the mechanism involved in FC-99-induced alleviation of lupus nephritis (LN) in MRL/lpr lupus mice.*

Methods: *Mice were divided into FC-99 group given intraperitoneal injection of FC-99, LN group, and normal control group (NC group) with an equivalent volume of normal saline. The injection lasted for 20 weeks. Mice urine was collected weekly from week 9. Urine protein content, renal pathological changes, relative expression levels of tumor necrosis factor-α (TNF-α), as well as monocyte chemoattractant protein (MCP-1) and interleukin-6 (IL-6) mRNA were determined with urine protein ELSA kit, H & E staining, and RT-PCR, respectively. The dendritic cells (DCs) were divided into control group (no treatment), lipopolysaccharide group (100 ng/ml LPS daily) and FC-99 group (50 μmol FC-99 for 120 min, and 100 ng/ml LPS daily). The expression levels of major histocompatibility complex II (MHCII), CD40, CD80 and CD86 were determined using flow cytometry; while the expressions of interleukin-1 receptor-associated kinase 4 (IRAK4), phospho-interleukin-1 receptor-associated kinase 4 (P-IRAK4), p38 and phosphorylated p38 (P-p38) by were determined with western blot.*

Results: *The urine protein content of the 3 groups ranked in a descending order was: LN group > FC-99 group > NC group ($p<0.05$). There were no obvious renal pathological changes in the NC group, but there were glomerular sclerosis, tubule-interstitial fibrosis, tubular atrophy, glomerular leukocyte infiltration and tubule-interstitial mononuclear cell infiltration in the FC-99 and LN groups. However, the lesions were milder in the FC-99 group than in the LN group. Relative expressions of TNF-α, MCP-1 and IL-6 mRNA in the kidney tissues of FC-99 group and NC group were significantly lower than those in the LN group ($p<0.05$). The expression levels of MHCII, CD40, CD80 and CD86 in DCs of control and FC-99 groups were significantly lower than those in the LPS group ($p<0.05$). Relative expressions of P-IRAK4 and P-p38 protein in 3 groups were in the order LPS group > FC-99 group > control group ($p<0.05$).*

Conclusion: *Inhibition of LPS-induced activation of the MAPK pathway in DCs is involved in the alleviation of LN in MRL/lpr lupus mice by FC-99.*

Keywords: *FC-99, DCs, MAPK pathway, LN.*

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Introduction

Systemic lupus erythematosus (SLE) is a category of autoimmune disease (AID) that usually occurs in young females, which mechanism is tissue injury caused by immune complex composed of antibodies produced by over-activation of B cells and autoantigen from multiple systems and organs[1-2]. Lupus nephritis (LN) is one of that most common complications of SLE[3]. Dendritic cells (DCs) are widely distributed heterogeneous cells, with antigen-presenting function[4]. Multiple studies show that DCs are closely correlated with the occurrence of SLE[5-6]. It has been reported that the immunosuppressor FC-99 inhibits the development of the disease and protects patients from injury in inducible enteritis and sepsis[7]. The present study was aimed at investigating the mechanism of onset of LN in MRL/lpr SLE, and the mechanism involved in FC-99-induced alleviation of LN in MRL/lpr lupus mice.

Materials and Methods

Experimental animals

Twelve (12) MRL/lpr SLE mice and 21 normal mice were bought from Pengli Biotech (Shanghai) Co., Ltd; Animal permission No: SYXK Hu/2014-0012). Mice DCs were bought from Shanghai Fengshou Biotech Co. Ltd.

Instruments and reagents

The instruments and reagents used, and their sources were: cell incubator (Chongqing Songlang Electronic Instruments Co., Ltd, type: DHP-9052); low-temperature refrigerated centrifuge (Germany Eppendorf company, type: MiniSpin plus); inverted microscope (Japan Plympus company, type: IX73); high temperature sterilization oven (Shanghai Boxun Medical Molecular Devicesiological Instrument Incorporated Company, type: BXM-50VD); multifunction ELISA (American Molecular Devices company, type: SpectraMax iD5); flow cytometry (FC) (American Beckman Coulter Company, type: CytomicsTM FC 500) and real-time fluorescence PCR instrument (American Applied Biosystems company, type: Applied Biosystems). The others were super clean bench (Suzhou Jiabao Purification Engineering Co. Ltd, type: JB-CJ-1500FX); electrophoresis apparatus (Beijing LiuYi Biotech Co., Ltd, type: DYCZ-20H); Vilber LouRMAT (Changchun Junyihuahao Tech Co., Ltd, type: JY02G); cell culture medium (American Scien Cell); fetal calf serum (American ZETA LIFE Company); and urine protein ELISA kits (England Abcam Company).

Methods

All mice were given one week of adjustable feeding in animal laboratory of our hospital before normal trials without diet limitation. During adjustment period, the laboratory temperature and humidity were maintained at 23 ± 2 °C and 55 ± 5%, respectively, in an environment with 12-h light/day. The MRL/lpr SLE mice were randomly divided into FC-99, LN group and normal control (NC) groups (21 mice/group). Mice in the FC-99 group were given abdominal injection of FC-99 at a dose of 10 μg/g. Mice in LN group and NC group were given equivalent volume of normal saline injection. Mice were given injection once a week in the first eight weeks, and twice a week from the ninth week. All mice were sacrificed by cervical dislocation at the end of the 20th week. Beginning from the 9th week, mice urine was collected weekly. Urine protein level was measured using urine protein ELISA; and renal pathological conditions of mice in the 3 groups were determined using H & E staining. The relative renal expressions of mRNA of various inflammatory factors were determined with RT-PCR. Mice DCs were divided into control group, lipopolysaccharide (LPS) and FC-99 group. The LPS group received LPS at 100 ng/ml and were cultured for 24 h. Cells in FC-99 group were given pretreatment for over 120 min using 50 μmol FC-99, followed with LPS at a dose of 100 ng/ml and culturing for 24 h. The levels of expression of surface activation markers of DCs in the 3 groups were determined using flow cytometry, while the expression levels of various MAPK proteins in DCs were determined using Western blot.

Observation indices

The observation indices were urine protein, renal pathological conditions and relative expression levels of tumor necrosis factor α (TNF-α), monocyte chemoattractant protein 1 (MCP-1) and interleukin-6 (IL-6) in kidney tissue in the 3 mice groups; expression level of activation markers i.e. major histocompatibility complex II (MHCII), CD40, CD80 and CD86 on DC surface in various groups; expression levels of interleukin 1 receptor-associated kinase 4 (IRAK4), phosphorylation interleukin 1 receptor-associated kinase 4 (P-IRAK4), p38 and p38 phosphorylation p38 (P-p38).

Statistical analysis

Measurement data are presented as mean ± standard deviation (SD), and were statistically analyzed with Student's t-test. The statistical analysis was done with SPSS version 21.0. Values of $p<0.05$ were considered significant.

Results

Comparison of proteinuria in mice in the three groups

The results in Figure 1 show that urine protein level of mice in FC-99 group and LN group were significantly higher than that in the NC group ($p<0.05$), but urine protein level in the FC-99 group was significantly lower than that of the LN group after treatment with FC-99 ($p<0.05$).

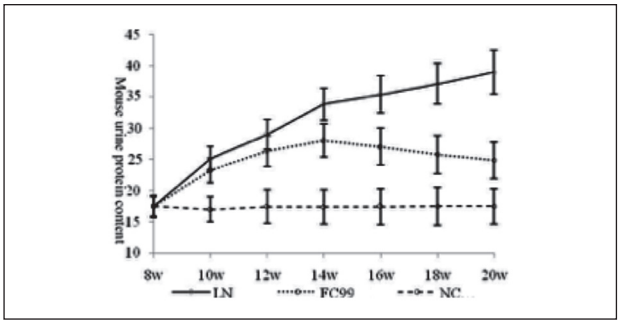

Figure 1: Urine protein levels of mice in the three groups.

Comparison of renal pathological conditions of mice in the three groups

There were no obvious pathological changes in renal tissue of mice in the NC group. In contrast, pathological changes such as glomerular sclerosis, renal tubular interstitial fibrosis, renal tubular atrophy, glomerular leukocyte infiltration and tubular interstitial mononuclear cell infiltration occurred in mice kidney in the FC-99 and LN groups. The renal pathological changes in mice in the FC-99 were mitigated, when compared with mice in the LN group. These results are shown in Figure 2.

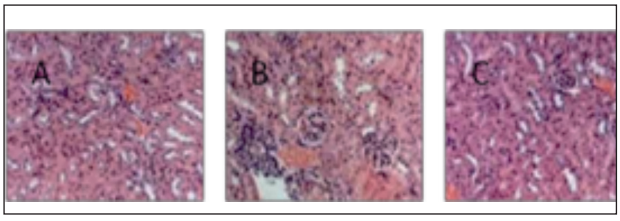

Figure 2: Renal pathological changes in mice in the three groups. A: NC group; B: LN group; C: FC-99 group.

Comparison of relative expressions of TNF-α, MCP-1 and IL-6 mRNA in renal tissue of mice in the three groups

As shown in Table 1, the relative expression levels of TNF-α, MCP-1 and IL-6 mRNA in renal tissue of mice in FC-99 group and NC group were significantly decreased, relative to corresponding expressions in the LN group ($p<0.05$). However, there were no significant differences in the relative expression levels of TNF-α, MCP-1 and IL-6 mRNA in renal tissue of mice between the FC-99 group and the NC group ($p>0.05$).

Group	TNF-a mRNA	MCP-1 mRNA	IL-6 mRNA
FC-99 (n=21)	1.73±0.59#	1.21±0.30#	1.28±0.41#
LN (n=21)	14.57±4.86	5.34±1.51	6.29±1.35
NC (n=21)	0.97±0.11#	0.83±0.09#	1.04±0.13#
F	153.43	165.75	275.75
p	<0.001	<0.001	<0.001

Table. 1: Relative expressions of TNF-α, MCP-1 and IL-6 mRNA in renal tissue of mice in the three groups.
#p > 0.05, compared with LN group.

Comparison of expression levels of surface activation markers of CDs in the three groups

The expression levels of MHC II, CD40, CD80 and CD86 in DCs of the control group and FC-99 group were significantly decreased, when compared with DCs in LPS group ($p<0.05$). However, there were no significant differences in the expression levels of MHC II, CD40, CD80 and CD86 on CDs between the FC-99 group and the control group ($p>0.05$). These results are depicted in Figure 3.

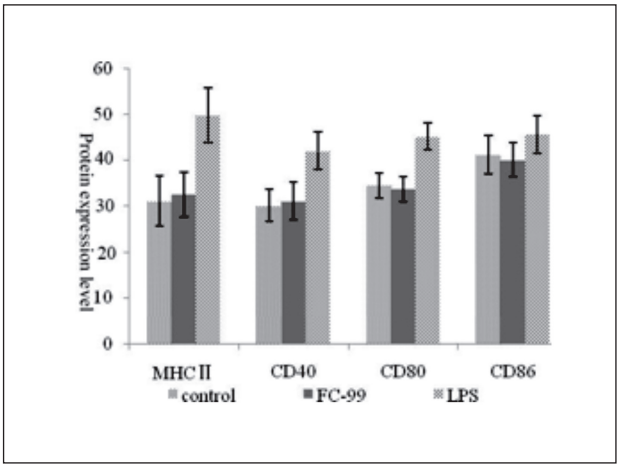

Figure 3: Comparison of expression levels of MHC II, CD40, CD80 and CD86 in DCs of the three groups.

Relative expression levels of IRAK4, P-IRAK4, p38 and P-p38 proteins in cells in the three groups

As shown in Figure 4, there were no significant differences in relative expression levels of IRAK4 and p38 in the three groups ($p>0.05$). However, the relative expression levels of P- IRAK4 and P-p38 proteins were significantly lower in the control and FC-99 groups than in the LPS group ($p<0.05$); while relative expression levels of P-IRAK4 and P-p38 proteins in the DCs in control group were significantly decreased ($p<0.05$).

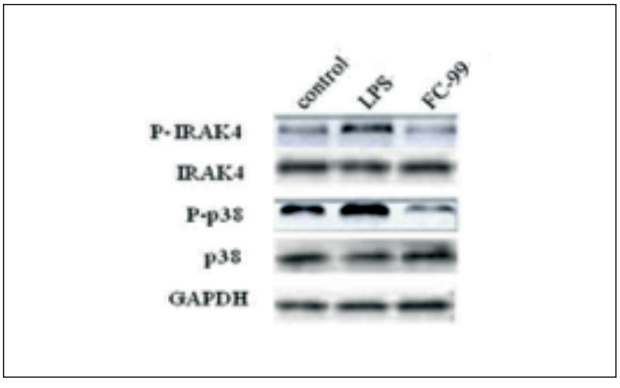

Figure 4: Relative expression levels of IRAK4, P- IRAK4, p38 and P-p38 proteins in cells in the three groups.

Discussion

Systemic lupus erythematosus (SLE) is a complex autoimmune disease induced by heredity, infection, hormone and environmental stimulation. Typically, the face of the patient manifests skin lesion of butterfly erythema, in addition to symptoms in kidney, digestive system, respiratory system, nervous system, heart and skeletal system[1-8]. The major cause of death in SLE patients is lupus erythematosus. At present, there are no radical treatments for SLE. The treatment relies mostly on immune-suppressors such as methotrexate and cyclophosphamide; antiperiodic drugs and glucocorticoids. However, the patients usually experience disease relapse due to severe and multiple adverse reactions[9]. Therefore, it is necessary to investigate and evolve new ways of inhibiting the pathogenesis of LN and SLE.

The FC-99 used in this study is a synthetic version of FC, an indole alkaloid extracted from Aspergillus fumigatus. Studies have demonstrated that FC-99 inhibits macrophage signal transduction, regulates differentiation of mononuclear macrophagocytes, and relieves symptoms of sepsis in mice[7-10]. Currently, DCs are the most functional antigen-presenting cells, and they regulate immunity by activating T cells, and processing and presenting antigens. They express antigen-presenting molecules such as MHC I and MHC II as well as co-stimulators such as CD40, CD80 and CD86 on their surfaces[11]. Under non-activation state, the expression levels of MHC II, CD40, CD80 and CD86 on DC surface are extremely low. However, a large amount of antigen-presenting molecules and co-stimulators are expressed when DCs become activated. One of these (MHC II) forms a complex with tumor antigen, thereby activating CD4+T cells. Activated DCs secrete chemotactic factors, and the chemotactic factors activate more DCs in a paracrine fashion. The activated DCs upregulate the surface expression levels of CD40, CD80 and CD86, thereby presenting antigens to CD8+T and activating CD8+T cells and CD4+T cells[12-13].

Urinary protein usually occurs in renal injury caused by nephritis and nephrotic syndrome. Urinary protein is used for evaluating renal pathological conditions in clinics[14]. The TNF-α, MCP-1 and IL-6 are inflammatory factors, which enhance the occurrence and development of LN. The key protein of intracellular signal conduction pathway, IRAK4, has tyrosine kinase activity and serine or threonine kinase activity[15]. Mitogen-activated protein kinase (MAPK) is important for cell signaling. The physiological function of this pathway is to conduct extracellular signals into cell nucleus across the cell membrane[16]. Studies have shown that IRAK4 mediates inflammatory reactions by regulating the p38MAPK pathway. Indeed, IRAK4 influences multiple MAPK pathways, inhibits IRAK4 expression, and lowers P-p38 expression level[17].

This study found that urinary protein level of mice in the LN group was higher than that of mice in the F-99 and NC group. Glomerular sclerosis, renal tubular interstitial fibrosis, renal tubular atrophy, glomerular leukocyte infiltration and tubular interstitial mononuclear cell infiltration occurred in mice kidney in FC-99 and LN groups, but renal pathological changes in mice in the FC-99 group were mitigated, relative to pathological changes in the LN group. In addition, relative expression levels of TNF-α, MCP-1 and IL-6 mRNA of renal tissues of mice in FC-99 and NC groups were lower than those of mice in the LN group, which indicates that FC-99 relieved the onset LN in MRL/lpr SLE mice. This study also found that the expression levels of MHC II, CD40, CD80 and CD86 in DCs in the control and FC-99 groups were significantly decreased, when compared to corresponding expressions in the LPS group; relative expressions of P- IRAK4 and P-p38 proteins in the control and FC-99 groups were lower than those in the LPS group, and relative expressions of P- IRAK4 and P-p38 proteins of DCs in control group were lower than corresponding expressions in the FC-99 group. These results indicate that FC-99 inhibits DC activation and p38MAPK pathway activation-induced by LPS.

Conclusion

The results obtained in this study show that FC-99 alleviates LN in MRL/lpr SLE mice by inhibiting DC activation and LPS-induced p38MAPK pathway activation.

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