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The new complement inhibitor CRIg/FH ameliorates lupus nephritis in lupus-prone MRL/lpr mice

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Abstract

Background: Inhibition of complement inhibition is recognized as a promising treatment for lupus. The safety and efficacy of a novel complement inhibitor, CRIg/FH, in the treatment of lupus nephritis in a mouse model of MRL/ lpr were observed. Method: 12 weeks old MRL/lpr female mice were randomly divided into treatment group and control group. 8 MRL/ lpr mice in every treatment group were intraperitoneally injected with complement inhibitor CRIg/FH at 10mg/kg(A1), 5mg/kg(A2) and 2mg/kg(A3). Mice in control group B1 were intraperitoneally injected with 2ml normal saline. Mice in control group B2 received daily steroid gavage. 8 C57BL/6J mice in B3 group were normal control group. Plasma biochemical indexes and immunological indexes were measured at 16 and 20 weeks. Renal histopathological examination was performed. Result: Complement inhibitor CRIg/FH reduces the proteinuria of MRL/lpr mice and prevented renal failure. The proteinuria level of group A1-A3 were significantly lower than those in the group B1 and B2($P<0.05$). At 20 weeks serum creatinine levels of group A1, A2 and A3 were significantly lower than those in the control group B1 ($P<0.05$). In the treatment group A1, A2 and A3, the deposition of complement MAC, C1q and C3d was reduced compared with control group B1. Complement inhibitor CRIg/FH reduced complement activation and autoantibody production in MRL/LPR mice: At 20 weeks the blood levels of C3, C4 and C5a in group A1, A2 and A3 were significantly higher than those in the control group B1 ($P<0.05$). Blood anti-double-stranded DNA levels of group A1, A2 and A3 were also significantly lower than those in the control group B1 at 20 weeks. ($P<0.05$). Conclusion: CRIg/FH has a better therapeutic effect on lupus nephritis in MRL/lpr mice. The protective effect in lupus nephritis is manifested in many aspects, including reducing proteinuria, decreasing serum creatinine and urea nitrogen levels, and reducing renal tubular inflammation. Our findings confirm that complement inhibitors can treat lupus nephritis in a dose-dependent manner. Its application prospect in patients with lupus is expected.

Background

Complement plays an important role in many autoimmune diseases and inflammatory diseases, especially in the immune pathogenesis of systemic lupus erythematosus (SLE) [1]. The pathogenesis of systemic lupus erythematosus (SLE) is characterized by the production of multiple autoantibodies and complement plays a key role in the pathogenesis of lupus. Multiple factors are involved in the immune pathogenesis of lupus, including the disorder of innate immune system, the disturbance of apoptotic cell clearance, and the abnormality of cytokines. All of the above can lead to the formation of a large number of circulating immune complexes (CIC), which eventually activate the complement system, causing inflammation and the consumption of complement proteins [2-4].

Traditional treatment of SLE currently include the use of nonsteroidal anti-inflammatory drugs, antimalarial drugs, glucocorticoid and immunosuppressants, these drugs play an important role in relieving symptoms of disease, control and improve the prognosis, but due to individual differences, some patients curative effect is not very desirable, and drug adverse reaction is increasingly valued. In

recent years, various new biotherapies for autoimmune diseases have provided new ideas for the treatment of SLE and refractory LN [5-6].

The complement system plays an important role in the immunopathogenesis of systemic lupus erythematosus. Recent studies have also found that complement system plays an important role in the pathogenesis of lupus. The absence of C1q, C2, C3 and C4 components in the classical complement pathway is closely related to the occurrence, development and prognosis of SLE. More than 90% of patients with homozygote defect of C1q develop SLE or SLE-like syndrome. Approximately 10-30% of patients with C2 homozygous defect develop SLE [7-9]. Recent studies have shown that reduced levels of MBL in the blood may contribute to other immune diseases, such as systemic lupus erythematosus.[10]. Aditya [11] et al found that the blood MBL levels were significantly high in SLE patients compared to healthy controls ($P < 0.0001$). MBL levels were variable in different clinical categories of SLE.

As the complement system is the main cause of the occurrence and development of systemic lupus erythematosus, complement-targeted therapy has been regarded as a promising method in the drug research and development of systemic lupus erythematosus in recent years, attracting more and more attention. The first generation of complement inhibitors is mainly targeted at drugs of complement activators, the first used in clinic is the monoclonal antibody-Eculizumab, which mainly prevents the cleaving of complement C5 into C5a and C5b. The disadvantage is that it is the systematic inhibition of complement at the level of C5, which is non-targeted and easy to lead to opportunistic infection. Moreover, it cannot inhibit the activation of early C3 level of complement, which has certain treatment limitations, and has not been introduced in China. The biggest difficulty in treating the complement system lies in how to maintain the complement system as a key response of the immune monitoring system, and at the same time to treat the abnormal complement activation. Therefore, in the pathological state, specific targeted complement inhibitor factors are applied to achieve therapeutic inhibition at the local site of abnormal complement activation. Due to the limitations of anti-C5 treatment, researchers have focused on the development of complement inhibitors for the early stage of complement activation. Among which the inhibition strategy against the core component C3 of complement shows potential therapeutic prospects. However, all three complement pathways are blocked and the body is left vulnerable to possible infection. Therefore, the second generation of targeted complement inhibitors is the focus of current research. CRIg/FH, a targeted complement inhibitor of CRIg, is an effective C3 blocker complement inhibitor [12]. Targeting activated C3 products (C3b/iC3b) rather than the natural C3 in the circulatory system can avoid systematic inhibition of complement and only target specific sites [13–14].

Therefore, this study intends to use the MRL/lpr lupus mouse model of lupus and the new complement inhibitor CRIg/FH for treatment, so as to clarify the therapeutic effect, clinical manifestations, blood biochemical and immunological indexes and renal pathological changes of the complement inhibitor in lupus mice. To clarify the therapeutic effect of this drug on MRL/lpr in mice with lupus nephritis and its application prospects in lupus patients.

Methods

Mice

All the animal experiments in this study were approved by Ethics Committee of Children's Hospital of Fudan University(ID: ECCHFU(2016)173)

MRL/lpr mice (female, 37.7±1.6g, 8 weeks old), SPF grade, purchased from Shanghai Slack Laboratory Animal Co.Ltd., all the experimental animals are kept in the same condition. The experimental breeding place is the Experimental Animal Science Department of Fudan University, MRL/lpr mice receive regular feeding, weekly urine retention test urine protein / creatinine. Treatment was given at 12 weeks of age2-week-old MRL/lpr female mice were randomly divided into experimental group and control group. The experimental group was divided into 3 groups according to the dose of CRIg/FH. Group A1: 8 MRL/lpr mice received intraperitoneal injection of complement inhibitors CRIg/FH 10mg / kg twice a week; Group A2: 8 MRL/lpr mice received intraperitoneal injection of complement inhibitors CRIg/FH 5mg/kg twice a week. Group A3: 8 MRL / lpr mice received intraperitoneal injection of complement inhibitors CRIg/FH 2mg/kg twice a week. The control group was divided into control group B1 in which 8 MRL/lpr mice received intraperitoneal injection of saline (NS) twice a week and control group B2 in which 8 MRL/lpr mice received daily steroid gavage (18.2 mg/kg/d) and control group B3 in which clinical parameters of 8 C57BL/6J mice were observed as normal control.

General conditions such as mental state, food intake, mouse activity, hair removal, etc were recorded. 24 hours urine was collected weekly to test urine protein/creatinine. Blood samples were collected every 4 weeks through the tail vein to test blood urea nitrogen, serum creatinine, C3, C4, C5a and A-ds-DNA.

Histopathological analysis

The mice were sacrificed by intraperitoneal injection of anesthesia at 20 weeks and kidneys were removed. They were cut perpendicularly along the longitudinal axis of the renal hilum, fixed with 10% formalin, dehydrated, embedded in paraffin, and then cut into slices(4μm) for further HE staining. C3d, Mac and C1q staining were detected using a C3d Ab (R&D AF2655), a rabbit polycolonal anti-C5b-9 Ab (Abcam; ab55811), and anti C1q Ab (Abcam; ab71089). Alexa Fluor 488 goat anti-mouse IgG (Invitrogen; A11029) and Alexa Fluor 488 goat anti-mouse IgM mu chain (Abcam; ab150121) were used for immunofluorescence detection of IgG and IgM in kidney. Renal pathology indicators were scored by two experienced renal pathologists back-to-back according to the activity index of renal tissue in lupus nephritis.

Statistical analysis

Most analyses and calculations were performed using Prism® version 5 and SPSS Statistics version 19.0. Values, which are expressed as the mean ± SEM, were compared by one-way analysis of variance. Statistical significance was set at $P<0.05$.

Results

General conditions

In the control group, B1 mice began to depilate at 16 weeks of age. The whole-body hair was obviously sparse, and there was lupus-like rash. The lymph nodes in the axillary fossa were obvious, and the food intake and activity of the mice also decreased significantly.

In the experimental group, MRL/lpr lupus mice only had a small amount of hair loss, and the food intake and activity of the mice did not decrease significantly, and no significant difference was observed between the different therapeutic dose groups. The mice injected with complement did not have obvious side effects such as allergic rash, anaphylactic shock, nausea, vomiting or hair loss.

CRIg / FH protect renal function and reduce proteinuria in MRL / lpr mice

Complement inhibitor treatment significantly reduced serum creatinine and urea nitrogen levels in lupus mice. Blood urea nitrogen level of the experimental group A1 was (11.8 ± 2.5) umol/L and the control group B1 was (24.6 ± 1.2) umol/L. Serum creatinine level of group A1 was (109.8±20.6) umol/L, while that of control group B1 was (194.7±11.2) umol/L. Blood urea nitrogen level in group A1 , A2 , and A3 were significantly lower than those in the control group B1 ($P<0.05$). Blood urea nitrogen level in group A1 and A2 were significantly lower than that in the control group, Group B2 ($P<0.05$). At 20 weeks blood urea nitrogen level in group A1 and A2 were also significantly lower than those in the control group B1 and B2 ($P<0.05$). Blood urea nitrogen level in group A1 was significantly lower than that in group A3 ($P<0.01$), and there was no significant difference between group A1 and A2 ($P=0.961$). At 16 weeks serum creatinine level in group A1, A2, and A3 were significantly lower than those in control group B1 and control group B2 ($P<0.05$). At 20 weeks serum creatinine level in group A1, A2, and A3 were also significantly lower than that in group B1 ($P<0.05$). Serum creatinine level in group A1 and A2 were significantly lower than that in group B2 ($P<0.05$) (Figure 1.a\b). The level of proteinuria in treatment groups were slightly elevated, while the level of proteinuria in both B1 and B2 were significantly elevated. At 16 weeks the level of proteinuria in group A1 was significantly lower than that of the control group B1 ($P<0.05$). At 18 weeks the level of proteinuria in groups A1, A2, and A3 were significantly lower than that of the control groups B1 and B2 ($P<0.05$). Mice in groups B1 and B2 had a significant increase in urinary protein/creatinine, which were 30.6 mg/umol and 27.6 mg/umol respectively. (Figure 1.c)

CRIg / FH blocks complement activation in MRL / lpr mice

The level of serum C3, C4, and A-ds-DNA and C5a were detected by ELISA. At 16 weeks the serum A-ds-DNA level in group A1 was 21.9±4.1 IU/ml, and that in group B1 was 39.5±1.4 IU/ml. The serum A-ds-DNA level in groups A1, A2 and A3 were significantly lower than that in group B1 ($P<0.05$). At 20 weeks the serum A-ds-DNA level in group A1 was 25.9 ±3.3 IU/ml, and that in group B1 was 45.2±4.4

IU/ml. The serum A-ds-DNA level in groups A1, A2, and A3 were also significantly lower than that in group B1 (P<0.05). (Figure 2.a)

At 16 weeks the serum C3 level in group A1 was 129.7±23.3 ug/ml , and the serum C3 level in group B1 was 63.1±10.2 ug/ml. The serum C3 levels in groups A1, A2 and A3 were significantly higher than that in group B1 (P<0.05). The serum C3 level in groups A1 and A2 were significantly higher than that in group B2 (P<0.05). At 20 weeks the serum C3 level in group A1 was 121.4±16.9 ug/ml, and the serum C3 level in group B1 was 50.6±15.9ug/. The serum C3 level in groups A1, A2, and A3 were also significantly higher than that in group B1 (P<0.05). The serum C3 level in group A1 was significantly higher than that in group B2 (P<0.01) (Figure 2.b).

At 16 weeks the serum C4 level in group A1 was 162.7±11.4 ug/ml, and the serum C4 level in group B1 was 64.9±11.9 ug/ml. The serum C4 level in the groups A1, A2 and A3 were significantly higher than that in group B1 (P<0.05). The serum C4 level in groups A1 and A2 were significantly higher than that in group B2 (P<0.05). At 20 weeks the serum C4 level in group A1 was 170.1±14.5 ug/ml, and the serum C4 level in group B1 was 45.5±4.1 ug/ml. The serum C4 level in groups A1, A2, and A3 were also significantly higher than that in group B1 (P<0.05). The blood C4 levels in the experimental groups A1 and A2 were also significantly higher than those in the control group B2 (P<0.05). (Figure 2.c)

At 20 weeks the serum C5a level in group A1 was 18.9±3.0 ng/ml, and the serum C5a level in group B1 was 55.7±13.0 IU/ml. The serum C5a level in groups A1, A2, and A3 were also significantly lower than that in group B1 (P<0.05). (Figure 2.d)

CRIg/FH improves lupus nephritis in MPL/lpr mice

All surviving mice were sacrificed at 20 weeks and frozen slices were subjected to immunohistochemistry staining for each complement activation index. The deposition of complement MAC, C1q, and C3d in treatment groups A1, A2, and A3 were significantly reduced compared with group B1 in which complement MAC, C1q and C3d deposits were obvious. The deposition of immunoglobulin IgM and IgG were not significantly improved in treatment groups. (Figure 3.a)

The activity index of lupus nephritis was scored by two experienced renal pathologists back-to-back according to modified NIH lupus nephritis activity and chronicity scoring system proposal[15]. 7 cases in group A1 were LN Ⅱ, 1 case was LN Ⅲ. While all the cases in group B1 were LN Ⅳ-G and 7 cases in group B2 were LN Ⅳ-S and 1 case was LN III. The average activity index in A1, A2 and A3 was lower than that in group B1 (P <0.05). The activity index of group A1 was lower than that of group B2 (P<0.05). However, there was no statistical difference between group A2, A3 and B2. The chronic index of group A1, A2 and B2 was 0. Some mice in group A3 and group B1 had elevated chronic activity index, and the chronic index of group B1 was significantly higher than that of group A3 (P < 0.05). (Figure 3.b) Table 1

Discussion

To explore the role of the new C3 level complement inhibitors in systemic lupus erythematosus (SLE), we use the most classic lupus mice model, that is called simply MRL/lpr mice model, its characteristics are similar to those of human systemic lupus erythematosus (SLE). This mouse developed lupus-like rash at three months, glomerulonephritis, vasculitis and arthritis caused by the presence of serum autoantibodies and the deposition of immune complex. 4 months later, it would show renal lesions, with massive proteinuria and progressive deterioration of renal function [7-8].

The complement inhibitor CRIg/FH used in this experiment is the second generation complement inhibitor, and its inhibitory site is at the C3 level. It can bind the C3b subunit through the complement regulatory protein on the surface of macrophages, and inhibit complement activity at the level of C3 and C5 invertase. Once C3b conditioning particles bind to CRIg on the surface of macrophages, the extracellular domain of CRIg and the complement inhibitory domain of factor H are indirectly connected by connecting peptides, playing a targeted role in blocking complement activation at the C3 level, and inhibiting the complement cascade reaction through the selective action of factor H in the alternative pathway[14,16,17].

Qian Qiao[12] et al have found that CRIg/FH can specifically bind to the surface of red blood cells activated by complement, effectively protecting PNH defective red blood cells from complement-mediated hemolysis. This study also found that CRIg/FH efficiently attenuates MPGN in a rat Thy-1N model. Chao Hu et al found that CRIg/FH ameliorates renal ischemia reperfusion injury via activation of PI3K/AKT signaling.

Monoclonal antibodies against C5 levels have been shown to effectively improve lupus nephritis in NZB/W F1 mouse models of lupus[18]. Eculizumab has also been used in some patients with systemic lupus erythematosus, especially in patients with severe refractory LN, low complement, can significantly improve patients with low complement and renal function [19]. In recent years, some complement inhibitors at C3 level, such as Crry-Ig, CR2-Crry and CR2-FH, have also been proved to have certain curative effects in animal experiments on lupus mice, including improving proteinuria and alleviating renal function damage [20]. Because both CRIg and FH domains are derived from human proteins, the recombinant CRIg-FH will not produce strong immune response when applied to the treatment of human diseases and has the advantage of low immunogenicity[12]. Our experiments also found that the treatment of CRIg/FH in lupus mice did not cause observable side effects such as rash and anaphylactic shock. Whether CRIg/FH, a complement inhibitor targeting C3, also plays a role in lupus mice requires further study.

In this study, we found that the serum indices of lupus mice in each different dosage complement inhibitor groups were significantly better than those of control group B1 and B2. Blood C3 and C5a in complement inhibitor treatment group (10mg/kg) were close to normal mice, but the blood A-ds-DNA level was still higher than normal mice. The SLEDAI score, which measures SLE activity, includes blood C3,C4 and blood A-ds-DNA[21]. CRIg can specifically bind to C3b, iC3b and C3c. Therefore, when the complement is activated, it can inhibit complement activity on C3b-regulated target cell surface, while when

complement is inhibited, it can dissociate from target cell surface with C3c degradation product, this is more in line with the physiological needs of the body, compared with the continuous deposition of CR2-C3d on the surface of target cells after complement inhibition[22]. This indicates that complement inhibitor CRIg/FH can not only treat the primary disease of lupus, but also may maintain the original anti-infection effect of complement without excessive intervention.

In this study, it was found that complement inhibitor CRIg/FH can reduce the kidney injury of MRL/lpr mice. Through the detection of urinary protein, we found that the treatment of complement inhibitors had a therapeutic effect on the improvement of urinary protein in lupus mice, and the therapeutic effect was related to the dosage of complement inhibitors. The improvement of urinary proteinuria in the higher dosage group was better than that in the lower dosage group. Serum creatinine, urea nitrogen and other blood biochemical tests showed that complement inhibitors also had therapeutic effects on alleviating the progression of renal function, delaying the progress of renal failure. In addition, the improvement of renal function in experimental group A1 (CRIg/FH 10mg/kg) was more obvious, and the serum creatinine level in complement inhibitor group (10mg/kg) was close to that of normal mice. The acute activity index (AI) [23] of lupus in the treatment group was significantly lower than that in the control group, pathological types were generally mild, mostly LN II, and the control were mostly IV-G and IV-S. Immune complex disease is recognized as the basic pathogenesis of lupus nephritis. In SLE patients, apoptotic disorders, loss of self-tolerance and dysfunction of cell clearance system lead to the accumulation of various autoantibodies and free nucleosomes, thus forming a large number of circulating immune complex (CIC) deposited in the kidney [24]. CIC deposition is the initial link of occurrence of LN. With blood circulation to the kidney and deposition, it stimulates complement cascade reaction, promotes the proliferation and activation of glomerular mesangial cells, releases a variety of inflammatory factors, thus causes glomerular diseases [25]. In general, CIC can activate complement and fix C3b to form CIC-C3b complex, which binds to CR1 (C3b receptor) on the erythrocyte membrane and circulates with red blood cells to the liver and then is consumed by macrophages [26]. In patients with LN, the complement level and the erythrocyte membrane receptor decreased relatively during the active stage of disease, which led to a significant decrease in the clearance rate of CIC and accelerated its deposition in the kidney, thus promoting the occurrence of LN. In this study, it was found that the renal pathology of mice treated with complement inhibitors was significantly improved, and the deposition of MAC, C1q and C3d was significantly reduced by immunofluorescence staining. It is speculated that the treatment of complement inhibitors can reduce the activation of complement and the formation of MAC through C3 level.

Despite a lot of efforts and attempts were made by researchers on complement inhibitors or regulation in recent years, many promising drugs have encountered insurmountable bottlenecks in clinical trials, so the success rate is extremely low. On the one hand, because the cascade reaction of complement participation is as complex as the network, and there are many diseases involved in complement. In the design of complement-specific drugs, finding the best target of complement inhibition and controlling the degree of complement inhibition are the key to the design. On the other hand, early C5 blockers blocking the formation of MAC in PNH therapy have been widely used in clinic. However, because their inhibition of

complement system is extensive and systematic, rather than targeted, all complement activities are inhibited, resulting in some patients with decreased immunity and opportunistic bacterial meningitis [27].

Targeted new complement inhibitor CRIg/FH can exert targeted complement inhibition at C3 level, and its role in lupus patients is particularly important. In addition, because both CRIg and FH domains are derived from human proteins, the recombinant CRIg-FH will not produce strong immune response when applied to the treatment of human diseases and has the advantage of low immunogenicity. Our experiments also found that the treatment of CRIg/FH in lupus mice did not cause observable side effects such as rash and anaphylactic shock.

Conclusion

We have demonstrated that CRIg/FH, a new complement inhibitor, can play an effective role in the classical MRL/lpr model of lupus nephritis mice at the level of complement C3. The protective effects of lupus nephritis are manifested in many aspects, including reducing proteinuria, serum creatinine and urea nitrogen levels, and reducing tubular inflammation. In addition to directly blocking the complement pathway of complement, whether CRIg/FH can improve the inflammation of kidney and alleviate the proliferation of mesangial cells through other pathways needs to be further clarified. In addition, we found that the anti-ds-DNA titer of SLE mice decreased, which implies the new complement inhibitor may directly or indirectly act on the acquired immune system, improving the immune imbalance of the disease. It also suggests that the clinical value of the drug in treating SLE is not only limited to the complement system. Further experiments are needed to confirm the relationship between complement and the acquired immune system in SLE.

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Figure And Table Legends

Figure 1

Differences in Scr(a) ,BUN(b) levels among the 6 groups at 16 week and 20 week *P < 0.05

c.Pro/cr from 15 to 20 weeks are shown and levels in groups of 2mg,5mg,10mg show the same tendency as normal control.

Figure 2

Differences in anti-ds-DNA(a),C3 levels(b), C4 levels(c) among the 6 groups at 16 week and 20 week *P < 0.05Differences in C3a level among the 6 groups at 20 week

Figure 3

Complement inhibitor CRIg/FH ameliorates nephritis in MPL-lpr mice

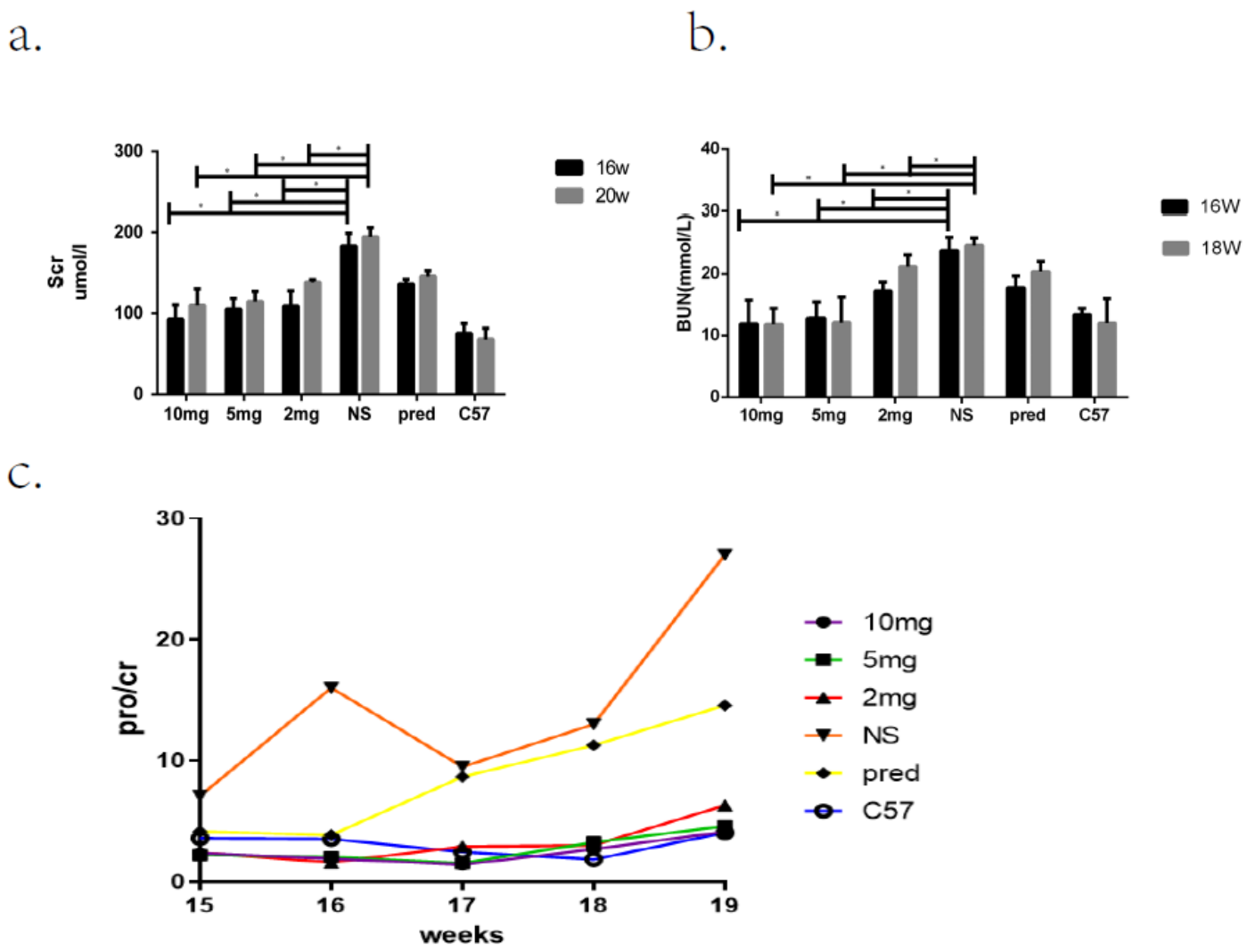
a.Representative kidney sections stained for IgG, IgM(original magnification *200) are shown no significant differences were observed in 2mg,5mg,10mg, pred and normal saline group.

b.Representative kidney sections stained with PAS (original magnification *200) from 6 groups are shown.

Table 1

The average activity index in different groups

Figures



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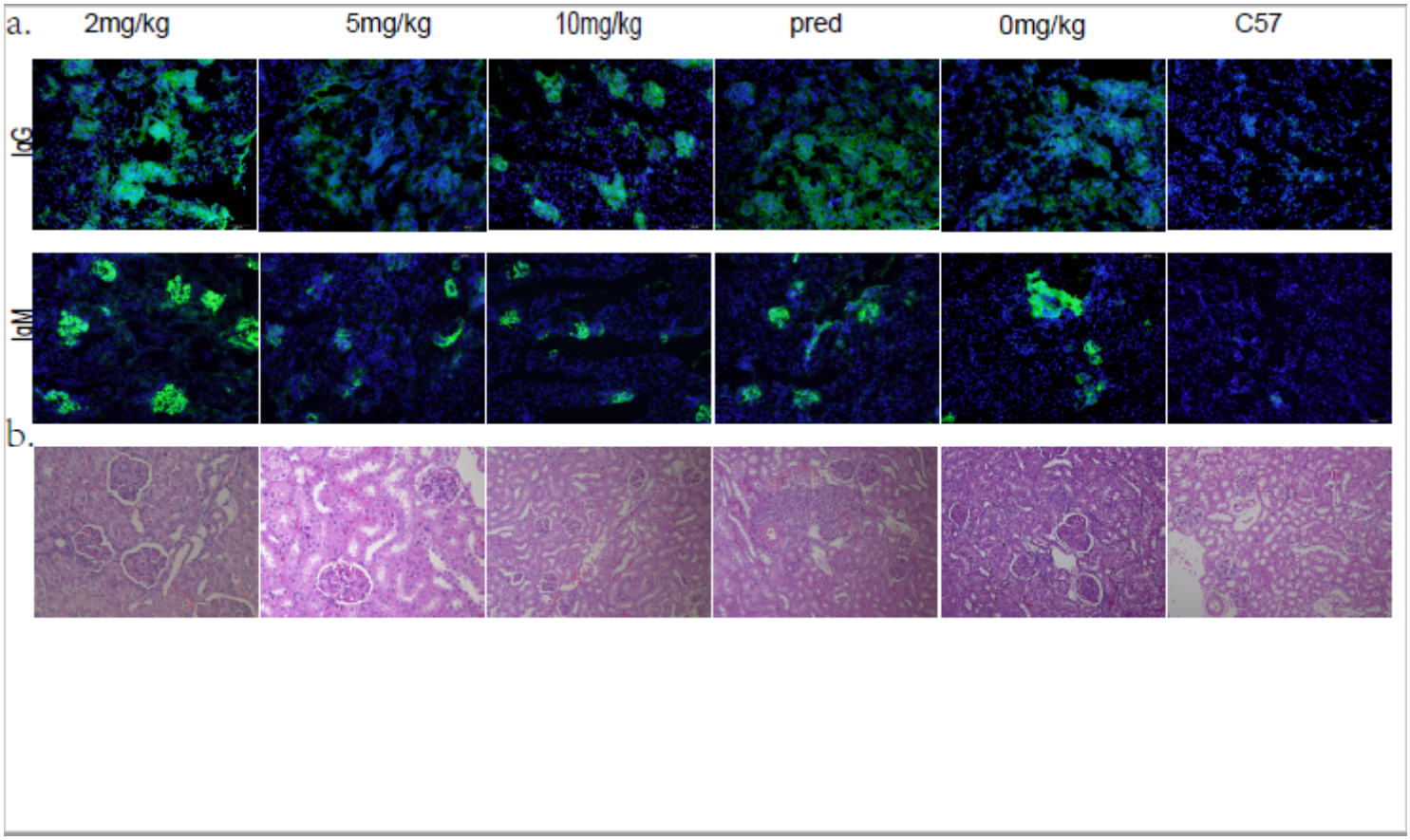

Figure 2

Differences in anti-ds-DNA(a),C3 levels(b), C4 levels(c) among the 6 groups at 16 week and 20 week *P < 0.05 d. Differences in C3a level among the 6 groups at 20 week

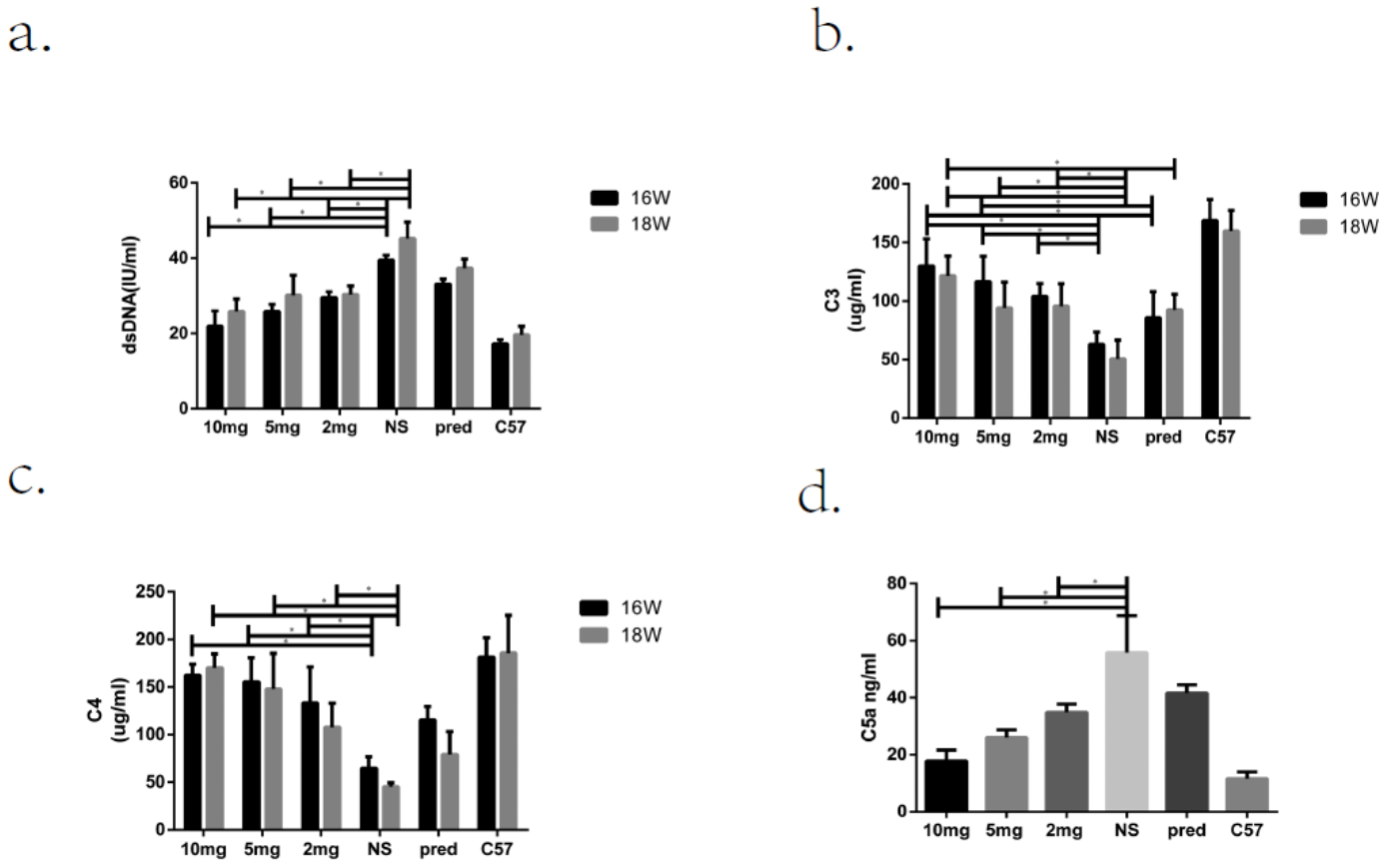

Figure 3

Complement inhibitor CRIg/FH ameliorates nephritis in MPL-lpr mice a. Representative kidney sections stained for IgG, IgM(original magnification *200) are shown no significant differences were observed in 2mg,5mg,10mg, pred and normal saline group. b.Representative kidney sections stained with PAS (original magnification *200) from 6 groups are shown.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.pdf
- supplement2.pdf