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The Association Between the Decreased Expression Levels of FOXJ1 and the Activation of NF-κB Pathway in Interstitial Lung Disease of MRL/Lpr Mice

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ABSTRACT

Background: Pulmonary manifestations of systemic lupus erythematosus (SLE) are appearing in 4-5% of patients involving lung in almost half of the cases during the disease course.

Objective: We compared the autoimmune pulmonary inflammation in the lung tissue of mice to determine the association between decreased expression levels of Forkhead Box J1 (FOXJ1) and the activation of the NF-κB pathway in autoimmune pulmonary inflammation of MRL/Lpr mice.

Methods: The female BALB/c mice (n=6) and MRL/Lpr mice (n=30) were divided into 5 groups including: a control group (BALB/c), and five MRL/Lpr mice groups (8W, 12W, 16W, 24W, and 32W). The infiltration of the inflammatory cells was determined in lung tissue by performing the histological analysis. The western blotting was used to examine the expression levels of the age-related FOXJ1, and p50 and p65 proteins in the lungs of MRL/Lpr mice. The expression levels of MMP2 and MMP9 were determined via immunohistochemistry and immunofluorescence.

Results: There were severe infiltrates of lung cells with high levels of tracheal damage, perivascular injury and interstitial inflammatory cell infiltration when the MRL/Lpr mice from 16w to 32w comparing to the 8w old healthy MRL/Lpr mice in the control group ($p<0.05$). Moreover, the reduced expression levels of FOXJ1 were associated with the activation of the NF-κB pathway in interstitial lung disease of MRL/Lpr mice via the modulation of p50 and p65. In addition, the expression levels of MMP2 and MMP9 pro-inflammation factors increased in the lungs of the MRL/Lpr mice from 16w to 32w.

Conclusions: The expression level of FOXJ1 might be an indicator of the degree of lung disease in lupus-prone mice.

Keywords: Forkhead-box j1, Interstitial Lung Disease, Systemic Disease, Systemic Lupus Erythematous

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INTRODUCTION

Systemic lupus erythematosus (SLE), a persistent inflammatory disease, is categorized based on the autoantibody production and B and T cells debilitation and the systemic clinical manifestations which result from the complicated relationship between genetic and environmental factors (1). Pulmonary manifestations may produce symptoms in 4-5% of patients. Moreover, lungs are involved in almost half of the cases during the development of the disease (2). Interstitial lung disease (ILD) is considered one of the major symptoms of the involvement of the respiratory system in SLE. Research has shown that the incidence of clinically important ILD in SLE ranges from 1% to 15% (3). ILD frequently emerges in hospitalized SLE patients because of the severity of their disease, the likelihood of their multiple organ system involvements, and their serologic and radiographic evaluation. However, subclinical ILD shows to be substantially more frequent than clinically recognizable ILD (4). The ILD is an important cause of SLE-related mortality (5). Bertoli et al., (3) found that the occurrence of pulmonary disorders like ILD increased as a result of the increase in the duration of SLE. Consequently, the study on ILD which strives to recognize, diagnose and estimate the level of ILD may hopefully provide a better understanding of SLE-ILD, issue clear monitoring guidelines, and formulate effective treatment strategies (6-9).

A number of the recently published studies (10-12) have reported that the Fox (forkhead) family includes transcription factors that contribute to various biological systems and result in the development of autoimmune diseases (10-14). It has the distinctive Forkhead DNA binding domain and functions as an activator or inhibitor of gene transcription (15). FOXJ1 as a part of the forkhead-box(FOX) gene family suggested that it can inhibit the spontaneous trigger of T and B cells by repressing the NF-κB activity (10-12). This issue highlights the fact that FOXJ1 attenuates autoimmune and inflammatory diseases. Research has shown that in patients with SLE, the tolerogenic peptide hCDR1 ameliorates lupus, is associated with the downregulation of pathogenic cytokines and the upregulation of FOXJ1 in peripheral blood mononuclear cells (16). Lin and colleagues (17) observed that FOXJ1-deficient mice showed inflammation in various organs. Moreover, as they stated, the FOXJ1 expression reduced in lupus-prone mice with severe ILD. The overexpression of the FOXJ1 protects against murine lupus (18). Nonetheless, the relevant studies have not surveyed the friendship between the FOXJ1 expression and the degree of infiltrates of ILD (17, 18). Therefore, there is no sufficient information on the effect of FOXJ1 on the development of ILD utilizing the modulation of the NF-κB, and MMPs.

To determine the association between the deregulated FOXJ1 and SLE-related ILD, we compared the normal female mice (BALB/c mice) with the MRL/lpr female mice which is one of the generalized autoimmune illnesses like SLE (19). In addition, we looked into the link between FOXJ1 expression levels and the severity of ILD in MRL/lpr mice.

MATERIALS AND METHODS

Animal Care

The female BALB/c mice (control group, n=6) and the MRL/lpr mice (n=30) were preserved under specific pathogen-free (SPF) facilities. Food and water were prepared for them ad libitum at Experimental Animal Center of the Nantong University, School of Medicine. The animals were kept at normal light/dark cycle at 21°C. The mice were organized into 5 groups including 8W, 12W, 16W, 24W, and 32W (n=5 mice in each group). The Ethics Committee of The First People's Hospital of Nantong granted this study ethical approval.

Western Blotting Analysis

The whole protein was eluted with boiling

buffer. Firstly, the elution of proteins was differentiated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was moved to polyvinylidene difluoride filter (PVDF) substrates. Secondly, the samples were impeded for 1 hour using Tris-buffered saline-tween (TBST) with 5% non-fat dry milk (NFDM). The the samples were incubated with 24 h with monoclonal antibodies (all of which were purchased from Santa Cruz, Santa Cruz, CA, USA) against FOXJ1 (anti-mouse, 1:500), MMP9 (anti-mouse, 1:500), MMP2 (anti-rabbit, 1:500), p65 (anti-rabbit, 1:500), or p50 (anti-rabbit, 1:500), and GAPDH (anti-mouse, 1:1,000) at 4°C. Thirdly, the membranes were washed with TBST and were incubated with the horseradish peroxidase[HRP]-conjugated goat anti-rabbit or goat anti-mouse IgG (1:2,000; Pierce, Rockford, IL, USA) for an additional 2 h. Finally, all of the western blots were visualized employing enhanced chemiluminescence, then the films were scanned. The quantification of protein expression was performed via an image analysis software (Scion Image software).

Immunohistochemistry and Immunofluorescence

First, the mouse lung tissues were placed overnight into 10% buffered formalin (4°C). Second, they were incubated with 20% sucrose (4°C) for 2–3 days. Third, these tissues were incubated with 30% sucrose (4°C) for 2–3 days. Fourth, the tissues were fixed in O.T.C. compound after the treatments which were provided utilizing sucrose solutions. Fifth, the tissues were cut into 6 μm frozen cross-sections. The samples were then blocked using 10% normal goat serum and then kept for 10 min at 25°C. Sixth, after blocking and washing the sections, we incubated the sections with monoclonal mouse anti-MMP9, or rabbit anti-MMP2 antibodies 24 h at 4°C. Seventh, the sections were incubated with HRP-labeled secondary antibody (Vector Laboratories, Burlingame, CA, USA). Eighth, the tissue samples were then visualized via diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA, USA). Cell groups with a dark or moderate brown color were considered positive. On the other hand, the cells without color were counted as negative. Ninth, the MMP-9 expression levels were examined randomly in five high-power fields (HPFs) (magnification×400). Tenth, the slides were surveyed at ×400 magnifications applying a Leica light microscope (Wetzlar, Germany). Eleventh, to carry out the immunofluorescence assay, the secondary anti-mouse or anti-rabbit fluorochromes Alexa Fluor 488 (Thermo Fisher Scientific, Waltham. MA, USA) was used to perform immunofluorescence based on the Thermo Fisher Scientific's staining protocol. Finally, using confocal microscopy, the positively labeled cells were observed and counted in different locations of the same tissue slices.

Evaluation of Pneumonitis

The tissues were placed overnight into 10% buffered formalin and were moved to 70% ethanol until they were fixed in paraffin. The tissue slides were provided and were stained using hematoxylin and eosin (H&E). The histopathological examinations were performed by an independent pathologist exploiting 4 semi-quantitative (lining layer cell number) scores: (0—under three layers; 1—between three to four layers; 2—between five to six layers; and 3—above six layers). The index of perivascular and peribronchiolar injuries was calculated based on the total number of all scores per section divided by the number of all sections. The infiltration of inflammatory cells into the tissues was evaluated and scored according to the following scoring system: 0=no recruitment of inflammatory cells; 1=less than 10 cells were present in the tissue 2=less than 20 cells had been observed, and 3=more than 20 had been observed. All of the sections were evaluated by a pathologist. The pathologist, who was not given any information about the trial groups, assessed five sections per animal (20).

Statistical Analysis

All of the data were summarized as means ±SEM. The Student's t-test was made use of to examine the changes between the two groups in the case of the normally dispersed data on the variables. On the other hand, the Mann–Whitney U test was utilized to investigate the differences between the aforementioned groups in the case of the data which were not normally distributed. A p-value less than 0.05 is statistically significant. Each experiment contained of least triplicates per condition. The statistical analysis was conducted through the IBM SPSS 19.0 software (IBM Corporation, Somers, NY, USA).

RESULTS

Evaluation of Autoimmune Pulmonary Inflammation in the Lung

According to research, the progression of lung inflammatory infiltration becomes apparent with time in MRL/lpr mice aged between 10-12 weeks (19). There was a positive correlation between lesion severity in the pulmonary interstitial and the increase in age. Fibrosis of pulmonary interstitial space and atelectasis were declared in the 32w group. We studied the increase of lung inflammatory reactions in MRL/lpr, and BAB/c mice. The MRL/lpr mice demonstrated a marked inflammation with increasing age. Consequently, the mice aged 24 weeks were sacrificed and their lungs were provided for histopathological analysis. The recruitment of inflammatory cells into the perivascular space (1.5±0.16, 1.71±0.12, 1.76±0.21) and peribronchial injuries and the alveolar area (1.5±0.2, 1.72±0.15, 1.75±0.13) of MRL/lpr mice (which ranged in age from 16w to 32w) were significantly greater than the above-mentioned lesions and the alveolar area of BAB/c mice, P<0.05. The infiltration of the lungs in MRL/lpr mice, that ranged in age from 16w to 32w, (1.51±0.14,

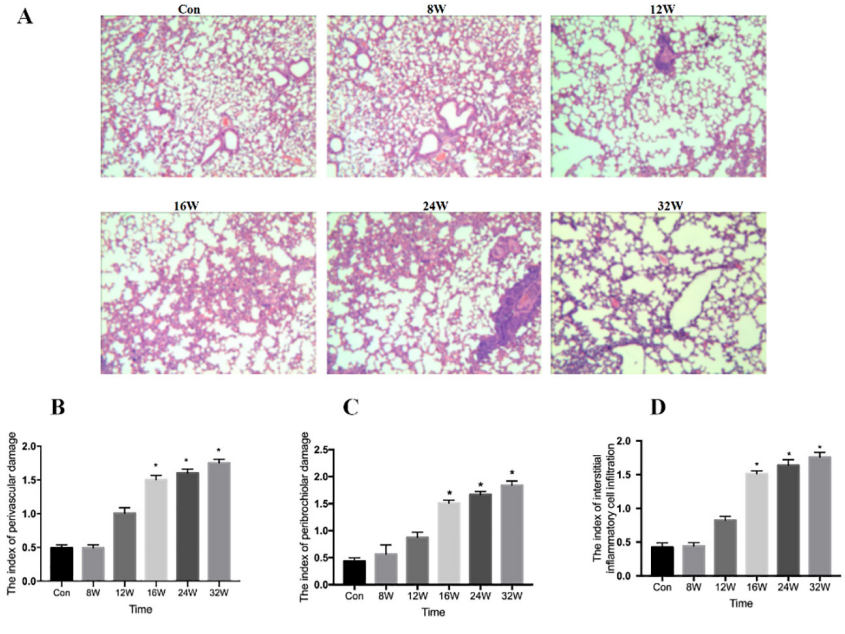

Figure 1. The MRL/lpr mice developed lung inflammation. On the other hand, there was an autoinflammation of the lungs in the normal MRL/lpr mice. Each group was comprised of mice who were of different ages. Three representative animals were examined in each group. (A) The histological analysis indicated the inflammation infiltration in the lung tissue. The inflammation infiltration in the lung began when the MRL/lpr mice reached the age 16w and was noticeable when they were at the age 32w. (B-D) Moreover, it began when they reached the age 16w. The MRL/lpr mice had severe infiltrates of the lungs with high levels of tracheal damage, perivascular injury, and interstitial inflammatory cell infiltration when they ranged in age from 16w（1.06±0.08 to 32w（1.75±0.04）(*, P<0.05 compared with the healthy MRL/lpr mice in the control group who were at the age 8w).

1.58±0.17, 1.76±0.20, Mann-Whitney U-test, P<0.05) was absent in their normal MRL/lpr counterparts which were at the age 8w (Figure 1A-1D).

FOXJ1 Prevents Autoimmune Pulmonary Inflammation by Directly Regulating Anti-inflammatory Regulators of the NF-κB Pathway

The histopathological examination, which was conducted as early as 12–16 weeks after the reconstitution, revealed the existence of systemic autoimmune inflammation, including moderate and severe infiltrates of the lung. We used the western blot to corroborate our findings and displayed that the NF-*κB* could be triggered via inflammatory molecules and the activated molecule had the p65 subunit. FOXJ1 inhibited the NF-*κB* activation by regulating the IκBβ, particularly the RELA (p65) subunit. The relevant studies have indicated that increased the NF-*κB* activity is involved in lupus-like diseases. However, there is not adequate information on the link between the expression levels of the NF-*κB* and the ILD of lupus. More importantly, we found that the expression levels of FOXJ1 decreased in the pulmonary tissue of the MRL/lpr mice which were at the age 16 w (0.7±0.31). Moreover, it meaningfully decreased in the lungs of the MRL/lpr mice which were at the age 32w (0.55±0.30) and were tested employing the western bolt assay in comparison with the MRL/lpr mice which were at the age 8w (P<0.05, Figure 2A-2B). As expected, the activity of p50 and p65 subunits gradually increased in the lungs of MRL/lpr mice which ranged in age from 16w to32w compared with their BAB/c counterparts which were at the age 8w in our study. More specifically, the expression levels of p65 and p50 markedly elevated in the case of the mice which were at the age 32w (0.76±0.02, 1.12±0.03, respectively) in our study (Student's t-test, Figure 2C-2D). This result indicated that the increased levels of p65 were accompanied by the decreased expression levels of FOXJ1. Overall, the data showed that FOXJ1 prevented autoimmune pulmonary inflammation by directly regulating anti-inflammatory regulators of the NF-*κB* pathway.

The Pro-inflammation Factors of MMP2/MMP9 Increased in MRL/lpr Mice

Research has shown that the NF-κB signaling enjoys a significant role to play in the pathophysiology of various immune system-related diseases which increase the production of pro-inflammatory mediators such as MMPs (21). Based on the findings, the MMPs expression increased in individuals suffering from primary Sjögren's syndrome

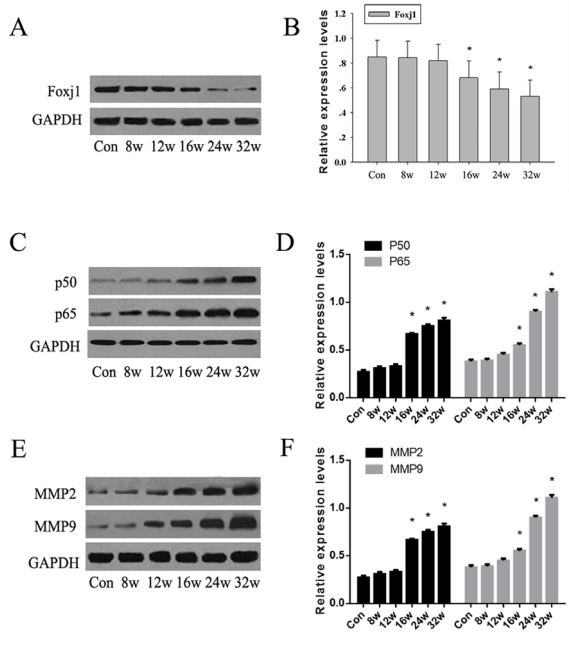

Figure 2. The expression levels of FOXJ1 and its associated protein in the lungs of MRL/lpr mice. (A) The age-related decrease in the expression levels of FOXJ1 protein in the lungs of MRL/lpr mice was detected by western blotting. (B) Relative ratio to GAPDH. (C) The increased age-related levels of the expression of p50 and p65 proteins in lung tissue of MRL/lpr mice were detected by western blotting. (D) Relative ratio to GAPDH. (E) Tissue expression of MMP2, and MMP9 proteins, which increased in the lungs of MRL/lpr mice, were detected by western blotting. (F) Relative ratio to GAPDH. The deregulation of FOXJ1 negative was associated with the increased levels of p50, p65, MMP2, and MMP9. Meanwhile, the upregulation of MMP2, and MMP9 positive was associated with the enhanced levels of p50 and p65 in the lungs of MRL/lpr mice which ranged in age from 16wto32w.

and the patients with SLE who suffered from lung diseases (10). Also, we showed that MMP2, and MMP9 overexpressed in the lungs of MRL/lpr mice which ranged in age from 16w to 32w in comparison with their normal MRL/lpr counterparts who were at the age 8w ($P<0.05$, Figures 2). The expression levels of MMP2, and MMP9 reached their highest levels in the case of the MRL/lpr mice which were at the age 32w (0.75±0.03, 1.1±0.02, respectively). This result is in line with the considerably more lung infiltrate showed in the histopathological analysis. The immunohistochemistry and immunofluorescence assays were carried out to investigate the localization and tissue expression extent of MMP2, and the MMP9 in the lung tissues of MRL/lpr mice during the development of the disease (Figures 3 and 4, respectively). The expression levels of MMP2, and the MMP9 increased in the lungs of MRL/lpr mice which ranged in age from 16w to 32w in comparison with the healthy mice in the control group

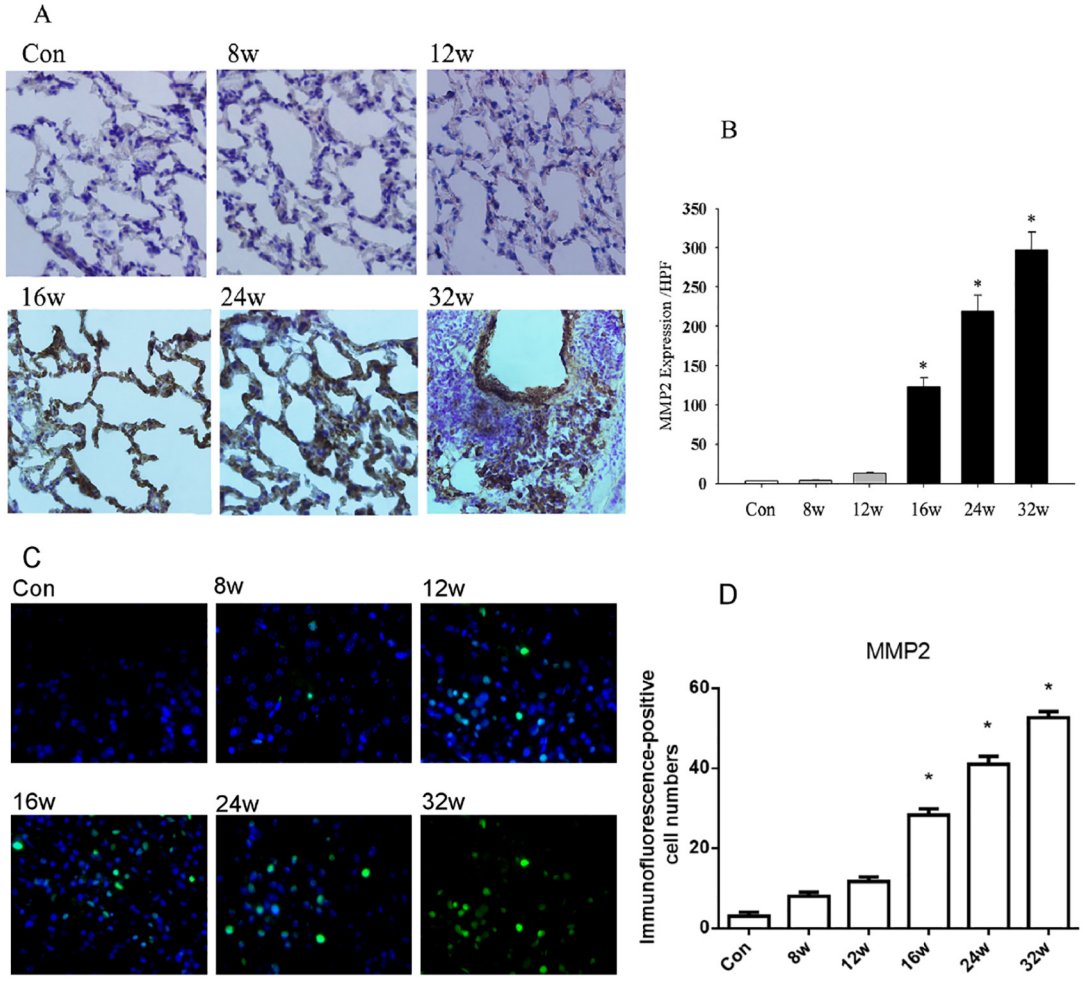

Figure 3. The pro-inflammation factor MMP2 was elevated in MRL/lpr mice. (A) Immunohistochemistry for MMP2 in the lungs of MRL/lpr mice and the normal mice. Staining did not reveal inflammation when the MRL/lpr mice and the normal mice ranged in age from 8wto12w. The punctuate localization of MMP2 was mainly expressed in alveolar epithelial cells, endothelial cells, and interstitial cells and was sporadically expressed in bronchiolar epithelial cells of the MRL/lpr mice after they reached the age 16w. (B) The semi-quantitative number of MMP2 in the positively stained cells in lungs (*, $P<0.05$ in comparison with the healthy MRL/lpr mice in the control group which were at the age 8w). (C) At the same time, the immunofluorescence showed an increased MMP2 positive staining among inflammatory cells infiltrate, in perivascular and around bronchiolar epithelial cells of the lungs of the MRL/lpr mice which ranged in age from 16w to 32 w in comparison with the healthy MRL/lpr mice in the control group which were at the age 8w. (D) The semi-quantitative number of MMP2 in the positively stained cells in lungs (*, $P<0.05$ compared to the healthy MRL/lpr mice in the control group which were at the age 8w).

which were at the age 8w (P<0.05).The semi-quantitative analysis of the degree of lung infiltration in MRL/lpr mice showed that FOXJ1 downregulated MMP2, and MMP9 expression levels via NF-κB. These issues highlighted the fact that there was a potentially positive association between the expression levels of MMP2, and MMP9 and the degree of ILD in MRL/lpr mice (Figures 1, 3, 4) via the FOXJ1 mediated activity of the NF-κB signaling pathway.

DISCUSSION

In our study, we demonstrated that the forkhead member FOXJ1 played a vital role in the maintenance of immune homeostasis, which coordinates the regulation of the activity of the key inflammatory transcription factor the NF-κB in ILD of MRL/lpr mice. The expression levels of FOXJ1 in the lungs of the MRL/lpr mice with ILD decreased as they grew older. Furthermore, based on

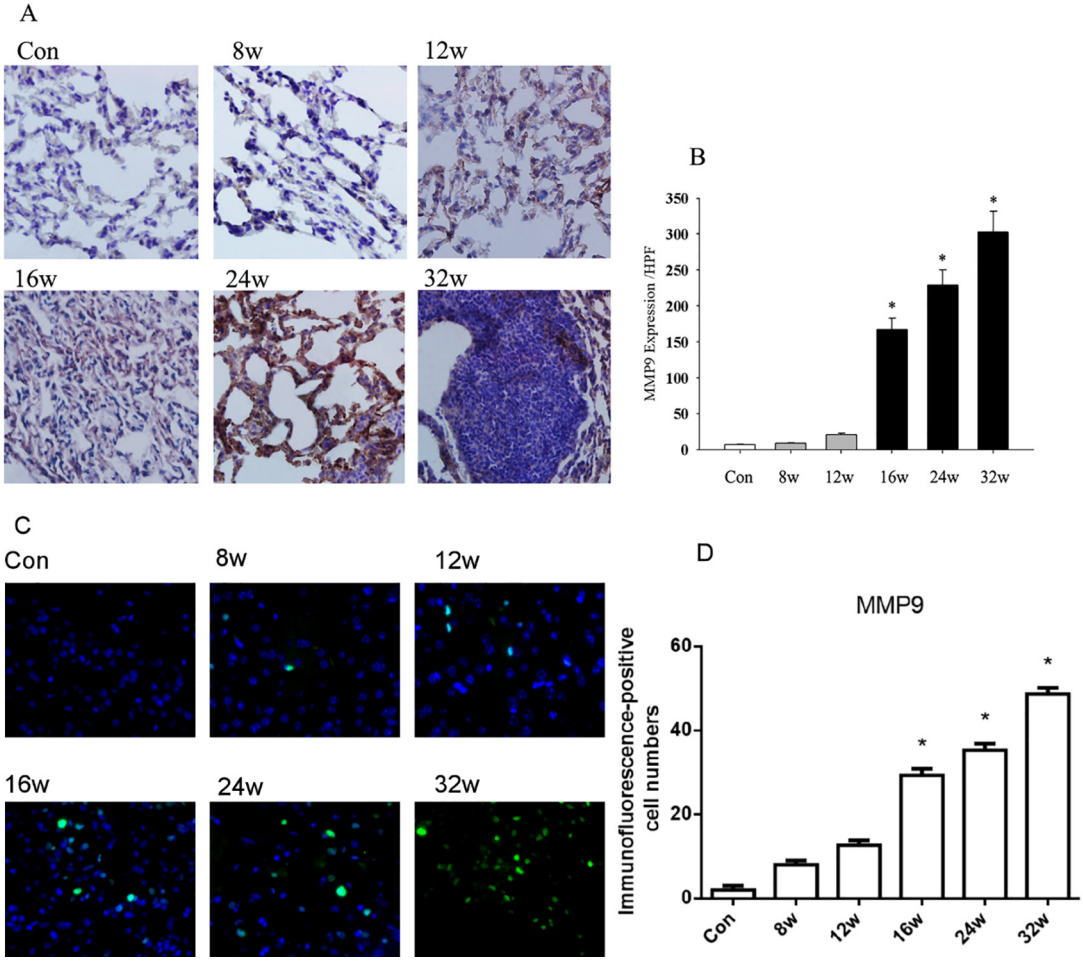

Figure 4. The pro-inflammation factor MMP9 was elevated in MRL/lpr mice. (A) Immunohistochemistry for MMP9 in the lungs of the MRL/lpr mice and normal mice. Staining did not reveal inflammation when the MRL/lpr mice and the normal mice were at the age 8w. There was strong MMP9- staining in the alveolar septa of the MRL/lpr mice when they were at the age 16w. The alveolar septa retained MMP9-positive staining when the mice were at the age 24w and had a small amount of interstitial MMP9. When the mice were at the age 32w, their lungs had more interstitial infiltration and there was a significant increase in the interstitial MMP9 in the areas of fibrosis. (B) The semi-quantitative number of MMP9 in positively stained cells in the lungs (*, P<0.05 in comparison with the healthy MRL/lpr mice in the control group when they were at the age 8w). (C) The immunofluorescence showed an increase in MMP9 positive staining of the inflammatory cell infiltration, in perivascular spaces, in the vessels' walls, and on the pulmonary bronchial epithelium in the MRL/lpr mice when they were at the age 16w in comparison with their counterparts in the control group. (D) The semi-quantitative number of MMP9 in positively stained cells in lungs (*, P<0.05 in comparison with the healthy MRL/lpr mice in the control group when they were at the age 8w).

the results, the upregulated activity of the NF-κB might induce the expression levels of the pro-inflammatory mediators such as MMP2, and MMP9 which are involved in the pathogenesis of ILD in MRL/lpr mice. FOXJ1 immunomodulates the pathogenesis of SLE-ILD by inhibiting the activity of the NF-κB. Therefore, it is suggested that the lower levels of FOXJ1result in more severe damage to the lungs. It can be stated that the low levels of FOXJ1 in peripheral blood of SLE patients may indicate the ILD. This issue might suggest that FOXJ1 is a novel diagnostic index regarding SLE patients with ILD.

There is not a large body of data at this time to make a case for using HRCT findings of ILD as a basis for the treatment of patients with an asymptomatic SLE. Consequently, conducting clinical research and identifying serologic markers of ILD constitute the basis for treating and determining the overall prognosis of the disease.

In our study, lung inflammation was manifested through high levels of perivascular injury, and peribronchiolar and perivascular mononuclear cell infiltration from the age 16w to the age 32w in MRL/lpr mice (Figure 1A-1D). The degree of ILD in MRL/lpr mice became more serious as the result of their increasing age. The expression levels of FOXJ1 reduced in the lungs of MRL/lpr mice because of their increasing age which affected these levels as the mice reached age 16w (Figure 2A-2D). On the other hand, the expression levels of FOXJ1 were negatively associated with the degree of ILD accompanied by the levels of perivascular injury, tracheal damage, and interstitial inflammatory cell infiltration (Figure 2A and 2B).

Research has highlighted the fact that the optimal NF-κB inhibition in FOXJ1-deficient T cells in vivo requires FOXJ1 (17). Moreover, FOXJ1 can inhibit spontaneous autoimmunity, in part by antagonizing the NF-κB activity in lupus-prone mice (22). In our study, the activity of the NF-κB (p65 and p50) increased in the lungs of MRL/lpr mice which ranged in age from 16w to 32w following the degree of ILD which was observed in the histopathological analysis in comparison with their normal MRL/lpr counterparts which were at the age 8w. These results suggest that FOXJ1 has protective effects against ILD since it downregulates the levels of the NF-κB activity in MRL/lpr mice. The previous studies have reported that the production of pro-inflammatory mediators like MMPs, is responsible for the NF-κB, which plays a crucial role as a pro-inflammatory transcription factor in the active stage of autoimmune diseases like rheumatoid arthritis and SLE (20, 23). In human idiopathic pulmonary fibrosis, the levels of MMP-2 and MMP-9 increased in the lungs (24) and BAL fluid (25) of affected patients. Research has shown that, the levels of MMPs increased in indivduals suffering from primary Sjögren's syndrome and the ones suffering from SLE (11). The immunohistochemistry staining showed that the tissue expression level of MMP2, and MMP9 increased in the lungs of MRL/lpr mice which ranged in age from 16w to 32w in comparison with the normal MRL/lpr mice which were at the age 8w. Moreover, the NF-κB p65 and p50 regulated the extent of MMP2, and MMP9 tissue expression and the expression of these factors positively associated with the degree of lung infiltration at different ages of mice. Finally, our results suggests that the levels of MMP2, and MMP9 were related to the degree of ILD in MRL/lpr mice because FOXJ1 mediated the activity of the NF-κB.

CONCLUSION

We surveyed the expression levels of FOXJ1, the activity of the NF-κB, and the degree of ILD in MRL/lpr mice, which spontaneously inhibitis the production of MMP2, and MMP9. We hypothesized that the FOXJ1 protein may attenuate lupus in MRL/lpr mice. Moreover, we averred that the levels of FOXJ1 protein might indicate the degree of ILD in lupus-prone mice. More specifically, FOXJ1 protein

may indirectly reflect the severity of ILD in SLE patients. Consequently, it is proposed that the upcoming research should emphasize the detection of the levels of FOXJ1 in blood circulation. These results are significant for the detection and treatment of SLE patients with ILD.

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Conflicts of Interest: None declared.

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