A role of oestrogen in aggravating SLE-like syndrome in C4-deficient mice

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Summary

Background: **As one of the epigenetic factors, oestrogen is considered to be a predisposing factor that is associated with a susceptibility to autoimmune disease development in women including systemic lupus erythematosus (SLE). Here, we proposed that oestrogen is also imparted in a post-lupus symptomatic enhancement as studied in the C4-deficient *($C4^{-/-}$)* mice model known to develop SLE-like symptoms.**

Methods: **Fifty-six C4 knockout mice were ovariectomised (OVX) to eliminate the effect of endogenous feminine hormones followed by 17-β oestradiol (E_2) administration in both dose- and time-dependent manners. Histopathological features of kidneys and spleens were studied by histological and immunofluorescent staining. The relative expression levels of IgG and IgM were measured densitometrically on their immunoreactive bands and the level of IgG-anti-double stranded (ds) DNA was measured by ELISA.**

Results: **E_2-treated mice displayed a gradual increase in immune complex deposition (both IgG and IgM) in glomeruli and proximal convoluted tubules. An increased reactivity of autoantibodies against dsDNA correlated with increasing doses and longer exposure to E_2 treatments. In addition, enlargement of the spleen (splenomegaly) was also observed in E_2-treated mice.**

Conclusions: **Our results support the hypothesis that oestrogen aggravates severity of the SLE-like symptoms in C4-deficient mice. *(Asian Pac J Allergy Immunol 2015;33:339-48)***

Keywords: *$C4^{-/-}$mice, ovariectomised, 17-β oestradiol, systemic lupus erythematosus, immune complex, glomerulonephritis*

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Submitted date: 27/1/2015
Accepted date: 7/5/2015

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that is ten times more common in women than men. In SLE patients, many organs are affected, including the joints, kidneys, lungs, skin, haematopoietic system and central nervous system.[1] The aetiology is presently unclear; it may involve genetic, hormonal and environmental factors, as well as a deficiency in complement components. The existence of complement-deficient SLE provides a crucial clue into the significance of early complement components for protecting animals against autoimmune diseases. Deficiency in the classical complement components C1, C2 and C4 is strongly associated with SLE. Human complement deficiencies of C1q, C2 and C4 are genetic risk factors for SLE.[2] Patients show progressive accumulation of the immune complex in kidneys and subsequent glomerulonephritis (GN) that is associated with anti-double stranded DNA (anti-dsDNA) autoantibodies. Lupus GN is one of the causes of morbidity and mortality in SLE. In experimental animals, C4-deficient mice display some features of a lupus-like disease by which they spontaneously develop anti-ds DNA antibodies,[3] as well as sharing many features of human diseases.[4] Although many factors contribute to the aetiology of SLE, the deficiency of C4 complement together with administration of sex hormones aggravating autoimmune diseases has yet to be investigated.

Generally, women have higher levels of serum immunoglobulin than men.[5,6] This finding correlates with the fact that women have a somewhat higher incidence of autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and Sjogren's syndrome. In addition, the

higher endogenous level of oestrogen in women may also explain the relationship of these sex-related autoimmune diseases. The effects of oestrogen on regulating the immune system have been studied in normal and autoimmune-prone mice. The administration of 17-ß oestradiol (E_2) has shown to increase the production of autoantibodies in the non-autoimmune BALB/C and C57BL/6 mice through promoting B cell hyperactivity in the spleen and secreting plasma cells.[7,8] In castrated NZB/NZW (B/W) F_1 mice, the level of autoantibodies and glomerulonephritis was enhanced when they were treated with E_2. On the other hand, a reduction in autoantibodies or lower prominence of glomerulonephritis and improved survival has been obtained with 5α-dihydrotestosterone treatment.[9] In contrast to castrated MRL-lpr mice, oestrogen did not exacerbate the signs and symptoms of autoimmune disease. This discrepant information of oestradiol-treatment pointed to the fact that sex hormones may influence autoimmune disease differently.[10] As a result, more information about the effect of oestradiol and its exacerbation of autoimmune symptoms is required before implicating it in other mammals, particularly women. We proposed here that there are aggravating effects of oestrogen in SLE symptoms in mice lacking C4 complement. The outcomes of this study may provide a pre-caution of oral contraception use or therapy with oestrogen replacement in postmenopausal SLE women who are deficient in C4 complement.

Methods

Animal handling and oestrogen administration

C4-deficient *($C4^{-/-}$)* mice were bred and maintained at the Institute of Molecular Biosciences, Mahidol University. Parental female and male C4-deficient mice purchased from Jackson Laboratory (Bar Harbor, ME, USA) were housed in individual ventilated cages with 12/12 hr light:dark cycles and fed a commercial diet. Only female mice were used in the experiments. Selective knockout of C4 was confirmed by PCR analysis according to the Jackson Laboratory protocol. All experiments on animals were carried out in accordance with the Ethical Guidelines of Laboratory Animals in Research, National Research Council, and were approved by the Animal Care and Use Committee, Institute of Molecular Biosciences (COA.NO.MB-ACUC 2011/001).

Fifty-six *$C4^{-/-}$* mice were ovariectomised according to the protocol(Deb et al., 2005) at 7 weeks old. *$C4^{-/-}$* mice were divided into four groups which included 7 mice in each group: (1) ovariectomised (OVX) mice with sex hormone replacement of 5 µg 17-ß oestradiol (E_2), (2) OVX mice with sex hormone replacement of 15 µg E_2, (3) OVX mice without sex hormone replacement and (4) sham-operated mice, which underwent the same surgical procedure but without ovary removal. Two groups of OVX mice with sex hormone administration were subcutaneously injected with E_2 every day for 6 months and 9 months after ovariectomy.

Histopathological and indirect immunofluorescence studies

The kidneys and spleens were removed at the end of oestrogen administration. Tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA). They were sectioned at 5 µm thickness and stained with Haematoxylin and Eosin (H&E) or Periodic-acid- Schiff (PAS). The kidney sections were observed under a light microscope for the degree of glomerular hypercellularity, thickening of glomerular basement membrane, and the presence of crescents or fibrosis. These parameters were combined for a total glomerular pathology score with a maximum score of 12. Each kidney section was determined using the following scoring, 1) <10% of glomeruli affected; 2) 11-25% of glomeruli affected; 3) 26-50% of glomeruli affected; and 4) >50% of glomeruli affected. For each individual animal, at least 50 glomeruli or 10 randomly selected cortical areas were counted to assess glomerular abnormalities. Interstitial changes were also graded separately. Scores from 1-4 were assigned to these changes and combined together to make a final renal score.[27] Histopathology of kidneys and spleens were compared with C57BL/6 mice (n = 10).

For indirect immunofluorescence staining, the aldehyde-fixed spleens and kidneys were processed for cryosectioning. Briefly, the fixed tissues were transferred to 30% sucrose, embedded in TissueTek (Sakura Fenetex. Torrance, CA, USA) and frozen on dry ice prior to storage at -70°C until use for immunofluorescence staining. Five-micron-thick tissues were blocked with either 5% normal goat serum or 4% BSA (room temperature, 30 min). They were then incubated with 1:1,000 (v/v) FITC-labelled goat anti-mouse IgG in blocking solution (for kidneys) or with 1:500 (v/v) Alexa 647-labelled rat anti-mouse B220 (for spleens) and washed three times with 0.1% PBS-Tween 20 (PBST). For spleens, the sections were also counterstained with FITC-peanut agglutinin (room temperature, 1hr).

They were mounted with anti-fade mounting medium (Sigma-Aldrich), topped with coverslips and then viewed by an Olympus FLUOVIEW FV 1000 confocal laser scanning microscope. The amount of immune complexes deposited in the glomeruli of the kidneys and in the germinal centre of spleen were densitometrically measured for fluorescent intensity in at least 8 randomised areas of 0.3×0.3 mm^2 in each acquired image. The mean and standard deviation were then calculated for the data collected.

Serum autoantibody measurements by ELISA

Anti-dsDNA antibodies in the collected sera were assessed by indirect ELISA. Blood samples in each group were collected by heart puncture and the sera were separated by centrifugation and kept at -70^{o}C until further analysis. Ninety six-well polystyrene plates (Costar, Corning incorporated, NY, USA) were coated (4^{o}C, overnight) with nitrocellulose-filtered salmon sperm DNA (Sigma-Aldrich) in Tris/EDTA. One hundred microlitres of sera was added at a dilution 1:100 in 0.1% BSA-PBST and allowed to stand (37^{o}C, 1 hr) for the formation of immune complexes, followed by washing with 0.1% PBST and blocking with 1% BSA-PBS (37^{o}C, 1 hr). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Sigma) at 1:1000 was added and incubated (37^{o}C, 2 hr), washed with PBST and exposed to TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate (room temperature, 30 min). The reaction was stopped by adding 2N H_2SO_4. The optical density (OD) was read at 450 nm with a microplate reader.

Western blotting and densitometric analysis

Proteins from spleens and kidneys were extracted in a lysis buffer and measured for their protein concentrations by Bradford protein assay. Proteins were separated by 10% sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose membrane. The transferred proteins were blocked with 5% skim milk or 4% BSA in 0.1% Tween-20/Tris-buffered saline (TBST) for 1 hr. Separated IgG or IgM were detected with a HRP conjugated-anti-mouse IgG or IgM at the dilution of 1:1000 in TBST. Visualisation of the immune complexes was performed with an enhanced chemiluminescence (ECL; Amersham Pharmacia, Piscataway, NJ, USA) and exposed on X-ray films.

For densitometric analysis, each band density was obtained from the scanned images in the same boxed pixel areas using the ImageJ software (NIH, Bethesda, MD, USA). The density information from 8-10 replications was used for calculations and expressed as mean and standard error of the mean (mean ± SEM). Statistical analyses in the differences of IgG anti-dsDNA levels, kidney and spleen weights were performed by *t*-test or one-way analysis of variance (ANOVA) test following by Newman-Keuls test. A p-value < 0.05 was considered to denote a significant difference between the analysed groups.

Results

E_2 administration enhanced severity of glomerulonephritis

Generally, mice lacking complement C4 or those treated with E_2 after OVX were prone to having a higher weight or larger size kidneys than OVX mice (Figure 1A), suggesting the significance of both endogenous and exogenous oestrogen in enhancing glomerulonephritis. It was clear that kidney weight was significantly increased in OVX mice treated with 15 µg E_2 for 9 months compared to OVX mice, sham-operated group ($p < 0.01$, ANOVA), and other E_2 treatment groups following the same duration ($p < 0.05$, ANOVA) (Figure 1A). Similar trends of increases in kidney weight were also notable in E_2 treatment at 6 months; however, there were no significant differences in kidney weight between any groups in this period. Kidney histopathological studies in Figures 1B and 2A confirmed that OVX mice treated with 15 µg E_2 for 9 months developed many signs of glomerulonephritis, including inflammatory cell infiltrates (arrows), glomerular enlargement (inset; Figure 1B), hypercellularity (Figure 2A, H&E staining) and glomerular basement membrane thickening (Figure 2A, PAS staining) that was clearer than in other treatments and sham control groups. However, all groups showed glomerular hypercellularity when compared to wild type (Figure 2A). In fact, these kidney pathological signs were also observed in the 12 month-old *C4*$^{-/-}$ more than in the younger mice used in this study (data not shown). Moreover, kidney sections were analysed for renal pathology (Figure 2). The glomerular scores were significantly increased in OVX mice treated with 15 µg E_2 for 6 months, whereas the interstitial scores trended toward higher in OVX mice treated with 15 µg E_2 for 6 months, but there were no significant differences between groups following the same duration (Figure 2B). A similar trend of increased glomerular scores was

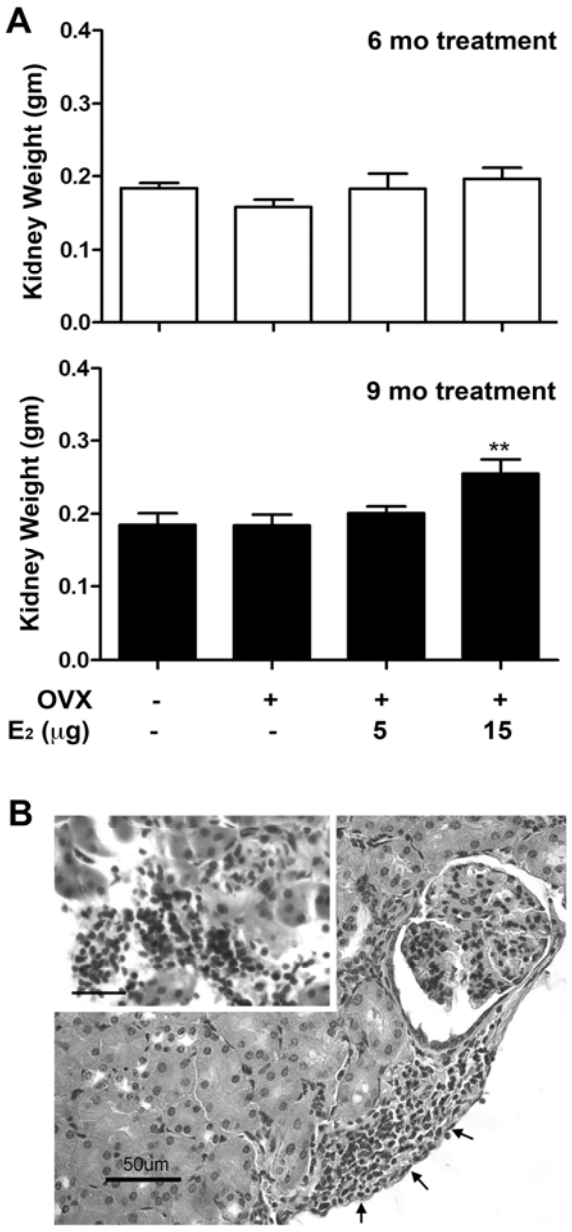

Figure 1. Kidney weight in *C4*$^{-/-}$ mice is shown by histograms (A), all data is representative of mean ± SEM from 7 mice per group. Kidney weight increased significantly in OVX mice treated with 15 μg E_2 for 9 months compared with OVX mice for the same time period (** indicates $p < 0.01$, one-way ANOVA), while the experiment at 6 months exhibited no significant differences between groups. The *C4*$^{-/-}$ mice developed autoimmune glomerulonephritis with mesangial cell hypercellularity and infiltrated inflammatory cells as shown by the arrows (B).

also shown in OVX mice treated with 15 μg E_2 for 9 months, while the OVX mice treated with E_2 had higher interstitial scores compared to OVX mice, but this was not significantly different (Figure 2B). These results indicate that glomerulonephritis spontaneously develops in mice lacking complement C4 but can be aggravated in a shorter time by the exogenous administration of oestrogen.

Immunofluorescence staining of renal cryosections revealed an increase in IgG and IgM deposition in all groups of female *C4*$^{-/-}$ mice at 6 and 9 months (Figure 3). The results showed that OVX mice treated with oestrogen (Figures 3C, D, G, H, K and L) had an apparently higher intensity of immune complexes for IgG and IgM in glomeruli, compared with the OVX mice group (Figures 3B, F and J). The OVX mice treated with 15 μg E_2 at 9 months (Figures 3H and L) showed the highest intensity of IgG and IgM staining comparing to those treated with lower doses (5 μg) of E_2 for the same duration (Figures 3G and K) and those treated for a shorter time (6 months) (Figures 3C and D). Together, the amount of IgG and IgM deposited in glomeruli was likely dependent on both dosage of E_2 and duration of treatment.

Deposition of IgG and IgM was further validated by Western blot analysis, as shown in Figure 4. Kidney protein extracts from representative *C4*$^{-/-}$ mice in each group was detected with anti-mouse IgG (H+L; as shown by 25 and 50 kDa) and anti-mouse IgM (μ chain; as shown by 50 and 75 kDa). For both IgG and IgM, their deposition levels in the kidney tissues were much lower in the OVX groups than in those with E_2 administration and the sham control groups. When the OVX animals were exposed to oestrogen, a dose-dependent, linearised increase of 50/75 kDa IgM (Figure 4A) and 25/50 kDa IgG (Figure 4B) was apparent in the 6-month E_2 treated groups. In the 9-month treatment, a non-linearised increase in band intensity was pronounced, namely, 75 kDa IgM and 25 kDa IgG were drastically increased with 5 μg E_2 treatments and became constant with 15 μg E_2 treatment, whereas a drastic increase of 50 kDa IgM was observed with 15 μg E_2 treatment. Nevertheless, "overall" increases of total IgG and IgM were notable compared to those of OVX mice.

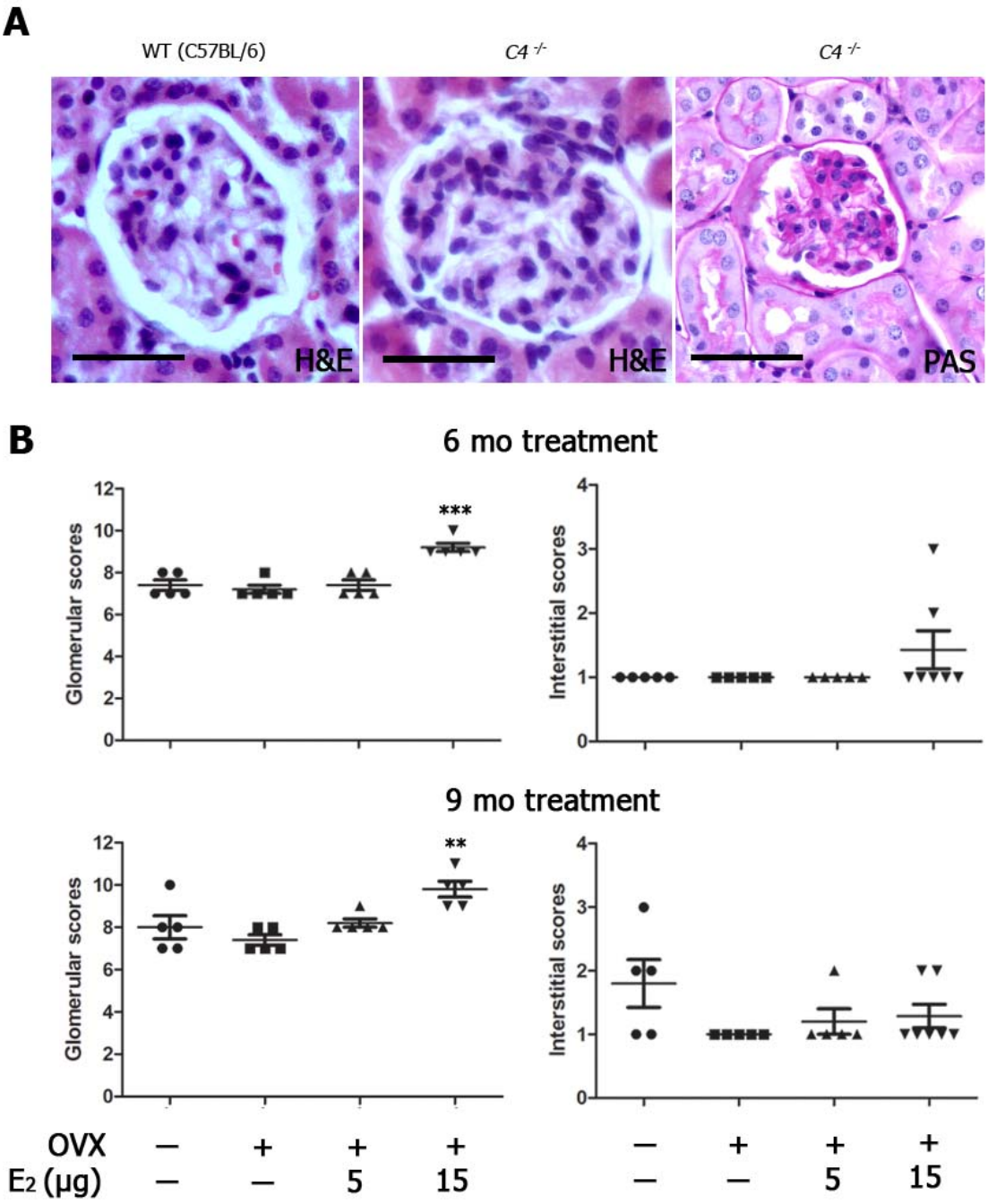

Figure 2. Renal pathology in *C4*$^{-/-}$ mice. Histological appearance of representative glomeruli when stained with H&E and PAS (A). *C4*$^{-/-}$ mice stained with H&E show increased glomerular hypercellularity (middle panel) when compared with wild type (left panel), while the kidney sections stained with PAS show thickening glomerular basement membrane (right panel). Data is representative of 5 mice per group. Scale bar = 50 µm and magnification, 400x. Glomerular scores and interstitial scores (B) were determined from various groups (mean ± SEM from 5-7 mice per group)

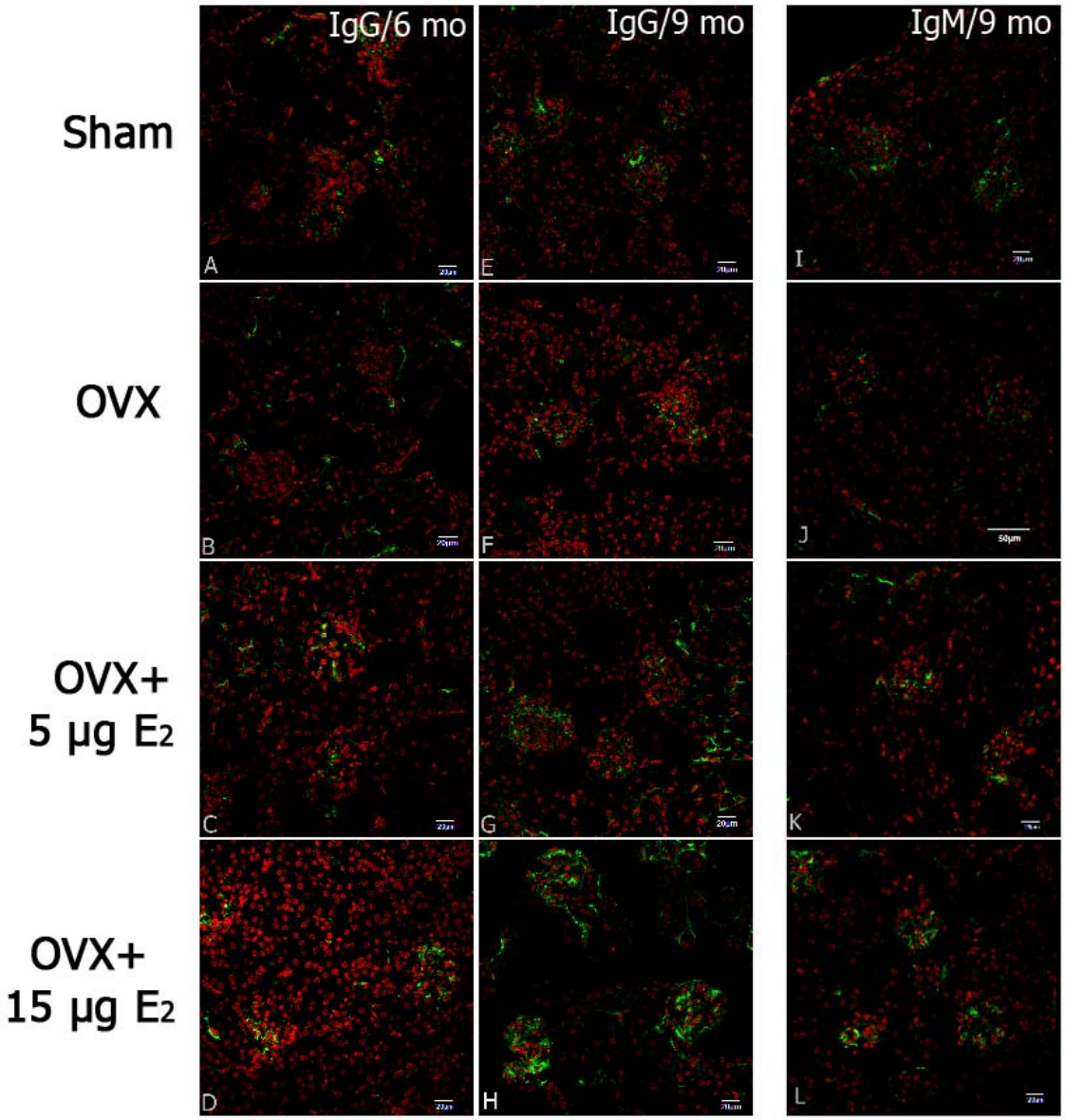

Figure 3. Immunofluorescence staining for IgG and IgM in representative glomeruli of *C4*$^{-/-}$ mice, kidney sections were stained with FITC-anti-mouse IgG/IgM (green color) and To-Pro-3 (red color). OVX mice treated with estrogen (C, D, G, H, K and L) show increased glomerular damage due to immune complex deposition compared with the sham-operated groups (A, E and I) and OVX mice (B, F and J). Magnification, 400x.

Effect of oestrogen on autoantibody levels in C4*$^{-/-}$ *mice

Due to the fact that the level of autoantibodies directly reflects tissue damage or forms immune complexes that elicit inflammation and damage in tissues such as the kidney, serum antibody to dsDNA is known to be an important diagnostic marker for SLE.[3] The serum level of anti-dsDNA antibody in the *C4*$^{-/-}$ study group was slightly higher but not significantly different in OVX mice treated with 5 µg E_2 at 6 months compared with the corresponding OVX group after the same period (Figure 5). As expected, a significant increase ($p < 0.01$) in IgG anti-dsDNA level was detected in OVX mice treated with 15 µg E_2 for 9 months compared with the other groups following the same duration (Figure 5). Moreover, there was a significant difference ($p < 0.01$) in the level of IgG anti-dsDNA in OVX mice treated with 15 µg E_2 for different durations as well as at different doses of E_2 for 9 months ($p < 0.01$).

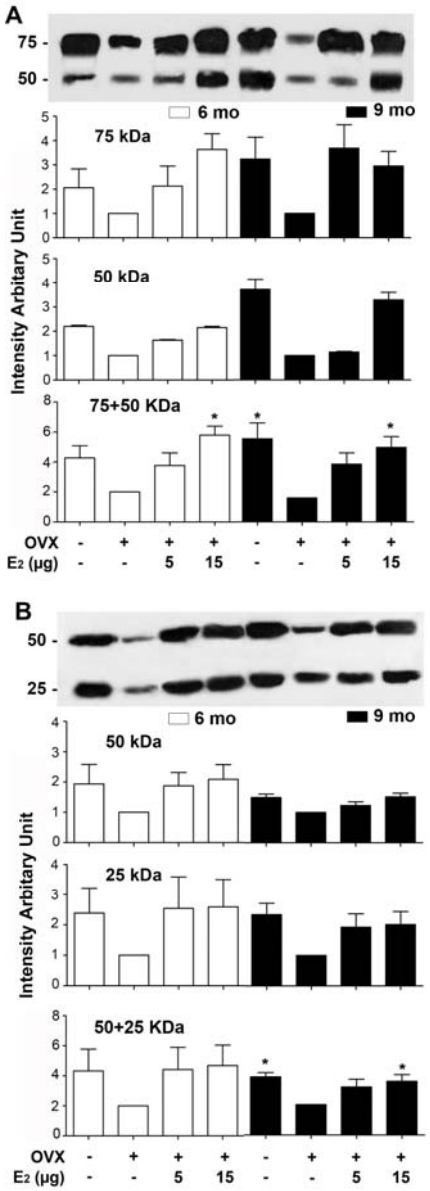

Figure 4. Western blot analysis using kidney protein extract from representative *C4*$^{-/-}$ mice and histograms showing the relative levels of IgG and IgM in each group. Western blot of IgM with anti-mouse IgM (μ chain) shows the expression of a heavy chain at about 50 and 75 KDa (A) and IgG with anti-mouse IgG (H+L) illustrates the expression of a light chain (25 KDa) and a heavy chain (50 KDa) (B). Not only is the expression of IgG decreased in the OVX group, but additionally the expression of IgM when compared between groups of the same duration. There are significantly increased expression of total IgG and IgM (light + heavy chains) (* indicates $p < 0.05$, ANOVA) in OVX mice treated with 15 μg E_2 for 9 months and the sham-operated group when compared with OVX mice of the same duration. There are significantly increased expression of total IgM, but no difference of total IgG between the groups at 6 months.

Splenomegaly in E_2 treated mice

We also checked for splenomegaly as this is known to be one of the markers for SLE and is associated with the phenotype of *C4*$^{-/-}$ mice.[12] In our study, this abnormality was observed in *C4*$^{-/-}$ mice, both those treated with oestrogen and without (Figure 6A). The spleen weights showed significant increases in OVX mice treated with 5 μg E_2 for 9 months compared to non-treated animals ($p < 0.01$) or those treated with the same dose of E_2 for 6 months ($p < 0.05$). The increase in spleen weight was even more pronounced in OVX mice administered with a higher dosage of oestrogen (15 μg) for 6 and 9 months in comparison with OVX mice for the same duration. At 9 months, the spleen weight was significantly higher in OVX mice treated with oestrogen compared to the OVX group (Figure 6A); however, there was no difference between the groups that received different doses of oestrogen.

In many cases of autoimmunity, persistent germinal centre (GC) formation may contribute to the development of disease because GC is a site of Ig Class switch and is thought to be an important site of immune dysregulation in autoimmune disease.[13] In contrast with splenomegaly, histological studies of spleens showed that there was either absent or poor fluorescent staining in the GCs in the OVX mice treated with any doses of oestrogen (Figure 6B, lower row). It is possible that the oestrogen-induced increased anti-dsDNA antibody levels did not result from the increased expression of B cell numbers in the spleen, although *C4*$^{-/-}$ mice displayed splenomegaly.

Discussion

Lupus nephritis is a major complication of SLE that is associated with a high rate of morbidity and mortality; this is considered to be important both diagnostically and prognostically.[14,15] *C4*$^{-/-}$ mice exhibited increased immune complex deposition and glomerular abnormalities similar to those found in *C1q* $^{-/-}$ mice.[16,17] In the present study, increased levels of immune complexes, both IgM and IgG within the glomerular were found in all experimental groups, especially in OVX mice treated with oestrogen. It has been shown that a deficiency in C4 complement impaired the clearance of the immune complex, leading to the deposition of the excess immune complex in the glomeruli, similar to those mice lacking early complement components (C1q, C1r, C1s, and C2), which also

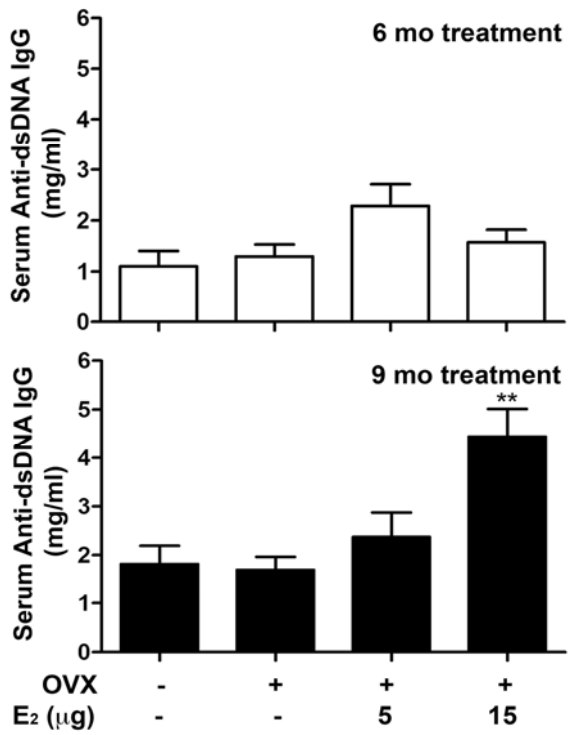

Figure 5. The level of IgG anti-dsDNA in *C4*$^{-/-}$ mice is shown by histograms. All data is representative of mean ± SEM from 7 mice per group. There are significantly increased levels of IgG anti-dsDNA (** indicates $p < 0.01$, ANOVA) in OVX mice treated with 15 μg E_2 for 9 months when compared with OVX mice of the same duration, while there is no difference of IgG-dsDNA level between the groups at 6 months.

showed glomerulonephritis, as mentioned above. In fact, the deposition of IgG is known to be associated with the severity of glomerulonephritis.[18] One of the possible explanations of this phenomenon is that the IgG isotype has a high-affinity for dsDNA.[19] Immunoglobulin G of anti-dsDNA antibody is thought to be the major source of immune complex because of the cellular damage within the tissues. Glomerulonephritis is characterised by glomerular enlargement, hypercellularity, glomerular basement membrane (GBM) thickening, and inflammatory cell infiltration.[3] In addition, this phenomenon of lupus nephritis was well described in a study reported by Tacky et al. (2004). At 10 months old, female *C4*$^{-/-}$ mice showed a predominantly mesangial cell deposition of IgG and C3 and renal enlargement with hypercellularity.[20] Our histological study also agreed well with the lupus-like nephritis indicated by glomerular enlargement, thickening of GBM, cell infiltrates and IgG depositions (Figures 1, 2 and 3)

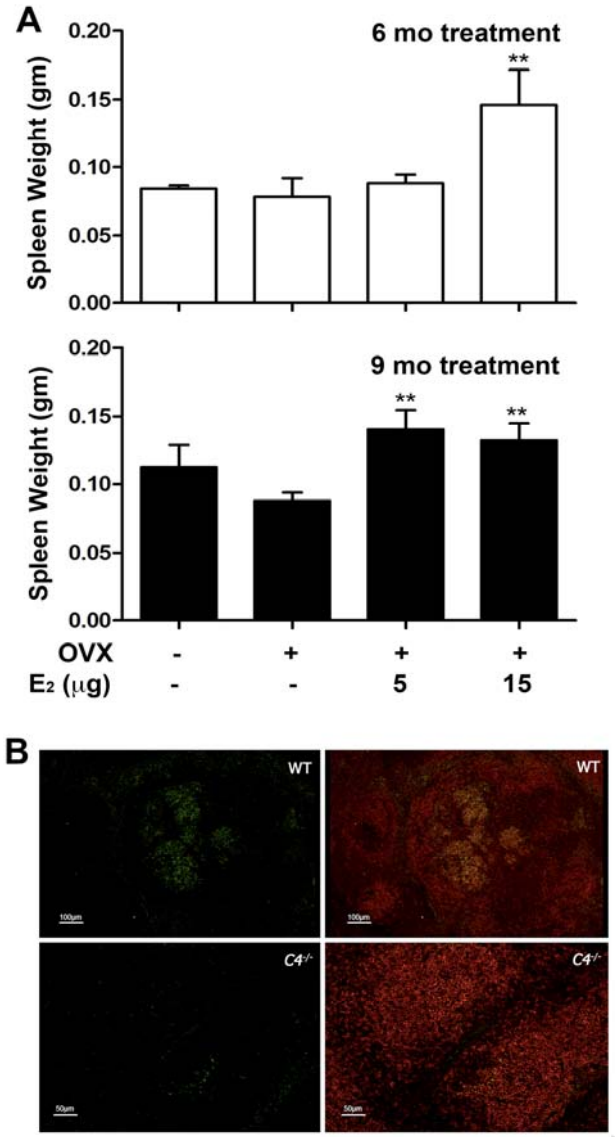

Figure 6. Spleen weight in *C4*$^{-/-}$ mice is shown by histograms, all data is representative of mean ± SEM from 7 mice per group (A). Immunofluorescence staining of spleen sections with B220 (red) and PNA (green), cells stained with both reagents appear yellow. Sections obtained from the wild type reveal PNA-positive germinal center formation (B, upper row) whereas *C4*$^{-/-}$ mice exhibit a poor germinal center (B, lower row).

Anti-dsDNA antibody is thought to participate in the pathogenesis of lupus nephritis by directly or indirectly binding with renal antigens.[14,21] At least two possibilities of anti-dsDNA antibody depositing in the kidney have been described. One possibility is the ability of anti-dsDNA, which can directly bind to glomerular matrix via electrostatic interaction between a negatively charge glomerular matrix and positively charged DNA–anti-dsDNA complex.[22]

We found here a higher level of anti-dsDNA antibodies in the sera (Figure 5) and the positive immunoreactivity of the deposited IgG in the glomerular matrix (Figure 3). The other possibility is the intrinsic non-DNA glomerular antigens, such as laminin, heparin sulphate, type IV collagen of GBM, alpha-actinin, and ribosomal P protein of mesangial cells, which are recognisable by anti-DNA antibodies.[23-25] Similar to IgG, the deposition of IgM was also present to a greater extent in *C4*$^{-/-}$ mice (Figure 3). It is feasible that IgM is a part of the natural antibody detection of foreign-antigens or self-antigens and is produced during the autoimmune response.[26] In addition, *C4*$^{-/-}$ mice with the impaired clearance of immune complexes in blood circulation would unsurprisingly have IgM deposition within glomerular matrix. We believe that oestrogen administration contributes to an aggravation of lupus-like nephritis. Higher doses and longer oestrogen exposures led to greater deposition of immune complex and anti-dsDNA antibodies. In fact, oestrogen is known to exert its effect by activation of the oestrogen receptor (ER) α, not ERβ, as demonstrated in the in case of female *ERα*$^{-/-}$, with significant protection from renal disease being seen in lupus-prone mice and the development of spontaneous GC.[13,27] In addition, ERα gene polymorphism is associated with the expression of IL-10, IL-4, IL-2 and IFN-γ in SLE patients. The imbalance of Th1/Th2 cells in SLE patients occurred due to the decrease in Th1 and predominant Th2 response that subsequently activates B cells producing antibodies in SLE patients.[28] Oestrogen stimulates auto-reactive B cell survival producing autoantibodies to both self- and non-self-antigens and breaks B cell tolerance via the up-regulation of Bcl-2.[6,7] It has been demonstrated that several autoimmune mouse strains develop GC in the spleen because GC are necessary for the generation of memory B cells and sites of Ig Gene V-region hypermutation.[29] In contrast, GC formation was significantly reduced in mice with deficiencies in early components of complements or complement receptors 1 and 2.[30] This was confirmed by our results showing that the formation of GC was either absent or poor in all groups of C4 knockout mice, even in those treated with high doses of oestrogen. Taken together, our results suggest an enhancement of oestrogen in aggravating SLE-like symptoms in mice lacking the complement system.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Acknowledgements

This research was supported by Mahidol University Research Fund (Projects 02011854-0005 and 02011868-0004) and National Science and Technology Development Agency (NSTDA), Ministry of Science and Technology, Thailand. The authors express appreciation to Mr. Charles Williams for critically reading the manuscript. We gratefully acknowledge the excellent technical assistance with the mouse experiment from Rittrong Unjana.

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