Impairment of CD4+CD25+ regulatory T cells in C4-deficient mice

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Summary

Objective: **To investigate the association between deficiencies of early components in the classical complement pathway and the development of SLE.**

Methods: **Forty inbred C57BL/6J mice and 40 knockout C4 complement gene (C4KO) mice, which included 10 mice in each age group (2, 4, 6, and 8 months) were used. The enumeration of CD4+CD25+ Tregs frequencies in bone marrow, spleen and peripheral blood from both normal and C4KO groups were performed by flow cytometry. The expression levels of Foxp3 and TGF-β in the same tested tissues were measured using real time PCR. The antinuclear antibodies (ANA) were semi-quantitatively measured using ELISA.**

Results: **We report decreased frequencies of CD4+CD25+ Tregs and reduced expression levels of Foxp3 and TGF-β, which efficiently program the development and function of Tregs, in lymphoid tissues and peripheral blood of C4KO mice. In this study, C4KO mice have higher titers of ANA than those of normal mice. Higher frequencies of mice positive for ANA are also found in older mice.**

Conclusions: **The deficiency of the C4 gene induces the decreased numbers of Tregs that further increase the production of ANA resulting in the development of an autoimmune disorder. The outcomes of our study help us to understand the association between the deficiency of C4 in the classical complement pathway and development of autoimmune disorder via the role of Tregs.** ***(Asian Pac J Allergy Immunol 2011;29:220-8)***

Key words: *Antinuclear antibody (ANA), C4 deficiency, CD4+CD25+ regulatory T cells, Forkhead box P3 (Foxp3), Systemic lupus erythematosus (SLE), Transforming growth factor (TGF)-β*

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Introduction

Systemic lupus erythematosus (SLE) is one of the autoimmune diseases identified with a typical loss of immunotolerance to self-antigens and the production of autoantibodies against the cell's nuclear components.[1] The pathogenesis of SLE is associated with genetic and environmental factors, as well as abnormalities in phagocytic function and immunoregulation, including hyperactivated B and T cells.[2,3]

The etiology and pathogenesis of SLE are not clearly understood. However, several genetic factors are related to SLE[4]; the deficiencies of early components in the classical complement pathway may substantially represent this association. Most individuals with deficiency of either C1 or C4, whether partial or complete can develop SLE.[5] Even though genetically determined deficiencies in complement are rarely found, acquired deficiencies of components of the complement system are common[6] and considered to arise from complement consumption by immune complexes. The influence of complement deficiency in SLE pathogenesis is still unknown. Thus, in humans, the early components in the classical pathway, which are involved in complement activation, especially C1 and C4, may suppress incipient autoimmunity.

Additionally, deficiencies in components of the classical complement pathway lead to the production

Table 1. Specific primer sequences used in the measurement of the expression levels of transcription factor Foxp3, cytokine TGF-β and the housekeeping gene (β-actin gene).

Gene Name	Forward Primers (5' – 3')	Reverse Primers (3' – 5')
Foxp3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT
TGF-β	CACCGGAGAGCCCTGGATA	TGCCGCACACAGCAGTTC
β -actin	AGGTCATCACTATTGGCAACGA	ATGGATGCCACAGGATTCCA

of autoantibody and the development of SLE by impairing the induction and maintenance of immune tolerance during negative selection in the thymus.[7,8] Regulatory T cells (CD4+CD25+ T cells or Tregs) play an important role in preventing autoimmunity in SLE patients by suppressing self-reactive T cells. The forkhead box P3 transcription factor (Foxp3) gene has major responsibility for the development and function of CD25+ Treg cells.[9,10] Though there is a marked distinction between the importance of immunosuppressive cytokines in vivo and CD25+ Treg cell suppression in vitro, many cytokines are involved by acting as mediators of inhibition, such as interleukin (IL)-10 and transforming growth factor (TGF)-β.[11]

To understand the relationship between C4 deficiency, Treg expression and autoantibody production, our study focused on the quantification of Tregs in lymphoid tissues and peripheral blood of C4KO mice compared with normal mice of different ages. Moreover, we also detected the expression levels of transcription factors Foxp3 and cytokine TGF-β of Tregs by using a real time quantitative polymerase chain reaction (PCR) in order to confirm Treg expression in mouse tissues.

Methods

Animal model and tissue collection

Forty of inbred C57BL/6J mice (normal group; National Laboratory Animal Center, Mahidol University, Thailand) and 40 knockout C4 complement gene C57BL/6J mice (C4KO; Jackson Laboratory, USA), which included 10 mice in each age group (2, 4, 6, and 8 months) were used throughout this study. The protocols were approved by Animal Research Ethics Committee of Institute of Science and Technology for Research and Development (Present: Institute of Molecular Biosciences), Mahidol University, Thailand.

Five hundred microliters of EDTA-blood and another 500 μl of clotted blood specimens were collected from each mouse tail vein. Peripheral blood mononuclear cells (PBMCs) were separated from EDTA-blood by using the density gradient centrifugation method, whereas clotted blood samples were centrifuged to separate serum.

Mice were euthanatized by using carbon dioxide gas for 30 to 45 seconds. After operation, bone samples were collected from femurs and humeruses, which were cut from surrounding tissues and washed with sterile normal saline solution. Bone marrow cells were then flushed with sterile normal saline solution using a 30G needle attached with 1-ml syringe.

Spleens were also collected after euthanasia. Thirteen to fifteen milligrams of mouse spleen tissue were homogenized in a microtube using a pestle homogenizer for 1 minute. The homogenized tissue was then kept in normal saline solution and stored at -70°C until it was used.

Flow cytometry

A lysed-washed procedure was employed for specimen staining with monoclonal antibodies. Briefly, the red blood cells were lysed with 1 ml of lysis buffer. Then, the mixture was centrifuged for 5 minutes at 2,000 rpm and the supernatant was discarded. One hundred microlitres of bone marrow cells, splenocytes and PBMCs were labeled with 2 different monoclonal antibodies, CD4-PE and CD4-FITC (Becton Dickinson, USA). Subsequently, the mixture was centrifuged at 1,500 rpm for 5 minutes at room temperature. After discarding the supernatant, the stained cells were washed with phosphate buffer saline (PBS) and re-centrifuged. The supernatant was discarded and the stained cells were resuspended and fixed in 1% paraformaldehyde. The samples were run on a FACSCalibur (Becton Dickinson, USA); data from a forward and side scatter (FSC/SSC) gating strategy was analyzed by using WinMDI version 2.9 (Windows Multiple Document Interface for Flow Cytometry).

Real time quantitative PCR

Mouse mRNAs from bone marrow cells, splenocytes and PBMCs were extracted using RNeasy kit (Qiagen, Germany). Extracted mRNA

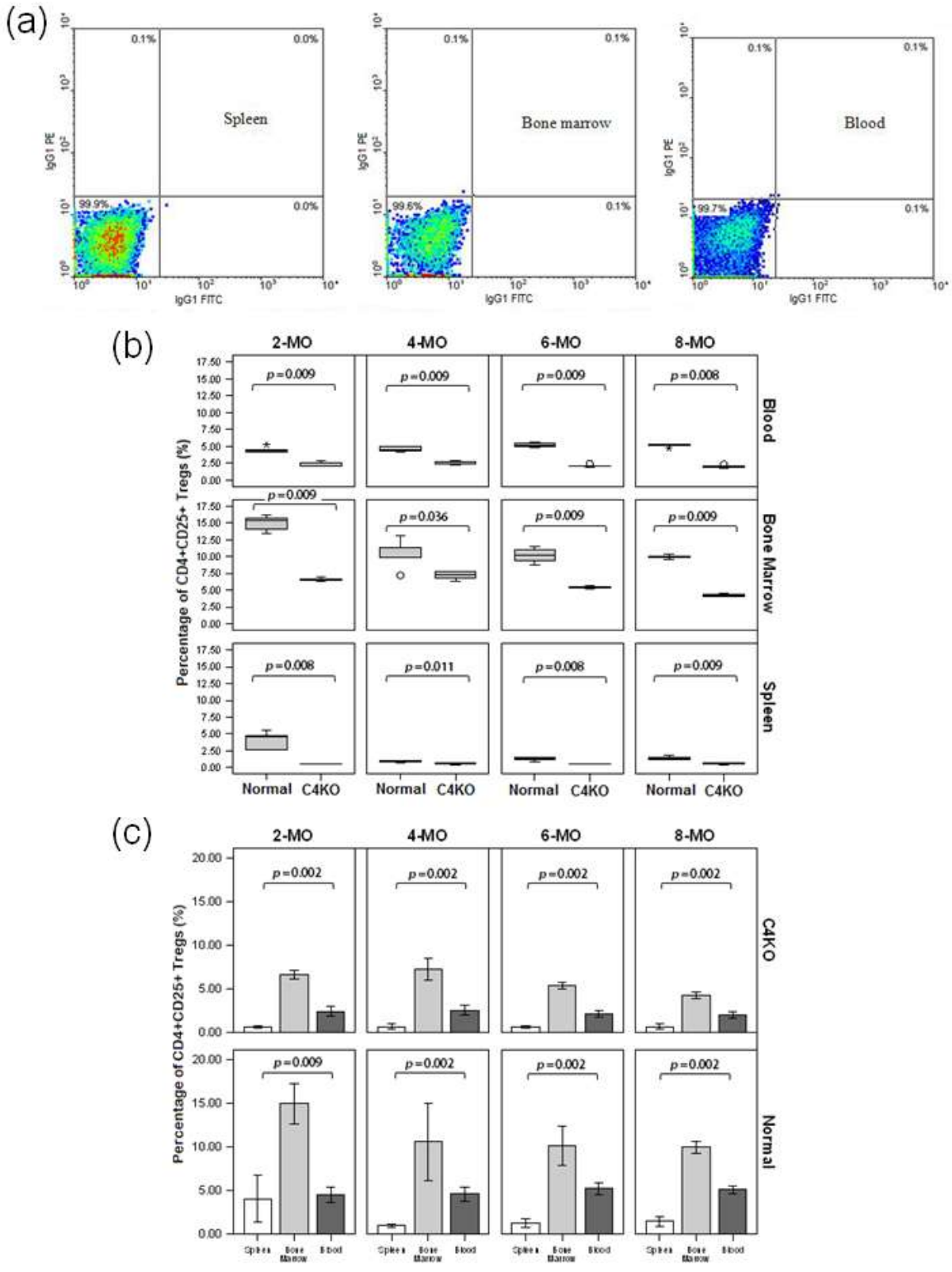

Figure 1. Frequencies of CD4+CD25+ Tregs found in blood, bone marrow and spleen of normal (N=40) and C4KO mice (N=40). PBMCs separated from EDTA blood, bone marrow cells and splenocytes were stained with fluorescent-labeled monoclonal antibodies specific to CD4 and CD25. The stained samples were then analyzed using FACSCalibur instrument with WinMDI software, version 2.9. (a) Examples of isotype control dot plot represent CD4-CD25- negative control obtained from PBMCs, bone marrow cells, and splenocytes. (b, c)

was subjected to real time quantitative PCR for the detection of transcription factors Foxp3 and TGF-β using SuperScript™ III Platinum®SYBR® Green One-Step qRT-PCR Kit (Invitrogen, USA) with specific primers (Table 1.).

Amplification of mRNA was performed by the following steps; cDNA synthesis at 50°C for 30 minutes, initial denaturation at 95°C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 15 seconds. The data were expressed as relative values to a housekeeping gene (β-actin gene).

Enzyme-linked immunosorbent assay (ELISA)

Antinuclear antibodies (ANA) were semi-quantitatively measured using an Anti-dsDNA Antibodies ELISA kit (Alpha Diagnostic International, USA). Briefly, 100 µl of serially diluted sera (from 1:20 to 1:640) were incubated on an antinuclear-coated microwell plate at 37°C for 30 minutes. Subsequently, microwell plates were washed 3 times with washing buffer. Goat anti-mouse IgG horseradish peroxidase (HRP) conjugated and tetramethylbenzidine (TMB) was then added as a conjugate and substrate, respectively. The reaction was stopped using stop solution (H_2SO_4) and the absorbance was measured as the optical density (OD) at 450 nm. Any samples with a specific absorbance equal to or more than 0.260 (as recommended by manufacturer), which was directly converted into antibody titer equal to 1:160 (predicted antibody titer using standard regression equation), could be considered positive.

Statistical analysis

Differences between normal mice and C4KO mice were analyzed with the Kruskal-Wallis test for continuous variables and Pearson Chi-square test for categorical variables using SPSS Statistics 18.0. A P-value of < 0.05 was considered to be statistically significant.

Results

Enumeration of regulatory T cells by flow cytometry

The enumeration of CD4+CD25+ Tregs frequencies in bone marrow, spleen, and peripheral blood of mice were performed by flow cytometry. An IgG isotype control served as an appropriate negative population for each sample (Figure 1a.). In every age group, the percentage of CD4+CD25+ Tregs in the normal group was higher than those in C4KO mice for every tissue tested (Figure 1b.).

In both the normal and C4KO groups, the bone marrow had the highest frequencies of CD4+CD25+ Tregs compared with the spleen and peripheral blood. A higher percentage of CD4+CD25+ Tregs were found in blood compared to spleen (Figure 1c.). Additionally, in the normal group, the highest percentage of CD4+CD25+ Tregs was found in bone marrow and spleen of 2-month-old mice. Moreover, we also found a statistically significant difference in CD4+CD25+ Treg frequencies between all tested tissues in every age group (Figure 1c.).

Expression levels of transcription factor Foxp3, and cytokine TGF-β

The expression levels of transcription factor Foxp3 and cytokine TGF-β in bone marrow, spleen, and peripheral blood obtained from both normal and C4KO groups were measured using primers specific to *Foxp3* and *TGF-β* genes and real time quantitative PCR to confirm the consistency of CD4+CD25+ Treg frequencies in tested tissues. This method is currently widely used by many research groups [12-14].

As previously found in CD4+CD25+ Treg enumeration, in every age, the expression levels of *Foxp3* and *TGF-β* in all tested specimens of normal group were higher (than those in C4KO group for each age group ($P<0.05$) (Figure 2a. & 2b.). However, no significant difference in *Foxp3* and *TGF-β* gene expression levels was found between age groups of either normal or C4KO mice for any of the tested tissues.

Comparable levels of *Foxp3* and *TGF-β* expression were found in bone marrow and peripheral blood of both normal and C4KO groups (Figure 2a. & 2b.); however, they were higher than those in spleen.

TGF-β expression in spleen was higher than *Foxp3* in both groups. Additionally, there was statistically significant difference in expression levels of both genes in all tested tissues of both animal model groups.

Detection of autoantibodies in mice models

ANA levels in 17 of 40 normal mice were greater than or equal to 1:160, whereas a greater proportion of C4KO mice (25/40) showed positive result for ANA (Figure 3a.). Moreover, nearly half of the ANA positive C4KO mice (11/25) had ANA titers greater than or equal to 1:640, which were not found in normal group (Figure 3b.).

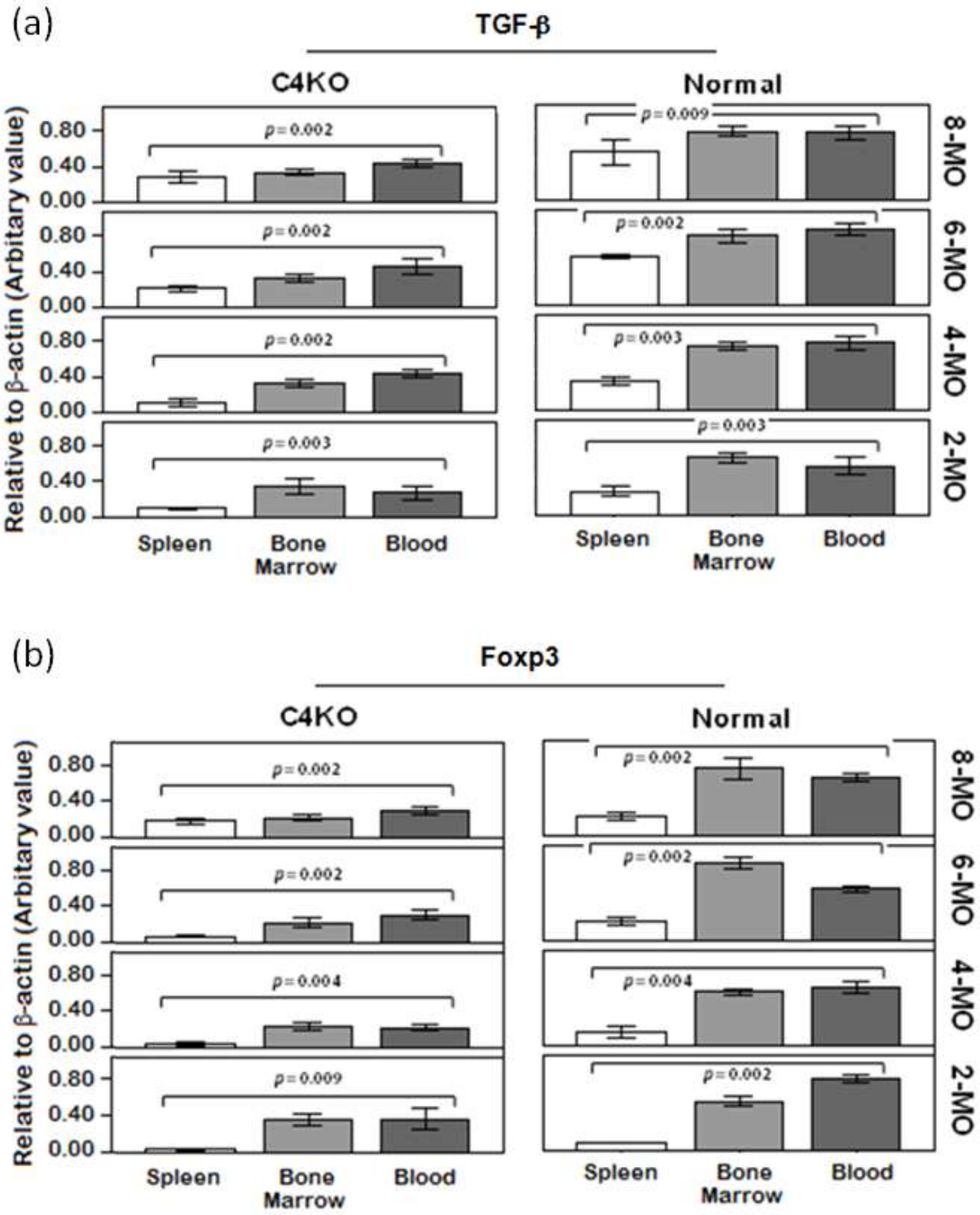

Figure 2. Expression levels of cytokine TGF- β (a) and transcription factor Foxp3 (b) in the spleen, bone marrow, and peripheral blood of C4KO and normal mice shown versus different age groups (2-, 4-, 6-, and 8-month-old). The extracted mRNA from splenocytes, bone marrow cells, and PBMCs of mice were amplified and detected using specific primers with real time quantitative PCR. MO, month-old.

Our result showed significant differences between ANA levels of normal and C4KO mice (P=0.036) (Figure 3b.). Positive ANA titers were increased in the elderly group of mice. Forteen of 17 mice (82.4%) in the normal group and 18 of 25 in C4KO mice (72.0%) with positive ANA, were either 6 or 8 months old (Figure 3b.).

Discussion

Our study investigated the effects of genetic variations in the classical complement pathway that could induce autoimmunity in mice, using complement component C4 knockout mice (C4KO) compared with normal mice of identical genetic background. The *C4* genes in mice and humans are Sex limited protein (Slp). C4A generally plays an important role in immune complex degradation, whereas C4B is responsible for hemolytic activity. Therefore, immune complexes accumulate in the case of C4A deficiency. These immune complexes can circulate in blood vessels and cause inflammation in lymphoid tissues and peripheral blood.[15,16] Several studies have demonstrated the association between a deficiency of C4A (C4AQ0) and the severity of SLE. Renal disorder, serositis and the presence of anti-dsDNA antibodies in SLE patients with C4AQ0 are more severe than those in SLE patients without C4A deficiency.[17-19] An important role for Tregs in self-tolerance induction, which is correlated to autoimmune production, has been suggested.[20,21] Our study provided information about Treg frequencies and the expression levels of transcription factor Foxp3 and cytokine TGF-β, which are related to Treg development in lymphoid tissues and peripheral blood using a murine model with and without C4 deficiency. Bone marrow and spleen are representative of primary and secondary lymphoid organs, respectively, whereas blood cells represent of cell components in the peripheral circulation.

Significantly lower percentages of circulating $CD25^{high}$ in SLE patients compared with healthy individuals were reported by Suen J-L *et al.* [22] Our study showed decreased frequencies of CD4+CD25+ Tregs in bone marrow, spleen and peripheral blood of C4KO mice compared with normal mice for all age groups tested, which suggests that the decreased frequencies of CD4+CD25+ Tregs in C4KO mice also correlated with the impairment of immune tolerance from deficiency of complement. In addition, we reported the higher frequencies of CD4+CD25+ Tregs in bone marrow of either normal or C4KO mice compared with peripheral blood and spleen. The CD4+CD25+ Treg frequencies in the peripheral blood of this murine model were also higher than those in spleen. Our results are compatible with the previous findings in humans that bone marrow is a major reservoir for CD3+CD4+CD25+ T cells and the homeostasis of Tregs from peripheral blood to bone marrow, but not to spleen, after their origination in thymus.[23,24] The presence of CD4+CD25+ Tregs in the spleen as found in our study is concordant with an earlier study which demonstrated phenotypic and function heterogeneity of splenic CD4+CD25+ Tregs.[25] The highest frequencies of CD4+CD25+ Tregs were present in both the bone marrow and spleen of 2-month old mice, compared with other age groups. However, this finding needs further investigation.

We have demonstrated significantly higher expression levels of transcription factor Foxp3 and cytokine TGF-β in the tissues of normal mice compared with C4KO mice in all age groups. However, there was no significant difference in expression levels of Foxp3 and TGF-β between age groups. These findings in our study, that is the reduced expression levels of Foxp3 and TGF-β and the decreased frequencies of Tregs in C4KO mice, possibly demonstrates the malfunction of Tregs in C4KO mice because of the deficiency of an important component in the classical complement pathway. Our results confirmed those of former studies [9,26,27] concerning the association between deficiency of C4 and impairment of Treg development, as well as the expression levels of transcription factor Foxp3 and TGF-β as products of CD4+CD25+ Tregs.

Although our findings are different from those of a previous study in which Foxp3 expression was higher in bone marrow compared to peripheral blood, the comparable levels of Foxp3 and TGF-β expression in bone marrow and the periphery that we present here are still consistent with the trafficking of Tregs between bone marrow and the periphery.[23] This discrepancy in Foxp3 expression may be due to the different methods used (RT-PCR and real time quantitative PCR) for measuring the expression levels of Foxp3 in those tissues.

Two populations of Tregs are present in spleens, one being $CD4+CD25^{high}$ Tregs, which demonstrate highly suppressive activity with high expression levels of transcription factor Foxp3 and cytokine TGF-β, and the other being $CD4+CD25^{low}$ Tregs, which express low suppressive activity with low

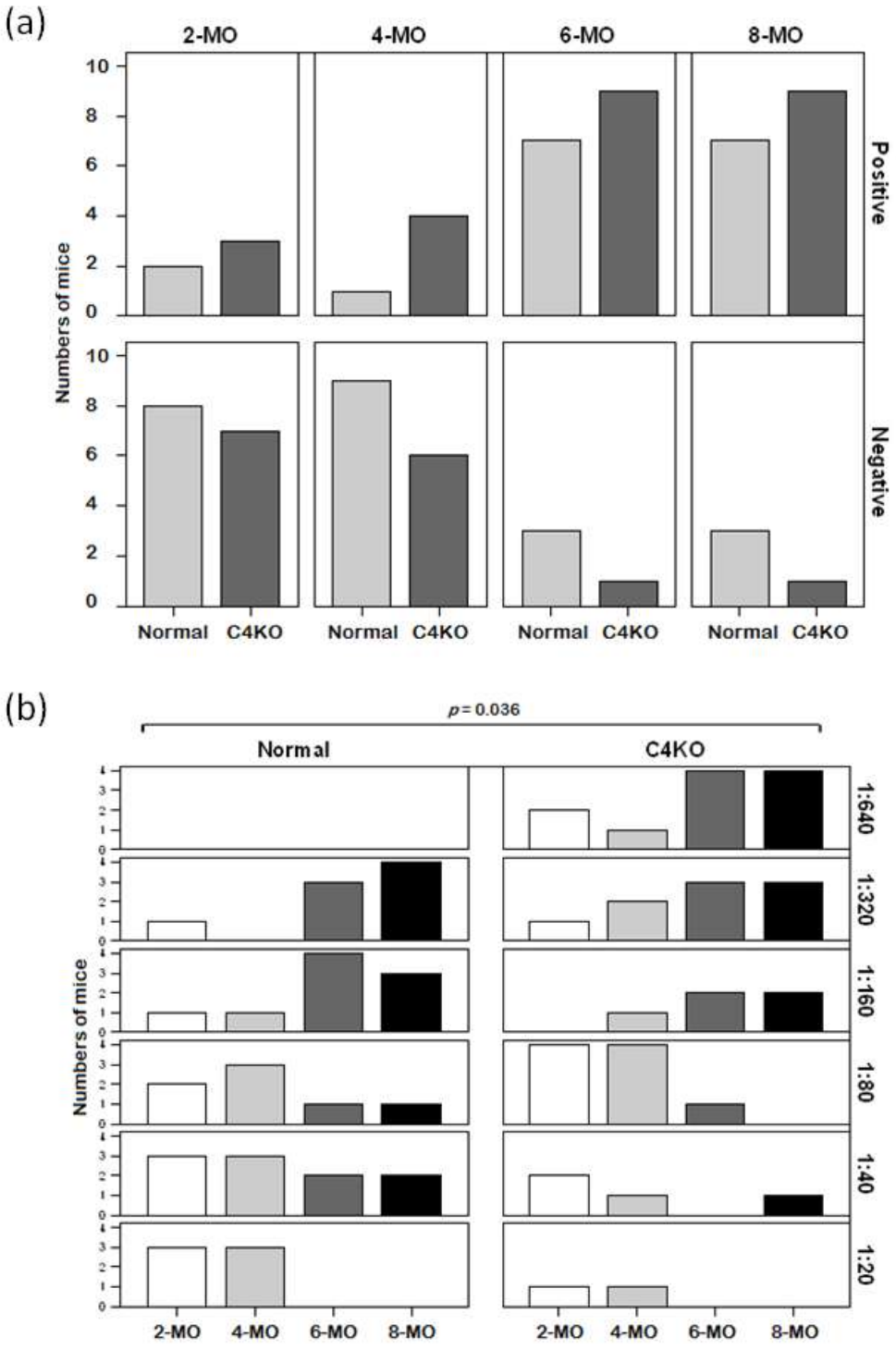

Figure 3. (a) Numbers of positive (titer $\geq$ 1:160) and negative (titer < 1:160) ANA mice in different age groups (2-, 4-, 6-, and 8-month-old) shown versus the normal and C4KO groups. (b) Comparison of ANA titers in normal and C4KO mice shown versus different age groups (2-, 4-, 6-, and 8-month-old). ANA titers in serum specimens obtained from each mouse were detected using ELISA method. MO, month-old.

levels of Foxp3 and TGF-β expression.[25] The discordant expression levels between Foxp3 and TGF-β in spleen found in our study may possibly indicate the need for further studies.

The numbers of C4KO mice with positive ANA are higher than those in the normal group (62.5% and 42.5%, respectively). This finding is consistent with the decreased frequencies of Tregs in C4KO mice compared to normal mice and suggests that the deficiency of complement C4 might diminish the clearance of dying cells, thus potentially establishing a source of autoantigens, which can activate T and B cells resulting in the production of autoantibodies as previously proposed.[28-31] Additionally, nearly 50% of ANA positive C4KO mice had higher ANA titers (1:640) compared to ANA positive normal mice (data not shown). Additionally, the combination of higher titers of ANA found in approximately 50% of ANA positive C4KO mice compared to ANA positive normal mice and lower frequencies of Tregs found in C4KO mice implies a relationship between quantity of Tregs and severity of the autoimmune disorder.[10,32,33] Our results demonstrate a relationship between C4 deficiency, reduced frequencies of Tregs and the presence of ANA. The positive ANA present in the normal group of mice is consistent with previous findings that ANA can be found in healthy individuals.[34-37] Our study also indicated that both C4KO and older mice in the normal control (6 or 8 months of age) had significantly higher titers of ANA compared with younger mice, which agrees with the findings of the previous study by Chen Z *et al*.[6] Our findings suggest that increased levels of autoantibodies within the older population of animal models are induced by immune system malfunction, which can also be found in older individuals.

In summary, our study investigated the numbers of CD4+CD25+ Tregs in both primary and secondary lymphoid organs, including blood. The primary lymphoid organ, bone marrow, is the major source of CD4+CD25+ Tregs. The deficiency of the C4 gene, which plays an important role in the classical complement pathway, induces the decreased numbers of Tregs that further increase the production of antinuclear antibodies resulting in the development of an autoimmune disorder. Although the deletion of C1q or C4, which are important components in the classical complement pathway, was previously found to be a cause of autoimmune disease,[30,38] the outcomes of our study help us to understand the association between the deficiency of C4 in the classical complement pathway and development of autoimmune disorder via the role of Tregs. Further studis in animal models of autoimmune disease (e.g. Lupus, EAE and RA) using C4 deficient mice should be conducted.

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