

Serum Antibodies and Cytokines in C4-deficient Mice and Their Responses to Exercise

Supawan Visetnoi¹, Runglawan Chawengkirtikul², Sansanee C. Chaiyaroj², Yindee Kitiyanant^{3,4} and Chumpol Pholpramool^{1,4}

SUMMARY Psychological stress is believed to be one of the predisposing factors for systemic lupus erythematosus (SLE), whereas physical stress such as exercise has never been reported to be related. We measured the circulating levels of antibodies (IgM, IgG, anti-dsDNA IgG), Th1 (IFN- γ), Th2 (IL-4, IL-6), and of pro-inflammatory (TNF- α , IL-1 β) and anti-inflammatory (TGF- β) cytokines of C4^{-/-} female mice at rest, after acute exercise and after exercise training, using an antibody-capture ELISA. Prior to the exercise, the C4^{-/-} mice had higher levels of IgG and anti-dsDNA IgG but lower levels of IFN- γ , IL-1 β , IL-6 and IL-4 than wild-type C57BL/6 (B6) mice. A single bout of exercise to exhaustion increased serum IgG, TNF- α , IL-1 β and TGF- β in the B6 mice but only TGF- β in the C4^{-/-} mice was increased. We conclude that exhaustive or moderate exercise has no effect on the levels of serum antibodies and cytokines and is thus unlikely to promote the onset of SLE.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a breakdown of self tolerance, B cell hyperactivity, and the production of autoantibody and immune complexes leading to an inflammation of many organs.¹ The mechanism underlying this disease is not clearly understood. However, there is strong evidence that SLE is closely linked to a deficiency of classical pathway of complements.² The loss of self tolerance when complement is deficient has been explained by an impaired clearance of immune complexes² and an inadequate regulation of B cells.³ Cytokine profiles in SLE patients have been used to determine disease progression and deterioration.⁴ The immune system

seems to be skewed towards an overproduction of anti-dsDNA antibody as a result of a shift in the Th1/Th2 balance.⁴ Th1 cells primarily produce IFN- γ and IL-2 which are mainly responsible for cellular immunity, whereas Th2 cytokines such as IL-4, IL-6 and IL-10 rather enhance humoral immunity and promote antibody production *via* B cell activation.⁵ In addition, the ratio of Th1/Th2 cytokines has been shown to correlate with the progression of the dis-

From the ¹Department of Physiology, ²Department of Microbiology, ³Department of Anatomy, Faculty of Science, Mahidol University, Bangkok, ⁴Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand.
Correspondence: Chumpol Pholpramool
E-mail: sccpp@mahidol.ac.th

ease.⁴ The role of the Th1/Th2 balance in the pathogenesis of the disease has also been demonstrated in lupus-prone animal models. Serum levels of circulating IFN- γ and IL-4 in (NZB x NZW) F1 mice were significantly higher than those in the wild-type mice.⁶ Similarly, in MRL/*lpr* mice the disease has been associated with the Th1 cytokines IFN- γ ⁷ and TNF- α ⁸ as well as the Th2 cytokine IL-4.⁸ Additionally, glomerulonephritis in (NZB x NZW) F1 mice treated with anti-IFN- γ or anti-IFN- γ receptor-antibodies was abrogated, suggesting a dominant role of Th1 cytokines.⁹ In contrast, the administration of recombinant IL-6 in (NZB x NZW) F1 lupus prone mice led to an acceleration of renal disease such as glomerulonephritis¹⁰ suggesting an important role of the Th2 cytokines. Although complement component (C1q, C4 and C2) deficiencies have been closely associated with SLE in humans² and C4-deficient mice,¹¹ the roles of Th1 and Th2 as well as pro- and anti-inflammatory cytokines have not yet been elucidated in an animal model using C4-deficient mice.

Alterations in circulating cytokines and antibodies including Th1 and Th2 cells have been demonstrated during different forms of exercise. During acute strenuous exercise in humans, the circulating levels of IL-6, IL-1 β and TNF- α were substantially increased.^{12,13} In contrast, the IFN- γ level was not altered¹⁴ or reduced.¹⁵ Acute exhaustive and chronic exercise in humans caused changes in the distribution and the cytokine contents of Th1 and Th2 lymphocytes in favor of the latter.¹⁵ On the other hand, moderate exercise training leads to a different result, *i.e.* the immune response was enhanced promoting a resistance to infection.¹⁶ The immunomodulation by exercise has also been shown in animal studies. In aged mice, 8 weeks moderate exercise training increased antigen-specific cytokines and Th1-associated cytokines but not Th2 or IgM antibodies,¹⁷ whereas voluntary wheel-running exercise was reported to prolong the serum IgG half life in mice.¹⁸

Beneficial effects of exercise training in SLE patients have also been reported. Most studies have shown that exercise improves fatigue in SLE patients and does not exacerbate the disease.¹⁹ Whether exercise accelerates or delays the onset of lupus is not known at present. Therefore, the present study investigated the effects of different intensities of exercise

on the cytokine profiles, as well as its correlation with disease activity using C4-deficient mice which displayed a mild form of SLE.

MATERIALS AND METHODS

Mice

C4-deficient (C4 knockout, C4^{-/-}) female mice derived from a mixed 129/Sv backcross with C57BL/6 were purchased from the Jackson Laboratory, Bar Harbor, ME, USA, and bred at the Institute of Molecular Biosciences, Mahidol University. C57BL/6 (B6) mice were purchased from the National Laboratory Animal Center, Nakhon Pathom, Thailand. Female C57BL/6 and C4 knockout mice, 8-9 weeks old, weighed between 18-22 g, were randomly assigned into exercise and sedentary groups. Female mice were used in this study because SLE has been shown to affect more female than male patients²⁰ and mice.^{21,22} They were housed and maintained in a conventional hygienic facility under a reversed cycle of 12 hours light-dark at 20-22°C at the Faculty of Science, Mahidol University, for at least one week prior to experimentation. The protocols were approved by the Animal Care and Use Committee of the Faculty of Science, Mahidol University.

Measurements of serum antibodies

To determine the IgM, IgG and anti-dsDNA IgG concentrations in the sera, enzyme-linked immunosorbent assay (ELISA) was performed. Total IgM and IgG were assayed in 96-well polystyrene EIA/RIA plates (Costar, Corning Incorporated, NY, USA) coated with rabbit anti-mouse IgM or IgG (DakoCytomation, Denmark) in PBS coating buffer for 18 hours at 4°C. The plates were then blocked with dry skimmed milk in PBS. Thereafter serially diluted mouse sera in blocking agent containing Tween-20 were added, followed by horseradish peroxidase-conjugated rabbit anti-mouse IgM or IgG (DakoCytomation, Denmark, and Pharmingen, San Diego, CA, USA). Color was developed and the absorbance was read at 450 nm.

For anti-dsDNA IgG detection, Immulon 2 polystyrene microtiter plates (Dynatech, Chantilly, VA, USA) were coated with 100 μ g/ml filtered salmon sperm DNA (Sigma-Aldrich) in Tris EDTA,

and dried for 18 hours at 37°C.²³ The plates were blocked in PBS containing 0.05% Tween-20 (PBST) and 1% BSA for 2 hours at 37°C. Mouse sera diluted 1:100 in 0.1% BSA in PBST were applied (1 hour at 37°C), followed by horseradish peroxidase conjugated-goat anti-mouse IgG (DakoCytomation, Denmark) diluted 1:2,000. The plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution, and stopped by adding 5 N H₂SO₄. The absorbance at 450 nm was measured using a microplate reader (Thermo Electron Corporation, Multiskan Ex, USA).

Mouse serum IL-1 β , IL-4, IL-6, IFN- γ , TNF- α and TGF- β were analyzed using Quantikine cytokine kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Exercise protocols

Prior to the exercise protocols, all mice were acclimatized to treadmill running for 10-15 minutes at a speed of 15 meters/minute for 3-4 days. Each strain of mice was randomly assigned to one of two groups: exercise (Ex, n = 8-16) and non-exercise (Sedentary, n = 8-16). The mice in the exercise group were randomly assigned to one of following exercise programs.

For a single bout of exercise, mice were assigned to a single bout of exhaustive running on a treadmill at the speed of 28 meters/minute²⁴ with an 8% grade; the speed was gradually increased and reached 28 meters/minute within 40 minutes whereas the gradient was slowly increased to 8% during the first thirty minutes. The mice were allowed to run until intrinsic fatigue set in, which was determined by a failure to maintain the pace. An air puff was used to encourage the mice to run when they did not respond to the increases in speed and gradient. During the exercise program, all mice in the non-exercise group were also brought to the exercise room and were exposed to the same environment as the exercise group.

For exercise training, the mice were assigned to run at a moderate intensity with a speed of 22 meters/minute for 30 minutes at 0% grade, 5 days a week for 8 weeks.^{17,24} All exercise programs were conducted at the end of the 12 hour light-dark cycle.

Blood sample and organ collections

In the group that performed a single bout of exhaustive exercise, blood samples were collected prior to, immediately after, and one hour after exercise session. The mice were then euthanized by an overdose of sodium pentobarbital, and the kidneys were collected by dissection and fixed for further histological analysis. In the exercise training group, blood samples were taken prior to, four weeks after, and eight weeks after training. Blood samples and kidneys were collected 24 hours after the last exercise session in order to avoid the effects of the last bout of exercise. The kidney from one side was fixed in 10% buffered formalin for histological studies, and the other kidney was snap frozen for immunofluorescence analysis.

Statistical analyses

All results were expressed as means \pm standard errors (SE). The statistical analysis was conducted using the statistical software package GraphPad Prism version 4.00 for Windows (GraphPad software, San Diego California USA; www.graphpad.com). The Student's *t*-test was employed for comparing the baseline values between the C4^{-/-} and B6 mice. One way analysis of variance was used to test the effect of exercise, and when the variance was significant the Tukey post hoc test was applied to identify the difference. The level of significance was set at $p < 0.05$.

Immunofluorescence microscopy

To detect the deposition of IgM, IgG and C3 immune complexes, kidneys were snap frozen in an O.C.T. compound (Tissue Tek[®], Sakura Finetek, Torrance, CA, USA) on dry ice and kept at -70°C.^{21,22} Frozen sections (7 μ m thick) were cut and nourished with 0.1 M glycine. After one hour of blocking with 1% goat serum in PBS, the slides were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgM (μ chain), IgG (whole molecule) and C3 (Cappel, ICN Biomedicals, Ohio, United States) diluted to 1:200. This step was performed in a humidified dark chamber. Slides were then mounted with mounting media (DAPI, Vectashield, Vector Laboratories, CA, USA) and viewed under a confocal laser-scanning microscope (Model FV1000, Olympus, Tokyo, Japan) operated with Fluoview 1.4.

Histology

Kidneys were fixed in buffered formalin, dehydrated in serially graded ethanol, and embedded in paraffin. The sections (4 μ m thick) were stained with hematoxylin and eosin, and viewed under a light microscope.²⁵

RESULTS

Antibody and cytokine profiles before exercise

Fig. 1 shows the baseline concentrations of the total IgM, IgG and anti-dsDNA IgG of the two mouse strains before subjecting them to an exhaustive bout of exercise. There was a trend to higher levels of IgM in $C4^{-/-}$ compared to B6 mice (Fig. 1). The $C4^{-/-}$ mice showed significantly higher levels of IgG and anti-dsDNA IgG than the wild-type mice (Fig. 1). In contrast, the serum levels of IL-1 β , IL-6 were significantly lower whereas the TNF- α and TGF- β levels were not different to those of the B6 mice (Fig. 2). Additionally, the levels of IL-4 and IFN- γ were significantly lower in the $C4^{-/-}$ mice (Fig. 3).

Effects of an exhaustive bout of exercise

Immediately after exhaustive exercise, both IgM and IgG levels were not altered in $C4^{-/-}$ and B6 mice (Table 1). However, anti-dsDNA IgG was elevated in the B6 but not in the $C4^{-/-}$ mice. By one hour post-exercise, the mean IgG level in B6 mice was increased whereas other antibodies were at pre-exercise levels in all groups. A bout of exhaustive exercise increased most cytokines in B6, but not in $C4^{-/-}$ mice. Thus, TNF- α and IL-1 β were elevated one hour post-exercise whereas IFN- γ was enhanced at the onset of exercise but returned to the baseline value one hour post-exercise in B6 mice (Table 1). However, a transient increase in IFN- γ immediately after exercise was also noted in $C4^{-/-}$ mice. Also, the TGF- β concentration in $C4^{-/-}$ mice and B6 were enhanced immediately and one hour post exercise respectively (Table 1).

Effects of exercise training on antibodies and cytokine production

By four weeks of exercise training, increases in serum IgM and IgG levels, but not anti-dsDNA IgG, were observed in both groups of animals (Table

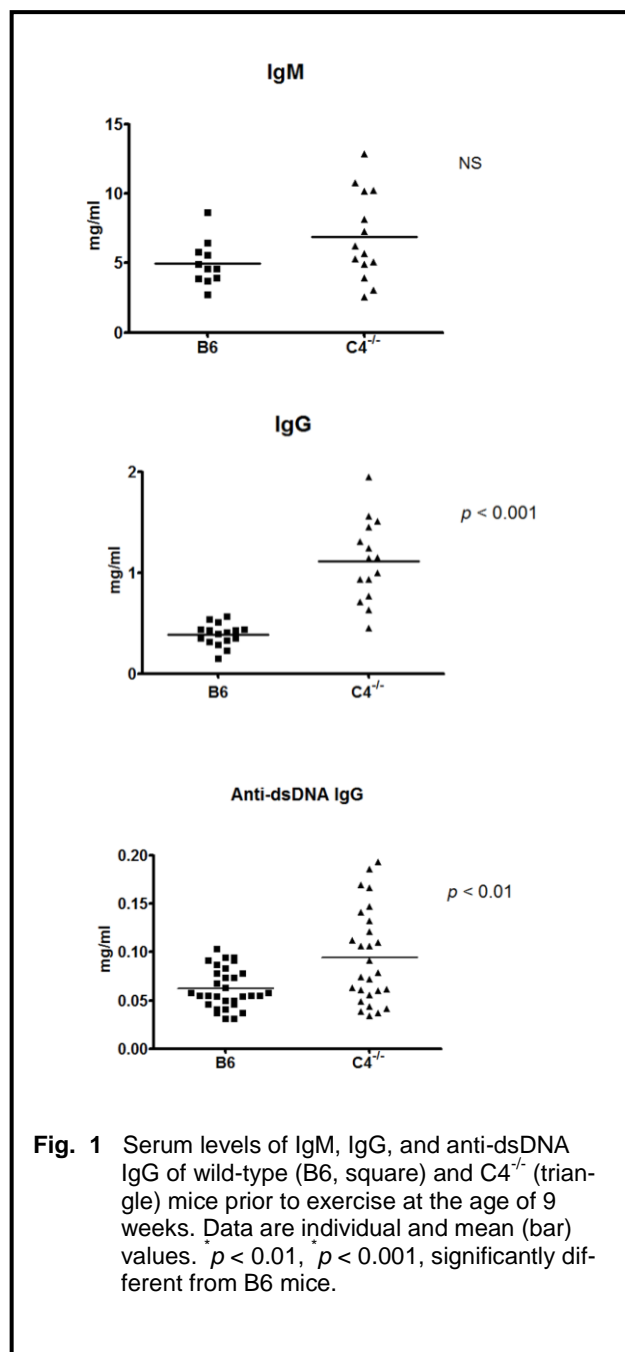
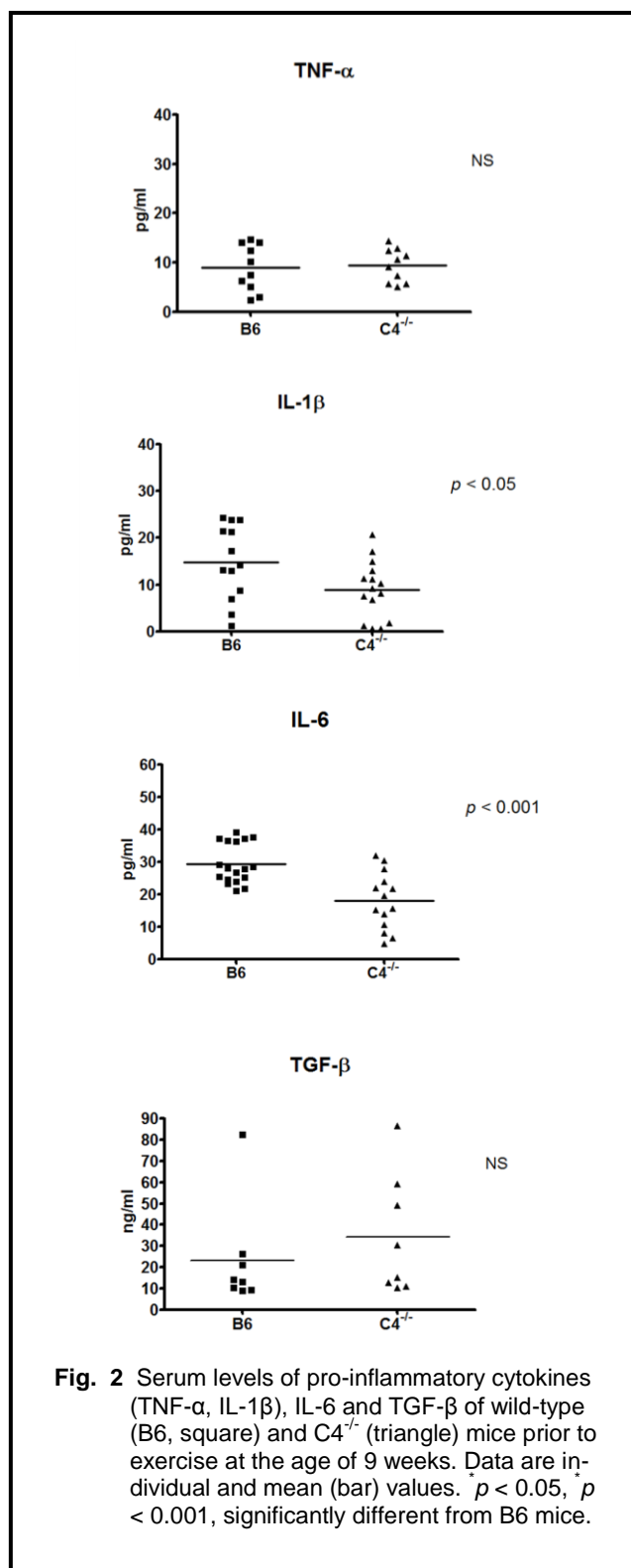
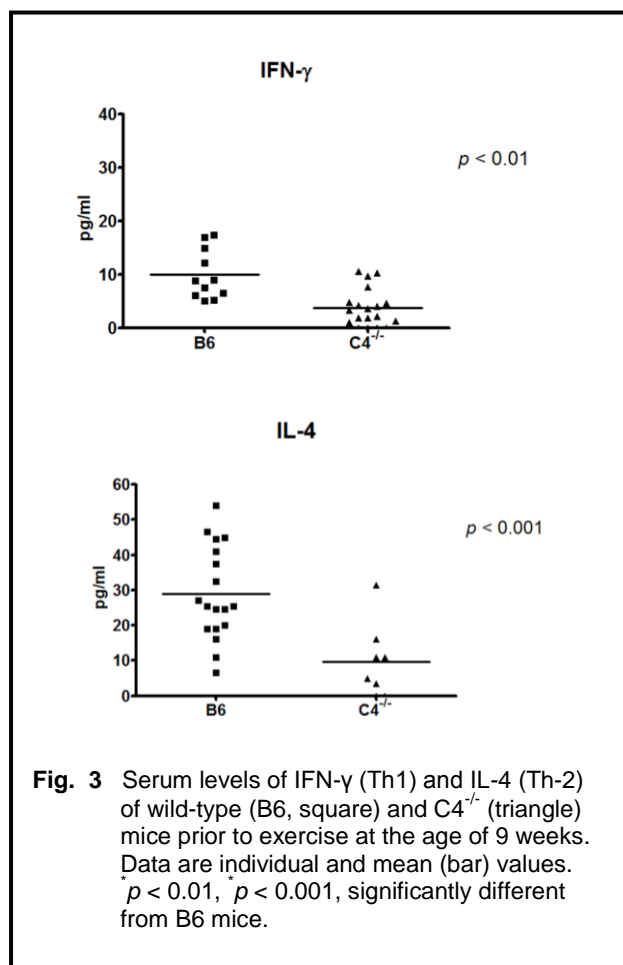


Fig. 1 Serum levels of IgM, IgG, and anti-dsDNA IgG of wild-type (B6, square) and $C4^{-/-}$ (triangle) mice prior to exercise at the age of 9 weeks. Data are individual and mean (bar) values. $p < 0.01$, $p < 0.001$, significantly different from B6 mice.

2). The levels of antibodies after training were not statistically different in the exercise and non-exercise groups. Both wild-type and knockout mice seemed to have an age-related rise in IgM and IgG concentrations but this could not be observed with anti-dsDNA IgG. The increases in IgM and IgG persisted to the end of the 8th week of exercise training. On the other hand, there were no changes in most cytokines in both groups, except for IFN- γ and IL-4 which were decreased in $C4^{-/-}$ mice. The suppression of IFN- γ in



C4^{-/-} mice persisted until the end of the training period (Table 2). Although a decrease in IFN- γ was also observed in the control C4^{-/-} mice, there was a



trend of lower concentrations of IFN- γ in exercising mice. Levels of TGF- β were elevated by the end of the training in both strains (Table 2). In contrast, opposite response of IL-4 was observed in B6 and C4^{-/-} mice, *i.e.* the former showed a decrease whereas the latter exhibited an increase.

DISCUSSION

Vigorous physical exertion is often associated with the suppression of many immune cells and functions, while mild exercise seems to have an opposite effect on the immune system.²⁶ To our knowledge, this is the first study that attempted to determine the effect of exercise on the levels of antibodies, Th1, Th2 and other cytokines and on the onset of SLE, using C4^{-/-} mice as an animal model.

We investigated Th1 and Th2 cytokines in B6 and C4^{-/-} mice prior to and after different modes of exercise. The serum baseline levels of both types of cytokines prior to a single bout of exercise were

Table 1 Effects of single bout of exhaustive exercise on antibodies and cytokines

	Before exercise		Immediately after		1 hour after	
	B6	C4 ^{-/-}	B6	C4 ^{-/-}	B6	C4 ^{-/-}
Antibodies (mg/ml)						
IgG	0.38 ± 0.03	1.12 ± 0.10 ^{***}	0.39 ± 0.03	1.24 ± 0.15 ^{***}	1.22 ± 0.14 ^{†††}	0.97 ± 0.14
IgM	4.98 ± 0.48	6.88 ± 0.84	6.09 ± 0.55	8.07 ± 1.67	6.21 ± 1.52	6.29 ± 0.73
Anti-dsDNA IgG	0.062 ± 0.004	0.095 ± 0.009 ^{***}	0.093 ± 0.006 [†]	0.067 ± 0.007	0.062 ± 0.011	0.11 ± 0.009
Cytokines (pg/ml)						
IFNγ	9.9 ± 1.4	3.7 ± 0.8 ^{***}	17.7 ± 1.3 ^{††}	10.4 ± 1.4 [†]	7.8 ± 1.2	5.4 ± 1.3
TNF-α	8.9 ± 1.5	10.7 ± 1.1	5.9 ± 0.9	13.7 ± 2.0	26.5 ± 8.9 ^{††}	9.8 ± 1.6 [†]
IL-1β	14.8 ± 2.2	8.9 ± 1.6	24.7 ± 3.0 [†]	8.71 ± 2.3 [†]	22.9 ± 2.8 [†]	19.8 ± 4.9
IL-6	29.5 ± 1.3	18.0 ± 2.4 ^{**}	37.0 ± 4.0	23.3 ± 4.5 [†]	25.0 ± 1.9	17.6 ± 1.0
TGF-β (ng/ml)	23.2 ± 8.7	34.3 ± 9.9	48.6 ± 10.6	70.0 ± 8.1 [†]	46.9 ± 6.6 [†]	48.8 ± 8.6

Values are means ± SEM (n = 5-9); ^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001 compared to B6; [†]p < 0.05, ^{††}p < 0.01, ^{†††}p < 0.001 compared to before exercise.

Table 2 Changes in antibodies and cytokines after training

	Four weeks of training				Eight weeks of training			
	B6		C4 ^{-/-}		B6		C4 ^{-/-}	
	Exercise	Control	Exercise	Control	Exercise	Control	Exercise	Control
Antibodies (mg/ml)								
IgM	5.26 ± 2.60 [†]	3.61 ± 1.4	3.89 ± 1.021 ^{†††}	5.39 ± 1.71 ^{††}	6.70 ± 2.60 [†]	3.48 ± 1.70	5.58 ± 0.98 ^{†††}	6.82 ± 1.59 ^{†††}
IgG	0.65 ± 0.12 ^{††}	0.65 ± 0.11 ^{†††}	0.47 ± 0.17 [†]	0.67 ± 0.22 ^{††}	1.28 ± 0.24 ^{††††}	0.82 ± 0.14 ^{††}	0.85 ± 0.18 ^{††††}	0.86 ± 0.23 ^{††}
Anti-dsDNA IgG	0.019 ± 0.007	0.00080 ± 0.0095	0.007 ± 0.003	0.0064 ± 0.005	0.014 ± 0.013	0.00052 ± 0.014	0.011 ± 0.004	0.0064 ± 0.009
Cytokines (pg/ml)								
IFN-γ	0.20 ± 0.87	0.76 ± 0.84	-3.44 ± 0.99 ^{††††}	-3.54 ± 1.18 ^{††}	0.44 ± 0.51	1.79 ± 1.12	-4.50 ± 0.70 ^{†††††}	-2.45 ± 0.79
IL-6	11.68 ± 16.73	-7.12 ± 4.69	15.09 ± 12.85	24.30 ± 13.53	13.05 ± 8.14	16.80 ± 27.01	38.71 ± 20.19	17.30 ± 6.71
IL-4	-4.56 ± 6.72	-4.75 ± 5.68	-5.50 ± 1.87 [†]	2.62 ± 13.23	-18.91 ± 4.90 [†]	-12.47 ± 4.25 [†]	8.75 ± 4.33 ^{†††}	15.06 ± 9.95
TGF-β (ng/ml)	-0.87 ± 15.93	-8.01 ± 6.40	-4.84 ± 3.24	11.84 ± 8.73	31.33 ± 6.05 [†]	8.68 ± 4.55	34.94 ± 8.39 ^{†††}	26.15 ± 5.67

Values are means ± SEM (n = 5-11); ^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001 compared to B6; [†]p < 0.05, ^{††}p < 0.01, ^{†††}p < 0.001 compared to before exercise, ^{††††}p < 0.05, ^{†††††}p < 0.01 compared to 4 weeks.

significantly lower in the C4^{-/-} mice than in the B6 animals. Our results showed that exhaustive exercise increased IgG and anti-dsDNA IgG antibodies, Th1 cytokines (IFN- γ) as well as TNF- α , IL-1 β and TGF- β , either immediately or within 1 hour following exercise in B6 mice. In contrast, most cytokines in the

C4^{-/-} group, except TGF- β , were not altered after exercise. Increased levels of pro-inflammatory cytokines, TNF- α and IL-1 β after prolonged exercise are very common, possibly as a result of muscle injury.²⁷ An elevation in IFN- γ may reflect an increase in NK cell activation by exercise.

Typically, an increase in immunoglobulins after exercise implies an enhanced antibody response, *i.e.* a rapid stimulation of the secondary immune response. This finding of increased total IgG after strenuous exercise is concordant with one study in which total IgG raised immediately after exercise was ceased.²⁸ During strenuous exercise, several hormones, *e.g.* cortisol and noradrenaline are released, thus directly affecting the antibody class switch and production. In addition, hemoconcentration and a decrease in hepatic clearance during exercise may contribute to a rise in IgG. However, this should also be accompanied by a rise in all antibodies including IgM which was not the case. On the other hand, a decrease in anti-dsDNA IgG detected after exercise was probably due to a very low level of anti-dsDNA IgG found in mice during this particular age compared to the concentration of IgG, thus a rise in anti-dsDNA IgG in the circulation may be slower than that of IgG. The plasma levels of IgG were elevated after exercise training for 8 weeks, which was, however, probably not due to the effect of exercise since similar changes in these antibodies were also observed in the sedentary control mice of both strains.

The variation in the baseline of some cytokines as well as antibodies prior to exhaustive and repetitive exercise was probably due to either the short half life or the rapid turnover of cytokines before they could be detected, or due to the paracrine nature of their activities. Also, the age of the mice before the experiments varied between 8-9 weeks and mice at various ages may display different cytokine levels despite their identical genetics. Another factor that influences such variations is the environment in which the mice have been nurtured such as a shift in the light-dark cycle that may disturb an immune response, *e.g.* antibody production.²⁹ In addition, a significant elevation of TGF- β detected following exercise could reflect a sign of accumulative muscle damage. It is important to note that measurement of TGF- β in blood circulation might not represent the level of TGF- β in muscle tissues, thereby may not reflect the degree of tissue repair.

TGF- β and IL-6 enhance Th17 development and differentiation while IL-4 and IFN- γ inhibit it.³⁰ An increase in IL-17 promoted inflammation causes the symptoms of autoimmune diseases.³¹ Thus, we

speculated that there might be an increase in IL-17 in the C4^{-/-} mice, as our data showed significantly lower levels of IFN- γ and IL-4 in C4^{-/-} mice at a resting state compared to those of the B6 group which favor Th17 differentiation and the production of IL-17 in C4^{-/-} mice. However, the level of IL17 was not measured in this study, since there are still some contradictory findings regarding the fundamental basis of biology in both, mice and humans.³¹

In addition, no evidence of deposition of antibodies in the renal glomeruli of C4^{-/-} mice was found (data not shown). C4^{-/-} murine models have been shown to display an elevation in anti-dsDNA antibody levels, signs of renal pathology,²¹ and to have an age-related rise in anti-dsDNA antibody.^{21,22} This may explain why we did not detect any change in anti-dsDNA IgG levels or immune complex depositions in the kidneys. Also, glomerulonephritis could not be found in the C4^{-/-} in one study.²¹ It may be concluded that exercise has no deleterious effects on the development of SLE in C4^{-/-} mice as the levels of anti-dsDNA IgG were not altered by exercise.

The C4 deficient mice used in the present study displayed a mild form of lupus and contained a single gene defect which was responsible for the presence of Complement C4 which may not be comparable the human counterparts. Unlike the murine models, the SLE pathogenesis in human can be influenced by a number of factors other than genetics such as environment and stress which vary in each individual. But the complement C4 pathway is critical and has affected a large number of people worldwide. Therefore, the study of physical stress in this model is beneficial and can be used as suggestive evidence representing those suffering from SLE as a result of a lack in early complement components. However, the effect of exercise on the symptoms of this disease in human could be different from the murine models. Since the onset of SLE in human may involve multiple gene defects, a murine model that mimics a severe human lupus can be used in order to investigate whether exercise provokes any changes in polygenic models.

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