RESEARCH ARTICLE

Effects of conventional immunosuppressive therapy on functional and pathological features of CNS lupus in NZB/W mice [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract
Neurological involvement is one of the most devastating complications of the disease, systemic lupus erythematosus (SLE). To understand the effect of the drugs, cyclophosphamide (CY) and prednisolone (PD) on CNS manifestations, the New Zealand Black/White (NZB/W) lupus mice, were given a cocktail of both drugs by intraperitoneal injections daily from 22 to 44 weeks of age. The treatment prolonged survival (10% of the treated 20 NZB/W mice died compared to 50% of the 30 NZB/W mice, with no mortality in the control NZW mice). Real-time PCR analysis showed a three- to fifteen-fold increase in the expression of GFAP, vimentin and syndecan4 in the cerebral cortex of 44 week NZB/W mice. These alterations were prevented by CY and PD treatment. Immunostaining revealed increased GFAP expression in NZB/W mice compared to congenic, nondiseased NZW mice, which was prevented by treatment. In addition, concomitant changes were observed in the expression of extracellular matrix proteins, collagen IV and fibronectin. To determine the impact of these alterations on the neurological manifestations of SLE, behavior was studied in these mice. The NZB/W mice were spontaneously less active in the open field and exhibited a decrease in distance traveled (58% of control, p<0.01) and ambulatory measurements (52% of control, p<0.01). They took more time (8.8±1.2min) to escape from the maze compared to the control NZW mice (2.6±0.8min). Even more striking was that the behavioral deficits were alleviated in these mice by CY and PD treatment. These results support the hypothesis that increased astrogliosis and altered extracellular matrix proteins may be two of the critical factors that mediate lupus brain disease.

Keywords
Systemic lupus erythematosus, central nervous system, NZB/W mice, behavior, astrogliosis, cyclophosphamide
This article is included in the Lupus nephritis and neuropsychiatric lupus collection.

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**Competing interests:** No competing interests were disclosed.

**Grant information:** This work was supported by National Institutes of Health grants R01DK041873 and R01DK055357, and by the Arthur M. Morris Endowed Chair to RJQ.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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**How to cite this article:** Alexander J, Jacob A and Quigg RJ. Effects of conventional immunosuppressive therapy on functional and pathological features of CNS lupus in NZB/W mice [version 1; peer review: 1 approved, 1 approved with reservations] F1000Research 2015, 4:163 (https://doi.org/10.12688/f1000research.6568.1)

**First published:** 23 Jun 2015, 4:163 (https://doi.org/10.12688/f1000research.6568.1)
**Introduction**

Systemic lupus erythematosus (SLE) is an autoimmune disease in which one third of patients exhibit neuropsychiatric (NP) disturbances. Patients with NP-SLE have a variety of behavioral and cognitive impairments. These are likely to track with pathological alterations occur in the brain, however subtle. A challenge in defining the pathogenesis of SLE is its complexity, involving affecting many systems and pathways.

Animal models have proven invaluable in our understanding of SLE. Lupus mice mirror many of the findings seen clinically and they can be manipulated to help determine underlying pathophysiological mechanisms. Arguably the most accurate SLE model occurs in females of the F1 cross between New Zealand Black and New Zealand White mice (NZB/W). Among the finest examples of translational research occurred over a several decade period in work done at the National Institutes of Health by Alfred Steinberg, James Balow and colleagues; they first showed cyclophosphamide (CY) and methylprednisolone (PD) were efficacious in NZB/W mice, followed by classical studies in human SLE with this therapy remaining the “gold standard” by which all else is compared.

NZB/W mice have an increase in anxiety behavior and decreased exploratory behavior, which is increased with advancing age, indicating these behaviors were related to the development of autoimmune disease. In studies using the MRL/pr lupus mouse model, these mice explored the open field less, spent more time at home-base, had impaired exploratory activity and defecated less in comparison to congenic MRL/+ controls. Lupus mice have a progressive disease over time, including NP-SLE, which does contrast to the periods of disease quiescence punctuated by intermittent flares seen in human SLE. Of course, the clinical disease is typically treated aggressively. Although the mice differ in this aspect from humans, they give us insights and a better understanding of the underlying mechanisms that lead to disease and enable the development of therapeutic strategies.

Several alterations have been observed affecting the central nervous system (CNS) in lupus mice. The cross-talk between the peripheral immune system and the CNS predicts that, particularly when there is an alteration of the blood-brain barrier (BBB) and peripheral immune signals could result in glial cell activation. Activated astrocytes could secrete factors that promote neuronal survival, and could also initiate an inflammatory response, leading to neuronal death, which could result in behavioral changes.

The CNS responds to injury by the process of gliosis which involves astrocytes, oligodendroglial precursors and meningeal cells. Astroglia are the most abundant glial cells in the CNS. They play a crucial role in maintaining normal brain physiology, integrity of the BBB and are a key component of the CNS response to injury and disease. Astrocytes contain abundant intermediate filaments dispersed in the cell body and organized as thick bundles in astrocytic processes. Depending on the region of the brain, astrocytic intermediate filaments are either homopolymers of glial fibrillary acid protein (GFAP) or heteropolymers of GFAP and vimentin. Reactive astrogliosis, in which astrocytes undergo hypertrophy or proliferation along with other histological and enzymatic changes, is a prominent feature in CNS inflammation. Alterations in astrocytes could affect brain circuits that include interactions between neurons and astrocytes, thereby impacting behavior. Activation of astrocytes could lead to increased expression of the proteoglycan, syndecan, to provide a supporting environment for axons to regenerate at the site of brain injury. Coupling of astrocytic and neuronal activities gives rise to membrane potential instability and oscillations. The presence of autoantibodies to GFAP in serum correlated with NP manifestations in SLE patients.

Although there has been progress in our understanding of the immunology and phenotype of lupus brain disease, our current therapy is still imperfect. The continued use of non-specific and potent immunosuppressive agents like CY and PD is less than ideal. There remains a balance between drug toxicity and efficacy. Pulse doses of intravenous CY are often used for NP-SLE. Treated patients show considerable clinical and electrophysiological improvement of cerebral function. Children with severe neuropsychiatric lupus also showed a favorable response to this treatment. In a similar manner, CY treatment alleviated symptoms in lupus mouse models including reduced leukocyte (CD45) infiltration in MRL/pr mice and prevention of behavioral changes such as floating in the forced swim test.

Here we evaluated the effect of conventional CY/PD treatment on clinicopathological features of NP-SLE such as anxiety disorder and cognitive dysfunction. Our studies show that alterations in gliosis and extracellular matrix proteins tracked with behavioral changes in NZB/W mice, both of which were ameliorated by CY/PD treatment.

**Materials and methods**

**Mice**

New Zealand Black and New Zealand White mice were from Jackson Laboratories (Bar Harbor, ME) and crossed in-house to obtain NZB/W F1 mice. Mice were maintained in 12 h light and dark cycles (lights off at 6:00 p.m., lights on at 6:00 a.m.) and a temperature-controlled environment (21 ± 1°C). All studies were approved by the Animal Care and Use Committee at the University of Chicago.

**Experimental plan**

A total of 60 female mice were divided into three groups: 1) NZB/W mice treated from 22–44 weeks of age with CY/PD (each 3 mg/kg/d in saline) given once daily via intraperitoneal (i.p.) injection (n=20); 2) NZB/W mice treated identically, but without CY/PD (i.e., receiving saline vehicle only i.p.) (n=30); and, 3) NZW mice serving as controls (n=10).

Following behavioral testing at 44 weeks of age, animals were euthanized. For this, animals were first anesthetized with inhalational isoflurane. Animals were documented to be unresponsive to
behavioral testing

**Testing:** All mice in a testing group were cage changed on the same day and no testing was performed until 1 day after a cage change. All behavioral testing was executed during the day, when the mice are normally active, with testing carried out in a behavioral testing room under normal light. To ensure the 5 min open field test was accurately measuring the activity of the mice, open field activity was measured over a 60 min period.

**Preliminary workup:** A series of preliminary observations of general health, home cage behaviors and neurological reflexes (eye blink, response to tail pinch and righting reflex) were first conducted for each mouse to avoid spurious false positives. All mice tested were groomed (the appearance of its fur and whiskers is noted), and moved around the cage normally.

**Open-field analysis:** Mice were assessed using an open-field activity monitor (Med Associates Inc., St Albans, VT, USA) for a period of 60 min. The testing chamber was wiped clean with water and then with ethanol between each test subject. The chambers were kept in a room used only for behavioral studies so that no movement or sound disturbs the behavior of the mice. Each subject was placed in the center of an open field apparatus. Measurements (calculated total distance traversed, number of movements, horizontal activity, vertical active and resting time and the beam-break counts for stereotyped behaviors) were recorded by accompanying software (Med Associates Inc, Activity Monitor, version 5.1) and calculated using Excel software (Microsoft, 2007). Ambulatory count and episodes were also recorded. Ambulatory count was defined as the number of beam breaks while the mouse is ambulating, while ambulatory episodes are the number of times the mouse begins ambulating (from a resting position). Data were collected over a 60-min period.

**Maze:** A circular maze having three chambers of 4, 8 and 12 inches was constructed. The mice were placed in the middle chamber. The time taken by the mice to escape from the maze was recorded.

**Quantitative RT-PCR**

Real-time quantitative (q) RT-PCR was performed on RNA from cerebral cortices dissected from NZW, NZB/W and CY/PD-treated NZB/W mice (n=10 each). RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cleaned (RNeasy Mini Kit, Qiagen, Valencia, CA, USA). Total RNA (1 µg) from each sample was reverse transcribed with random hexamer primers using M-MuLV reverse transcriptase (Life Technologies). Ten ng of cDNA and gene-specific primers were added to SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTag DNA polymerase, dNTPs with dUTP and optimal buffer components; Applied Biosystems, Foster City, CA, USA) and subjected to PCR amplification (one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min) in a TaqMan 5700 Sequence Detection System (Applied Biosystems). For each transcript, real-time PCR was conducted three times in duplicate using each of the RNA samples. The amplified transcripts were quantified with the comparative C_{ΔΔ}T method using GAPDH RNA as the control (http://docs.appliedbiosystems.com/pebiiodocs/04303859.pdf). The primers were designed using the Primer Express software (Applied Biosystems) based on the GenBank accession numbers; the sequences (5’−3’) are as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Accession Number</th>
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<tr>
<td>Syndecan-4</td>
<td>Forward: 5'-TGCTGGCGCGTCCGGATGACTTTG-3'</td>
<td>Reverse: 5'-CTGCGAAGACCTCTAGTTCTC-3'</td>
<td>NM_011521.2</td>
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<tr>
<td>Fibronectin</td>
<td>Forward: 5'-ATGTGGACCCCTCCTGATAGT-3'</td>
<td>Reverse: 5'-GCAGCTGATTTCAGCAAAGG-3'</td>
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<tr>
<td>Vimentin</td>
<td>Forward: 5'-CGGCTGCGAGAGAAATTGC-3'</td>
<td>Reverse: 5'-CCACTTTCCGTTCAAGGTCAG-3'</td>
<td>NM_011701</td>
</tr>
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<td>GFAP</td>
<td>Forward: 5'-GCGCTCTCAATGCTGCTTCA-3'</td>
<td>Reverse: 5'-TCTGCCCTCGCCCTGAGTT-3'</td>
<td>X02801</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Forward: 5'-CAAGCATAGTGGTCCGAGTC-3'</td>
<td>Reverse: 5'-AGGCAGGTCAAGTTCTACG-3'</td>
<td>J04694</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-GGTGAGTGTCGCTGGTGAAGC-3'</td>
<td>Reverse: 5'-TTGGGCTCACCCTTCAAGGTG-3'</td>
<td>NM_008084</td>
</tr>
</tbody>
</table>
Immunofluorescence (IF) microscopy

Sections of 6 µm thickness were incubated with a blocking solution for 30 min, then with the primary antibodies overnight at 4°C. After washing in PBS, the sections were incubated in secondary fluorescence-conjugated antibodies (1:200) for 1 h at room temperature, washed in PBS and cover slips placed on them. Polyclonal antibodies used and their respective dilutions in PBS were: FITC-conjugated antibodies to mouse C3 (1:200, Cappel, 55500); rabbit anti-GFAP (1:200 Dako, CA, USA, Z0334), rabbit anti-collagen IV (1:60, Sigma, SAB4500369), anti-fibronectin (1:60, Sigma, F3648) followed by goat anti-rabbit-Alexa Fluor 594 (A-21207, 1:100), 488 (A-11034, 1:100) respectively (Molecular Probes). Negative controls were generated by omitting either primary or secondary antibodies. Images were acquired with a Zeiss microscope. The sections were photographed maintaining the exposure time constant (Axiocam version 3.1; Zeiss).

Sample size and statistical analysis

Statistical analyses were performed using Minitab software (v. 17.1, State College, PA, USA). The number of control NZB/W mice included an estimated 40–60% mortality by 44 weeks and the positive effects of CY/PD treatment\(^6\), with the resultant informative censoring of data\(^3\). Power analyses were done for one-way ANOVA with three levels, assuming \(\alpha = 0.05\) and \(\beta = 0.2\) (power = 0.8). Sample sizes of 10 and 20 were sufficient to identify changes of 1.0 and 1.5 times the SD.

The data from each mouse in the study are presented graphically; group means are also depicted. Statistical significance was determined by one-way ANOVA; a P-value < 0.05 was used to reject the null hypothesis of no differences among the level means. Tukey’s method was then used for pairwise comparisons, with resultant P-values presented in the appropriate section.

Results

Spontaneous mortality in NZB/W lupus mice is lessened by CY/PD treatment

The study was begun with 50 female NZB/W as well as 10 NZW control mice. At 22 weeks of age, 30 of NZB/W mice began treatment with CY and PD, which was administered during the full evolution of autoimmune disease, until 44 weeks when they were sacrificed. At 30 weeks the first untreated NZB/W mouse was found dead. Thereafter, there was progressive mortality, such that by the end of the study at 44 weeks only 15 NZB/W mice (50% of the starting number) remained. There were only two dead among the NZB/W that were treated (10% of the starting number) and no mortalities among the NZW controls (Figure 1).

NZB/W lupus mice have impaired behavior that is ameliorated by CY/PD treatment

A series of preliminary observations were first conducted prior to proceeding with testing\(^3\). These included assessments of general health, home cage behavior and neurological reflexes (eye blink, response to tail pinch, and righting reflex). All mice tested had appropriate grooming of fur and whiskers, and moved around the cage normally. The NZB/W mice had tail pinch reflex and their body weight remained the same.

Open field behavior in rodents is considered to be a fundamental index of their general behavior and inability to escape from a maze is considered as a sign of anxiety. As shown in Figure 2A, on average, control NZW mice traversed over 3,000 cm over 60 min. Untreated NZB/W mice had considerable hypoactivity compared to NZW control mice, which was largely prevented with CY/PD treatment (Figure 2A). Similar patterns were observed in ambulatory and stereotypic (i.e., animal rearing/vertical movement). The number of jumps did not differ between the groups (Supplementary Dataset 1). Control NZW mice escaped from the maze within 1–3 min, while untreated NZB/W mice demonstrated anxious/timid behavior by staying close to the walls of the maze, with all requiring more than 6 min to escape, including two animals that remained within the maze at 10 min (Figure 2B). Treatment with CY/PD reduced but did not fully reverse this phenomenon (Figure 2B).
Transcriptional alterations in brains of NZB/W lupus mice are prevented by CY/PD treatment

Upregulation of astrocytic intermediate filaments is a crucial step in astrocyte activation or astrocytosis. Therefore, we performed qPCR on cDNA derived from the brains of the mice in this study. As shown in Figure 3, the mRNA for GFAP, vimentin and syndecan 4 were all increased in NZB/W mouse brains. Treatment with CY/PD from 22 to 44 weeks of age almost completely prevented this upregulated expression of these genes (Figure 3).

Astrogliosis in NZB/W lupus mouse brains is reduced by CY/PD treatment

The upregulated expression of GFAP mRNA in brains of NZB/W mice suggested the presence of astrogliosis (reactive astrocytosis). We therefore examined GFAP protein expression by IF microscopy. In NZW mice, there was low level expression of GFAP in the cortex (Figure 4A). In NZB/W mice, many GFAP-positive activated glia were observed in the cortex (Figure 4B), as well as the...
Figure 3. **Upregulated expression of GFAP, vimentin and syndecan 4 mRNA in brains of NZB/W mice is prevented by CY/PD treatment.**

RNA of mouse brains was reverse transcribed to cDNA and then subjected to real-time qPCR with gene-specific primers as described in Methods. Data are presented as gene expression relative to GAPDH. Each point represents data from NZW mice and NZB/W mice treated with either saline or CY and PD from 22 to 44 weeks of age. In all three measurements, the means were different in the three groups by ANOVA. *P < 0.05 vs. NZW and NZB/W-T.

Figure 4. **Astrogliosis occurs in NZB/W lupus mouse brains which is ameliorated by treatment with CY/PD.** Representative sections from NZW, untreated NZB/W and CY/PD-treated NZB/W brains were stained for GFAP. Inset, is an astrocyte at 60x under oil. Note the more intense GFAP staining, thicker, shorter processes and swollen soma of astrocytes in the NZB/W sections compared with the controls and treated mice. CY/PD treatment significantly reduced the number of reactive GFAP-expressing astroglia in these brains.

**Extracellular matrix protein accumulation in NZB/W lupus mouse brains is reduced by CY/PD treatment**

Since syndecan 4 associates with fibronectin and collagen IV, we determined their expression in the brains of NZB/W mice and it followed the same pattern of expression as the others, with and without treatment. Fibronectin (Figure 5A) and collagen IV (Figure 5B) were localized around the microvasculature. CY/PD treatment prevented the increase in extracellular matrix protein expression in lupus brains.

**Dataset 4. Raw data for Figure 5**

http://dx.doi.org/10.5256/f1000research.6568.d49716

Gene expression profiles of Fibronectin and Collagen IV assessed by qRT-PCR in relation to GAPDH.

**Dataset 5. Behavioural data**

http://dx.doi.org/10.5256/f1000research.6568.d49718

Tabled data from behavioural testing.

The number of GFAP-positive glia was reduced qualitatively by CY/PD treatment which tended to match the changes observed at the mRNA level for this protein.
Discussion

SLE is a complex, autoimmune disease, and considerable work has been done in the field. Yet, the mechanism underlying the CNS pathology, with its tight regulation and specialized microenvironment, is not completely understood. It is a complex, systemic disease that might be mediated by the synergistic action of many factors. As expected in the setting of an autoimmune disease, IgG deposits were significantly increased in NZB/W lupus mice compared to the NZW controls, which was prevented by CY and PD treatment. Using the microarray technique which gives insight into global mRNA changes in brain, we observed increased expression of GFAP, vimentin and syndecan 4 genes in the cortex, which was prevented by CY and PD treatment.

Astrocytes play a major role in regulating immune responses within the CNS and express major histocompatibility complex molecules required for antigen presenting cellular activity\(^3\). During neurodegeneration, astrocytes are activated and release both proinflammatory cytokines and chemokines\(^4\). Our results give insight into the potential contributions of intrinsic astroglial cellular hyperresponsiveness in the development of NP-SLE. Recent studies have shown that GFAP levels in SLE patients with CNS manifestations were 3-fold higher than those patients without CNS involvement, which was prevented by CY treatment. Furthermore, the GFAP level in these patients significantly correlated with MRI abnormalities\(^5\). A significant positive correlation was observed between anti-GFAP serum antibodies and NP manifestations\(^6\).

Figure 5. Accumulation of fibronectin (A) and collagen IV (B) occurs in NZB/W lupus mouse brains which is reduced by CY/PD treatment. Representative cryosections were immunostained with rabbit anti-mouse fibronectin and detected using Alexa 594 labeled anti-rabbit antibody (A) and rabbit anti-mouse collagen and detected using Alexa 488 labeled anti-rabbit antibody (B). Fibronectin and collagen IV was localized mainly around the microvasculature. RNA of mouse brains was reverse transcribed to cDNA and then subjected to real-time qPCR with primers for fibronectin and collagen IV as described in Methods. Data are presented as gene expression relative to GAPDH. Each point represents data from NZW mice and NZB/W mice treated with either saline or CY and PD from 22 to 44 weeks of age. CY and PD treatment reduced the expression of these extracellular matrix molecules in these brains to levels observed in control NZW brains. In both measurements, the means were different in the three groups by ANOVA. *P < 0.05 vs. NZW and NZB/W-T.
Although we cannot exclude a possible relation between health deterioration and the behavioral abnormalities, we believe these studies are important as many of the changes were also observed in preclinical mice of 6 weeks of age. The NZB/W mice display more anxiety behavior, less activity, and less exploratory behavior than non-autoimmune female NZW mice, prior to and during disease emergence at 6 and 12 weeks of age, respectively. The behavioral alterations observed prior to clinical disease suggest the participation of a genetic component to these differences. Concomitant with our findings of increased expression of astrogliosis and alteration in extracellular matrix proteins, the NZB/W mice showed significantly less activity than the NZW mice, based on the open-field behavior. Furthermore, they also demonstrated increased anxiety. These changes in behavior were prevented by treatment with CY/PD. These findings were similar to the alleviation of neuropsychiatric behavior observed in SLE patients treated with CY/PD.

Finally, this is the first attempt to understand the effect of CY/PD treatment in experimental NP-SLE, in a comprehensive manner. The treatment significantly alleviated both molecular and behavioral alterations in these mice, similar to humans. Future investigations elucidating the precise signaling mechanisms by which astrocyte activation occurs, prevent regeneration of neurons and contribute to the pathology observed in brain, in SLE, will reveal a critical location for therapeutic intervention in NP-SLE. In addition, GFAP in the cerebrospinal fluid could be used as indicators of brain damage and should be used as a follow-up tool in these patients.

Data availability

**F1000Research:** Dataset 1. Raw data for Figure 2, 10.5256/f1000research.6568.d4971

**F1000Research:** Dataset 2. Raw data for Supplementary Figure S1, 10.5256/f1000research.6568.d4971

**F1000Research:** Dataset 3. Raw data for Figure 3, 10.5256/f1000research.6568.d4971

**F1000Research:** Dataset 4. Raw data for Figure 5, 10.5256/f1000research.6568.d4971

**F1000Research:** Dataset 5. Behavioural data, 10.5256/f1000research.6568.d4971

Author contributions

JJA and AJ performed the studies. JJA and RJQ planned and designed the experiments and analyzed data. JJA, AJ, and RJQ wrote the manuscript.

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by National Institutes of Health grants R01DK041873 and R01DK055357, and by the Arthur M. Morris Endowed Chair to RJQ.

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References


Page 9 of 13
Christopher Reilly
Edward Via College of Osteopathic Medicine, Blacksburg, VA, USA

The article is straight forward written showing the effects of immunosuppressive therapy on animal behaviour in mice with lupus. A couple questions remain. Is there a dose effect of drug on brain function? How much of the compounds actually get across the BBB to effect the cells. Finally a more detail analysis of the sections of the brain that were analysed would also make the manuscript better.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
We thank the reviewer for constructive criticisms.

Response: When studying pharmacological agents introduced into circulation and the brain, an important aspect is transport across the BBB. Both cyclophosphamide and prednisolone cross the BBB. The concentration of how much crosses the BBB and is there a dose effect is a kinetic study to be addressed in the future. Our current study shows that when the agents were administered as given to patients, they had a beneficial effect similar to that observed in patients strengthening the translational viability of the model and the studies. The sections of the brain used for analysis in the present study were the cortical regions of the brain.

Competing Interests: No competing interest

Trine Jørgensen
Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

This is a well-powered and inclusive study, providing evidence that treatment with cyclophosphamide and prednisolone effectively limits signs of CNS lupus in a well-known spontaneous mouse model of lupus; the NZBxNZWF1 model. Despite a few missing words and misspellings, the manuscript is well-written and the conclusions are supported by the data.

Minor comments:

In discussion it is claimed that “the microarray technique..” was used for determining relevant gene transcript levels in brain tissue, however in the methods section a real-time RT-PCR analysis is described. Please comment and correct accordingly.

Please read the manuscript, in particular the introduction, for complete sentences and rewrite as needed.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes
If applicable, is the statistical analysis and its interpretation appropriate? 
Yes

Are all the source data underlying the results available to ensure full reproducibility? 
Yes

Are the conclusions drawn adequately supported by the results? 
Yes

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Author Response 15 Jan 2018**

**Jessy Alexander,** University at Buffalo, Buffalo, USA

We thank the reviewer for the positive response.

**Response:** The transcript levels were determined by qRT-PCR and the discussion has been corrected accordingly.

**Response:** The manuscript has been read thoroughly and corrections made accordingly.

**Competing Interests:** No competing interests

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