ARTHRITIS & RHEUMATOLOGY
Vol. 68, No. 11, November 2016, pp 2728–2739
DOI 10.1002/art.39791


# Mitochondrial Dysfunction in the Liver and Antiphospholipid Antibody Production Precede Disease Onset and Respond to Rapamycin in Lupus-Prone Mice

Zachary Oaks, Thomas Winans, Tiffany Caza, David Fernandez, Yuxin Liu, Steve K. Landas, Katalin Banki, and Andras Perl

***Objective.*** **Antiphospholipid antibodies (aPL) constitute a diagnostic criterion of systemic lupus erythematosus (SLE), and aPL have been functionally linked to liver disease in patients with SLE. Since the mechanistic target of rapamycin (mTOR) is a regulator of oxidative stress, a pathophysiologic process that contributes to the development of aPL, this study was undertaken in a mouse model of SLE to examine the involvement of liver mitochondria in lupus pathogenesis.**

***Methods.*** **Mitochondria were isolated from lupus-prone MRL/lpr, C57BL/6.lpr, and MRL mice, age-matched autoimmunity-resistant C57BL/6 mice as negative controls, and transaldolase-deficient mice, a strain that exhibits oxidative stress in the liver. Electron transport chain (ETC) activity was assessed using measurements of oxygen consumption. ETC proteins, which are regulators of mitochondrial homeostasis, and the mTOR complexes mTORC1 and mTORC2 were examined by Western blotting. Anticardiolipin (aCL) and anti–$\beta_2$-glycoprotein I (anti-$\beta_2$GPI) autoantibodies were measured by enzyme-linked immunosorbent assay in mice treated with rapamycin or mice treated with a solvent control.**

***Results.*** **Mitochondrial oxygen consumption was increased in the livers of 4-week-old, disease-free MRL/lpr mice relative to age-matched controls. Levels of the mitophagy initiator dynamin-related protein 1 (Drp1) were depleted while the activity of mTORC1 was increased in MRL/lpr mice. In turn, mTORC2 activity was decreased in MRL and MRL/lpr mice. In addition, levels of aCL and anti-$\beta_2$GPI were elevated preceding the development of nephritis in 4-week-old MRL, C57BL/6.lpr, and MRL/lpr mice. Transaldolase-deficient mice showed increased oxygen consumption, depletion of Drp1, activation of mTORC1, and elevated expression of NADH:ubiquinone oxidoreductase core subunit S3 (NDUFS3), a pro-oxidant subunit of ETC complex I, as well as increased production of aCL and anti-$\beta_2$GPI autoantibodies. Treatment with rapamycin selectively blocked mTORC1 activation, NDUFS3 expression, and aPL production both in transaldolase-deficient mice and in lupus-prone mice.**

***Conclusion.*** **In lupus-prone mice, mTORC1-dependent mitochondrial dysfunction contributes to the generation of aPL, suggesting that such mechanisms may represent a treatment target in patients with SLE.**

The pathogenesis of systemic lupus erythematosus (SLE) is incompletely understood, which limits the development of effective treatments (1). However, as recently recognized, T cells in patients with SLE (2–4) and in lupus-prone mice exhibit activation of the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1), which can be reversed by rapamycin treatment, with demonstrated clinical efficacy (5). The activation of mTORC1 has been attributed to oxidative stress, both inside (6) and outside the immune system (7). Moreover,

Supported in part by the NIH (National Institute of Allergy and Infectious Diseases grant AI-072648 and National Institute of Diabetes and Digestive and Kidney Diseases grant DK-078922) and the Central New York Community Foundation.

Zachary Oaks, BS, Thomas Winans, BS, Tiffany Caza, MD, PhD, David Fernandez, MD, PhD, Yuxin Liu, BS, Steve K. Landas, MD, Katalin Banki, MD, Andras Perl, MD, PhD: State University of New York, Upstate Medical University, Syracuse.

Address correspondence to Andras Perl, MD, PhD, Department of Medicine, State University of New York, 750 East Adams Street, Syracuse, NY 13210. E-mail: perla@upstate.edu.

Submitted for publication March 8, 2016; accepted in revised form June 9, 2016.

oxidative stress has been widely implicated in the immunogenicity of phospholipid antigens (8). The production of antiphospholipid antibodies (aPL) is primarily directed against $\beta_2$-glycoprotein I ($\beta_2$GPI; also recently designated as apolipoprotein H [Apo H]) (9).

Production of aPL represents a diagnostic criterion for SLE (10), and these autoantibodies elicit a significant condition known as the antiphospholipid syndrome (APS), which can occur in patients either with or without lupus (11,12). In a recent retrospective study of patients with APS nephropathy, who underwent renal transplantation and were either treated with rapamycin (also known as sirolimus) or left untreated, 7 (70%) of 10 patients treated with rapamycin had a functioning allograft 144 months after transplantation, in comparison to only 3 (11%) of 27 patients not treated with rapamycin (13). The efficacy of rapamycin was ascribed to its abrogating effects on mTOR activation in renal vascular endothelial cells. Interestingly, the majority of patients with APS in that study also had SLE (16 [57%] of 28) (13). However, it has not been disclosed whether any of the patients who benefited from rapamycin in that study (13) met the diagnostic criteria for SLE (14,15) or APS (11). Moreover, mTOR activity has not been measured in organs other than the kidney or within the immune system (13), the latter of which is considered to be the principal mediator of autoimmunity in patients with APS and SLE (1,12).

In a recent longitudinal study of patients with SLE, we observed a significant prevalence of liver disease, which was remarkably associated with the production of aPL (16). This finding is consistent with the data reported in meta-analyses of liver involvement in patients with APS (17,18). Interestingly, treatment with rapamycin, which blocks the activation of mTORC1, prevented liver disease in our cohort of lupus patients (16). We therefore undertook the present study to examine the role of the liver in mTOR activation and its association with APS in mice that spontaneously develop SLE.

The current study documents alterations in mitochondrial homeostasis in 4-week-old MRL/lpr mice, relative to age-matched control mice, preceding the onset of proteinuria and renal disease, with the changes characterized by depletion of the mitophagy initiator dynamin-related protein 1 (Drp1) and activation of mTORC1, as well as an increased production of anticardiolipin (aCL) and anti-$\beta_2$GPI autoantibodies. In addition, mice lacking transaldolase (TAL), a mouse strain that exhibits mitochondrial oxidative stress in the liver (19), also showed increased oxygen consumption, depletion of Drp1, and activation of mTORC1, as well as overexpression of NADH:ubiquinone oxidoreductase core subunit S3 (NDUFS3), a pro-oxidant subunit of electron transport chain (ETC) complex I, and increased production of aCL and anti-$\beta_2$GPI autoantibodies. Treatment with rapamycin in vivo, both in lupus-prone mice and in TAL-deficient mice, blocked the activation of mTORC1, restored Drp1 to normal levels, and diminished the expression of NDUFS3 in the liver, and, importantly, also abrogated the production of aCL and anti-$\beta_2$GPI autoantibodies.

## MATERIALS AND METHODS

**Mice.** Autoimmunity-resistant C57BL/6 (B6) mice (as negative controls) and lupus-prone strains of C57BL/6.lpr (lpr), MRL, MRL/lpr, NZW, and (NZB/NZW)F1 mice (20) were obtained from The Jackson Laboratory. Baseline studies were performed in the mice at age 4 weeks, an age at which none of the lupus-prone strains produces antinuclear antibodies (ANAs) or any signs of disease (20). As described previously (5), because the onset of SLE is rapid in these mouse models, 4-week-old MRL/lpr mice were separated into 2 treatment groups. One group received 0.2% carboxymethylcellulose (CMC) alone (a solvent control for rapamycin; n = 4), and one group received 1 mg/kg rapamycin in CMC (n = 8). CMC or rapamycin was injected intraperitoneally in the left lower quadrant of the abdomen 3 times per week. Mice were treated for a total of 10 weeks, starting at age 4 weeks. Blood was drawn from the mice biweekly to obtain serum for testing of antibodies. The animals were killed at age 4 weeks or after completion of treatment. Mice with heterozygous deletion of transaldolase (TAL$^{+/-}$) were created and fully backcrossed for >10 generations onto the C57BL/6 strain, as earlier described (19). All of the animal experiments were approved by the Committee on the Human Use of Animals and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Measurement of ETC activity in mitochondria.** ETC activity was measured in the absence or presence of substrates specific for the individual ETC complexes I, II, and IV, using Oxygraph, a Clark-type $O_2$ electrode (21). During the assay of each ETC complex, 150 $\mu M$ ADP and 150 $\mu M$ Pi was added, to attain state 3 respiration. When the ADP was exhausted, state 4 respiration was attained. After achievement of a stable rate for state 4 respiration, 2 $\mu M$ carbonylcyanide m-chlorophenylhydrazone was added, to measure uncoupled $O_2$ consumption.

Maximal ETC capacity was determined according to the rate of $O_2$ consumption of uncoupled mitochondria (22). Oxidative phosphorylation, which is a key element of bioenergetics, was measured as maximum ADP-stimulated respiration or state 3 respiration. State 4 is the respiratory state obtained in isolated mitochondria after state 3, when added ADP is phosphorylated completely to ATP, driven by electron transfer from defined respiratory substrates to $O_2$. Conventionally, ADP stimulation is expressed as the respiratory control ratio (ratio of state 3 respiration to state 4 respiration), which is frequently used as an index of coupling for diagnosis of mitochondrial defects (22). Each measurement was performed in duplicate, and the mean values were used as the result for individual experiments. Change in mitochondrial transmembrane potential ($\Delta\Psi$m) was assessed using a JC-1 carbocyanine dye fluorescent probe, while mitochondrial mass was assessed using MitoTracker green and nonylacridine orange fluorescent probes (21).

**Western blot analyses.** Liver and kidney protein lysates were prepared by sonication in 300 μl of lysis buffer (20 m*M* Tris HCl, 150 m*M* NaCl, 1 m*M* $Na_2$-EDTA, 1 m*M* EGTA, 1% Triton, 2.5 m*M* sodium pyrophosphate, 1 m*M* $\beta$-glycerophosphate, 1 m*M* $Na_3VO_4$, 1 $\mu$g/ml leupeptin, 1 m*M* phenylmethylsulfonyl fluoride). Splenocytes, CD4+ T cells, CD8+ T cells, and B cells were directly dissolved in lysis buffer. The protein lysates (40 μg each) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted to nitrocellulose. Rabbit polyclonal Rab4A and Drp1 antibodies and mouse monoclonal antibodies to S6 kinase (anti-S6K) and phosphorylated S6K (anti-pS6K) were obtained from Santa Cruz Biotechnology. Antibodies to $pDrp1^{S616}$, $pDrp1^{S637}$, Akt, and $pAkt^{S473}$ were purchased from Cell Signaling Technology. Rabbit monoclonal antibodies to Rab4A, NDUFS3, succinate dehydrogenase complex flavoprotein subunit A of ETC complex II, and mitochondrial cytochrome c oxidase subunit 1, as well as complex I immunocapture antibodies, were obtained from Abcam. Antibodies to $\beta_2$GPI/Apo H were purchased from R&D Systems, while $\beta$-actin was purchased from Millipore.

**Analysis of aPL by enzyme-linked immunosorbent assay (ELISA).** For ELISA, 96-well plates were coated with cardiolipin (100 ng/well), $\beta_2$GPI (100 pg/well), phosphatidylethanolamine (PE; 100 ng/well), or phosphatidylserine (PS; 100 ng/well) in 0.01*M* $NaHCO_3$ (pH 9.55) (23). Sera were incubated with antigen in phosphate buffered saline (PBS) with 0.1% Tween 20 at 100-fold dilution for 1 hour. The plates were then washed 6 times with 0.1% Tween 20/PBS, and incubated with peroxidase-conjugated secondary antibodies (diluted 2,000-fold) directed against the heavy and light chains of mouse IgG. After washing 6 times with 0.1% Tween 20/PBS, plates were developed with 3,3′,5,5′-tetramethylbenzidine, and the optical density (OD) was read at 405 nm, 450 nm, and 630 nm. Results are expressed as the fold change in OD at 630 nm relative to that in wells developed with the secondary anti-mouse antibody alone.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism software. Results are expressed as the mean ± SEM of individual experiments. Pairwise repeated-measures analysis of variance (ANOVA), two-way ANOVA, and Student's *t*-tests were used for analyses of the results. *P* values less than 0.05 were considered significant.

## RESULTS

**Occurrence of mitochondrial dysfunction and activation of mTORC1 in the liver prior to disease onset in lupus-prone mice.** Previous studies have demonstrated that mTOR is a sensor of metabolic stress (24), including mitochondrial oxidative stress (25), that accompanies cell survival, growth, and proliferation (26). To investigate whether mitochondrial dysfunction is confined to the immune system in SLE, we assessed the ETC activity of liver mitochondria from lupus-prone MRL/lpr mice, the lpr and MRL parental mouse strains, and B6 negative control mice, all matched for age and sex. We conducted the studies in mice at age 4 weeks, well before the onset of SLE, which, in MRL/lpr mice, is a pathologic process that has been characterized by progressive production of ANAs and development of proteinuria and nephritis from age 10 weeks onward (5).

Interestingly, mitochondria from the livers of MRL/lpr mice had increased $O_2$ consumption through ETC complex II (Figure 1A and Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39791/abstract). Moreover, the state 3:state 4 respiratory control ratio was reduced in MRL/lpr mice relative to the B6 controls and lpr and MRL mice (Figure 1B). Mitochondrial dysfunction was also found to be elevated in MRL/lpr mice, as indicated by an increase in the $\Delta\Psi$m relative to mitochondrial mass (Figure 1C).

To evaluate the impact of mitochondrial dysfunction emanating from the liver relative to the immune system, the rate of oxygen consumption by hepatocytes was compared to that by CD4+ T cells and CD19+ B cells. We found that in sera isolated from 4 C57BL/6 mice and studied in parallel, the rate of overall $O_2$ consumption by hepatocytes (mean ± SEM 6,745 ± 3,119.2 amoles/minute) was 25-fold greater than that by CD4+ T cells (264 ± 11.4 amoles/minute; $P < 0.05$) and B cells (270 ± 31.8 amoles/minute; $P < 0.05$) (data not shown). This robust difference in metabolic activities supports the notion that hepatocytes are a dominant source of oxidative stress in SLE.

Given that Rab4A-mediated Drp1 depletion reduces mitophagy and causes the accumulation of oxidative stress–generating mitochondria in lupus T cells, we examined the potential contribution of this mechanism to mitochondrial dysfunction in the liver. Expression of Rab4A was increased in lupus-prone mice, showing a 3.3-fold increase in MRL mice ($P = 0.003$) and a 4.5-fold increase in MRL/lpr mice ($P = 0.016$) compared to B6 controls (Figure 2A). In turn, Drp1 expression was moderately decreased in the livers of MRL/lpr mice compared to B6 controls (decrease of 24%; $P = 0.04$) (Figure 2B). Notably, 2 functionally distinct phosphorylation sites exist in the Drp1 protein (27,28). Interestingly, $pDrp1^{S616}$ levels were reduced by 41% in MRL mice ($P = 0.0003$) and by 40% in MRL/lpr mice ($P = 0.03$) (Figure 2B). Levels of $pDrp1^{S637}$ were unchanged in lpr, MRL, and MRL/lpr mice in comparison to B6 controls (Figure 2B).

In analyses of Rab GTPase proteins as controls, the expression of Rab5 was found to be increased 1.8-fold in MRL/lpr mice ($P = 0.009$) (results in Supplementary Figure 2, http://onlinelibrary.wiley.com/doi/10.

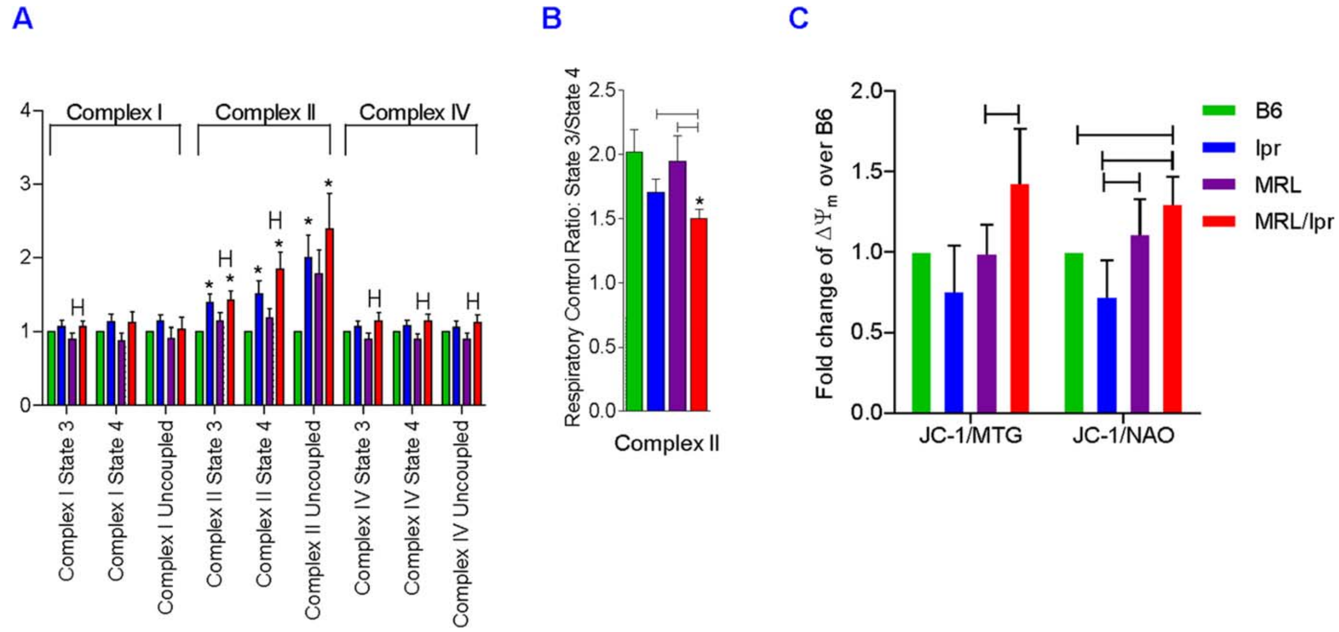


**Figure 1.** $O_2$ consumption rates and respiratory control ratios at complex II of the electron transport chain (ETC) in mitochondria isolated from the livers of 4-week-old C57BL/6 (B6), C57BL/6.lpr (lpr), MRL, and MRL/lpr mice. **A,** Cumulative analyses of $O_2$ consumption rates through ETC complexes I, II, and IV. During the assay of each ETC complex, 150 $\mu M$ ADP and 150 $\mu M$ Pi was added to attain state 3 respiration, and when the ADP had been exhausted, state 4 respiration was attained. After achievement of a stable rate for state 4 respiration, 2 $\mu M$ carbonylcyanide m-chlorophenylhydrazone was added to measure uncoupled $O_2$ consumption, which is an indicator of maximal ETC capacity (22). In all experiments, $O_2$ consumption was normalized to that of the autoimmunity-resistant B6 control strain (set as 1.0 for each ETC complex), which was studied in parallel with the lupus-prone strains. **B,** Respiratory control ratio (state 3:state 4 respiration) at ETC complex II of mitochondria isolated from the livers of 4-week-old mice. **C,** Change in mitochondrial transmembrane potential ($\Delta\Psi$m) over mitochondrial mass as a measure of mitochondrial hyperpolarization in the livers of MRL/lpr mice relative to the MRL and lpr parental strains. The $\Delta\Psi$m was detected using a JC-1 carbocyanine dye fluorescent probe, while mitochondrial mass was assessed using MitoTracker green (MTG) and nonylacridine orange (NAO) fluorescent probes. Results are the mean ± SEM in ≥4 mice per group. $P < 0.05$ versus B6 controls (*) or between individual mouse strains (brackets), based on 2-tailed unpaired *t*-tests.

1002/art.39791/abstract). The expression of Rab4A relative to $\beta$-actin was also increased, albeit to a lesser extent, in the kidneys of 4-week-old MRL mice (1.5-fold increase; $P = 0.005$), lpr mice (1.25-fold increase; $P = 0.012$), and MRL/lpr mice (1.8-fold increase; $P = 5 \times 10^{-6}$) compared to B6 controls (data not shown).

Consistent with its role as a sensor of mitochondrial dysfunction (25) and metabolic stress (7), mTORC1 activity was increased, as indicated by elevated levels of pS6K$^{T389}$ relative to $\beta$-actin in the livers of 4-week-old MRL/lpr mice compared to B6 controls (increase of 2.5-fold; $P = 0.026$) and compared to the parental lpr mouse strain (increase of 1.7-fold; $P = 0.034$) (Figure 3). Of note, S6K protein levels were increased in all lupus-prone mice, the MRL/lpr mice and the parental MRL and lpr strains, relative to B6 controls (Figure 3). In contrast, mTORC2 activity was diminished in MRL and MRL/lpr mice, as evidenced by a reduction in the levels of pAkt$^{Ser473}$ (Figure 3). Unlike the findings in the liver, mTORC1 activity was not elevated in the kidneys (data not shown) or in the immune system (thymus or spleen) of lupus-prone mice at age 4 weeks (5).

Interestingly, the prevalence of hepatocellular carcinoma (HCC) has been found to be increased in patients with SLE, with an elevated standardized incidence ratio of 2.6 (29). The genetic mutation in lpr mice inactivates the CD95 cell death receptor, which not only predisposes to the development of lupus-like autoimmunity, both in mice and in humans, but also confers susceptibility to malignancies, such as HCC (30). Compared to B6 control mice, markedly increased numbers of mitotic figures and binucleated cells were noted in lupus-prone lpr and MRL/lpr mice, and, interestingly, these features were also observed in MRL mice (data not shown).

It appears that this mutation in lpr mice contributes to mTORC1 activation. However, the lpr mice did not show changes in mTORC2 activity, which was diminished in the livers of MRL and MRL/lpr mice.

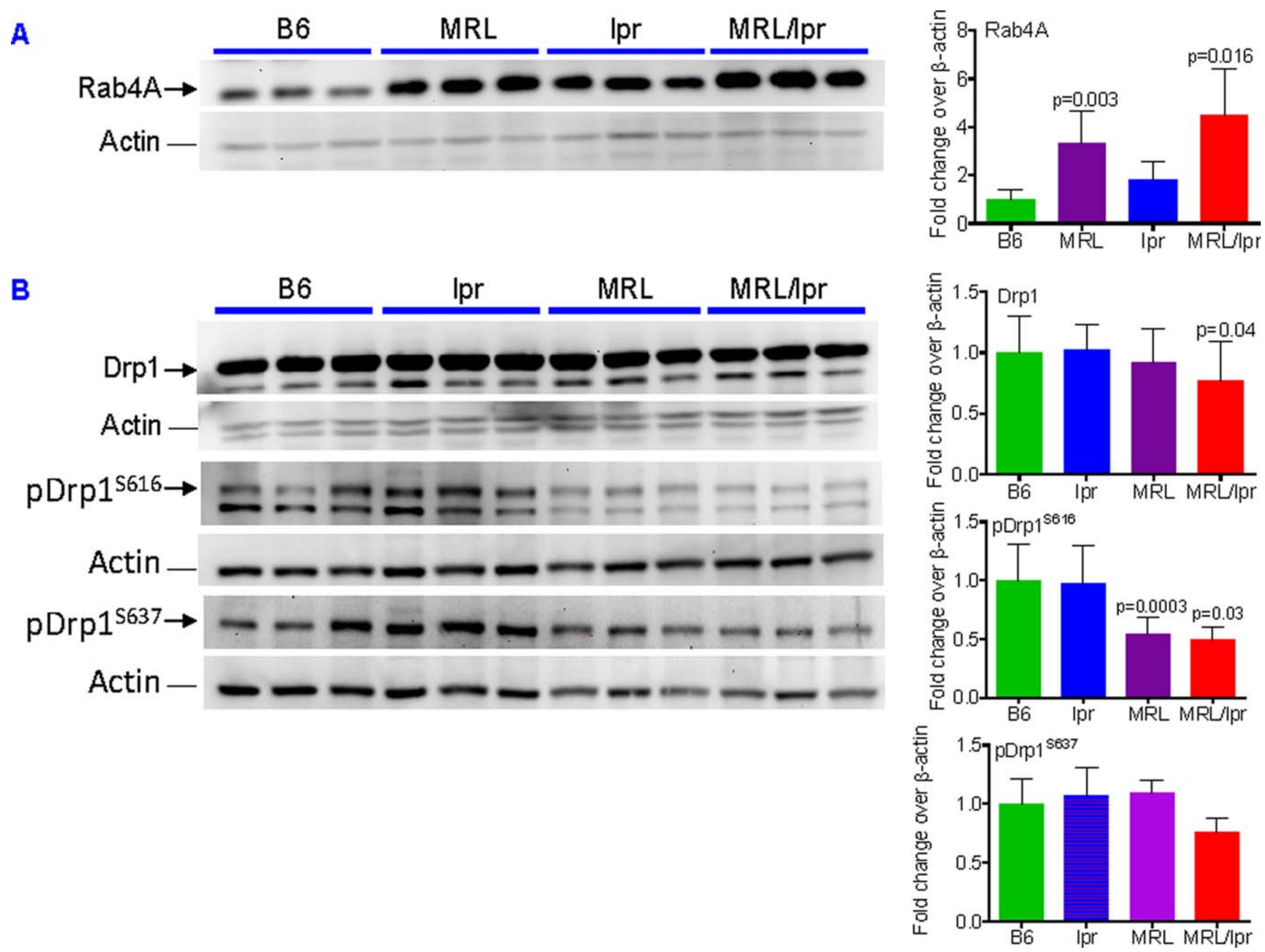


**Figure 2.** Increased expression of Rab4A and depletion of dynamin-related protein 1 (Drp1) in MRL/lpr mice. Western blot analyses were performed to assess Rab4A expression (**A**) and the expression of Drp1, pDrp1$^{S616}$, and pDrp1$^{S637}$ (**B**) in the livers of 4-week-old C57BL/6 (B6), MRL, C57BL/6.lpr (lpr), and MRL/lpr mice. Left, Representative blots are shown. Right, Cumulative analyses of the fold change in expression relative to $\beta$-actin are shown. Results are the mean ± SEM of 5 mice per strain. *P* values are versus B6 controls.

Moreover, Rab4A overexpression and Drp1 depletion were also absent in the livers of lpr mice. Along these lines, pDrp1$^{S616}$ levels were reduced in MRL and MRL/lpr mice but not in lpr mice. These findings are consistent with the notion that lupus susceptibility factors are mainly carried by the MRL strain and that these factors are accelerated by the genetic mutation in the lpr strain.

**Changes in production of aCL and anti-$\beta_2$GPI antibodies in response to mTORC1 blockade by rapamycin in lupus-prone mice.** Given that mTORC1 was activated in the livers of lupus-prone mice at age 4 weeks, before the onset of ANA production and nephritis, we examined the impact of rapamycin treatment subsequent to the abrogation of disease development at age 14 weeks (5). Treatment with rapamycin profoundly blocked mTORC1 activity in the livers of mice at age 14 weeks, as evidenced by a 50% reduction in pS6K levels ($P = 0.017$) and 62% reduction in S6K protein levels ($P = 0.003$) compared to control mice treated with CMC solvent alone. In contrast, mTORC2 activity was unaffected by rapamycin treatment (Figure 4).

We next examined whether mTORC1 activation in the mouse livers was associated with aPL production. As shown in Figure 5A, MRL, lpr, and MRL/lpr mice each exhibited markedly enhanced production of aCL and anti-$\beta_2$GPI antibodies relative to B6 controls. The levels of aCL and anti-$\beta_2$GPI were increased 8.5-fold ($P = 0.007$) and 8.3-fold ($P = 0.008$), respectively, in MRL/lpr mice compared to B6 controls at age 4 weeks, and both were further increased in MRL/lpr mice at age

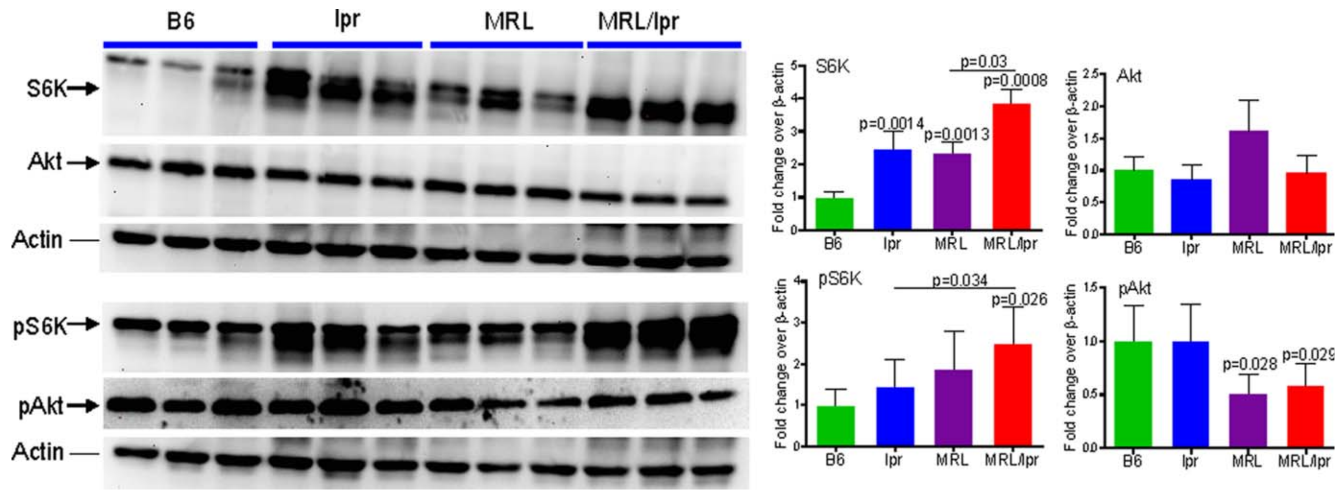


**Figure 3.** Increased activation of mechanistic target of rapamycin complex 1 (mTORC1) and diminished activation of mTORC2 in the livers of 4-week-old MRL/lpr mice. The mTORC1 and mTORC2 signature substrates (26), unphosphorylated S6 kinase (S6K) and phosphorylated S6K ($pS6K^{T389}$) and unphosphorylated Akt and phosphorylated Akt ($pAkt^{Ser473}$), were quantified by Western blotting, relative to $\beta$-actin, in liver protein lysates from 4-week-old C57BL/6 (B6), C57BL/6.lpr (lpr), MRL, and MRL/lpr mice. Left, Representative blots are shown. Right, Cumulative analyses of the fold change in expression relative to $\beta$-actin are shown. Results are the mean ± SEM in 5 mice per strain. *P* values are versus B6 controls.

14 weeks (increase of ~10.7-fold relative to B6 controls). In contrast, the production of ANAs was only evident in the very same serum from MRL/lpr mice at age 11 weeks (5).

Production of aPL was also evaluated in another lupus-prone strain, female (NZB/NZW)F1 mice, at age 4 weeks. These mice develop ANAs at ages 4–5 months and nephritis after the age of 8–10 months (20). Interestingly, aPL production was similar between B6 mice and (NZB/NZW)F1 mice at age 4 weeks (results in Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.39791/abstract). However, compared to the parental NZW mouse strain, levels of aCL and anti-$\beta_2$GPI were increased in 4-week-old (NZB/NZW)F1 mice 3.6-fold ($P = 6 \times 10^{-7}$) and 3.5-fold ($P = 2 \times 10^{-6}$), respectively. Production of aCL and anti-$\beta_2$GPI antibodies further increased in (NZB/NZW)F1 mice by ages 10 weeks and 30 weeks, and in (NZB/NZW)F1 mice by age 10 weeks, aPL production exceeded that in B6 controls (see Supplementary Figures 3A and B).

Treatment of MRL/lpr mice with rapamycin between the ages of 4 weeks and 14 weeks completely abrogated the development of ANAs and nephritis (5). As shown in Figure 5B, the generation of aCL and anti-$\beta_2$GPI antibodies was suppressed by 85% ($P = 0.012$) and by 84% ($P = 0.014$), respectively, in MRL/lpr mice upon treatment with rapamycin, in comparison to MRL/lpr mice treated with CMC solvent alone. Production of aPL was also abrogated upon rapamycin treatment in (NZB/NZW)F1 mice between the ages of 4 weeks and 30 weeks (see Supplementary Figure 3C).

Cardiolipin is localized to the inner mitochondrial membrane, along with additional phospholipids, such as PS and its decarboxylated product, PE (31). Interestingly, the levels of anti-PE antibodies and anti-PS antibodies were also increased in MRL/lpr mice compared to B6 controls at age 4 weeks (increase of 4.3-fold [$P = 0.012$] and increase of 4.5-fold [$P = 0.017$], respectively), and these were further enhanced in MRL/lpr mice by age 14 weeks (increase in anti-PE of 36-fold [$P = 0.004$] and increase in anti-PS of 38-fold [$P = 0.003$]). Importantly, treatment with rapamycin in vivo profoundly reduced the production of anti-PE antibodies (decrease of 68%; $P = 0.015$) and anti-PS antibodies (decrease of 70%; $P = 0.014$) in MRL/lpr mice at age 14 weeks. Likewise, (NZB/NZW)F1 mice showed increased production of both antibodies, and these elevations were reversed by rapamycin treatment (data not shown).

**Effects of mTORC1 blockade by rapamycin on NDUFS3 expression and production of aCL and anti-$\beta_2$GPI in lupus-prone mice.** Increased expression of HRES-1/Rab4 (also designated as Rab4A by NCBI; http://www.ncbi.nlm.nih.gov/gene/5867) mediates the depletion of Drp1 and the accumulation of oxidative stress–generating mitochondria in T cells (5) and HeLa cells (32). Moreover, the expression of Rab4A is partially controlled by mTORC1 (3). Therefore, we examined the impact of rapamycin on Rab4A and Drp1 expression, in terms of their role as potential mediators of mitochondrial dysfunction in the liver. Surprisingly, the profound blockade of mTORC1 activity enhanced the expression of Rab4A 2.5-fold ($P = 0.006$) and

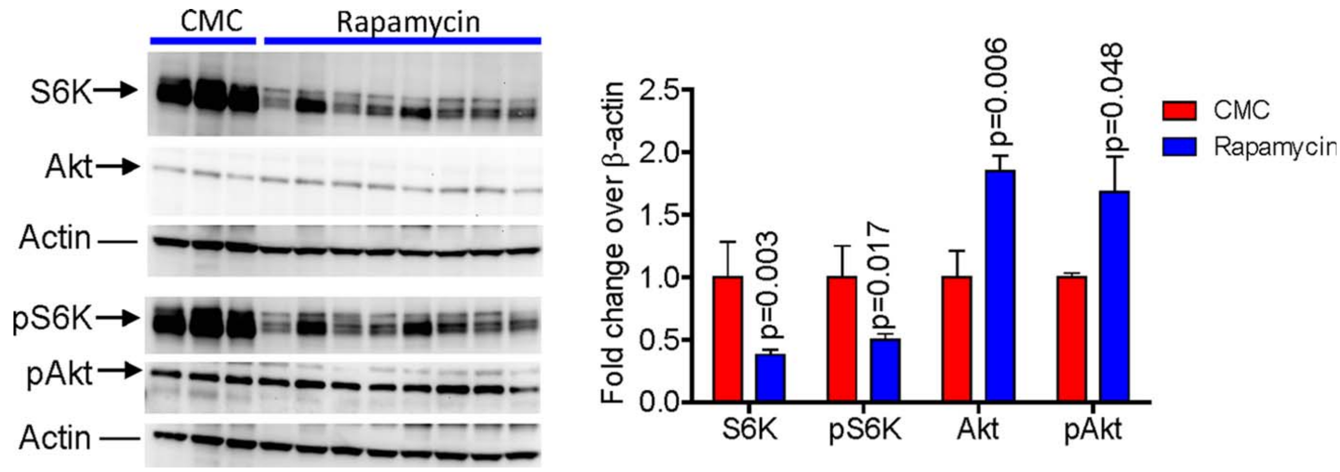


**Figure 4.** Blockade of mechanistic target of rapamycin complex 1 (mTORC1) activity in the livers of MRL/lpr mice by treatment with rapamycin, administered in vivo at ages 4–14 weeks. Western blot analyses of the expression of unphosphorylated S6 kinase (S6K) and unphosphorylated Akt, as well as phosphorylated S6K (pS6K$^{T389}$) and phosphorylated Akt (pAkt$^{Ser473}$), were performed in mice at age 14 weeks, after treatment with rapamycin (n = 8) or with a solvent control of 0.2% carboxymethylcellulose (CMC; n = 3). Left, Representative Western blots are shown. Right, Cumulative analyses of the fold change in expression relative to $\beta$-actin are shown. *P* values are versus CMC-treated controls.

increased the expression of Drp1 by 85% (*P* = 0.020) in MRL/lpr mice (Figure 6A).

Moreover, treatment of MRL/lpr mice with rapamycin profoundly diminished the expression of NDUFS3, with a reduction as deep as 22% from baseline (*P* = 0.0002) (Figure 6B). In contrast, expression of another subunit of ETC complex I, NDUFS1, and components of ETC complexes II and IV were increased in the livers of rapamycin-treated mice (Figure 6B). Thus, the selectively reduced expression of NDUFS3, which promotes oxidative stress (33), may be attributed to the reversal of Drp1 depletion and may account for the retention of healthier mitochondria in the liver of rapamycin-treated mice.

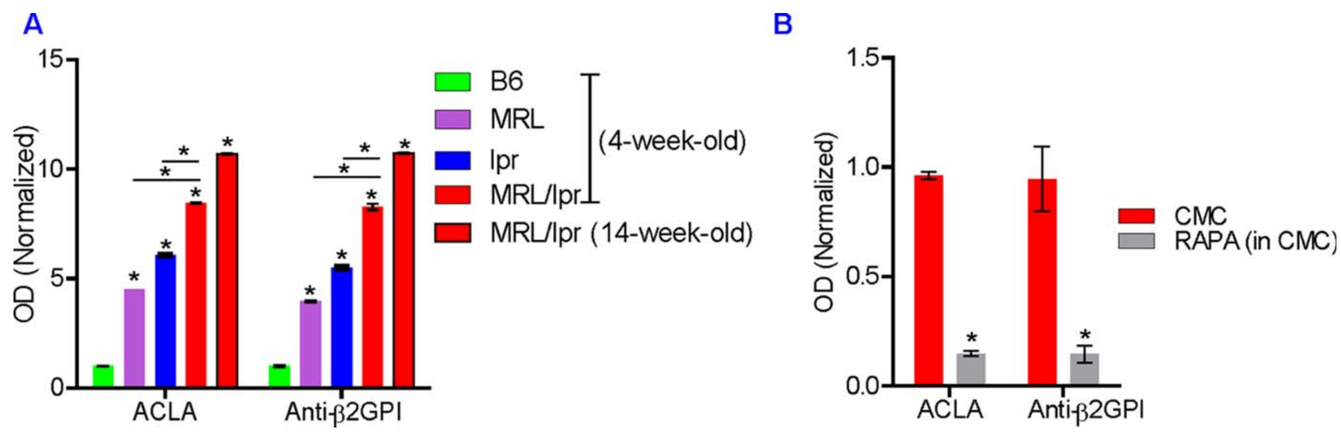


**Figure 5.** Increased production of anticardiolipin antibodies (ACLA; aCL) and anti–$\beta_2$-glycoprotein I (anti-$\beta_2$GPI) antibodies dependent on activation of mechanistic target of rapamycin complex 1 in lupus-prone mice. **A,** Production of aCL and anti-$\beta_2$GPI antibodies in 4-week-old MRL, C57BL/6.lpr (lpr), and MRL/lpr mice and 14-week-old MRL/lpr mice relative to C57BL/6 (B6) controls (n = 4–8 animals per strain). Results are the mean ± SEM fold change in OD at 630 nm relative to that in wells developed with secondary anti-mouse antibodies alone and normalized to the values in B6 controls (set as 1.0). $P < 0.05$ versus B6 controls (*) or between individual strains (horizontal lines), by 2-tailed *t*-test. **B,** Blockade of the production of aCL and anti-$\beta_2$GPI antibodies by rapamycin (RAPA) treatment in MRL/lpr mice. Mice were treated 3 times weekly with intraperitoneal injections of 0.2% carboxymethylcellulose (CMC) (a solvent control for rapamycin; n = 3) or 1 mg/kg rapamycin (n = 8). Treatment was started at age 4 weeks and antibody production was tested at age 14 weeks. Results are the mean ± SEM fold change in OD at 630 nm relative to CMC-treated control MRL/lpr mice (set as 1.0). * = $P < 0.05$ versus CMC-treated controls, by 2-tailed *t*-test.

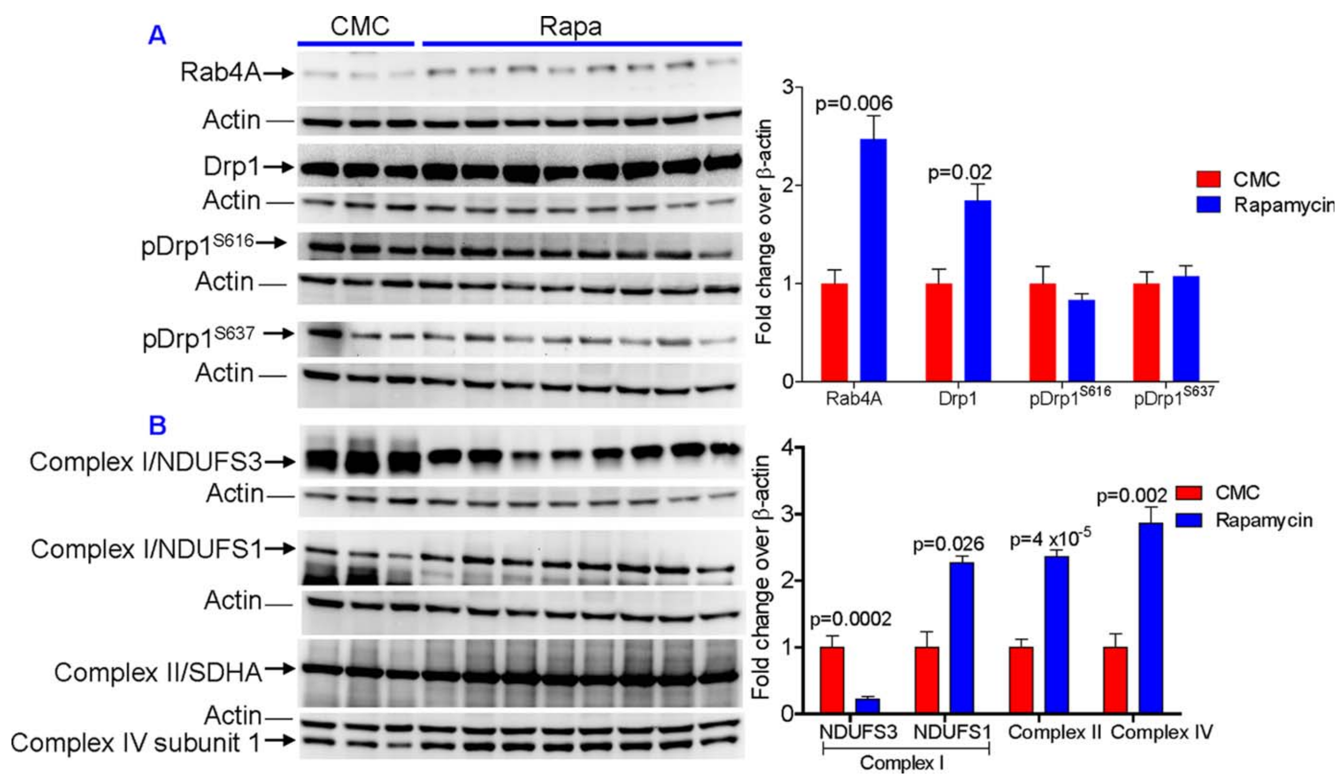


**Figure 6.** Reversal of dynamin-related protein 1 (Drp1) depletion and selective blockade of the expression of NADH:ubiquinone oxidoreductase core subunit S3 (NDUFS3) of electron transport chain (ETC) complex I by rapamycin (Rapa) treatment in the liver mitochondria of MRL/lpr mice. Female mice were treated 3 times weekly with intraperitoneal injections of 0.2% carboxymethylcellulose (CMC) (a solvent control for rapamycin; n = 3) or 1 mg/kg rapamycin (n = 8). Treatment was begun at age 4 weeks and continued through age 14 weeks. Western blot analyses (left) and cumulative analyses of the fold change in expression relative to β-actin (right) were performed to assess the expression of Rab4A, Drp1, $pDrp1^{S616}$, and $pDrp1^{S637}$ (**A**) and ETC complex I (subunits NDUFS3 and NDUFS1), ETC complex II (succinate dehydrogenase complex flavoprotein subunit A [SDHA]), and ETC complex IV (mitochondrial cytochrome c oxidase subunit 1) (**B**). *P* values are versus CMC-treated controls, by unpaired 2-tailed *t*-test.

**Activation of mTORC1 and initiation of aCL and anti-$\beta_2$GPI production in the presence of oxidative stress in the livers of TAL-deficient mice.** TAL deficiency causes oxidative stress in the liver, a process that creates a predisposition to progression of inflammation, resistance to CD95/Fas-mediated apoptosis, and development of HCC (19). Interestingly, the prevalence of HCC is increased in patients with SLE (29). Recently, liver disease was found to be associated with APS in our lupus cohort (16), a finding consistent with that in meta-analyses of liver involvement in patients with APS (17,18). TAL is overexpressed in the T cells of SLE patients, which may be related to protection against oxidative stress (3). The involvement of TAL in the pathogenesis of lupus was further supported by our observations of increased expression of TAL in the livers and spleens of MRL/lpr mice (results in Supplementary Figures 4A and B, http://onlinelibrary.wiley.com/doi/10.1002/art.39791/abstract). Therefore, we examined whether oxidative stress, which emanates from the liver, can activate mTORC1 and thus predispose to the production of aPL.

Mitochondria from the livers of $TAL^{-/-}$ mice exhibited increased ETC activity (data not shown). Among the ETC subunits, NDUFS3 was overexpressed in the livers of $TAL^{-/-}$ mice compared to wild-type ($TAL^{+/+}$) littermates (each n = 5) (2.78-fold increase; $P = 0.002$) (data not shown). Other ETC complexes did not exhibit such changes. Similar to the findings in MRL/lpr mice, Rab4A expression was increased in the livers of $TAL^{-/-}$ mice compared to $TAL^{+/+}$ mice (increase of 1.7-fold; $P = 0.049$), while Drp1 levels were reduced in $TAL^{-/-}$ mice, to a mean ± SEM 76 ± 8% of the levels in $TAL^{+/+}$ mice ($P = 0.017$) (data not shown). Moreover, there was evidence for activation of mTORC1 in $TAL^{-/-}$ mice. In particular, phosphorylation of 4E-BP1 in the livers of $TAL^{-/-}$ mice was increased in comparison to wild-type controls (increase of 2.39-fold; $P = 0.032$). Interestingly, TAL deficiency did not affect the levels of $pS6K^{S389}$, $pAkt^{S473}$, or total Akt (data not shown).

In accordance with an underlying role of oxidative stress and mTORC1 activation in the liver, the production of aCL and anti-$\beta_2$GPI antibodies was increased in $TAL^{-/-}$ mice compared to $TAL^{+/+}$ control mice matched for age and sex (data not shown). Importantly, treatment with rapamycin for 10 weeks abrogated the production of aPL in $TAL^{-/-}$ mice (data not shown).

## DISCUSSION

The present study provides evidence to support the concept that mitochondrial dysfunction is controlled by mTORC1 in the livers of 4-week-old lupus-prone mice preceding the development of similar metabolic changes within the immune system, since mTOR activation is known to be absent at this age both in MRL/lpr mice and in (NZB/NZW)F1 mice (5). These observations in mouse models of lupus are consistent with the findings from epidemiologic studies that document the occurrence of aPL prior to the manifestations of clinical disease in patients with SLE (34,35).

Moreover, the generation of aPL in TAL-deficient mice and its responsiveness to rapamycin treatment indicate that mitochondrial oxidative stress and mTORC1 activation in the liver constitute a trigger of pathogenesis. These mice do not develop nephritis (data not shown), which suggests that the metabolic defect due to inactivation of TAL is insufficient to cause lupus, at least by itself. Nevertheless, the involvement of TAL in lupus pathogenesis, as a potential protector against oxidative stress, is supported by its increased expression in the livers and spleens of MRL/lpr mice. These considerations raise the possibility that TAL deficiency, which has been recently recognized as a cause of oxidative stress–driven liver disease in children, may rarely contribute to SLE (36).

This study not only newly documents mTORC1-dependent mitochondrial dysfunction in the liver as an early event in lupus pathogenesis, but also offers insights into the underlying mechanisms. Similar to that in the immune system (5), expression of Rab4A was found to be far greater in the livers of 4-week-old MRL, lpr, and MRL/lpr mice than in autoimmunity-resistant B6 controls. Polymorphic haplotypes of the Rab4A (i.e., HRES-1/Rab4) genomic locus, which influence gene expression (37), have been associated with predisposition to SLE (38). Although the precise cause of its overexpression in lupus-prone mice is still being investigated, the overexpression of Rab4A in T cells of patients with SLE was found to be driven by oxidative stress (3). The overexpression of Rab4A may reflect an overall activation of the endocytic recycling machinery, which targets surface proteins such as CD4 (37), CD3$\zeta$ (3), CD2AP (3), and endosome-bound Drp1 for lysosomal degradation (5).

Similar to the findings in T cells from SLE patients (3) and lupus-prone mice, expression of Rab5 was also increased, albeit to a lesser extent, in the livers of MRL/lpr mice. This is suggestive of an enhanced traffic of internalizing endosomes, which are regulated by Rab5 and contribute to the formation of autophagosomes (26). The age-related delay of Rab5 overexpression relative to that of Rab4A in lupus-prone MRL, MRL/lpr, and (NZB/NZW)F1 mice indicates that activation of Rab4A may represent an upstream event.

Importantly, Rab4A-regulated Drp1 depletion appears to underlie a disturbed mitochondrial homeostasis, which results in the accumulation of oxidative stress–generating mitochondria (5,32). Indeed, the markedly elevated expression of Rab4A occurred with a loss of Drp1 in MRL/lpr mouse livers. Moreover, beyond a moderate reduction in the levels of Drp1, the phosphorylated isoform $pDrp1^{S616}$ was considerably depleted in MRL and MRL/lpr mice, which may play a key role in deficient mitophagy, since this posttranslational modification allows the translocation of Drp1 to the mitochondrial membranes and the fission of mitochondria to occur (27). Notably, serine-616 of Drp1 is phosphorylated by ERK-1 (39), which is also regulated via endosomal traffic by Rab4A (40). Given the multifactorial pathogenesis of SLE, an oxidative stress–induced deficiency of ERK-1, which has been demonstrated in T cells (41), may also contribute to lower expression of $pDrp1^{S616}$ in the liver.

Mitochondrial dysfunction in the livers of lupus-prone mice was characterized by elevation in the $\Delta\Psi$m or mitochondrial hyperpolarization, which is consistent with findings in the T cells of SLE patients (42) and mice (5). Mitochondrial hyperpolarization was accompanied by increased ETC activity through ETC complex II and a diminished respiratory control ratio, all of which point to an underlying mechanism of reverse electron transfer from ETC complex II to ETC complex I that can generate oxidative stress (43).

Our studies identified NDUFS3, a subunit of ETC complex I, as a regulatory checkpoint of therapeutic impact. First, it was found to be up-regulated in the livers of TAL-deficient mice, which exhibited increased aPL production. Second, and perhaps most importantly, it was down-regulated in the livers of MRL/lpr mice treated with rapamycin, which completely abrogated the production of aCL and anti-$\beta_2$GPI. These results are consistent with a recently uncovered role of mTORC1 in regulating the expression of NDUFS3 (33), which in turn controls the generation of electron transport–dependent oxidative stress (33). Although NDUFS3 is pinpointed as a therapeutically relevant checkpoint for genetic and pharmacologic interventions, via TAL and mTORC1, its role in mitochondrial dysfunction in SLE remains unclear.

Alternatively, similar to the findings in the T cells of patients with SLE (5), the coordinate changes in Rab4A and Drp1 expression indicate that oxidative stress may originate from the defective elimination of damaged mitochondria. This is consistent with earlier findings showing that the depletion of Drp1 is a cause of inappropriate ETC complex I assembly and poorly coupled respiration (44). Interestingly, mTORC1 blockade interrupted the coordinate changes in Rab4A and Drp1 expression, both of which were increased in rapamycin-treated mice. Notably, treatment with rapamycin profoundly diminished the expression of NDUFS3, which has been identified as a source of oxidative stress (33). Whereas rapamycin reduced the activity of mTORC1, it reversed the depletion of mTORC2 in MRL/lpr mice. These findings are consistent with the observed effects of rapamycin on mTORC2 activity, as measured by $pAkt^{Ser473}$ levels, in the T cells of patients with SLE (45). Of note, $pAkt^{Ser473}$ can be a substrate of kinases other than mTORC2 (26). Thus, the beneficial effect of rapamycin on aPL may be attributed, at least partially, to selective blockade of mTORC1 in the immune system.

Interestingly, the levels of anti-PE and anti-PS antibodies were also increased in MRL/lpr and (NZB/NZW)F1 mice, and their production was blocked by rapamycin treatment in vivo. Of note, the generation of PE from PS is tightly linked to the use of mitochondrial membranes for autophagosome formation (32), which in turn is facilitated by Rab4A and opposed by mTORC1, as recently reviewed (26). Although further mechanistic studies are clearly warranted, the robust and concordant production of antibodies against these functionally linked constituents of the inner mitochondrial membrane prior to disease onset indicate that the abnormal composition and activity of the respiratory chain represent early events during the pathogenesis of lupus.

To systematically evaluate the signaling cascade involved in altered mitochondrial homeostasis, we conducted RNAseq analyses of the livers from 5 TAL-deficient mice as well as 5 wild-type mice matched for age and sex. Of the 24,606 RNA sequences assessed, 300 were found to be significantly affected by TAL deficiency at a false discovery rate $P$ value of $<0.05$. Confirmatory Western blot analyses revealed that the expression and activity of paraoxonase 1 were markedly reduced (decrease of $79.8 \pm 2.6\%$ [$P = 0.014$] and decrease of $49.9 \pm 0.7\%$ [$P = 0.008$], respectively) in the serum of TAL-deficient mice, and these were reversed by rapamycin treatment in vivo (data not shown). Paraoxonase 1 is synthesized by the liver, and it circulates in the blood bound to high-density lipoproteins. This enzyme degrades oxidized phospholipids, and its activity is reduced in patients with APS (46) and those with SLE (47). Therefore, paraoxonase represents a liver-derived protein that may modulate aPL production in lupus-prone mice.

In summary, the model of lupus pathogenesis proposed herein involves mTORC1-controlled mitochondrial dysfunction in the liver, with related generation of aPL and increased hepatocarcinogenesis in SLE (29). In support of this model, liver disease, which was defined as a ≥2-fold elevation in the levels of aspartate aminotransferase or alanine aminotransferase, was associated with the production of aPL in our SLE cohort (16) as well as in previous meta-analyses (17,18). Along these lines, HCC develops in the liver following chronic inflammation, which is driven by mitochondrial oxidative stress (19,36) and responds to treatment with rapamycin (48). The remarkable efficacy of rapamycin in abrogating the production of both aCL and anti-$\beta_2$GPI has immense relevance with regard to the treatment of patients with APS who currently require life-long anticoagulation therapy (12). The growing evidence to indicate that mTORC1 blockade by rapamycin extends life expectancy (49) supports the overall safety and benefits of this intervention. Nevertheless, thrombosis should be carefully evaluated as a clinical outcome of rapamycin treatment in patients with and those without SLE. Given that the initiation of aPL production may precede clinical disease in patients with SLE (34,35), the underlying role of liver disease and preventative treatment via blockade of mTORC1 clearly warrant further investigations.

## ACKNOWLEDGMENT

The authors thank Dr. Paul Phillips for continued encouragement and support.

## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Perl had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Oaks, Winans, Caza, Fernandez, Liu, Landas, Banki, Perl.

**Acquisition of data.** Oaks, Winans, Caza, Fernandez, Liu, Landas, Banki, Perl.

**Analysis and interpretation of data.** Oaks, Winans, Caza, Fernandez, Liu, Landas, Banki, Perl.

## REFERENCES


1. Tsokos GC. Systemic lupus erythematosus. N Engl J Med 2011; 365:2110–21.
2. Fernandez D, Bonilla E, Mirza N, Niland B, Perl A. Rapamycin reduces disease activity and normalizes T-cell activation–induced calcium fluxing in patients with systemic lupus erythematosus. Arthritis Rheum 2006;54:2983–8.
3. Fernandez DR, Telarico T, Bonilla E, Li Q, Banerjee S, Middleton FA, et al. Activation of mammalian target of rapamycin controls the loss of TCR$\zeta$ in lupus T cells through HRES-1/Rab4-regulated lysosomal degradation. J Immunol 2009;182:2063–73.
4. Lai ZW, Borsuk R, Shadakshari A, Yu J, Dawood M, Garcia R, et al. Mechanistic target of rapamycin activation triggers IL-4 production and necrotic death of double-negative T cells in patients with systemic lupus erythematosus. J Immunol 2013;191:2236–46.
5. Caza TN, Fernandez D, Talaber G, Oaks Z, Haas M, Madaio MP, et al. HRES-1/RAB4-mediated depletion of Drp1 impairs mitochondrial homeostasis and represents a target for treatment in SLE. Ann Rheum Dis 2014;73:1888–97.
6. Perl A. Oxidative stress in the pathology and treatment of systemic lupus erythematosus. Nat Rev Rheumatol 2013;9:674–86.
7. Sarbassov DD, Sabatini DM. Redox regulation of the nutrient-sensitive raptor-mTOR pathway and complex. J Biol Chem 2005;280:39505–9.
8. Ioannou Y, Zhang JY, Qi M, Gao L, Qi JC, Yu DM, et al. Novel assays of thrombogenic pathogenicity in the antiphospholipid syndrome based on the detection of molecular oxidative modification of the major autoantigen $\beta_2$-glycoprotein I. Arthritis Rheum 2011;63:2774–82.
9. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Antiphospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: $\beta_2$-glycoprotein I (apolipoprotein H). Proc Natl Acad Sci U S A 1990;87:4120–4.
10. Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum 2012;64:2677–86.
11. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295–306.
12. Lockshin MD, Erkan D. Treatment of the antiphospholipid syndrome. N Engl J Med 2003;349:1177–9.
13. Canaud G, Bienaime F, Tabarin F, Bataillon G, Seilhean D, Noel LH, et al. Inhibition of the mTORC pathway in the antiphospholipid syndrome. N Engl J Med 2014;371:303–12.
14. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
15. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus [letter]. Arthritis Rheum 1997;40:1725.
16. Liu Y, Yu J, Oaks Z, Marchena-Mendez I, Francis L, Bonilla E, et al. Liver injury correlates with biomarkers of autoimmunity and disease activity and represents an organ system involvement in patients with systemic lupus erythematosus. Clin Immunol 2015;160:319–27.
17. Asherson RA, Cervera R, Piette JC, Font J, Lie JT, Burcoglu A, et al. Catastrophic antiphospholipid syndrome: clinical and laboratory features of 50 patients. Medicine (Baltimore) 1998;77:195–207.
18. Ambrosino P, Lupoli R, Spadarella G, Tarantino P, di Minno A, Tarantino L, et al. Autoimmune liver diseases and antiphospholipid antibodies positivity: a meta-analysis of literature studies. J Gastrointestin Liver Dis 2015;24:25–34.
19. Hanczko R, Fernandez D, Doherty E, Qian Y, Vas G, Niland B, et al. Prevention of hepatocarcinogenesis and increased susceptibility to acetaminophen-induced liver failure in transaldolase-deficient mice by N-acetylcysteine. J Clin Invest 2009;119:1546–57.
20. Peng SL. Experimental use of murine lupus models. In: Perl A, editor. Methods in molecular medicine: autoimmunity methods and protocols. 1st ed. Totowa (NJ): Humana; 2005. p. 227–72.
21. Perl A, Hanczko R, Doherty E. Assessment of mitochondrial dysfunction in lymphocytes of patients with systemic lupus erythematosus. In: Perl A, editor. Methods in molecular medicine: autoimmunity methods and protocols. 2nd ed. Clifton (NJ): Springer; 2012. p. 61–89.
22. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. Biochem J 2011;435:297–312.
23. Monestier M, Kandiah DA, Kouts S, Novick KE, Ong GL, Radic MZ, et al. Monoclonal antibodies from NZW $\times$ BXSB F1 mice to $\beta_2$ glycoprotein I and cardiolipin: species specificity and charge-dependent binding. J Immunol 1996;156:2631–41.
24. Perl A, Hanczko R, Lai ZW, Oaks Z, Kelly R, Borsuk R, et al. Comprehensive metabolome analyses reveal N-acetylcysteine-responsive accumulation of kynurenine in systemic lupus erythematosus: implications for activation of the mechanistic target of rapamycin. Metabolomics 2015;11:1157–74.
25. Desai BN, Myers BR, Schreiber SL. FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction. Proc Natl Acad Sci U S A 2002;99: 4319–24.
26. Perl A. Activation of mTOR (mechanistic target of rapamycin) in rheumatic diseases. Nat Rev Rheumatol 2016;12:169–82.
27. Kashatus JA, Nascimento A, Myers LJ, Sher A, Byrne FL, Hoehn KL, et al. Erk2 phosphorylation of Drp1 promotes mitochondrial fission and MAPK-driven tumor growth. Mol Cell 2015;57:537–51.
28. Chang CR, Blackstone C. Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J Biol Chem 2007;282:21583–7.
29. Bernatsky S, Boivin JF, Joseph L, Rajan R, Zoma A, Manzi S, et al. An international cohort study of cancer in systemic lupus erythematosus. Arthritis Rheum 2005;52:1481–90.
30. Park SM, Rajapaksha T, Zhang M, Sattar H, Fichera A, Ashton-Rickardt P, et al. CD95 signaling deficient mice with a wild-type hematopoietic system are prone to hepatic neoplasia. Apoptosis 2008;13:41–51.
31. Vance JE. MAM (mitochondria-associated membranes) in mammalian cells: lipids and beyond. Biochim Biophys Acta 2014; 1841:595–609.
32. Talaber G, Miklossy G, Oaks Z, Liu Y, Tooze SA, Chudakov DM, et al. HRES-1/Rab4 promotes the formation of $LC3^+$ autophagosomes and the accumulation of mitochondria during autophagy. PLoS One 2014;9:e84392.
33. Miwa S, Jow H, Baty K, Johnson A, Czapiewski R, Saretzki G, et al. Low abundance of the matrix arm of complex I in mitochondria predicts longevity in mice. Nat Commun 2014;5:3837.
34. Tarr T, Muzes G, Pitlik E, Lakos G, Csepany T, Soltesz P, et al. Is the primary antiphospholipid syndrome a forerunner of SLE? Orv Hetil 2005;146:203–7. In Hungarian.
35. McClain MT, Arbuckle MR, Heinlen LD, Dennis GJ, Roebuck J, Rubertone MV, et al. The prevalence, onset, and clinical significance of antiphospholipid antibodies prior to diagnosis of systemic lupus erythematosus. Arthritis Rheum 2004;50: 1226–32.
36. Perl A, Hanczko R, Telarico T, Oaks Z, Landas S. Oxidative stress, inflammation and carcinogenesis are controlled through the pentose phosphate pathway by transaldolase. Trends Mol Med 2011;17:395–403.
37. Nagy G, Ward J, Mosser DD, Koncz A, Gergely P Jr, Stancato C, et al. Regulation of CD4 expression via recycling by HRES-1/ RAB4 controls susceptibility to HIV infection. J Biol Chem 2006;281:34574–91.

38. Pullmann R Jr, Bonilla E, Phillips PE, Middleton FA, Perl A. Haplotypes of the HRES-1 endogenous retrovirus are associated with development and disease manifestations of systemic lupus erythematosus. Arthritis Rheum 2008;58:532–40.
39. Serasinghe MN, Weider SY, Renault TT, Elkholi R, Asciolla JJ, Yao JL, et al. Mitochondrial division is requisite to RAS-induced transformation and targeted by oncogenic MAPK pathway inhibitors. Mol Cell 2015;57:521–36.
40. Roberts MS, Woods AJ, Shaw PE, Norman JC. ERK1 associates with $\alpha v\beta 3$ integrin and regulates cell spreading on vitronectin. J Biol Chem 2003;278:1975–85.
41. Li Y, Gorelik G, Strickland FM, Richardson BC. Oxidative stress, T cell DNA methylation, and lupus. Arthritis Rheumatol 2014;66:1574–82.
42. Gergely PJ Jr, Grossman C, Niland B, Puskas F, Neupane H, Allam F, et al. Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus. Arthritis Rheum 2002;46:175–90.
43. Quinlan CL, Orr AL, Perevoshchikova IV, Treberg JR, Ackrell BA, Brand MD. Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions. J Biol Chem 2012;287:27255–64.
44. Benard G, Bellance N, James D, Parrone P, Fernandez H, Letellier T, et al. Mitochondrial bioenergetics and structural network organization. J Cell Sci 2007;120:838–48.
45. Kato H, Perl A. Mechanistic target of rapamycin complex 1 expands Th17 and IL-4+ CD4-CD8- double-negative T cells and contracts regulatory T cells in systemic lupus erythematosus. J Immunol 2014;192:4134–44.
46. Lambert M, Boullier A, Hachulla E, Fruchart JC, Teissier E, Hatron PY, et al. Paraoxonase activity is dramatically decreased in patients positive for anticardiolipin antibodies. Lupus 2000;9: 299–300.
47. Delgado Alves J, Ames PR, Donohue S, Stanyer L, Noorouz-Zadeh J, Ravirajan C, et al. Antibodies to high-density lipoprotein and $\beta_2$-glycoprotein I are inversely correlated with paraoxonase activity in systemic lupus erythematosus and primary antiphospholipid syndrome. Arthritis Rheum 2002;46:2686–94.
48. Finn RS. Current and future treatment strategies for patients with advanced hepatocellular carcinoma: role of mTOR inhibition. Liver Cancer 2012;1:247–56.
49. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 2009;460:392–5.