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N-Acetylcysteine Reduces Disease Activity by Blocking Mammalian Target of Rapamycin in T Cells From Systemic Lupus Erythematosus Patients

A Randomized, Double-Blind, Placebo-Controlled Trial

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Objective. **Systemic lupus erythematosus (SLE) patients exhibit T cell dysfunction, which can be regulated through mitochondrial transmembrane potential ($\Delta\psi m$) and mammalian target of rapamycin (mTOR) by glutathione (GSH). This randomized, double-blind, placebo-controlled study was undertaken to examine the safety, tolerance, and efficacy of the GSH precursor *N*-acetylcysteine (NAC).**

Methods. **A total of 36 SLE patients received either daily placebo or 1.2 gm, 2.4 gm, or 4.8 gm of NAC. Disease activity was evaluated monthly by the British Isles Lupus Assessment Group (BILAG) index, the SLE Disease Activity Index (SLEDAI), and the Fatigue Assessment Scale (FAS) before, during, and after a 3-month treatment period. Mitochondrial transmembrane potential and mTOR were assessed by flow cytometry. Forty-two healthy subjects matched to patients for age, sex, and ethnicity were studied as controls.**

Results. **NAC up to 2.4 gm/day was tolerated by all patients, while 33% of those receiving 4.8 gm/day had reversible nausea. Placebo or NAC 1.2 gm/day did not influence disease activity. Considered together, 2.4 gm and 4.8 gm NAC reduced the SLEDAI score after 1 month ($P = 0.0007$), 2 months ($P = 0.0009$), 3 months ($P = 0.0030$), and 4 months ($P = 0.0046$); the BILAG score after 1 month ($P = 0.029$) and 3 months ($P = 0.009$); and the FAS score after 2 months ($P = 0.0006$) and 3 months ($P = 0.005$). NAC increased $\Delta\psi m$ ($P = 0.0001$) in all T cells, profoundly reduced mTOR activity ($P = 0.0009$), enhanced apoptosis ($P = 0.0004$), reversed expansion of CD4−CD8− T cells (mean ± SEM 1.35 ± 0.12-fold change; $P = 0.008$), stimulated FoxP3 expression in CD4+CD25+ T cells ($P = 0.045$), and reduced anti-DNA production ($P = 0.049$).**

Conclusion. **This pilot study suggests that NAC safely improves lupus disease activity by blocking mTOR in T lymphocytes.**

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease that often has debilitating and potentially life-threatening consequences. Although current therapies afford significant clinical improvement, they are increasingly costly, are not curative, and carry significant side effects (1–3). While the cause of SLE is unknown, its pathogenesis clearly involves cellular dysfunction of the immune system and the production of antinuclear autoantibodies (4). Existing data show that a

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natural antioxidant, glutathione (GSH), is depleted in the peripheral blood lymphocytes (PBLs) of patients with SLE (5–7). GSH regulates the elevation of mitochondrial transmembrane potential ($\Delta\psi m$) or mitochondrial hyperpolarization, which in turn activates the mammalian target of rapamycin (mTOR) in lupus T cells (6). The mTOR skews cell death signal processing, modulates T cell differentiation (8,9), and, in particular, inhibits the development of CD4+CD25+FoxP3+ Treg cells (10), which are deficient in patients with active SLE (11,12). Blockade of mTOR with rapamycin, a potent and expensive immunosuppressant, improved disease activity in murine lupus (13) and in patients with SLE (14). *N*-acetylcysteine (NAC), which acts as a precursor of GSH and as an antioxidant in and of itself, inhibited mTOR in vitro (15) and improved the outcome of murine lupus in vivo (16).

GSH is a tripeptide composed of cysteine, glutamic acid, and glycine. The availability of cysteine is rate-limiting for GSH synthesis (17). NAC is the acetylated form of L-cysteine; compared to other forms of cysteine supplementation, it has the advantages of resistance to oxidation and permeability through the cell membrane (17). NAC can effectively raise intracellular GSH levels in lymphocytes both in vitro (18) and in vivo (19). Although NAC is relatively inexpensive (~$150/kg), it is currently unavailable as oral medication by prescription or over the counter. However, NAC is used as an antioxidant, and it is widely accessible in health food stores. In a European study of idiopathic pulmonary fibrosis patients, "high-dose" oral NAC (1.8 gm/day) diminished disease severity and reduced the toxicity of pro-oxidant and immunosuppressant drugs (20) commonly used in patients with SLE (1). Similar doses of NAC improve muscle fatigue (21), which is reported to be the most disabling symptom in 53% of SLE patients (22). Therefore, we initiated this double-blind, placebo-controlled, phase I/II study to evaluate the metabolic, immunologic, and therapeutic impact of NAC in 36 SLE patients. Importantly, NAC was found to be safe, and it improved disease activity and fatigue by profoundly blocking mTOR and expanding the CD4+CD25+FoxP3+ T cell population.

PATIENTS AND METHODS

Clinical study design. Thirty-six SLE patients with stable disease were enrolled in a double-blind placebo-controlled trial of treatment with NAC (FDA Investigational New Drug application no. 101,320; clinicaltrials.gov identifier NCT00775476). The mean ± SEM age of the patients was 44.6 ± 1.8 years (range 25–64 years). (See Supplementary Table 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.) Of the 36 patients, 34 were women (30 white patients, 2 African American patients, and 2 Hispanic patients). Two patients were white men. Forty-two healthy subjects were individually matched at each patient blood donation for the patient's age within 10 years, sex, and ethnic background; patient and matched control cells were isolated and studied in parallel for immunologic studies. The mean ± SEM age of controls was 44.4 ± 1.7 years (range 22–63 years). Thirty-nine of the controls were women (36 white subjects, 2 African American subjects, and 1 Hispanic subject). Three controls were white men.

SLE patients were randomized to receive either placebo or NAC in 1 of 3 treatment arms of increasing doses: 600 mg, 1,200 mg, or 2,400 mg twice daily for 3 months. Twelve patients were enrolled in each dosing group; 9 received NAC while 3 received placebo. We used the following dose-progression rule: 6 of 8 patients with active disease had to tolerate each dose and show no worsening of SLE as defined in the Data Safety and Monitoring Plan to proceed to the next higher dose.

Inclusion criteria. Male and female patients older than 18 years of age were included in the study. Patients had to fulfill ≥4 of the 11 American College of Rheumatology diagnostic criteria for SLE (23) and have clinically stable disease while taking one of the following allowable immunosuppressants: prednisone (≤10 mg/day), antimalarials, azathioprine, or mycophenolate mofetil.

Exclusion criteria. Patients who were pregnant or lactating, had moderately serious or serious comorbidities (e.g., diabetes mellitus, congestive heart failure, chronic obstructive pulmonary disease, or chronic renal insufficiency), a history of chronic infections (e.g., human immunodeficiency virus, hepatitis B virus, hepatitis C virus, mycobacteria, or bronchiectasis), infections in the past month, a history of severe or recurrent infections, or were current smokers were excluded. Patients taking over-the-counter antioxidants that can enhance the effect of NAC were excluded. Alternatively, patients taking acetaminophen, which is metabolized by hepatic cytochrome P450 enzymes, primarily CYP2E1, to a toxic intermediate compound (*N*-acetyl-para benzoquine imide) requiring detoxification by hepatic GSH (24), were also excluded. Patients were allowed 1 daily dose of multivitamin containing ≤500 mg of vitamin C and ≤30 IU of vitamin E. Patients with an acute flare of SLE threatening vital organs and requiring intravenous cyclophosphamide treatment were excluded. Patients receiving biologic agents (rituximab, abatacept) and those enrolled in other clinical trials were also excluded.

Study materials. Identical-appearing capsules containing NAC or placebo (dextrose) were manufactured by the compounding pharmacy within the Department of Pharmacy at State University of New York Upstate Medical Center. Both NAC and dextrose were obtained from Spectrum Chemical Manufacturing Corporation. Each capsule contained 600 mg of NAC or placebo. All capsules were rolled in NAC to equalize smell. Each bottle contained the number of capsules needed for 32 days. The pills were counted when the bottles were returned to ascertain compliance. Randomization of patients was performed by the pharmacist. The principal investigator, biostatistician, and research staff remained

blinded with regard to the participants' treatment group assignments.

Study visits. Routine and lupus-specific clinical and laboratory data were acquired during 5 monthly visits. At visit 1, a baseline assessment was conducted before administration of the first NAC or placebo capsule. Three hours and 6 hours after administration of the first capsule, additional blood samples were obtained for GSH assay. Patients were provided with capsules for the first month of the study. Visit 2 took place after 1 month of treatment, and assessments were conducted before patients took that morning's capsule. Patients were provided with the second batch of monthly capsules. Visit 3 took place after 2 months of treatment, and assessments were conducted before patients took that morning's capsule. Patients were provided with capsules for the third month. Visit 4 took place after 3 months of treatment, and visit 5 took place after 4 months (at the end of a 1-month washout period).

For each patient visit, we obtained blood from healthy donors matched for age (within 1 decade), sex, and ethnicity, to be used as a control for flow cytometric measurement of mitochondrial function, T cell activation and death pathway selection, Ca^{2+} flux, production of nitric oxide (NO) and reactive oxygen intermediates (ROIs), activation of mTOR, and expression of FoxP3 in subsets of T cells and B cells. GSH was measured in whole blood and isolated PBLs by high-performance liquid chromatography (HPLC). Each patient provided 7 blood samples (visit 1 at 0 hours, visit 1 at 3 hours, visit 1 at 6 hours, visit 2 after 1 month, visit 3 after 2 months, visit 4 after 3 months, and visit 5 after 4 months [after 1 month of washout]). Forty-two healthy controls also donated blood for use as control samples for HPLC analysis of GSH, flow cytometry of live cells, and gene expression and signaling studies. We recorded ~384 flow cytometry data points for each of the 5 patient visits, both for the patients and the matched controls. DNA, RNA, and protein lysates obtained at each visit were saved and catalogued. Individual controls gave blood on multiple occasions.

Clinical outcomes and assessments. *Tolerance.* At each visit, patients were specifically asked about common side effects (nausea, bloating, and bad taste) seen in prior trials. These side effects were reviewed by our Data Safety and Monitoring Board (DSMB) biannually. Tolerance and safety were primary clinical outcomes.

Blinding. Patients were asked about the smell or taste of the capsules in order to ascertain whether they believed themselves to be in the NAC group or the placebo group.

Clinical assessments. A complete physical examination was performed before enrollment. A directed physical examination of the cardiovascular, respiratory, gastrointestinal, musculoskeletal, and neurologic systems, skin, head, neck, sinuses, and nasal and oral cavities was performed at each visit. SLE disease activity was assessed using the SLE Disease Activity Index (SLEDAI) (25) and the British Isles Lupus Assessment Group (BILAG) index (26). We documented concurrent use and dosage of other medications. Improvements in SLEDAI or BILAG disease scores were secondary clinical outcomes.

Fatigue. Fatigue was assessed by using a validated Fatigue Assessment Scale (FAS), a self-administered questionnaire that provides a subjective measurement of fatigue severity and has been shown to have a high degree of internal consistency, validity, and sensitivity to changes in clinical condition (27). Improvement in FAS score was a secondary clinical outcome.

Blood tests. Routine blood tests included complete blood cell count, liver and kidney function tests, urinalysis, and lupus-relevant laboratory tests, such as tests for anti–double-stranded DNA, C3, and C4.

Compliance. Details on patient compliance with study procedures are available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

Immunobiologic outcomes and assessments. The primary outcome was a measurable increase in GSH level in PBLs by HPLC (5). Secondary outcomes, including modulation of $\Delta\psi$m, ROI production (oxidative stress), apoptosis (5), mTOR activity (6), and FoxP3 expression (10), were assessed by flow cytometry.

Statistical analysis. Power and sample size requirements for this study were based on a Type I error rate of 0.05, 2-tailed testing, and a minimal power level of 0.80, using Sample Power software version 2 (SPSS). Estimates of effect size were based on our preliminary data (5) and the relevant literature to compare mean values of GSH across treatment groups (placebo, lowest NAC dose, medium NAC dose, and highest NAC dose). Our analysis suggested that administration of NAC to a minimum of 8 patients per treatment arm should have 83.7% power to detect a 42% elevation in intracellular GSH in SLE patients (mean ± SEM 3.60 ± 0.30 ng/μg protein) to levels found in normal donors (5.11 ± 0.50 ng/μg protein) (5). This study compared the longitudinal effects of 3 different doses of NAC and a placebo control condition before a 3-month intervention (visit 1), during the 3 months of treatment (visit 2 [after 1 month], visit 3 [after 2 months], and visit 4 [after 3 months]), and after the 3-month intervention (visit 5 [after 1 month of washout]). Thus, we used a double-blind longitudinal trial design comparing 4 groups with regard to data collected at intervals before, during, and after intervention.

Overall clinical effectiveness of NAC relative to placebo was analyzed with multilevel modeling as implemented in the Stata routine XTMIXED (StataCorp), with the 3 nested levels being drug group, subject within drug group, and study visit within subject. All models included fixed effects for drug group, study visit, and the drug group–by–study visit interaction, along with random intercepts at each design level. Our test for efficacy was the fixed effect for the drug group–by–study visit interaction, which if significant indicated that the change in outcome scores over time was significantly different among drug groups. A paired 2-tailed *t*-test was used to assess the effects of placebo, of each NAC dose, and of all NAC doses combined on the values for clinical indices and biomarkers recorded at visits 2–5 relative to those at visit 1. *P* values less than 0.05 were considered significant. Patients and controls were compared using an unpaired 2-tailed *t*-test.

RESULTS

Patient enrollment and tolerance of NAC. None of the patients in dosing group 1 (NAC 1.2 gm/day; n = 12) or dosing group 2 (NAC 2.4 gm/day; n = 12)

reported unpleasant smell or taste, and all 24 patients completed the treatment. Three patients dropped out of dosing group 3 (NAC 4.8 gm/day) (Supplementary Table 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131) due to heartburn after 19 days, nausea after 60 days, and nausea and headaches after 76 days. The third patient continued to take NAC after day 76 by reducing the original number of capsules to half and thus halving the original dosage to 2.4 gm/day. Since all 3 of the patients who reported intolerance were receiving NAC, in accordance with the Data Safety and Monitoring Plan, no higher dose was initiated. After the last study visit for dosing group 3, the study was unblinded on November 30, 2010.

Effect of NAC on disease activity. Lupus disease activity was measured by the SLEDAI and the BILAG index, and fatigue was evaluated by the FAS, at baseline (visit 1) as well as monthly during a 3-month intervention (visits 2–4) and after a 1-month washout period (visit 5) (Figure 1). Placebo or NAC dose 1 did not influence SLEDAI, BILAG, or FAS scores (Figure 1). NAC doses 2 and 3 reduced the SLEDAI score (Figure 1A). In NAC dosing group 3, we observed a significant increase in the number of patients in whom SLEDAI scores improved by 3 or more (2 of 9 patients in the placebo group; 3 of 9 patients in NAC dosing group 1; 4 of 9 patients in NAC dosing group 2; and 5 of 6 patients in NAC dosing group 3 [$P = 0.0406$ versus placebo, by Fisher's exact test]).

In the combined group of all patients treated with NAC, the SLEDAI score was improved from 5.3 at baseline (visit 1) to 3.5 after 1 month (visit 2; $P = 0.0013$) and to 3.7 after 2 months of treatment (visit 3; $P = 0.048$) (Figure 1A). In the combined group of patients treated with NAC dose 2 and those treated with NAC dose 3, the SLEDAI score improved from 5.78 at baseline on all followup visits (3.6 at visit 2 [$P = 0.0007$], 4.0 at visit 3 [$P = 0.0009$], 4.9 at visit 4 [$P = 0.0030$], and 4.4 at visit 5 [$P = 0.0046$]). Using multilevel modeling, the reduction in the SLEDAI score was greater in the NAC-treated group than in the placebo-treated group, as indicated by a significant visit-by-drug interaction (XTMIXED $z = -2.14$, $P = 0.033$). Among biomarkers of disease activity, the mean ± SEM anti-DNA level was reduced in the combined group of all patients exposed to NAC, from 78.9 ± 45.2 IU/ml at baseline to 19.5 ± 6.0 IU/ml after 1 month ($P = 0.049$). C3 and C4 were not affected.

In the combined group of all patients treated with NAC, the BILAG score improved from 26.2 at baseline

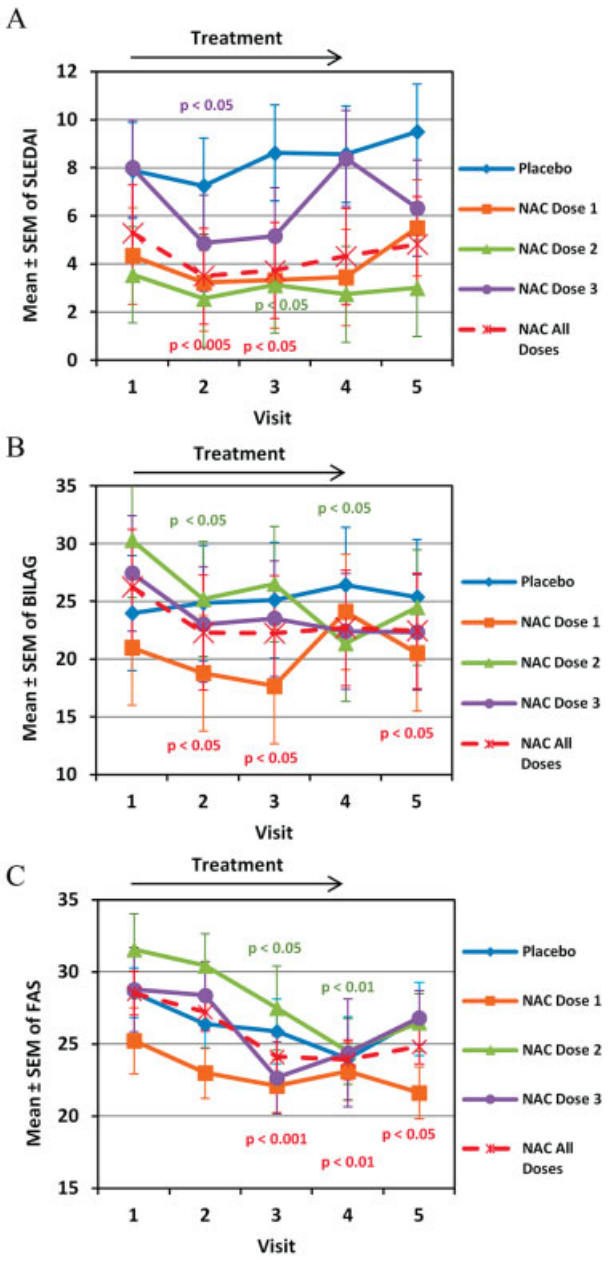

Figure 1. Effect of *N*-acetylcysteine (NAC) and placebo on disease activity in patients with systemic lupus erythematosus (SLE), as measured by **A,** SLE Disease Activity Index (SLEDAI), **B,** British Isles Lupus Assessment Group (BILAG) index, and **C**, Fatigue Assessment Scale (FAS) scores. A total of 36 SLE patients were randomized to receive placebo (n = 9), NAC 1.2 gm/day (NAC dose 1; n = 9), NAC 2.4 gm/day (NAC dose 2; n = 9), or NAC 4.8 gm/day (NAC dose 3; n = 9). The combined group of patients who were receiving any NAC dose (NAC all doses; n = 27) was also analyzed. *P* values indicate comparisons of pretreatment values (visit 1) to values after 1 month (visit 2), 2 months (visit 3), 3 months (visit 4), or 4 months (visit 5 [3 months of treatment followed by a 1-month washout period]), determined by paired 2-tailed *t*-test.

(visit 1) to 22.3 after 1 month (visit 2; $P = 0.0158$) and to 22.2 after 2 months (visit 3; $P = 0.0223$) (Figure 1B). In the combined group of patients treated with NAC dose 2 (2.4 gm/day) and those treated with NAC dose 3 (4.8 gm/day), the BILAG score was reduced from 28.8 at baseline to 24.2 after 1 month ($P = 0.029$) and to 21.7 after 3 months ($P = 0.009$). Among the BILAG components reflecting organ system involvement, the swollen joint count was reduced after 3 months of treatment in patients receiving NAC dose 3 (XTMIXED z = −2.0, $P = 0.046$) and in the combined group of patients receiving any NAC dose (XTMIXED z = −2.2, $P = 0.028$). The reduction in the BILAG score was also greater for NAC-treated groups relative to the placebo-treated group, as indicated by a significant visit-by-drug interaction in mixed model analysis (XTMIXED z = −2.62, $P = 0.009$). This analysis also showed a significant reduction in the BILAG score in patients receiving NAC dose 3 (4.8 gm/day; XTMIXED z = −2.19, $P = 0.029$).

In the combined group of all SLE patients treated with NAC, the FAS score was improved from 28.5 at visit 1 to 24.1 at visit 3 ($P = 0.0006$), 23.9 at visit 4 ($P = 0.005$), and 24.8 at visit 5 ($P = 0.034$) (Figure 1C). Mixed model analysis showed a reduction in the FAS score in NAC dosing group 2 relative to the placebo group (XTMIXED z = −2.08, $P = 0.038$).

Effect of NAC on GSH levels in whole blood and PBLs. At baseline (visit 1, 0 hours), GSH levels were similar in whole blood samples from lupus patients and those from healthy donors. In contrast, GSH levels were reduced in PBLs from lupus patients (Figure 2A). NAC treatment increased GSH levels in whole blood samples from SLE patients after 1 and 2 months of treatment (Figure 2B). In the PBLs of SLE patients, the GSH level was increased 6 hours after a single 1.2 gm dose of NAC ($P = 0.022$) (Figure 2C) and was increased 3 hours ($P = 0.032$) and 6 hours ($P = 0.003$) after a single 2.4 gm dose of NAC (Figure 2C). Although the difference in GSH levels between the 0-hour and 6-hour time points in the placebo group was not statistically significant ($P = 0.053$), we observed an upward trend that was attributed to the fact that the 0-hour sample was obtained after fasting (between ~8:00 AM and 9:00 AM), while the 6-hour post–NAC treatment sample was obtained after 1 or 2 meals (between 2:00 PM and 3:00 PM). These changes were consistent with diurnal variation and peaking of GSH levels in the early afternoon hours due to nutritional factors (28). The GSH level was increased in PBLs in the combined group of SLE patients treated with either NAC dose 2 or NAC dose 3 (NAC-all in Figure 2) for 3 months (visit 4; $P = 0.027$). After the 1-month washout period, GSH levels in PBLs dropped below baseline levels in lupus patients exposed to NAC dose 3 and in the combined group of those exposed to dose 2 or dose 3 (Figure 2C). Placebo did not influence GSH levels in whole blood or PBLs (Figure 2).

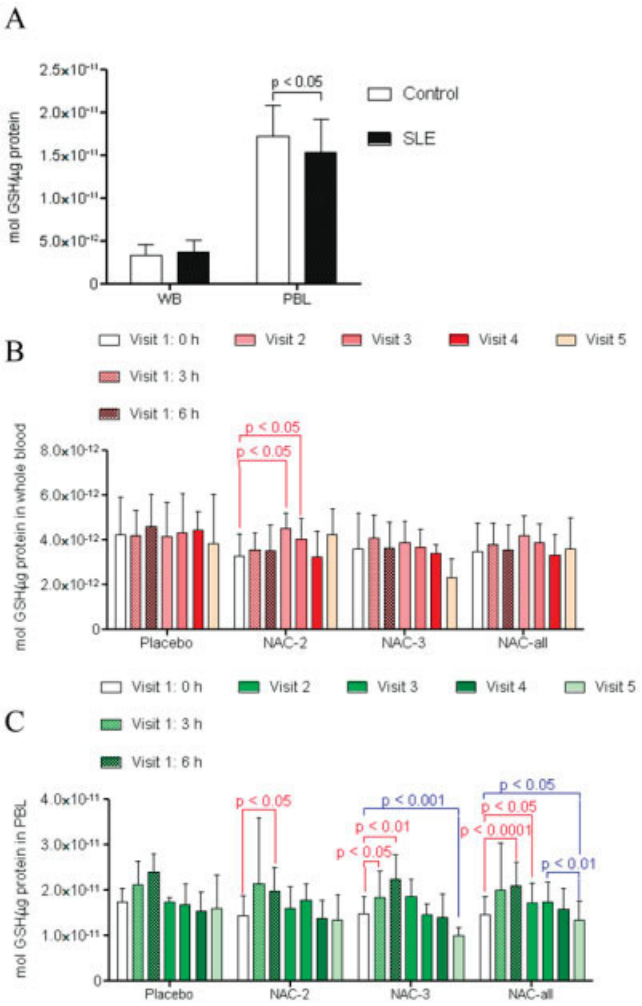

Figure 2. Effect of *N*-acetylcysteine (NAC) on glutathione (GSH) levels in whole blood (WB) and peripheral blood lymphocyte (PBL) samples from patients with systemic lupus erythematosus (SLE). **A,** High-performance liquid chromatography analysis of GSH levels in whole blood and PBL samples from untreated SLE patients (n = 36) and healthy controls matched for age, sex, and ethnicity (n = 42). **B,** Effect of NAC and placebo on GSH levels in whole blood samples from SLE patients treated as indicated. **C,** Effect of NAC and placebo on GSH levels in PBL samples from SLE patients treated as indicated. Values are the mean ± SEM. *P* values were determined by paired 2-tailed *t*-test.

NAC increases mitochondrial hyperpolarization, mitochondrial mass, and apoptosis of CD4−CD8− double-negative T cells in SLE patients. Consistent with the findings of previous studies (5,29), mitochondrial hyperpolarization was detected in T cells from lupus patients. Interestingly, NAC treatment progressively increased mitochondrial mass and hyperpolarization ($P = 0.0001$) of T cells (Figure 3A). Mitochondrial mass (Figure 3B) and H_2O_2 levels (Figure 3C) were increased in double-negative T cells after 3 months of NAC treatment and declined after washout. These changes were attributed to enhanced production of NO (mean ± SEM fold change 1.61 ± 0.17 after 3 months; $P = 0.002$) (Figure 3D). Mitochondrial mass was also robustly increased in double-negative T cells following CD3/CD28 costimulation (Figure 3E).

The mean ± SEM spontaneous apoptosis rate of double-negative T cells was progressively increased in NAC-treated patients, from 10.1 ± 1.3% at baseline to 15.2 ± 2.3% after 4 months ($P = 0.035$) (Figure 3F). The CD3/CD28-induced apoptosis rate was also increased in NAC-treated patients, from 16.0 ± 1.6% at baseline to 25.4 ± 2.6% after 2 months ($P = 0.0006$), 23.5 ± 2.1% after 3 months ($P = 0.0004$), and 22.7 ± 2.6% after 4 months ($P = 0.0313$) (Figure 3G). NAC moderated the expansion of double-negative T cells from 6.2 ± 0.5% at baseline to 5.3 ± 0.5% after 3 months ($P = 0.043$). The mean ± SEM 1.35 ± 0.12-fold expansion of CD4−CD8− double-negative T cells in patients with SLE relative to matched healthy controls ($P = 0.008$) was eliminated by NAC treatment. Mitochondrial homeostasis, oxidative stress, and apoptosis in T cell subsets from lupus patients were not affected by placebo (data not shown).

NAC blocks mTOR activation and stimulates FoxP3 expression in T cells from SLE patients. Suppression of mTOR by rapamycin (6) was associated with improved disease activity in SLE (14). In this study, increased mTOR activity was evidenced by a mean ± SEM 2.2 ± 0.45-fold greater prevalence of phosphorylated ribosomal protein S6 (p-RPS6^{high}) T cells in SLE patients ($P = 0.007$). The absolute frequency of

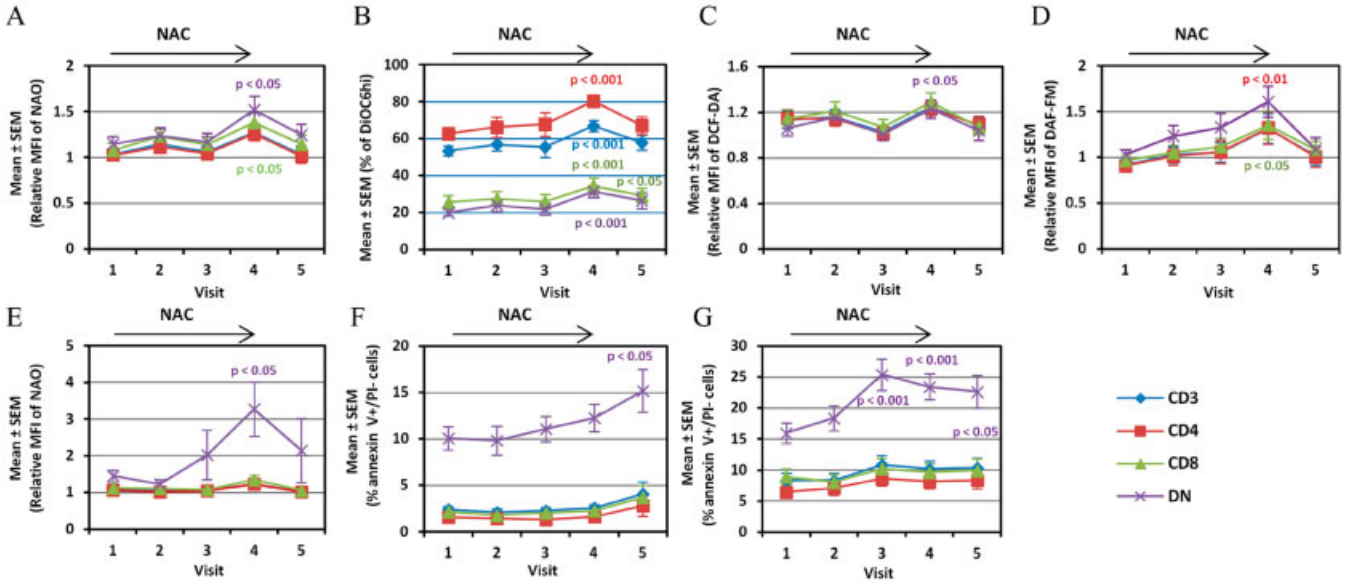

Figure 3. Mitochondrial homeostasis, oxidative stress, and apoptosis in T cell subsets in the combined group of systemic lupus erythematosus patients exposed to any dose of *N*-acetylcysteine (NAC). **A–C,** Effects of NAC on mitochondrial mass, determined by nonyl acridine orange (NAO) fluorescence (**A**), mitochondrial transmembrane potential, determined by 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) fluorescence (**B**), and H_2O_2 levels, determined by dichlorofluorescein diacetate (DCF-DA) fluorescence (**C**), in T cells allowed to rest in culture for 16 hours. **D** and **E,** Effects of NAC on nitric oxide production, determined by 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) fluorescence (**D**), and mitochondrial mass, determined by NAO fluorescence (**E**), in T cell subsets following CD3/CD28 stimulation for 16 hours. **F,** Spontaneous apoptosis rate, enumerated by the percentage of annexin V+/propidium iodide (PI)− T cells after culture for 16 hours. **G,** Activation-induced apoptosis, assessed following CD3/CD28 costimulation for 16 hours. *P* values indicate comparisons of pretreatment values (visit 1) to values after 1 month (visit 2), 2 months (visit 3), 3 months (visit 4), or 4 months (visit 5 [3 months of treatment followed by a 1-month washout period]), determined by paired 2-tailed *t*-test. MFI = mean fluorescence intensity; DN = double-negative.

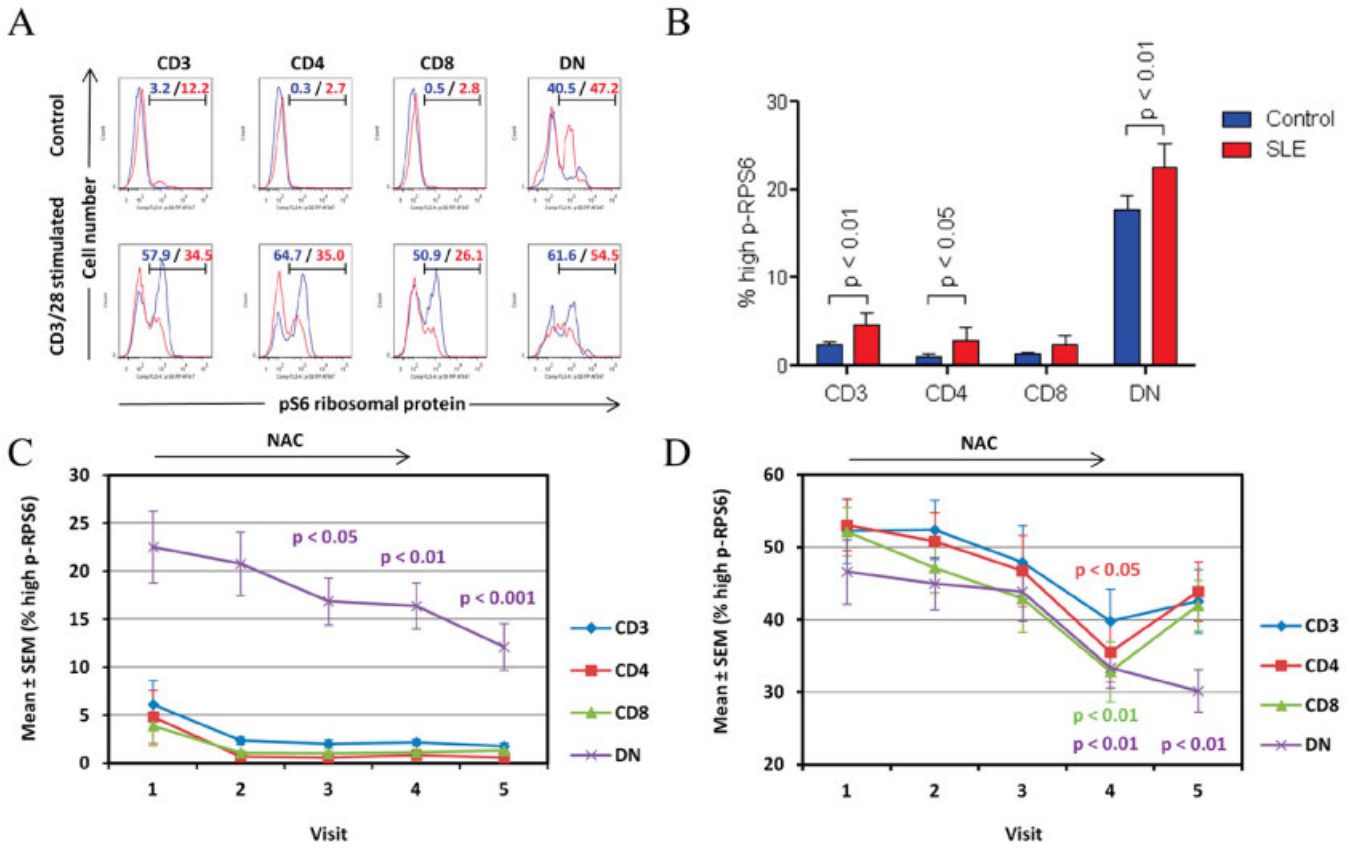

Figure 4. Detection of increased mammalian target of rapamycin (mTOR) activity via phosphorylation of ribosomal protein S6 (p-RPS6) in T cell subsets from systemic lupus erythematosus (SLE) patients and matched controls. **A,** Assessment of p-RPS6 in CD3+, CD4+, CD8+, and double-negative (DN) T cells from controls (blue histograms) and SLE patients (red histograms). Values are the percentage of cell populations with increased mTOR activity. **B,** Cumulative analysis of mTOR activity in T cell subsets in all SLE patients before treatment relative to all healthy controls. Values are the mean ± SEM percentage of cell populations with increased mTOR activity. *P* values were determined by unpaired 2-tailed *t*-test. **C,** Effect of *N*-acetylcysteine (NAC) on mTOR activity, measured by the prevalence of p-RPS6^{high} T cells, in the combined group of SLE patients exposed to any dose of NAC. **D,** Effect of NAC on CD3/CD28-induced mTOR activity, measured by the prevalence of p-RPS6^{high} T cells, in the combined group of SLE patients exposed to any dose of NAC. *P* values indicate comparison to pretreatment values (visit 1), determined by paired 2-tailed *t*-test.

p-RPS6^{high} T cells was greatest in the double-negative compartment (Figures 4A and B). NAC depleted p-RPS6^{high} cells in the double-negative T cell compartment, from 22.5 ± 3.7% at baseline to 16.9 ± 2.5% after 2 months ($P = 0.0104$), 16.4 ± 2.4% after 3 months ($P = 0.0095$), and 12.1 ± 2.4% after 4 months ($P = 0.0009$) (Figure 4C). CD3/CD28-induced mTOR activation was diminished in all T cells after 2 months and 3 months of NAC treatment, but rebounded after washout (Figure 4D). Placebo did not affect mTOR activity (data not shown). CD3/CD28-stimulated mTOR activity declined in double-negative T cells at visit 2 in the placebo group (data not shown); however, this effect did not progress and was not sustained over time and, therefore, it was not considered biologically significant.

Before treatment, FoxP3+ cells were reduced within the CD25+ T cell compartment in SLE patients relative to healthy controls ($P = 6.0 \times 10^{-5}$) (Figures 5A and B). FoxP3+ cells within the CD4+CD25+ T cell compartment were reduced in SLE patients compared to controls (37.8 ± 2.4% versus 47.2 ± 2.3%; $P = 9.1 \times 10^{-5}$) (Figure 5B), as were FoxP3+ cells within the CD8+CD25+ T cell compartment (10.7 ± 2.0% in SLE patients versus 26.7 ± 4.4% in controls; $P = 0.002$) (Figure 5B). Since rapamycin expanded CD4+CD25+ FoxP3+ T cells in patients with SLE (30), it was

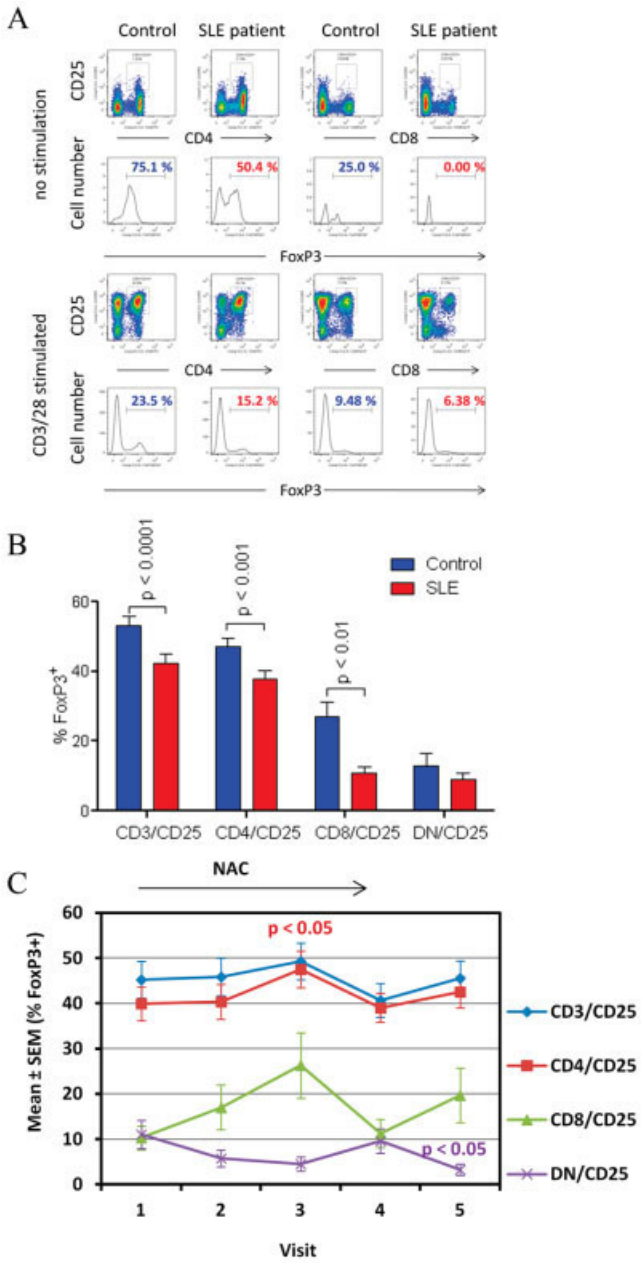

Figure 5. *N*-acetylcysteine (NAC) stimulation of FoxP3 expression in T cells from systemic lupus erythematosus (SLE) patients. **A,** Flow cytometric analysis of FoxP3 expression in CD4+CD25+ and CD8+CD25+ T cell subsets from SLE patients and control donors matched for age, sex, and ethnicity. Values are the percentage of FoxP3+ cells. **B,** Cumulative analysis of FoxP3 expression in CD25+ T cell subsets in SLE patients and matched controls. Values are the mean ± SEM. *P* values were determined by paired 2-tailed *t*-test. **C,** Effect of NAC on FoxP3 expression in CD25+ T cell subsets in the combined group of SLE patients exposed to any dose of NAC. *P* values indicate comparison to pretreatment values (visit 1), determined by paired 2-tailed *t*-test. DN = double-negative.

important to determine whether mTOR blockade by NAC affected FoxP3 expression. Indeed, the percentage of FoxP3+ cells within the CD4+CD25+ T cell compartment was increased in all NAC-treated patients ($P = 0.045$) (Figure 5C). FoxP3 expression was not affected in patients exposed to placebo (data not shown). CD4+CD25+FoxP3+ T cell populations were expanded in patients exposed to NAC 4.8 gm/day, from 3.2 ± 0.6% at baseline to 5.2 ± 0.9% after 2 months ($P = 0.018$). FoxP3 expression was also induced in CD25+ double-negative T cells in patients receiving NAC 4.8 gm/day, from 1.29 ± 0.23% at baseline to 2.24 ± 0.38% after 2 months of NAC treatment ($P = 0.0160$).

DISCUSSION

This double-blind, placebo-controlled phase I/pilot study provides evidence that NAC up to 2.4 gm/day is safe and tolerated by all SLE patients, while reversible nausea occurred in 33% of patients receiving NAC 4.8 gm/day. The low GSH level in PBLs, but not in whole blood, suggests that metabolic dysfunction in lupus (9) is confined to the immune system. NAC increased the GSH level in PBLs, and, most importantly, it improved disease activity in SLE patients through a newly identified molecular mechanism: disruption of the mitochondrial hyperpolarization/mTOR pathway in T cells (Figure 6).

Mitochondrial transmembrane potential is subject to regulation by oxidation–reduction equilibrium of ROIs, pyridine nucleotides (NADH/NAD + NADPH/NADP), and GSH (9). NAC may modulate mitochondrial hyperpolarization directly, via neutralizing ROIs, or indirectly, via sparing NADPH and promoting de novo GSH production. Interestingly, in the present study $\Delta\psi$m and mitochondrial mass were increased in T cells by NAC, particularly in the double-negative compartment, with reversal of these changes after washout. NAC-induced mitochondrial hyperpolarization occurred with a marked increase in NO production, which is required for mitochondrial biogenesis (29). In turn, NO production depends on the availability of NADPH; thus, increased NO production may have resulted from sparing of NADPH by NAC (9).

Since mTOR is a sensor of $\Delta\psi$m and oxidative stress in the T cells of lupus patients (6) and its blockade by rapamycin was associated with improvement of disease activity (14), blocking of mTOR may be critical for the mechanism of action of NAC. Similar to the effect

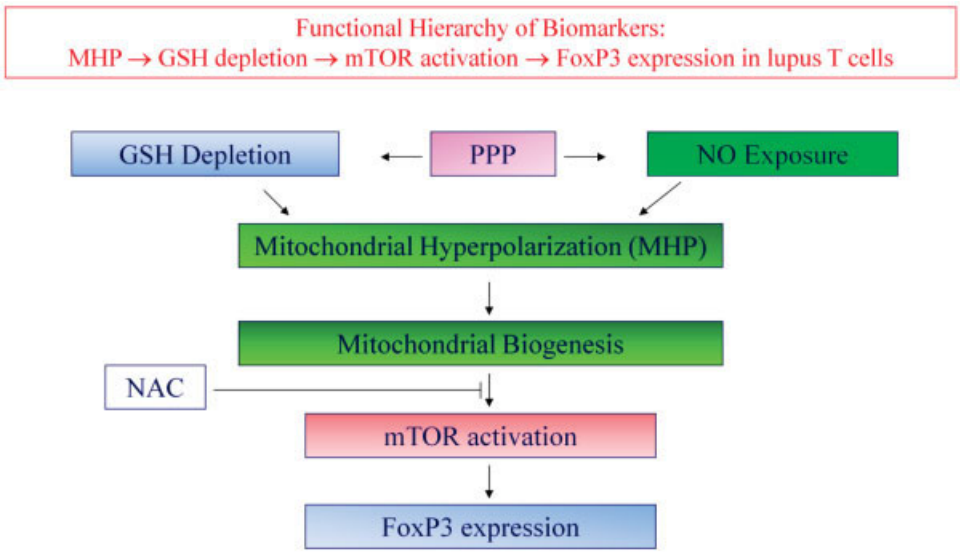

Figure 6. Schematic diagram of the functional hierarchy of metabolic biomarkers of T cell dysfunction in patients with systemic lupus erythematosus, depicting the proposed site of impact of *N*-acetylcysteine (NAC). Mitochondrial hyperpolarization (MHP) is caused by exposure to nitric oxide (NO). De novo synthesis of NO and maintenance of glutathione (GSH) in reduced form are both dependent on the production of NADPH by the pentose phosphate pathway (PPP). Mitochondrial hyperpolarization causes mammalian target of rapamycin (mTOR) activation, which in turn controls the expression of the transcription factor FoxP3.

of rapamycin (30), suppression of mTOR by NAC was accompanied by increased FoxP3 expression in CD4+ CD25+ T cells. These results suggest that the effect of NAC on the immune system is cell type–specific and that it occurs through disconnecting the activation of mTOR from the elevation of $\Delta\psi m$ in the T cells of lupus patients, similar to the effect of rapamycin (14). Such a direct inhibitory effect of NAC was confirmed by blocking of CD3/CD28 stimulation–induced mTOR activity in normal PBLs upon pretreatment by NAC in vitro (data not shown).

Mitochondrial hyperpolarization of the T cells of lupus patients, which most prominently affects double-negative T cells, was associated with resistance to activation-induced apoptosis (5). In a total of 27 SLE patients receiving daily NAC doses of either 1.2 gm, 2.4 gm, or 4.8 gm, both spontaneous and CD3/CD28-induced apoptosis of double-negative T cells were markedly increased, and the expansion of these cells was effectively reversed. The elimination of double-negative T cells, which are known to promote anti-DNA autoantibody production by B cells (31) and nephritis (32), is likely to contribute to reduced anti-DNA titers and to the efficacy of NAC.

The therapeutic importance of NAC for SLE is reflected by the achievement of clinical improvement within 3 months, as assessed by 2 validated disease activity scores; diminishing fatigue (21), which is considered the most disabling symptom in a majority of SLE patients (22); the absence of significant side effects; and the affordability of this medication. A monthly supply of 600-mg NAC capsules (120–240 capsules) costs $15–30 on the retail market. This sharply contrasts with the estimated average annual direct medical costs of ~$22,580 per patient in 2009 (33). Thus, the cost of NAC at $180–360/year would be negligible compared to the overall expense to society caused by the disease and the expected benefit in reducing the need for vastly more expensive medications burdened with potentially serious side effects. While this is a successful proof-of-concept study, it has limitations and warrants followup investigations in larger cohorts, including patients with severe lupus, with longer durations of treatment. In summary, the selectivity of NAC for mTOR activation in T cells provides a safe, inexpensive, alternative, mechanism-driven, and potentially synergistic approach to B cell blockade in SLE (1–3).

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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.

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