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# Oxidative stress and Treg depletion in lupus patients with anti-phospholipid syndrome☆

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**Abstract** Anti-phospholipid antibodies (APLA) represent a diagnostic criterion of systemic lupus erythematosus (SLE) and cause morbidity, termed anti-phospholipid syndrome (APS). Activation of the mechanistic target of rapamycin (mTOR) has been recently associated with APS. mTOR is a sensor of oxidative stress. Therefore, we examined mitochondrial mass, superoxide production, mTOR activity and FoxP3 expression in 72 SLE patients, twelve of whom also had APS, and 54 healthy controls by flow cytometry. Mitochondrial mass was increased in $CD4^-CD8^-$ double-negative (DN) T cells of SLE patients with APS (2.7-fold) in comparison to those without APS (1.7-fold; $p = 0.014$). Superoxide production was increased in all lymphocyte subsets of APS patients. $FoxP3^+$ cells were depleted within $CD4^+CD25^+$ Tregs in patients with APS (28.4%) relative to those without APS (46.3%, $p = 0.008$). mTOR activity was similar between SLE patients with and without APS. Thus, oxidative stress and Treg depletion rather than mTOR activation underlie APS in patients with SLE.


## 1. Introduction

The pathogenesis of systemic lupus erythematosus (SLE) is incompletely understood which limits the development of effective treatments [1]. As recently recognized, SLE patients exhibit activation of the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) in T cells that can be reversed with clinical efficacy upon treatment with rapamycin [2–5]. The activation of mTORC1 has been attributed to oxidative stress both outside [6,7] and within the immune system [3,5,8]. Oxidative stress originates in mitochondria of lupus T cells [8–10] which accumulate both in patients and mice with SLE [11]. In turn, oxidative stress is associated with depletion of the intracellular antioxidant, glutathione [9]. Importantly, the reversal of glutathione depletion with N-acetylcysteine (NAC) also blocked mTORC1

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with promising therapeutic efficacy in a randomized double-blind placebo controlled pilot study of SLE patients [5]. mTORC1 has been also identified as a metabolic sensor of oxidative stress in SLE [3,6,8] and principal driver of pro-inflammatory expansion of $CD4^{-}CD8^{-}$ double-negative (DN) T cells [1,4,12–14]. These findings are in line with recent discoveries that the metabolic pathways which control mTOR activation are key regulators of T-cell lineage specification both under physiological conditions and in autoimmunity [15–18].

Anti-phospholipid antibodies (APLA) represent a component of the diagnostic criteria for SLE [19–21]. They also contribute to significant pathologies, termed anti-phospholipid syndrome (APS), both in patients with or without SLE [22]. Oxidative stress has been recently implicated in driving the immunogenicity of phospholipid antigens [23–25]. In a retrospective study of 10 patients with APS nephropathy, who required renal transplantation and received treatment with rapamycin, also known as sirolimus, 7 of 10 patients (70%) had a functioning allograft 144 months after transplantation versus 3 of 27 patients treated without rapamycin (11%) [26]. The efficacy of rapamycin was attributed to mTOR activation in renal vascular endothelial cells. Interestingly, the majority of APS patients in this study also had SLE (16/28 = 57%) [26]. However, it has not been disclosed how many of the 7 patients who actually benefited from rapamycin [26] satisfied the diagnosis of SLE [19,20] or APS [22]. mTOR activity has not been measured within the immune system itself [26], which is considered to be the principal mediator of autoimmunity both in APS [24] and SLE [1]. Therefore, critical gaps exist in our knowledge about the role of mTOR activation and its relationship to oxidative stress in APS. In the present study, we examined mTORC1 activity and metabolic biomarkers of oxidative stress [4,5] in 72 SLE patients, 12 of whom also had APS, as well as in 54 healthy controls matched for age, gender, and ethnicity for each blood donation. The results indicate that oxidative stress and Treg depletion rather than mTOR activation underlie APS in patients with SLE.

## 2. Materials and methods

### 2.1. Human subjects

Peripheral blood lymphocytes (PBL) were isolated from 72 SLE patients. Each patient satisfied the criteria for a definitive diagnosis of SLE [19,20]. 12 SLE patients also satisfied the diagnostic criteria for APS [22]. Nine APS patients were Caucasian females, one was a Caucasian male, and one was an African-American female. Among the SLE patients without APS, two were Caucasian males, one was an African-American female, and the remaining were Caucasian females. The mean (±SEM) age of patients was 43.3 ± 1.5 years, ranging between 21 and 62 years. 54 healthy subjects were individually matched for each patient blood donation for age within ten years, gender, and ethnic background and their freshly isolated cells were studied in parallel as controls for flow cytometry studies. The mean (±SEM) age of controls was 45.6 ± 1.4 years, ranging between 20 and 62 years. 47 controls were females including 40 Caucasians, five African-Americans, and two Hispanics. 7 controls were Caucasian males.

### 2.2. Flow cytometry of oxidative stress, mTOR activity, and FoxP3 expression

T-cell subsets were analyzed by staining with antibodies to CD4, CD8, and CD25. B cell subsets were identified by CD19 and CD25 staining [4]. Mitochondrial transmembrane potential ($\Delta\psi_m$) was assessed with tetra-methyl-rhodamine methyl ester (TMRM, 100 nM, excitation: 543 nm, emission: 567 nm recorded in FL-2). Mitochondrial mass was evaluated with MitoTracker Green-FM (MTG, 100 nM; excitation: 490 nm, emission: 516 nm recorded in FL-1). Reactive oxygen intermediates (ROI) were assessed with superoxide-sensing hydroethidine (HE, 1 μM). All metabolic and mitochondrial sensor dyes were obtained from Invitrogen (Carlsbad, CA) and used as described earlier [4,27]. For detection of mTOR activity and FoxP3 expression, cells were permeabilized with Cytofix/CytopermPlus (eBiosciences) and stained with an AlexaFluor-488-conjugated antibody to pS6RP and AlexaFluor-647-conjugated antibody to FoxP3, as described earlier [5]. Each patient's cells were freshly isolated, stained and analyzed in parallel with a matched control. Mean channel fluorescence (MFI) values of patient samples were normalized to controls set at 1.0 for each analysis and expressed as fold changes. Frequencies of cell populations were compared as absolute values.

### 2.3. Statistics

Data were analyzed with t-test using the Prism software (GraphPad, San Diego, CA).

## 3. Results

Twelve of the 72 patients (17%) satisfied the diagnostic criteria for APS [22]. The accumulation of oxidative stress-generating mitochondria was significantly greater in DN T cells of SLE patients with APS (2.7-fold) in comparison to those without APS (1.7-fold; two-tailed $p = 0.014$; Fig. 1A). Elevation of $\Delta\psi_m$ or mitochondrial hyperpolarization, which drives the production of reactive oxygen intermediates (ROI) [8], i.e., oxidative stress, was as also increased in DN T cells of patients with APS ($TMRM^{hi}$: 25.3 ± 4.4%) relative to those without APS ($TMRM^{hi}$: 16.8 ± 1.5%; $p = 0.029$). While mitochondrial mass was only elevated in DN T cells (Fig. 1A), the intracellular production of superoxide, i.e., oxidative stress, was increased in all lymphocyte subsets of patients with APS in comparison to those without APS (Fig. 1B). In accordance with earlier findings [4,5], mTORC1 was markedly elevated in DN T cells relative to CD4 (8.5-fold, $p = 2.8 \times 10^{-9}$) or CD8 T cells of all SLE patients combined (9.4-fold, $p = 3.1 \times 10^{-10}$). However, mTORC1 activity was similar between SLE patients with and without APS (Fig. 2). No discrete population with elevated mTORC1 activation was detectable in $CD19^{+}$ B cells (data not shown). Given that Tregs are depleted in SLE patients [28], which, in turn contribute to autoantibody production [29], we examined their prevalence in patients with and without