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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 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.

Keywords

Systemic lupus erythematosus; Anti-phospholipid syndrome; Oxidative stress; Mechanistic target of rapamycin; mTOR; Treg

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 with promising therapeutic efficacy in a randomized double-blind placebo controlled pilot study

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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). 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 African American female, and the remaining were Caucasian females. The mean (\pm SEM) age of patients was 43.3 \pm 1.5 years, ranging between 21–62 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 (\pm SEM) age of controls was 45.6 \pm 1.4 years, ranging between 20–62 years. 47 controls were females including 40 Caucasians, five African-Americans, and two Hispanic. 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 (Ψ 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 μ M). All metabolic and mitochondrial sensor dyes were obtained from Invitrogen (Carlsbad, CA) and used as earlier described (4;27). For detection of mTOR activity and FoxP3 expression, cells were permeabilized with Cytotfix/CytopermPlus (eBiosciences) and stained with AlexaFluor-488-conjugated antibody to pS6RP and AlexaFluor-647-conjugated antibody to FoxP3, as earlier described (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 ψ_m or mitochondrial hyperpolarization, which drives the production of reactive oxygen intermediates (ROI) (8), i.e., oxidative stress, was also increased in DN T cells of patients with APS (TMRM^{hi} : 25.3 \pm 4.4%) relative to those without APS (TMRM^{hi} : 16.8 \pm 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 APS. The frequency of CD4⁺CD25⁺FoxP3⁺ Tregs was reduced in patients with APS (Fig. 3). In particular, FoxP3⁺ expression was diminished within CD4⁺CD25⁺ Tregs in SLE patients with APS (28.4%) relative to those without APS (46.3%, $p=0.008$; Fig. 3).

4. DISCUSSION

The present study reveals the accumulation of mitochondria in DN T cells and enhanced superoxide production in all lymphocytes subsets of SLE patients with APS. The prominent changes in DN T cells are consistent with earlier findings of their pro-inflammatory metabolic state (4;30) that generates oxidative stress in SLE (8). However, mTORC1 activity, which is responsive to oxidative stress (6;7), was similar within the immune system of SLE patients irrespective of APS status. On the one hand, these findings suggest that

activation of the mTOR pathway does not differentiate lupus patients with APS from those without APS. On the other hand, mTOR blockade may be equally beneficial for both groups of patients due to the underlying SLE. Indeed, rapamycin showed clinical efficacy both in mouse models (11;31) and patients with SLE (2;4;11). Therefore, the benefit from rapamycin treatment in APS nephropathy may be attributed to underlying lupus, given that the majority of patients in that study had SLE as well (16/28 = 57%) (26). Moreover, in accordance with mTOR being a sensor of mitochondrial dysfunction (32) and oxidative stress (3;6), its activation is responsive to antioxidant treatment in vitro (6;7) and in vivo (5). The enhanced production of superoxide in SLE patients with APS is consistent with earlier observations that oxidative stress spreads from DN T cells to other cells and through the bloodstream (8). Along these lines, oxidative modification triggers the immunogenicity of phospholipid antigens (23–25). Furthermore, these studies reveal a profound depletion of Tregs in SLE patients having concurrent APS. Given that antioxidant treatment with NAC blocked mTOR and reversed the depletion of Tregs in SLE patients (5), such intervention may be particularly effective in patients with APS. Therefore, follow-up clinical studies with NAC, which are clearly warranted to confirm its promising therapeutic efficacy in SLE, should include patients with APS.

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HIGHLIGHTS

- Anti-phospholipid antibodies (APLA) is a diagnostic criterion of SLE
- APLA cause anti-phospholipid syndrome (APS).
- Mitochondrial mass is increased in T cells of SLE patients with APS
- Oxidative stress is increased in SLE patients with APS
- Tregs are depleted in SLE patients with APS

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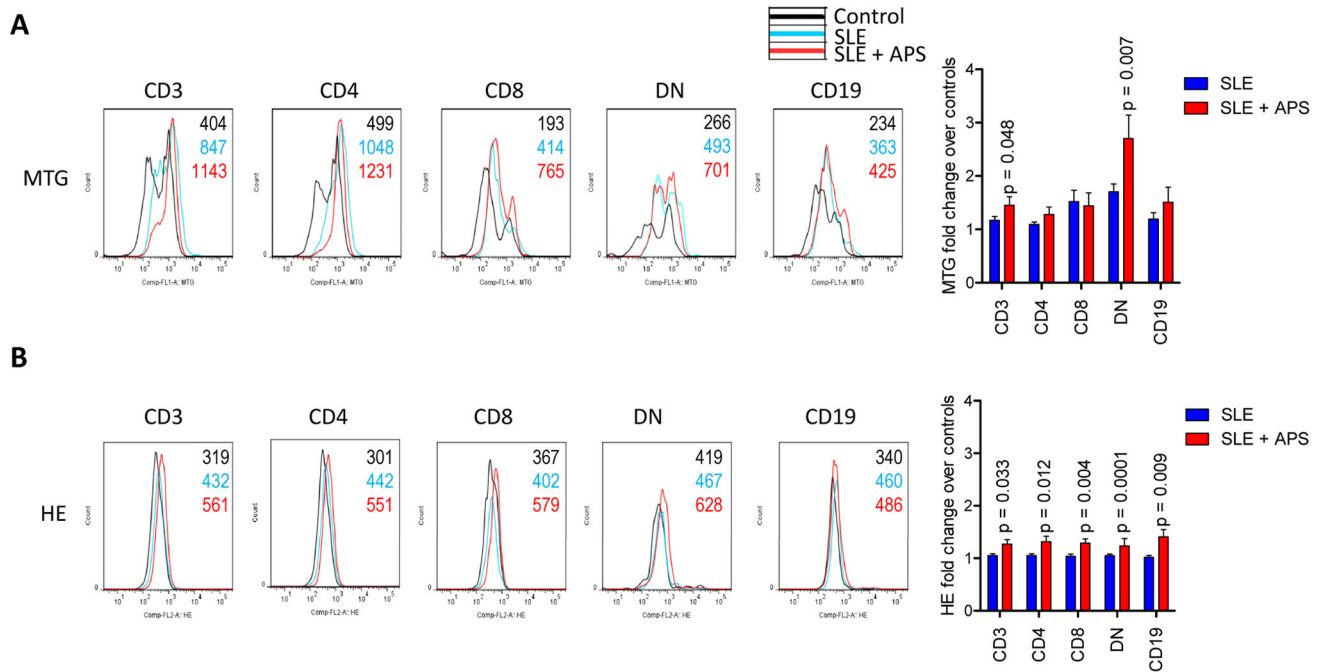


Figure 1.

Increased oxidative stress in SLE patients with APS. Peripheral blood lymphocytes (PBL) of 72 lupus patients were freshly isolated and studied in parallel with 54 healthy control subjects matched for age within 10 years, gender, and ethnicity for each blood donation, as earlier described (4;5). **A**, Mitochondrial mass was measured by MitoTracker Green (MTG) fluorescence in CD3⁺, CD4⁺, CD8⁺, and CD3⁺CD4⁻CD8⁻ double-negative (DN) T cells and CD19⁺ B cells. Left panels show histogram overlays of healthy (black) and SLE subjects with (red) and without APS (blue). Bar chart represents mean \pm SEM of fold changes relative to paired controls normalized at 1.0. **B**, Oxidative stress, that is, the production of reactive oxygen intermediates (ROI) was measured by hydroethidine (HE) fluorescence. Left panels show representative histogram overlays, while bar chart depicts the mean \pm SEM of fold changes relative to paired controls normalized at 1.0. p values reflect comparison between SLE patients with and without APS using unpaired t-test.

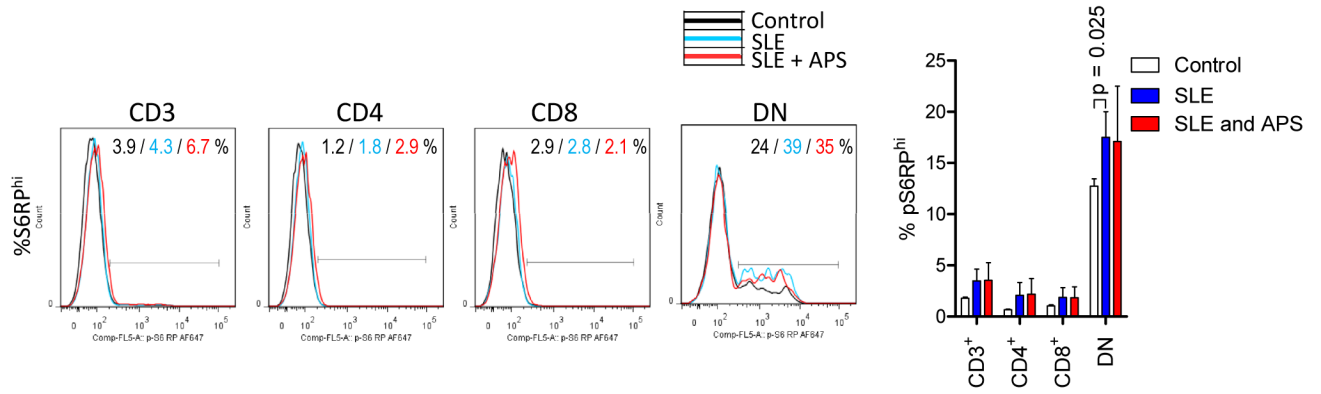


Figure 2.

Comparable mTOR pathway activity in lupus T cells of patients with or without APS. mTORC1 activity was measured by the frequency of cells with elevated intracellular phosphorylated S6 ribosomal protein levels (%pS6R^{ph}). Left panels show representative histogram overlays, while bar chart depicts the mean \pm SEM of fold changes relative to paired controls normalized at 1.0. p values reflect comparison between SLE patients with and without APS using unpaired t-test.

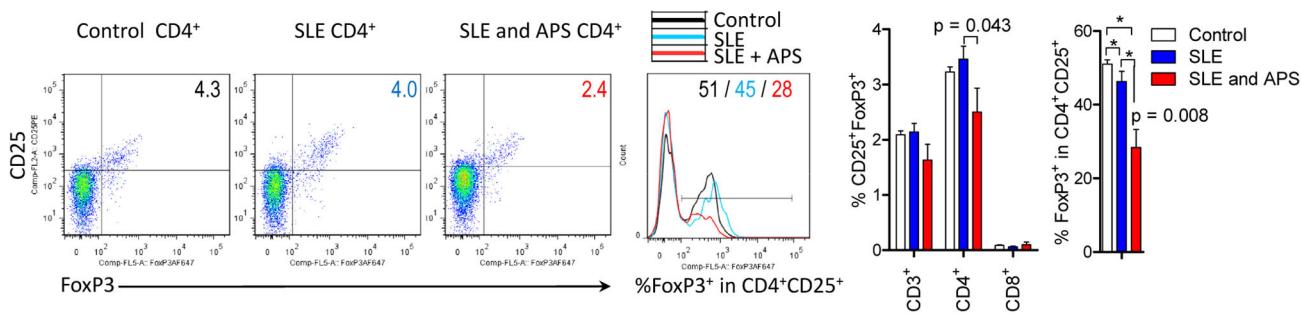


Figure 3. Treg depletion in SLE patients with APS. CD25⁺FoxP3⁺ Tregs were enumerated within CD4⁺ T cells and FoxP3 expression was also assessed by intracellular staining of permeabilized CD4⁺CD25⁺ T cells (4;5). * reflect p values < 0.05 comparing groups of subjects marked by brackets. Exact p values of 0.043 and 0.008 reflect comparisons between SLE patients with and without APS using unpaired t-test.