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Mechanistic Target of Rapamycin Activation Triggers IL-4 Production and Necrotic Death of Double-Negative T Cells in Patients with Systemic Lupus Erythematosus

Zhi-Wei Lai,* Rebecca Borsuk,* Ashwini Shadakshari,* Jianghong Yu,* Maha Dawood,* Ricardo Garcia,* Lisa Francis,* Hajra Tily,* Adam Bartos,* Stephen V. Faraone,† Paul Phillips,* and Andras Perl*‡,§

The mechanistic target of rapamycin (mTOR) is recognized as a sensor of mitochondrial dysfunction and effector of T cell lineage development; however, its role in autoimmunity, including systemic lupus erythematosus, remains unclear. In this study, we prospectively evaluated mitochondrial dysfunction and mTOR activation in PBLs relative to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) during 274 visits of 59 patients and 54 matched healthy subjects. Partial least square–discriminant analysis identified 15 of 212 parameters that accounted for 70.2% of the total variance and discriminated lupus and control samples (p < 0.0005); increased mitochondrial mass of CD3+CD4−/CD8− double-negative (DN) T cells (p = 1.1 × 10−22) and FOXP3 depletion in CD4+CD25+ T cells were top contributors (p = 6.7 × 10−7). Prominent necrosis and mTOR activation were noted in DN T cells during 15 visits characterized by flares (SLEDAI increase ≥ 4) relative to 61 visits of remission (SLEDAI decrease ≥ 4). mTOR activation in DN T cells was also noted at preflare visits of SLE patients relative to those with stable disease or healthy controls. DN lupus T cells showed increased production of IL-4, which correlated with depletion of CD25+/CD19+ B cells. Rapamycin treatment in vivo blocked the IL-4 production and necrosis of DN T cells, increased the expression of FOXP3 in CD25−/CD4+ T cells, and expanded CD25+/CD19+ B cells. These results identify mTOR activation to be a trigger of IL-4 production and necrotic death of DN T cells in patients with SLE. The Journal of Immunology, 2013, 191: 000–000.

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease of unknown etiology characterized by T cell and B cell dysfunction and anti-nuclear Ab production (1). Abnormal death signal processing plays a key role in driving anti-nuclear Ab production through the release of immunogenic nuclear materials from apoptotic (2) and necrotic cells (3, 4).

The mechanistic target of rapamycin (mTOR) is recognized as a sensor of mitochondrial dysfunction and effector of T cell lineage development; however, its role in autoimmunity, including systemic lupus erythematosus, remains unclear. In this study, we prospectively evaluated mitochondrial dysfunction and mTOR activation in PBLs relative to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) during 274 visits of 59 patients and 54 matched healthy subjects. Partial least square–discriminant analysis identified 15 of 212 parameters that accounted for 70.2% of the total variance and discriminated lupus and control samples (p < 0.0005); increased mitochondrial mass of CD3+/CD4−/CD8− double-negative (DN) T cells (p = 1.1 × 10−22) and FOXP3 depletion in CD4+CD25+ T cells were top contributors (p = 6.7 × 10−7). Prominent necrosis and mTOR activation were noted in DN T cells during 15 visits characterized by flares (SLEDAI increase ≥ 4) relative to 61 visits of remission (SLEDAI decrease ≥ 4). mTOR activation in DN T cells was also noted at preflare visits of SLE patients relative to those with stable disease or healthy controls. DN lupus T cells showed increased production of IL-4, which correlated with depletion of CD25+/CD19+ B cells. Rapamycin treatment in vivo blocked the IL-4 production and necrosis of DN T cells, increased the expression of FOXP3 in CD25−/CD4+ T cells, and expanded CD25+/CD19+ B cells. These results identify mTOR activation to be a trigger of IL-4 production and necrotic death of DN T cells in patients with SLE. The Journal of Immunology, 2013, 191: 000–000.
patients with active SLE (23, 24). Depletion of C3 and C4 (25) and increased anti-DNA Abs have long been associated with disease activity, particularly with renal flares (26). However, neither hypocomplementemia (27) nor changes in anti-DNA (28) predict future flares (27, 28). Therefore, we evaluated checkpoints of mitochondrial dysfunction, which may drive abnormal death signaling and anti-DNA production, as measures of disease activity in SLE. The present study reveals that mTOR activation causes increased production of IL-4 and necrosis of CD3+CD4+CD8+ double-negative (DN) T cells, mediates lineage skewing in T cell and B cell compartments, predicts flares, and, thus, serves as a mechanistically relevant target for treatment of SLE.

Materials and Methods

Human subjects

PBLs were isolated during 274 visits of 59 SLE patients and evaluated by flow cytometry in parallel with 214 PBL samples from 54 healthy controls. The mean (+ SEM) age of patients was 43.1 ± 1.6 y, with a range of 20–65 y. Fifty-six patients were females, including 49 whites, 6 African-Americans, and 1 Hispanic. Three patients were white males. Fifty-four healthy subjects were individually matched for each patient blood donation for age within 10 y, 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 39.1 ± 1.8 y, with a range of 20–62 y. Forty-seven controls were females, including 40 whites, 5 African-Americans, and 2 Hispanics. Seven controls were white males. SLE disease activity was assessed by the British Isles Lupus Assessment Group (BILAG) Disease Activity Index (29) and the SLE Disease Activity Index (SLEDAI) (30, 31). Preflare visits were defined as a change in SLEDAI < 4 relative to the preceding visit and followed by a flare characterized by ≥ 4 increase in SLEDAI on a subsequent visit within 129 ± 29 d (n = 15). Stable disease was defined as visits with SLEDAI change < 4 relative to the preceding and follow-up visits (n = 124). Remission visits were defined as a decrease in SLEDAI ≥ 4 relative to a preceding visit that occurred after a follow-up interval of ≥ 228 ± 24 d (n = 61). We documented concurrent use and dosage of medications. Routine blood tests included complete blood count, liver and kidney function test, urinalysis, and traditional lupus-relevant serological biomarkers, such as anti-dsDNA, C3, and C4.

Fourteen patients enrolled into the prospective study of rapamycin for the treatment of SLE (ClinicalTrials.gov Identifier: NCT00779194) were also investigated. The mean (+ SEM) age of these patients was 44.3 ± 4.2 y, with a range of 18–65 y. Twelve patients were white females, and two patients were white males. Seventeen healthy subjects were individually matched for each patient blood donation for age within 10 y, 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 37.4 ± 3.4 y, with a range of 20–63 y. Fourteen controls were white females, and three controls were white males. Assay of metabolic biomarkers in live cells by flow cytometry

We examined unstimulated cells and cells stimulated with CD3/CD28 for 16 h (9). T cell subsets were analyzed by staining with Abs to CD4, CD8, and CD25. B cell subsets were identified by CD19 and CD25 staining. Cell death pathway selection was monitored with Annexin V (AnnV)–FITC, AnnV–PE, or AnnV–Alexa Fluor 647 matched with emission spectra of propidium iodide (PI) to detect AnnV–PI+ apoptotic cells and PI–AnnV–PE+ necrotic cells (6). Δψm was assessed with positively charged cationic dyes (3,3'-dihexyloxacarbocyanine iodide [DiOC6], 40 nM, excitation: 488 nm, emission: 525 nm recorded in FL-1; tetramethylrhodamine methyl ester [TMRM], 50 nM, excitation: 543 nm, emission: 567 nm recorded in FL-2). MitoTracker Green FM (MTG, 100 nM; excitation: 490 nm, emission: 514 nm recorded in FL-1) or nonyl arylacridine orange (50 nM; excitation: 490 nm, emission: 540 nm recorded in FL-1). ROI were assessed with superoxide-sensing hydroethidine (1 μM) and H2O2-sensing dichlorofluorescein diacetate (1 μM). NO sensor 4-aminomethyl-2,7'-difluorofluorescein diacetate (1 μM, excitation: 495, emission: 515 nm recorded in FL-1). Cytosolic Ca2+ was assessed with Fluo-3 (1 μM, excitation: 506 nm, emission: 526 nm recorded in FL-1), and mitochondrial Ca2+ was assessed with Rhod-2 (1 μM, excitation: 506 nm, emission: 511 nm recorded in FL-2). All metabolic and mitochondrial sensor dyes were obtained from Invitrogen (Carlsbad, CA) and used as previously described (9, 13, 32, 33). We recorded up to 12 parameters simultaneously using a Becton Dickinson LSR II flow cytometer equipped with 20 mW solid-state Nd:YAG (emission at 355 nm), 20 mW argon (emission at 488 nm), 10 mW diode-pumped solid-state yellow-green (emission 561 nm), and 16 mW helium-neon (emission at 634 nm) lasers. Necrostatin-1 (34), necrostatin-5

FIGURE 1. Expansion of DN T cells with MHP and increased mitochondrial mass in PBLs of patients with SLE. Frequency of AnnV– cell subsets (A) and subpopulations with increased Δψm [(B) DiOC6 fluorescence; (C) TMRM fluorescence] and mitochondrial mass [(D) MTG fluorescence] were assessed in PBLs during 274 visits of 59 patients relative to 214 PBL samples from 54 healthy subjects. p < 0.05, unpaired two-tailed t test.
(35), and necrostatin-7 were obtained from BioVision (Milpitas, CA) and used to inhibit necrosis, as previously described (36). Each patient’s cells were freshly isolated, stained, and analyzed in parallel with a matched control. Mean fluorescence intensity (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.

Assessment of mTOR activity, FOXP3 expression, and cytokine production by flow cytometry

We examined unstimulated cells and cells stimulated with CD3/CD28 for 16 h. For detection of mTOR activity and FOXP3 expression, cells were permeabilized with Cytofix/Cytoperm Plus (eBioscience) and stained with Alexa Fluor 488– or Alexa Fluor 647–conjugated Ab to pS6RP (Cell Signaling, Beverly, MA; cat. no. 4851) and Alexa Fluor 647–conjugated Ab to FOXP3 (BioLegend, San Diego, CA; cat. no. 320014), as previously described (12). Intracellular cytokine production was measured after additional in vitro stimulation for 3 h with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence of 10 μg/ml brefeldin A (all from Sigma-Aldrich, St. Louis, MO), followed by fixation, permeabilization, and staining with Abs from BD Biosciences: FITC-conjugated anti–IFN-γ (cat. no. 554700), allophycocyanin-conjugated anti–IL-4 (cat. no. 560671), and PE-conjugated anti–IL-17a (cat. no. 560436). Relative fluorescence intensity (RFI) was calculated by comparison of MFI values of patients’ cells to healthy subjects’ cells, which were analyzed in parallel and normalized to 1.0.

Statistics

Pearson correlations between disease activity and biomarkers, partial least square–discriminant analysis (PLS-DA), principal component analysis, factor analysis, paired or unpaired \( t \) test with the Welch correction, and \( \chi^2 \) and Fischer exact tests were performed with the Statistical Package for the Social Sciences (SPSS, Chicago, IL), Stata (StataCorp, College Station, TX), or Prism (GraphPad, San Diego, CA) software. Multivariate analyses of biomarkers capable of discriminating between SLE and control subjects were performed using PLS-DA, which is a supervised method that uses a multivariate regression technique to extract, via linear combination of biomarkers (X), the information that can predict the subject group membership (Y). The classification and cross-validation were performed using the wrapper function offered by the caret package in Metaboanalyst software (37). A permutation test was performed to assess that the class discrimination was statistically significant. In each permutation, a PLS-DA model was built between the data (X) and the permuted class labels (Y) using the optimal number of components determined by cross-validation for the model based on the original class assignment. The ratio of the between-sum of the squares and the within-sum of squares for the class assignment prediction of each model was calculated. The PLS-DA model of 212 metabolic biomarkers was validated by a permutation test \( p \) value < 0.001, which generated components 1 and 2 accounting for 24.7 and 21% of the total variance in lupus and control PBLs. Validity of the PLS-DA model was retained at a permutation test \( p \) value < 0.000236, with correction for multiple comparisons (0.05/212). Comparison of lupus and control PBLs with principal component analysis and factor analysis revealed top differentiating biomarker sets similar to those identified by PLS-DA. Individual biomarkers were compared between control and lupus PBLs by the paired or unpaired \( t \) test with the Welch correction using Prism software. Medication use was compared between patient groups exhibiting flare and remission with the \( \chi^2 \) and Fischer exact tests using Prism software.

Supplemental materials include Supplemental Figs. 1–3 and Supplemental Table I.

Results

Prominent MHP and accumulation of mitochondrial mass in CD3+/CD4+/CD82 DN T cells of patients with SLE

We observed overall depletion of CD3+ T cells and expansion of CD19+ B cells in SLE patients (Fig. 1A). The prevalence of necrotic cells was moderately increased within CD3+, CD4+, CD8+, and FOXP3+ T cells from SLE patients as determined by mTOR activity and FOXP3 expression (Fig. 2A). The relative frequency of MHP-expressing T cells was significantly increased within CD4+ T cells from SLE patients as determined by the relative frequency of mTOR activity and CD4+ T cells (Fig. 2B). The relative frequency of MHP-expressing T cells was significantly increased within CD4+ T cells from SLE patients as determined by the relative frequency of mTOR activity and CD4+ T cells (Fig. 2C).

FIGURE 2. PLS-DA of 212 metabolic biomarkers in 274 lupus and 214 healthy control PBL samples. (A) Coefficient-based importance measures of the top 15 contributors to components 1, 2, and 3. (B) Three-dimensional score plot of PLS-DA using components 1, 2, and 3, accounting for 28.3, 29.2, and 12.7% of total variance, respectively. (C) Validation of PLS-DA by permutation tests.

Obtained Estimated
\( \chi^2 \) p = 0.004

\( p \) value
Mitochondrial potential and mass-sensing dyes detected discrete cell populations exhibiting MHP (DiOC6 and TMRM) and increased mitochondrial mass (MTG; Supplemental Fig. 1). MHP (%DiOC6: 1.9-fold, p = 5.9 × 10^{-15}, Fig. 1B; %TMRM: 1.8-fold, p = 2.1 × 10^{-22}, Fig. 1C) and mitochondrial mass were most prominently increased in DN lupus T cells (%MTG: 1.6-fold, p = 3.9 × 10^{-26}; Fig. 1D). Of note, B cells exhibited a modest decline in TMRM fluorescence, because cells exhibiting high TMRM fluorescence were depleted in the B cell compartment of SLE patients (Fig. 1C).

Overall, the population of cells with high mitochondrial mass was increased in the CD3+ T cell compartment (p = 1.1 × 10^{-8}), most considerably in CD3+ T cells of SLE patients (35.3 ± 1.0%) relative to controls (21.8 ± 0.7%, p = 3.9 × 10^{-26}). The PLS-DA model of 212 metabolic biomarkers identified 15 top contributors that accounted for 28.3, 29.2, and 12.7% of the total variance (Fig. 2A) and allowed discrimination between lupus and control PBLs (Fig. 2B), as validated by a permutation test p value < 0.0005 (Fig. 2C). Increased mitochondrial mass (%MTG) of DN T cells (FDR p = 1.1 × 10^{-22}) and reduced FOXP3 expression within CD4+CD25+ T cells (FDR p = 6.7 × 10^{-7}) were the most robust contributors to PLS-DA components 1–3 (Fig. 2A).

**mTOR activation of DN T cells correlates with diminished FOXP3 expression of CD4+/CD25+ T cells in patients with SLE**

Activation of mTOR, which serves as a sensor of ΔΨm in T lymphocytes (8), has been implicated in controlling the development of Tregs dependent on expression of the transcription factor FOXP3 (38). Therefore, mTOR activity was evaluated by intracellular staining of its downstream target, the phosphorylated form of the S6 ribosomal protein (pS6RP, Fig. 3A). pS6RP+ cells were expanded within the DN T cell subset (12.7 ± 0.7%) relative to CD4+ T cells (0.6 ± 0.07%, p = 4.1 × 10^{-13}), CD8+ T cells (1.0 ± 0.1%; p = 9.4 × 10^{-20}), or all T cells (1.8 ± 0.1%, p = 2.3 × 10^{-42}) in normal PBLs. The frequency of pS6RP+ DN T cells was increased in SLE patients (p = 0.007; Fig. 3B). Although the percentage of FOXP3+CD25+ cells within the CD3+/CD4+ compartment was not diminished, a discordant expression of FOXP3 and CD25 was observed between lupus and control T cells (Fig. 3C). The percentage of FOXP3+ cells within the CD3+/CD4+/CD25+ compartment was diminished in lupus patients relative to controls (p = 2.7 × 10^{-7}, Fig. 3A, 3C). In contrast, FOXP3 expression was significantly increased in CD4+/CD25+ T cells of SLE patients (p = 1.6 × 10^{-5}, Fig. 3A, 3C). Increased mTOR activity in DN T cells showed a moderate, but significant, correlation with diminished FOXP3 expression in CD4+/CD25+ T cells.

**FIGURE 3.** mTOR activation in DN T cells correlates with contraction of Tregs in SLE. (A) Detection of increased mTOR activity via pS6RP in T cell subsets from lupus and matched control donors (upper panels). The percentages of pS6RP+ cells are indicated for control (blue graphs) and lupus (red graphs) T cells. Dot plots and graphs of FOXP3 expression within CD25+ T cells, gating on CD3+CD4+ T cells in control and lupus PBLs (lower panels). (B) Cumulative analysis of mTOR activity in T cell subsets in 274 lupus and 214 control PBL samples. (C) Cumulative analysis of CD4+ T cells by expression of FOXP3 and CD25. (D) Correlation of mTOR activity in DN T cells with expression of FOXP3 in CD4+/CD25+ T cells of 264 lupus PBL samples.
DNA (each other (correlations are shown in Fig. 4). The SLEDAI (6.2 after correcting for multiple comparisons (0.05/212; representative p +0.450, BILAG (24.5 6 scores showed significant correlation (r = 0.316, C4 (r = 0.410, p = 1.6 × 10−10) with MHP of DN T cells, C3 (r = +0.249, p = 0.00009) and C4 correlated negatively with oxidative stress in CD3+ T cells (dichlorofluorescein diacetate: r = −0.234, p = 0.00002) and negatively with oxidative stress in CD8+ T cells (r = −0.239, p = 0.00017). Along these lines, C4 correlated positively with CD4+ (r = +0.268, p = 0.00001) and negatively with CD8+ (r = −0.234, p = 0.00014) T cell frequencies, MHP of DN T cells (%DiOC6 hi: r = −0.295, p = 0.00004; %TMRM hi: r = −0.316, p = 2.6 × 10−7), and mitochondrial mass of DN T cells (%MTG hi: r = −0.264, p = 0.00002) and B cells (%MTG hi: r = −0.274, p = 0.00001). Anti-DNA correlated positively with MHP (%DiOC6 hi: r = +0.380, p = 3.2 × 10−7; %TMRM hi: r = +0.410, p = 1.6 × 10−10), TMRM MFI: r = +0.517, p = 9.2 × 10−17) and mitochondrial mass (%MTG hi: r = +0.284, p = 0.00001; MTG MFI: r = +0.357, p = 3.6 × 10−3) of DN T cells and oxidative hyperactivity in CD4+/CD25+ lupus T cells pointed to the involvement of indirect mechanisms driving the diminished expression of FOXP3 in SLE.

Correlation of metabolic biomarkers with disease activity in SLE

We evaluated 212 parameters reflecting ΔΨm, mitochondrial mass, NO and ROI production, [Ca2+]i, and [Ca2+]m, and mTOR and FOXP3 expression in T cell and B cell subsets for correlation with disease activity; p values < 0.000236 were considered significant after correcting for multiple comparisons (0.05/212; representative correlations are shown in Fig. 4). The SLEDAI (6.2 ± 0.3) and BILAG (24.5 ± 6) scores showed significant correlation (r = +0.450, p = 1.4 × 10−14; Supplemental Table I). SLEDAI correlated with absolute values of traditional biomarker components C3 (r = −0.225, p = 0.00037), C4 (r = −0.237, p = 0.00016), and anti-DNA (r = +0.237, p = 0.00039; Fig. 4). C3 and C4 correlated with each other (r = +0.695, p = 1.4 × 10−38). Anti-DNA correlated with C3 (r = −0.191, p = 0.004) and C4 (r = −0.323, p = 6 × 10−7).

As shown in Supplemental Table I and for representative data in Fig. 4, SLEDAI correlated positively with the overall percentage of necrotic CD3+ (r = +0.254, p = 0.0001), CD4+ (r = +0.260, p = 0.00003), and DN (r = +0.230, p = 0.00019) T cells, as well as MHP (%DiOC6 hi: r = +0.264, p = 0.00022) and mitochondrial mass of DN T cells (%MTG hi: r = +0.254, p = 0.00003). SLEDAI correlated negatively with FOXP3+/CD25+/CD4+ T cells after CD3/CD28 costimulation (r = −0.229, p = 0.00018).

Among the serological components of SLEDAI, C4 correlated negatively (%TMRM hi: r = −0.316, p = 2.0 × 10−10) and anti-DNA correlated positively (%TMRM hi: r = +0.410, p = 1.6 × 10−10) with MHP of DN T cells, C3 (r = +0.249, p = 0.00009) and C4 correlated negatively with oxidative stress in CD3+ T cells (dichlorofluorescein diacetate: r = −0.234, p = 0.00022; Supplemental Table I). Likewise, C3 (r = −0.294, p = 3.3 × 10−7) and C4 correlated negatively with oxidative stress in CD8+ T cells (r = −0.239, p = 0.00017). Along these lines, C4 correlated positively with CD4+ (r = +0.268, p = 0.00001) and negatively with CD8+ (r = −0.234, p = 0.00014) T cell frequencies, MHP of DN T cells (%DiOC6 hi: r = −0.295, p = 0.00004; %TMRM hi: r = −0.316, p = 2.6 × 10−7), and mitochondrial mass of DN T cells (%MTG hi: r = −0.264, p = 0.00002) and B cells (%MTG hi: r = −0.274, p = 0.00001). Anti-DNA correlated positively with MHP (%DiOC6 hi: r = +0.380, p = 3.2 × 10−7; %TMRM hi: r = +0.410, p = 1.6 × 10−10), TMRM MFI: r = +0.517, p = 9.2 × 10−17) and mitochondrial mass (%MTG hi: r = +0.284, p = 0.00001; MTG MFI: r = +0.357, p = 3.6 × 10−3) of DN T cells and oxidative

FIGURE 4. Correlation of SLEDAI and traditional biomarkers of disease activity, such as C3, C4, and anti-DNA, with 212 metabolic, cell surface, and gene expression biomarkers during 274 visits of 59 patients with SLE. Because of missing data, the actual number of available data pairs is indicated for each comparison (n = 228–255). Correlation r values were considered significant at p < 0.000236 when corrected for multiple comparisons (0.05/212). Correlation p values > 0.000236 and p values < 0.000236 are indicated by blue and red symbols, respectively.
Increased necrosis, mTOR activation in DN T cells, and depletion of Tregs distinguish SLE patients in flare

To further evaluate the relationship of metabolic biomarkers with disease activity, we compared their performance during 15 patient visits with an increase ≥ 4 in SLEDAI (+5.3 ± 1.5) relative to 61 patient visits with a decrease ≥ 4 in SLEDAI (−7.8 ± 0.5, p = 3.9 × 10⁻³). During the remaining 198 patient visits, SLEDAI changes were <4. Although anti-DNA was increased at 605 ± 181 U/ml relative to 174 ± 47 U/ml (p = 0.003), unexpectedly, C3 and C4 levels were not different between flaring and remitting patients, possibly due to the relatively modest number of flaring patients. The individual doses and numbers of patients taking prednisone were similar between the groups in flare and remission (data not shown).

Flaring SLE patients exhibited increased necrosis of CD3⁺ (SLE: 1.4 ± 0.3%; control: 0.5 ± 0.07%; p = 0.0196), CD4⁺ (SLE: 0.9 ± 0.3%; control: 0.2 ± 0.2%; p = 0.0413), and DN (SLE: 9.8 ± 2.7%; control: 3.4 ± 0.5%; p = 0.0247; Fig. 5A, 5B) T cells. Patients in remission did not show increased frequency of necrotic cells within the T cell compartment (Fig. 5A, 5B).

In keeping with their increased rate of necrosis, DN T cells of flaring patients exhibited MHP (%TMRM hi SLE: 25.6 ± 0.3%; control: 3.4 ± 0.5%; p = 0.0196), increased mitochondrial mass (%MTG hi SLE: 33.4 ± 2.0%; control: 24.3 ± 0.9%; p = 0.0001), and DN T cells (%DiOC6⁺ SLE: 33.4 ± 2.4%; control: 15.6 ± 0.6%; p = 7.9 × 10⁻¹⁰; Fig. 5C), as well as increased mitochondrial mass in CD3⁺ (MTG MFI 1.3 ± 0.06-fold; p = 4.6 × 10⁻⁶), CD4⁺ (MTG MFI 1.3 ± 0.05-fold; p = 3.8 × 10⁻⁶), and CD8⁺ (MTG MFI 1.4 ± 0.1-fold; p = 0.001), and DN T cells (MTG MFI 1.8 ± 0.1-fold, p = 5.3 × 10⁻⁷; %MTG MFI 3.6 × 10⁻⁹; Fig. 5C). SLE patients in remission did not show increased mTOR activity in DN T cells relative to healthy controls (%pS6RP hi SLE: 12.3 ± 1.8%; control: 12.7 ± 0.7%; p = 0.83), but they did show diminished mTOR activity in all CD3 T cells relative to all patients upon study entry (61 SLE patient visits in remission %pS6RP, 1.6 ± 0.3%; all 59 SLE patients at baseline: 3.7 ± 0.9%; p = 0.037). FOXP3 expression within CD3⁺/CD4⁺/CD25⁺ T cells of SLE patients in remission remained low (42.7 ± 3.2%) in comparison with healthy controls (49.8 ± 1.3%, p = 0.043; Fig. 5D). mTOR blockers were being used in 3 of the 15 flare visits (rapamycin in two patients and NAC in one patient) and in 56 of 61 remission visits (rapamycin in 36 cases and NAC in 20 cases; Fischer exact test p value < 0.0001). No difference was found in the use of prednisone, mycophenolate, or hydroxychloroquine.
mTOR activation in DN T cells and discordant expression of FOXP3 and CD25 in CD4+ T cells predict flare in SLE

To identify metabolic biomarkers potentially predicting disease flares, we compared preflare parameters of 15 patient visits, which had preceded the increase in SLEDAI ≥ 4 by 129 ± 32 d with those of healthy controls, 124 stable SLE patient visits defined as showing SLEDAI changes < 4 compared with previous and subsequent visits, and 61 remission visits defined by a decrease ≥ 4 in SLEDAI relative to prior visits. Within the live compartment, reduced numbers of CD3+ T cells were noted in all SLE patients relative to healthy controls, whereas preflare patients had fewer CD4+ T cells and more CD8+ and DN T cells than did stable patients treated without mTOR inhibitors (Fig. 6A). Three-dimensional PLS-DA showed clear distinction of preflare and flare visits that was dominated largely by parameters of increased T cell necrosis (data not shown). Necrosis of DN T cells was reduced in preflare visits (3.5 ± 0.8%) relative to flare visits (9.8 ± 2.7%, p = 0.020, paired t test) but were unaffected in comparison with stable visits (4.4 ± 0.5%, p = 0.068; Fig. 6B). mTOR activity was increased remarkably in DN T cells at preflare visits (%pS6RP 30.5 ± 6.1%) compared with healthy controls (12.7 ± 0.7%, p = 0.0161) and stable (18.2 ± 1.2%, p = 0.032) or remission (12.3 ± 1.8%, p = 0.0004; Fig. 6C) SLE visits. mTOR blockers were only used during 2 of 12 preflare visits relative to 56 of 61 remission visits (p < 0.0001) and 75 of 124 stable patient visits (p = 0.0048). mTOR activity was lower in DN T cells of stable patients treated with mTOR inhibitors (11.0 ± 1.2%) relative to those without (21.7 ± 2.1%, p = 7.5 × 10^-6; Fig. 6C). However, mTOR activity was also increased in CD4+ and CD8+ T cells during preflare visits relative to stable visits without mTOR blockade (Fig. 6C). Upon CD3/CD28 costimulation, FOXP3 expression was “inappropriately” increased in CD4+/CD25− T cells in SLE patients at preflare visits (2.9 ± 0.65%).

**FIGURE 6.** mTOR activation in DN T cells and discordant expression FOXP3 and CD25 in CD4+ T cells predict flare in SLE patients with stable disease. Preflare visits were defined as visits preceding flare characterized by an increase ≥ 4 in SLEDAI on a subsequent visit within 129 ± 29 d (n = 15). Preflare visits were compared with visits of patients with stable disease, defined as having a change in SLEDAI < 4 relative to preceding and follow-up visits (n = 124). Within the stable visit group, treatment regimens included mTOR inhibitors, rapamycin, or NAC, in 75 cases; for the remaining 49 cases, patients were not treated with mTOR blockade. (A) Frequency of live (%AnnV− /PI−) cells in T cell compartments. (B) Assessment of necrosis (%PI+ cells) in CD3, CD4, CD8, and DN T cell compartments. The p values < 0.05 reflect comparison with flaring patients using a two-tailed t test. (C) Assessment of mTOR activity (%pS6RP cells) in CD3, CD4, CD8, and DN T cell compartments. The p values < 0.05 reflect comparison with preflare patients using a two-tailed t test. (D) Detection of FOXP3+CD25−CD4+ T cells following CD3/CD28 activation. The p values < 0.05 reflect comparison with preflare patients using a two-tailed t test.
relative to matched controls (0.8 ± 0.04%, p = 0.024) and SLE patients with stable disease (1.4 ± 0.12%, p = 0.0016; Fig. 6D).

**Increased IL-4 expression by DN T cells correlates with skewing of the B cell compartment and production of anti-DNA**

Because disease activity has been associated with B cell activation and production of anti-DNA, we investigated the possible influence of DN T cells on skewing of the B cell compartment via cytokine production. Within an expanded B cell compartment (Fig. 1A), the frequency of CD25+/CD19+ B cells was diminished in SLE patients (4.8 ± 0.3%) relative to controls (7.8 ± 0.4%, p = 2.7 × 10^{-10}). T cell activation markedly increased the frequency of CD25+/CD19+ B cells; however, they remained depleted in SLE (70.6 ± 1.6%) relative to control subjects (78.4 ± 0.4%, p = 4.9 × 10^{-5}). To understand the mechanism by which DN T cells may impact lupus disease activity, we investigated their production of Th1 (IFN-γ), Th2 (IL-4), and Th17 cytokines (IL-17α) in 26 patients who had not been exposed to mTOR inhibitor treatment with rapamycin or NAC. DN T cells harbored the highest frequency of IL-4–producing cells relative to other T cell subsets both in SLE and matched healthy subjects (p < 0.0001; Fig. 7A). IL-4 production was detected in 11.2 ± 1.7% of DN T cells in SLE patients relative to 6.9 ± 0.8% of DN T cells in healthy subjects (p = 0.007; Fig. 7A). Representative dot plots are shown in Supplemental Fig. 2. Following CD3/CD28 costimulation, IL-4–producing cells remained increased in the DN T cell compartment (p = 0.045, data not shown). IFN-γ production was reduced in CD8+ lupus T cells (p = 0.045, data not shown). Although the frequencies of IL-17α T cells were not increased significantly in SLE (Fig. 7B), moderately enhanced production of IL-17 was observed when comparing MFI of T cells of lupus patients with matched controls using the paired t test (Fig. 7C). IL-4 production by DN T cells correlated positively with anti-DNA (r = +0.601, p = 0.006; Fig. 7D) and CD25+/CD19+ B cells (r = +0.520, p = 0.009; Fig. 7E) and negatively with CD25+/CD19+ B cells (r = −0.446; p = 0.029; Fig. 7F). In contrast, IL-17 production by DN T cells did not correlate with anti-DNA (Fig. 7G) or frequencies of CD25+/CD19+ (Fig. 7H) or CD25−/CD19+ (Fig. 7I) B cells.

**Treatment with rapamycin reduces necrosis and IL-4 production of DN lupus T cells**

To determine whether mTOR has a controlling influence over lineage skewing and abnormal death signaling in SLE, we examined 14 patients who were enrolled in a prospective open-label study and

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**FIGURE 7.** Increased IL-4 production by DN T cells correlates with skewing of B cell subsets and anti-DNA in patients with SLE. (A) Intracellular production of IL-4 by CD3+, CD4+, CD8+, and DN T cells in 26 patients with SLE and 26 matched healthy subjects. The percentage of IL-4+ cells was increased among DN T cells relative to other T cell subsets in lupus and control PBLs (p < 0.0001). (B) Intracellular production of IL-17 by CD3+, CD4+, CD8+, and DN T cells in SLE and matched healthy subjects. The percentage of IL-17+ cells was increased among CD4+ T cells relative to other T cell subsets in lupus and control PBLs. There was no difference in the percentages of IL-17+ cells between lupus and control subjects using the paired t test. (C) Production of IL-4 and IL-17 by MFI of CD3+, CD4+, and DN T cells of lupus patients normalized to matched healthy controls set at 1.0 for each analysis and expressed as fold changes. (D) Correlation of the percentages of IL-4+ DN T cells with anti-DNA levels in SLE. (E) Positive correlation of IL-4+ DN T cell and CD25+ B cell frequencies. (F) Negative correlation of IL-4+ DN T cell and CD25− B cell frequencies. (G) Correlation analysis of the percentages of IL-17+ DN T cells and anti-DNA levels in SLE. (H) Correlation analysis of IL-17+ DN T cell and CD25− B cell frequencies. (I) Correlation analysis of IL-17+ DN T cell and CD25+ B cell frequencies. The p values < 0.05 reflect comparisons of lupus versus healthy subjects, paired two-tailed t test.
achieved therapeutic plasma levels of rapamycin at 8.7 ± 1.2 ng/ml after 126 ± 18 d. Preliminary analysis of this ongoing study revealed a significant improvement in disease activity, as measured by the reduction in SLEDAI from 11.8 ± 1.1 at baseline to 5.7 ± 1.0 (p = 0.0028) and in BILAG score from 29.7 ± 3.2 at baseline to 20.9 ± 2.4 (p = 0.027; Fig. 8A). mTOR activity, as measured by %S6RPh cells, was reduced in all T cell subsets of rapamycin-treated SLE patients relative to matched controls studied in parallel (p < 0.03; data not shown). Rapamycin-treated lupus patients exhibited diminished necrosis of CD4+ T cells (Fig. 8B). Necrosis of DN T cells was also diminished following CD3/CD28 costimulation (Fig. 8C). Rapamycin inhibited IL-4 production by DN T cells from lupus patients, as indicated by reduced IL-4 MFI, from 2.2 ± 0.5-fold to 0.9 ± 0.1-fold, relative to matched controls (p = 0.034; Fig. 8D). The percentage of IL-4+ DN T cells was not reduced significantly by rapamycin (from 15.4 ± 2.6% to 12.4 ± 1.9%; p = 0.26). Rapamycin did not significantly affect the frequency of IL-17+ DN T cells (pretreatment: 0.21 ± 0.12%, rapamycin treatment: 0.11 ± 0.03%; p = 0.33, two-tailed paired t test) or the IL-17 MFI in DN T cells from lupus patients (p = 0.59). In contrast, rapamycin increased the expression of FOXP3 in CD4+/CD25+ T cells (Fig. 8E). Within the B cell compartment, the frequency of CD25+/CD19+ cells increased from 1.9 ± 0.4% to 3.3 ± 0.7% in rapamycin-treated patients (p = 0.029; Fig. 8F). The role of programmed necrosis or necroptosis (39) was evaluated by necrostatins (34–36). Necrostatin-7, but not necrostatin-1 or necrostatin-5, reduced CD3/CD28 costimulation-induced necrosis of DN T cells in PBLs of healthy subjects from 4.3 ± 0.7% to 2.2 ± 0.3% (p = 0.013). None of the necrostatins reduced necrosis or IL-4 production of DN T cells in PBLs of eight patients with SLE (data not shown).

Discussion

Lupus pathogenesis involves abnormal activation and death signaling in the immune system (1) that culminates in the release of proinflammatory nuclear debris, mostly from necrotic cells (4, 6), driving the production of anti-nuclear auto-Abs with >100 known specificities (40). The present study provides evidence that metabolic biomarkers of T cell dysfunction correlate with disease activity in SLE patients. The mitochondrial and cell death signaling processes, such as MHP, increased mitochondrial mass, and necrosis, are most prominently exhibited by DN T cells and correlate with SLEDAI, contraction of Tregs, and activation of B cells. Necrotic cell death of DN T cells was strikingly increased in flaring SLE patients. mTOR, which is a sensor of ΔΨm and ATP depletion in T cells (8), is activated in DN T cells of flaring SLE patients. The correlation of SLEDAI with checkpoints of mitochondrial dysfunction in DN T cells reached or exceeded that with hypocomplementemia or increased anti-DNA. In contrast to mTOR activation, MHP and accumulation of mitochondria were observed in all T cells of SLE patients, even those in remission, which suggested that mitochondrial dysfunction was a cause, rather than a consequence, of mTOR activation and disease flares. Indeed, mTOR activation is downstream of mitochondrial dysfunction, as depicted in Supplemental Fig. 3, and evidenced by the induction of mTOR by NO (9), a trigger of MHP (13) and mitochondrial biogenesis (41), as well as the persistence of MHP and accumulation of mitochondria during mTOR blockade in rapamycin-treated patients (9, 11). The notion that altered mitochondrial homeostasis contributes to lupus pathogenesis is supported by the recent identification of a lupus susceptibility gene as estrogen-related receptor γ, which accounts for increased mitochondrial voltage-dependent anion channel protein levels in the spleen of lupus-prone Sle1c2 mice (42).

mTOR activation of DN T cells and expansion of FOXP3+/CD25+/CD4+ T cells have been identified as predictors of flare. Of note, FOXP3+/CD25+/CD4+ T cells are also expanded in new-onset SLE (43–45), suggesting a mechanistic role for this T cell subset in triggering disease activation. The clinical improvement in SLE following mTOR blockade both in mice (10) and humans (11, 12, 46) supports the notion that mTOR activation represents

FIGURE 8. Effect of rapamycin on biomarkers of disease activity in patients with SLE. (A) SLEDAI and BILAG disease activity scores in 14 SLE patients before and during rapamycin treatment of 126 ± 18 d monitored by therapeutic plasma levels of 8.7 ± 1.2 ng/ml. (B) Effect of rapamycin treatment on necrosis monitored by the prevalence of PI+ cells in CD3+, CD4+, CD8+, and DN T cell subsets. (C) Effect of rapamycin treatment on necrosis monitored by the prevalence of PI+ cells in CD3+, CD4+, CD8+, and DN T cell subsets following CD3/CD28 costimulation. (D) Effect of rapamycin on IL-4 expression in CD3+, CD4+, CD8+, and DN T cell subsets assessed by RFI in comparison with matched healthy controls. (E) Effect of rapamycin on FOXP3 expression in CD4+/CD25+ T cells assessed by RFI in comparison with matched healthy controls. (F) Effect of rapamycin on the frequency of CD25+/CD19+ and CD25+/CD19+ B cells in SLE patients. The p values were calculated using a paired t test.
a pathogenetically relevant biomarker, potentially causing, as well as predicting, flares and responsiveness to treatment. Measurement of MHP and mitochondrial mass, to a lesser extent, are dependent on cellular viability and require parallel analysis of healthy controls (47). In contrast, mTOR activity and FOXP3 expression can be measured in fixed and permeabilized cells that may be stored, shipped, and analyzed in batches, making these checkpoints of T cell activation potentially useful biomarkers of disease activity in the clinical setting.

In addition to apoptosis and autophagy, necrosis is one of three fundamental forms of cell death (48). Necrosis has long been considered a passive or accidental form of cell death, which is characterized by membrane damage and cellular swelling due to the depletion of ATP required for the activity of enzymes that maintain membrane integrity (4, 49). However, it became apparent recently that many necrosis pathways exist (50). In fact, several necrosis pathways may also be actively regulated; this is termed “necroptosis” (39). Although necroptosis was initially implicated in mouse ischemic brain injury (34), neurodegeneration, and infection (39), it has become apparent that necroptosis also occurs in cells of the immune system (50). The best-studied necroptosis pathway is triggered by TNF-α and transduced by the kinase, receptor-interacting protein-1 (51). However, necroptosis inhibitor necrostatins failed to reduce necrosis or IL-4 production of DN T cells in PBLs of patients with SLE. The lack of inhibition by necrostatins is in accordance with a central role for TNF-α in necroptosis (52) and the diminished production of this cytokine in patients with SLE (53). Of note, mTOR activation was recently found to promote necrotic cell death both in vivo (54) and in vitro (17). This is consistent with our findings shown in Fig. 8, indicating that in vivo treatment of SLE patients with rapamycin inhibited both necrosis and IL-4 production of DN T cells. Because necrotic materials are more potent than are apoptotic materials in triggering antinuclear Ab production in lupus-prone mice (7), inhibition of necrosis may have contributed to reduced anti-DNA production in rapamycin-treated SLE patients (11).

mTOR activation has been associated with the contraction of CD4+/CD25+/FOXP3+ Tregs in healthy subjects (22), type I diabetes patients (22), transplant recipients (55), and SLE patients (12, 46). Although inactivation of mTOR favors T cell differentiation into Tregs (38), recent findings indicate that mTOR may not be completely dispensable for Treg development in mice (56). In our study, the inverse correlation between elevated mTOR in DN T cells and diminished frequency of FOXP3+/CD25+/CD4+ T cells pointed to the involvement of indirect mechanisms. In particular, the enhanced secretion of IL-4 by DN T cells may block the differentiation of FOXP3+ Tregs from naive CD4+ T cells (57). The enhanced production of IL-4 by DN T cells may also promote the differentiation of Th2 cells and their production of IL-4, which are also mTOR dependent (58). The indirect effect of mTOR blockade may involve the expansion of CD25+/CD19+ B cells, which were found to expand Tregs (59) and to be depleted in SLE (60). Increased IL-4 production by DN T cells has also been implicated in B cell activation and anti-DNA production in SLE (61, 62). Interim analysis of our ongoing prospective trial indicated that rapamycin treatment lowered the production of IL-4 by DN T cells and expanded CD25+/CD19+ B cells and Tregs in SLE patients. In summary, the current study identified mTOR activation as a therapeutically targetable checkpoint of disease pathogenesis that may account for elevated production of IL-4 by DN T cells, thus causing Treg depletion, B cell activation, and increased anti-DNA production in SLE. mTOR activation warrants further characterization as a mechanism of pathogenesis and predictor of flares in SLE.

Disclosures

The authors have no financial conflicts of interest.

References

SUPPLEMENTAL MATERIALS

SUPPLEMENTAL FIGURES S1-S3 and TABLE S1

LEGENDS TO SUPPLEMENTAL FIGURES S1-S3

Fig. S1. Detection of $\Delta \psi_m$ (DiOC6 and TMRM), mitochondrial mass (MTG) and activation of mTOR (pS6RP) in DN T cells in healthy subject (black histogram), SLE patient in remission (blue histogram) and SLE patient in flare (red histogram).

Fig. S2. Increased production of IL-4 in DN lupus T cells. A, Representative dot plot analysis of DN T cells by gating on CD3+/CD4-/CD8- (DN) T cells. B, Measurement of intracellular IL4, IL-17, and IFN-γ production in permeabilized CD3+, CD4+, CD8+, and DN T cells.

Fig. S3. Functional hierarchy of metabolic biomarkers in SLE. MHP is caused by exposure to nitric oxide (NO). Synthesis of NO and maintenance of GSH in reduced form depend on the production of NADPH by the pentose phosphate pathway (PPP). MHP promotes mTOR activation which in turn controls the expression of the transcription factor FoxP3 and IL-4. Increased IL-4 production by DN T cells may account for diminished expression of FoxP3 by CD25+/CD4+ T cells.
Fig. S1
Fig. S2

A

Control

SLE

CD3

CD4

CD3

CD4

CD19

CD8

CD19

CD8

B

CD3

CD3^+4^+

CD3^+8^+

CD3^+4^- (DN)

Control

IL-4

SLE

Control

IL-17A

SLE

IFN-γ
Table S1. Correlation of SLEDAI, BILAG and traditional biomarkers of disease activity, such as C3, C4, and anti-DNA, with 212 metabolic, cell surface, and gene expression biomarkers during 274 visits of 59 patients with SLE. Correlation r values were considered significant at p < 0.000236 when corrected for multiple comparisons (0.05/212). Metabolic biomarkers with correlation p values below 0.000236 relative to at least one of the traditional disease activity measures are shown and highlighted in yellow (SLEDAI, BILAG, C3, C4 or anti-DNA).

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