Increased Levels of NF-ATc2 Differentially Regulate CD154 and IL-2 Genes in T Cells from Patients with Systemic Lupus Erythematosus

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Vasileios C. Kyttaris,*†++ Ying Wang,*++ Yuang-Taung Juang,*† Arthur Weinstein,‡ and George C. Tsokos2*†

T cells from patients with systemic lupus erythematosus (SLE) are characterized by heightened TCR-initiated free intracytoplasmic calcium responses. We demonstrate that activated T cells from SLE patients, but not from rheumatoid arthritis patients, displayed higher levels of the calcineurin-dependent transcription factor NF-ATc2 in the nucleus compared with control T cells. DNA NF-AT-binding activity was also increased, as was the amount of NF-ATc2 bound to the promoters of CD154 (CD40L) and IL-2 genes. Nevertheless, although high NF-ATc2 levels translated into higher CD154 transcription in SLE, IL-2 transcription was decreased. The absence of important transcriptional activators (AP-1, NF-κB) and the presence of transcriptional repressors (cAMP response element modulator) on the IL-2 promoter explain this dichotomous effect. The Journal of Immunology, 2007, 178: 1960–1966.

Systemic lupus erythematosus (SLE) is characterized by significant T and B cell signaling aberrancies that play a central role in the expression of disease pathology (1). In particular, T cells once activated through the TCR show earlier and higher than normal mobilization of calcium in the cytoplasm in part due to the preaggregation of lipid rafts and the substitution of TCR ζ-chain by the FcγR chain (2). This heightened calcium influx, however, does not lead to a uniform up-regulation of all calcium-dependent genes (such as IL-2 and CD154 (CD40L, CD40L) in SLE T cells for reasons that are not fully understood to date (3); in particular, whereas IL-2 production is decreased, CD154 is increased (4, 5).

In normal T cells, calcium influx leads to activation of the phosphatase calcineurin (6), which in turn dephosphorylates the transcription factor NF-AT (7). The dephosphorylated NF-AT molecule enters the nucleus and binds to promoters of many genes often in conjunction with other molecules, such as AP-1 (8) initiating gene transcription. Gene transcription ceases once NF-AT is rephosphorylated by the export kinases casein kinase-1 and glycogen synthase kinase-3 and is transported back to the cytoplasm (9).

Of the five known NF-AT isoforms, NF-ATc2 (also called NF-AT-1 or NF-ATp) accounts for 80–90% of total NF-AT in resting T cells and is rapidly dephosphorylated by calcineurin soon after TCR-Ag-MHC engagement, thus playing a vital role in the transcription of calcium-dependent early response genes (9). NF-ATc1 (NF-AT-2/NF-ATc) is expressed in immune cells following activation, and therefore plays a role in later stages of cellular activation (9, 10). NF-ATc3 is expressed mainly in thymocytes (9), NF-ATc4 plays a role in cardiac tissue hypertrophy, and NF-AT5 is important in immune cell osmotic stress response (9).

We hypothesized that the heightened calcium influx in SLE T cells (11) is associated with increased translocation of NF-ATc2 to the nucleus in the early stages of cell activation. In this study, we present evidence that NF-ATc2 is increased in the nuclei of activated SLE T cells and that it binds to the promoters of CD154 and IL-2 genes. Nevertheless, although it results in enhanced transcription of the CD154 gene, it fails to unregulate the expression of the IL-2 gene.

Materials and Methods

Study subjects

Twenty-five female patients diagnosed with SLE and fulfilling the American College of Rheumatology criteria diagnosed 30–50 ml of blood for our studies. The mean age of the SLE patients was 35 (20–61) years old, and the mean SLE disease activity index (SLEDAI) was 2.7 (0–8) (12). One patient donated blood on three separate occasions. A total of 62.9% of the patients was taking oral prednisone at a mean dose of 13.3 mg (2.5–40). Other immunosuppressive medications that the patients were on at the time of the study were as follows: hydroxychloroquine (81.4% of the patients), azathioprine (three patients), mycophenolate mofetil (five patients), methotrexate (three patients), leflunomide (one patient), and dapsone (one patient). One patient was receiving monthly i.v. Ig, with the last dose given 1 mo before the blood draw for the study. Prednisone was held for at least 12 h before the blood draw. Each patient was matched for age with a healthy female volunteer. In addition, eight patients with rheumatoid arthritis (RA) and matched controls were also analyzed as part of the study. The institutional review boards of all involved institutions approved the study protocol, and informed consents were obtained from all of the study subjects.

T lymphocyte isolation and stimulation

Peripheral venous blood was obtained from each study subject in heparin-lithium tubes. The specimens were processed between 6 and 20 h after
collection. The blood was incubated for 30 min with a tetrameric Ab mixture against CD14, CD16, CD19, CD56, and glycA that attaches non-T cells to erythrocytes. Ficoll-containing lymphoprep gradient (Nycomed) was subsequently used to separate these complexes from T cells. Using flow cytometry, we established that the purified cells were >98% positive for CD3. Where mentioned, the T cells were stimulated with either PMA (10 ng/ml) and calcium ionophore A23187 (ionophore) (0.5 μM/ml) or anti-CD3Ab (10 μg/ml) and goat anti-mouse cross-linking Ab (25 μg/ml). For the cyclosporin treatment experiments, purified T cells were incubated at 37°C for 1 h with cyclosporin A (EMD Biosciences) at a concentration of 100 ng/ml before stimulation.

**Antibodies**

Anti-NF-ATc2, anti-NF-ATc1, anti-actin, anti-c-fos, anti-GKLF (gun-enshrined Kruppel-like factor), anti-rabbit HRP, anti-goat HRP, and anti-mouse HRP Abs were purchased from Santa Cruz Biotechnology. Rabbit anti-heterogeneous nuclear ribonucleoprotein (hnRNP) serum was and anti-mouse HRP Abs were purchased from Santa Cruz Biotechnology.

**Protein purification and Western blotting**

Blood from 18 patients and 18 controls was used for protein extraction. The cells after treatment were washed initially on ice with a 200 μl lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA supplemented with freshly added 1 mM DTT, 0.5 mM PMSF, 2 mM aprotinin, 1 mM leupeptin, 10 mM NaF, and 2 mM Na3VO4) for 15 min. At the end of the incubation, Nonidet P-40 was added to the reaction mixture at a concentration of 0.6%. The reaction mixture was vortexed for 10 s and then subjected to centrifugation at 13,000 rpm for 15 s. The supernatant was saved as cytoplasmic extract. The pellet was resuspended in 25 μl of buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 2 mM aprotinin, and 1 mM leupeptin) and then shaken for 15 min at 4°C. After centrifugation for 5 min at 13,000 rpm, the supernatant was stored as nuclear extract. We followed the manufacturer’s instructions (ECL; Amersham) for the Western blotting. The film was scanned, and the density of each band was calculated with QuantityOne software (Bio-Rad).

**EMSAs**

Nuclear extracts (2 μg) were incubated with a radiolabeled dsDNA probe, 1 μg of poly(dI/dC), and potassium chloride in a binding buffer for 15 min at room temperature. The reaction mixture was then subjected to separation in 6% nondenaturing gel (InViogen Life Technologies). The dried gel was then autoradiographed. For supershift assays, the nuclear proteins were incubated with specific Abs at 4°C for 10 min before the probe, poly(dI/dC), and binding buffer were added. The reaction was further conducted for another 15 min at room temperature. The sequences of the probes were used as follows: NF-AT consensus-binding oligonucleotide, forward, 5'-GCAGCCGAGAGAAAGTTGTTTCATA-3' and reverse, 5'-TATGAAACAATTTTTCTCTTGTCG-3'. Mutated NF-AT consensus-binding oligonucleotide was as follows: forward, 5'-GCAGCCGAGAGAAAGTTGTTTCATA-3' and reverse, 5'-TGTGGGAAGCACTTAATTATCAAGTGTGGGAAGCACTTAATTATCAAGTGTGGGAAGCACTTAATTATCAAGTG-3'. Elf-1-binding oligonucleotide was as follows: forward, 5'-TCCGAAACCTTCAGGGCCCTTCTGCTTGACATA-3' and reverse, 5'-TTGTTCAACAGGCAGAAGCCCTCCGCA-3'.

**Chromatin immunoprecipitation analysis (ChIP)**

Four million T cells were used per investigation. The cells were fixed with 1% formalin for 10 min, washed, lysed, and sonicated. The DNA-protein complexes were immunoprecipitated with the desired Ab and extracted by protein A/G-Sepharose beads (Santa Cruz Biotechnology). After several washing steps, the cross-link between DNA and protein was reversed at 65°C, followed by protein digestion with protease K, and the DNA was extracted (QiaAmp DNA Extraction kit; Qiagen). The DNA was amplified with conventional PCR and real-time PCR, as described below, with primers flanking the CD154 and IL-2 promoters. The sequences used were as follows: CD154, forward, 5'-CAGTGTCCCTTGGCTTATTA-3' and reverse, 5'-AGTGCCACCTTACTGAGGA-3'; IL-2, forward, 5'-CTTGCCTTCTGCTTGACATA-3' and reverse, 5'-TGTGGCAAGGATTTGAGGATTA-3'.

**Preparation of mRNA, reverse transcription, PCR, and real-time PCR**

Two million T cells were used for extracting RNA (RNA Easy Mini kit; Qiagen). The RNA was treated with DNase I (Qiagen) and quantitated;

**FIGURE 1.** NF-ATc2 is up-regulated early after stimulation of T cells and represents the main isoform responsible for NF-AT-binding activity. T cells from a normal volunteer were treated with PMA/ionophore and the nuclear protein complexes were immunoprecipitated with the desired Ab and followed the manufacturer’s instructions (ECL; Amersham) for the Western blotting. The film was scanned, and the density of each band was calculated with QuantityOne software (Bio-Rad).
As shown in Fig. 1A, NF-ATc2 protein levels in the nucleus of T cells peaked 1 h after stimulation.

To confirm that NF-ATc2 was the main NF-AT molecule that contributes to NF-AT-binding activity in T cells in the early stages after stimulation, we performed EMSA using an NF-AT consensus-binding oligonucleotide and nuclear protein from PMA/ionophore-stimulated T cells. As shown in Fig. 1B, NF-AT DNA binding was specific and the binding activity was lost when key nucleotides were mutated. The oligonucleotide-protein complex was shifted with an anti-NF-ATc2 mAb. Although the addition of anti-NF-ATc1 Ab caused partial shift, the addition of either anti-NF-ATc3 or anti-c-fos Ab failed to shift the complex.

The data corroborate previous observations (9) that activation of T cells causes early translocation of NF-ATc2 to the nucleus, where it constitutes the major determinant of NF-AT DNA-binding activity.

We subsequently measured the levels of NF-ATc2 in the nuclei of T cells that had been stimulated with PMA/ionophore for 1 h, from 14 patients with SLE and matched controls. We found that stimulated SLE T cells had a 2-fold increase in the levels of NF-ATc2 in their nucleus compared with control T cells (SLE NF-ATc2 levels ± SEM: 2.1 ± 0.6-fold increase over control, p = 0.012; Fig. 2, A and B). As can be seen in Fig. 2A, specimens from stimulated T cells exhibit two bands, one of the same m.w. as the one from unstimulated T cells and one of lower m.w. The lower band is considered to represent the dephosphorylated form of NF-ATc2 (that is the form that binds to DNA) in the nucleus because it disappears in the presence of calcineurin (phosphatase) inhibitors in a dose-specific manner. Less likely, it may represent a variant of NF-ATc2 that is induced upon stimulation and reacts with the anti-NF-ATc2 Ab (14–16). As shown in Fig. 2, A and C, the intensity of the lower m.w. band was higher in SLE T cell nuclear extracts than controls. We analyzed the density of the lower m.w. band in eight pairs of SLE and controls in which this lower m.w. band was observed. We found that seven of eight SLE patients had significantly higher density of this band NF-ATc2 than controls (SLE lower m.w. NF-ATc2 band density ± SEM: 2.8 ± 0.7-fold increase over control, p = 0.0156).

As expected, most resting SLE as well as control T cells had undetectable NF-ATc2 protein in their nuclei, as determined by Western blotting. Interestingly though, in 4 of 14 SLE-control pairs that had detectable NF-ATc2, there was a trend for higher NF-ATc2 expression in resting SLE T cells than controls (SLE
NF-ATc2 levels ± SEM: 3.4 ± 1.1-fold increase over control, \( p = 0.12 \). There was no detectable difference in the cytoplasmic levels of NF-ATc2 between SLE and control T cells (data not shown).

Subsequently, we asked whether direct stimulation of T cells with an anti-CD3 Ab would also lead to higher NF-ATc2 levels in SLE. Indeed, as shown in Fig. 2, C and D, anti-CD3-stimulated SLE T cells displayed significantly higher NF-ATc2 levels than controls (SLE NF-ATc2:hnRNP ratio ± SEM: 3.2 ± 0.6-fold increase over control, \( p = 0.031 \)). In addition, stimulation of T cells from eight patients with mild to moderately active RA and eight controls showed no significant differences between the two groups (RA NF-ATc2:hnRNP ratio ± SEM: 0.47 ± 0.08; control NF-ATc2:hnRNP ratio ± SEM: 0.53 ± 0.07, Mann-Whitney \( U \) test, \( p = 0.79 \)) (Fig. 2E). These experiments demonstrate that activation of SLE T cells leads to a significantly higher translocation of NF-ATc2 to the nucleus than activation of normal T cells.

**Increased nuclear T cell content of NF-ATc2 is a feature of SLE disease activity**

To examine the effect of disease activity, disease manifestations, and medications used on NF-ATc2 nuclear translocation, we correlated the levels of NF-ATc2 with the disease activity of the patients, as determined by SLEDAI, as well as with the type and dose of immunosuppressive medications. As shown in Fig. 3, NF-ATc2 levels in the PMA/ionophore-activated T cells of non-active (SLEDAI: 0) patients were comparable to controls, whereas patients that had even minimally active disease (SLEDAI: 2–8) had a 2.8 ± 1.0-fold increase over controls (\( p = 0.0078 \)). On the contrary, there was no correlation between NF-ATc2 levels and immunosuppressive medications or specific disease manifestations (such as skin disease, neuropsychiatric lupus, nephritis, and arthritis). In particular, corticosteroid dose did not correlate with levels
NF-ATc2 from SLE T cells were extracted by immune precipitation. In Fig. 5, nuclear protein from SLE T cells had significantly higher binding activity to the NF-AT-specific oligonucleotide than nuclear protein from control T cells (SLE NF-AT-binding activity > control). Similarly, in Fig. 5, binding activity in SLE T cells. After stimulating T cells from SLE patients and normal controls with PMA/ionophore to the NF-AT-specific oligonucleotide de-phosphorylation (17) or control (ethanol). After 1 h of incubation, the cells were stimulated with PMA/ionophore for 3 h. Subsequently, the cells were lysed, and the mRNA was used for real-time RT-PCR with primers specific for the CD154, IL-2, and GAPDH genes.

Because NF-ATc2 binding to CD154 promoters is higher in stimulated SLE T cells than controls, we evaluated its effect on the transcription of CD154 and IL-2 genes. To this end, we incubated T cells from both SLE patients and normal controls with either cyclosporin A (a small peptide that blocks the calcineurin-induced NF-AT dephosphorylation) (17) or control (ethanol). After 1 h of incubation, the cells were stimulated with PMA/ionophore for 3 h. Subsequently, the cells were lysed, and the mRNA was collected and reverse transcribed to cDNA, which was in turn amplified with primers specific for the CD154, IL-2, and GAPDH genes. In Fig. 6A (representative of three independent experiments), we show that after stimulation, SLE T cells up-regulated CD154 transcription significantly more than controls (in all three experiments) in accordance with previous publications (5). In contrast, the expression of GAPDH was similar between SLE and controls (Fig. 6C).

Cyclosporin effect on CD154 and IL-2 transcription in SLE and control T cells. T cells from SLE patients and normal controls were incubated with either cyclosporin A (100 ng/µl) or control (ethanol) for 1 h and then stimulated with PMA/ionophore for 3 h. Subsequently, the cells were lysed and the mRNA was used for real-time RT-PCR with primers specific for the CD154, IL-2, and GAPDH genes.

A representative experiment (of three independent experiments) of real-time PCR with CD154-specific primers of one SLE and one control sample from stimulated T cells pretreated with cyclosporin or control. A representative experiment (of three independent experiments) of real-time PCR with IL-2-specific primers of one SLE and one control sample (same pair as in A) from stimulated T cells pretreated with cyclosporin or control. A representative experiment (of three independent experiments) of real-time PCR with GAPDH-specific primers of one SLE and one control sample (same pair as in A) from stimulated T cells pretreated with cyclosporin or control.

Pathophysiologic effects of heightened NF-ATc2 recruitment in the nucleus of SLE T cells

Because NF-ATc2 binding to CD154 and IL-2 promoters is higher in stimulated SLE T cells than controls, we evaluated its effect on the transcription of CD154 and IL-2 genes. To this end, we incubated T cells from both SLE patients and normal controls with either cyclosporin A (a small peptide that blocks the calcineurin-induced NF-AT dephosphorylation) (17) or control (ethanol). After 1 h of incubation, the cells were stimulated with PMA/ionophore for 3 h. Subsequently, the cells were lysed, and the mRNA was collected and reverse transcribed to cDNA, which was in turn amplified with primers specific for the CD154, IL-2, and GAPDH genes. In Fig. 6A (representative of three independent experiments), we show that after stimulation, SLE T cells up-regulated CD154 transcription significantly more than controls (in all three experiments) in accordance with previous publications (5). In contrast, the expression of GAPDH was similar between SLE and controls (Fig. 6C).

Incubation of both SLE and control T cells with cyclosporin A led to a significant decrease in the transcription of CD154 following stimulation (Fig. 6A, one of three independent experiments). Furthermore, the levels of CD154 transcription in stimulated, cyclosporin-treated T cells did not differ between SLE patients and controls. These data point to the fact that the higher NF-ATc2
nuclear translocation is responsible for the higher early transcription rate of CD154 in SLE T cells when compared with controls.

We observed a different effect of NF-AT on the expression of IL-2. Although NF-ATc2 binding to the IL-2 promoter was higher in SLE T cells than controls, IL-2 transcription (Fig. 6B, one of three independent experiments) was lower in T cells from patients with SLE, as demonstrated previously (18). Similar to CD154, treatment of T cells with cyclosporin A led to a significant decrease in IL-2 transcription. Therefore, although NF-AT is responsible to a significant level for the transcription of IL-2, just as it is for the transcription of CD154, other factors present (increased binding of the repressor cAMP-responsive element modulator (CREM) (19)), or absent (decreased binding of AP-1 (20) and NF-kB (21)) in SLE T cells interfere with the proper NF-AT-mediated IL-2 transcription in SLE (Fig. 7).

We conclude from this set of experiments that although NF-AT translocation to the nucleus is necessary (as demonstrated by cyclosporin inhibition) for the transcription of both CD154 and IL-2 genes, it is not sufficient for the transcription of IL-2 in SLE in which other transcription factors play a significant role.

Discussion

We present evidence that the main T cell isoform of NF-AT, NF-ATc2, translocates to the nuclei of SLE T cells at higher levels than controls following T cell activation. This translates into higher NF-ATc2 binding to the promoters of both CD154 and IL-2 genes. In turn, SLE T cells show higher rates of NF-AT-mediated transcription of the CD154, but not of the IL-2 gene. Blocking the calcium-dependent phosphatase calcineurin leads to a significant decrease in the transcription of both genes. These observations establish a link between the higher TCR-mediated free intracytoplasmic calcium response that is observed in SLE T cells following activation (2) and the expression of molecules that are involved in the pathogenesis of SLE.

Our results are consistent with multiple studies, which have established that SLE T cells have higher and more sustained surface expression of CD154 following stimulation when compared with controls (5, 22), which is partially reversed by cyclosporin especially if the drug is added during the early stages of stimulation (23, 24). Transcriptional, posttranscriptional, and posttranslational mechanisms may all contribute to the increased expression of CD154 by SLE T cells. In this study, we focused on the early transcriptional regulation of the CD154 gene and we show that early increased CD154 mRNA production is linked to the established (11) increased CD3-mediated free intracytoplasmic calcium response in SLE T cells. Because cyclosporin A cannot effectively abrogate the prolonged expression of CD154 if added in activated SLE T cells, other mechanisms and not just increased early nuclear translocation of NF-ATc2, such as phosphorylated ERK (23), also contribute to the abnormally high and prolonged expression of CD154 by SLE T cells. CD154 is a costimulatory molecule on the surface of T cells that is important for B cell activation and proliferation as well as Ig isotype switch. Enhanced CD40-CD154 interaction in SLE has been demonstrated to contribute to increased Ig and autoantibody production (5), and treatment with anti-CD154 Ab has been successfully attempted (25, 26). Due to significant expression of CD154 on other cells such as platelets, direct blocking of this molecule, although proving useful, also caused significant thromboembolic side effects. For this reason, it is important that CD154 blockade be attempted by specifically targeting the expression of CD154.

Consistent with previous data, and contrary to CD154 transcription, IL-2 production following in vitro activation is decreased in SLE T cells (4). The relative deficiency in IL-2 production in SLE contributes to the increased infection rate in these patients (27), decreased activation-induced cell death (28), and regulatory T cell numbers (29).

The dichotomous effect of NF-AT on these two genes can be explained by the fact that other transcription factors that are aberrantly regulated in SLE bind to the IL-2, but not to the CD154 gene promoter. Specifically, the transcriptional repressor CREM is expressed in increased amounts in SLE T cells, binds to the IL-2 promoter, and limits directly its transcription (18). CREM also binds to the promoter of c-fos and suppresses its transcription (20). Under normal conditions, c-fos forms together with c-jun, the dimeric transcription factor AP-1, a transcriptional activator of IL-2. AP-1 combines with NF-AT on the promoter of the IL-2 gene, leading to the initiation of transcription. Lack of AP-1 leads to inability of NF-AT to act as a transcriptional activator of IL-2 (30). Therefore, the CREM-mediated AP-1 deficiency in SLE T cells helps explain the null effect of up-regulated NF-AT on the transcription of IL-2 in SLE T cells. On the contrary, other transcription factors such as Egr-1 (31) and not AP-1 may act as molecular coactivators for the NF-AT-mediated CD154 transcription.
While searching for the underlying factors of the signaling abnormalities that have been described in SLE, our laboratory has shown that CREM recruitment to the promoter of IL-2 is caused by anti-TCR/CD3 autoantibodies present in the serum of patients with SLE (32). We have not been able to show such a serum-mediated effect on either calcium response (G. Tsokos, unpublished data) or NF-AT translocation (data not shown). These observations lead us to hypothesize that both exogenous factors (autoantibodies and/or cytokines) and intracellular mechanisms play a combined role in the expression of aberrant cell signaling observed in SLE T cells. We propose that chronic exposure of T cells to serologic factors leads to increase of IL-2 and c-fos promoters of resting T cells. Once these SLE T cells are further activated through their rewired TCR, calcium enters at much higher speed and quantity leading to increased calcineurin-mediated NF-AT translocation to the nuclei. In turn, this (coupled with other signaling abnormalities) leads to up-regulation of CD154 transcription and increased B cell activity. At the same time, the presence of CREM prevents the up-regulation of AP-1 (the molecular partner of NF-AT) and indirectly prevents NF-AT from initiating the transcription of the IL-2 gene, thus leading to deficient immune regulation and susceptibility to infections.

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Disclosures

The authors have no financial conflict of interest.

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