

## Prolonged Expression of CD154 on CD4 T Cells From Pediatric Lupus Patients Correlates With Increased CD154 Transcription, Increased Nuclear Factor of Activated T Cell Activity, and Glomerulonephritis

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**Objective.** To assess CD154 expression in patients with pediatric systemic lupus erythematosus (SLE) and to explore a transcriptional mechanism that may explain dysregulated expression of CD154.

**Methods.** Cell surface CD154 expression (pre- and postactivation) in peripheral blood CD4 T cells from 29 children with lupus and 29 controls matched for age, sex, and ethnicity was examined by flow cytometry. CD154 expression was correlated with clinical features, laboratory parameters, and treatments received. Increased CD154 expression on CD4 T cells from the SLE patients was correlated with CD154 message and transcription rates by real-time reverse transcription–polymerase chain reaction (RT-PCR) and nuclear run-on assays, respectively. Nuclear factor of activated T cell (NF-AT) transcription activity and mRNA levels in CD4 T cells from SLE patients were explored by

reporter gene analysis and real-time RT-PCR, respectively.

**Results.** CD154 surface protein levels were increased 1.44-fold in CD4 T cells from SLE patients as compared with controls in cells evaluated 1 day postactivation ex vivo. This increase correlated clinically with the presence of nephritis and an elevated erythrocyte sedimentation rate. Increased CD154 protein levels also correlated with increased CD154 mRNA levels and with CD154 transcription rates, particularly at later time points following T cell activation. Reporter gene analyses revealed a trend for increased NF-AT, but decreased activator protein 1 and similar NF- $\kappa$ B, activity in CD4 T cells from SLE patients as compared with controls. Moreover, NF-AT1 and, in particular, NF-AT2 mRNA levels were notably increased in CD4 T cells from SLE patients as compared with controls.

**Conclusion.** Following activation, cell surface CD154 is increased on CD4 T cells from pediatric lupus patients as compared with controls, and this increase correlates with the presence of nephritis, increased CD154 transcription rates, and increased NF-AT activity. These results suggest that NF-AT/calcineurin inhibitors, such as tacrolimus and cyclosporine, may be beneficial in the treatment of lupus nephritis.

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The interaction between CD154 and CD40 is important for B cell development, antibody production by B cells, germinal center formation, interleukin-12 (IL-12) production, CD8 T cell effector function, and optimal T cell-dependent antibody responses (1). As might be expected from its role as a driver of multiple effector functions throughout the immune system, the

expression of CD154 on CD4 T cells is normally very tightly regulated (2). Similar to activation-dependent CD4 T cell–derived cytokines, the tight regulation of CD154 in normal CD4 T cells likely occurs at the level of transcription (3). Disturbances in this regulation have been hypothesized to contribute to overexpression in patients with systemic lupus erythematosus (SLE) (4).

The role of CD154 overexpression on CD4 T cells in SLE was first demonstrated in lupus-prone mice (5–7). Numerous experiments have validated the role of this protein in the pathogenesis of SLE in humans. Overexpression of CD154 on T cells has also been demonstrated in adult SLE patients (8,9). Based on these findings, clinical trials were conducted to study the effect of anti-CD154 monoclonal antibody (mAb) in patients with SLE. Grammer and colleagues (10) reported that SLE patients treated with anti-CD154 mAb showed disappearance of CD38-expressing antibody-secreting cells, which are not found in normal individuals, and significant improvement in anti–double-stranded DNA (anti-dsDNA) levels, proteinuria, and disease activity scores. Though it was prematurely terminated due to unanticipated thromboembolic events, an open-label trial of anti-CD154 antibody in patients with proliferative lupus nephritis showed a significant reduction in anti-dsDNA antibody levels, an increase in serum C3, and the disappearance of hematuria (11). Nevertheless, treatment of SLE patients with anti-CD154 mAb is on hold, and other ways of disrupting dysregulated CD154 expression are under exploration.

Despite the strong evidence that CD154 overexpression on CD4 T cells contributes to the pathogenesis of SLE, it remains unclear why, or at what level, this increased and prolonged expression occurs. Examining SLE CD4 T cell lines exposed to anergy-inducing stimulation, Yi et al (12) found prolonged expression of strongly phosphorylated ERK, a member of the MAPK family; in addition, specifically inhibiting a kinase of ERK blocked the early and prolonged hyperexpression of CD154. In contrast, Cedeno and colleagues (13) found decreased enzymatic activity of ERK-1 and ERK-2 in resting SLE T cells, and suggested that there are upstream signaling defects that lead to MAPK activation in lupus T cells. Thus, the defect leading to dysregulated CD154 expression in SLE remains unclear.

Multiple experiments have suggested the integral role of the transcription factor nuclear factor of activated T cells (NF-AT) in the production of CD154 messenger RNA (mRNA) (14,15), and it is possible that the overexpression of CD154 on T cells in patients with lupus is mediated by NF-AT. Along these lines, in-

creased calcium flux, which is critical to NF-AT signaling, in lupus T cells has been well established (16). Examining this possibility, Fujii et al (17) recently observed skewed NF-AT1 activation (dephosphorylated) in patients with active SLE nephritis and pleuritis. Moreover, a recent article reported increased nuclear NF-AT1 levels and NF-AT binding to the CD154 promoter in SLE T cells, suggesting that increased CD154 transcription contributes to CD154 overexpression in SLE (18).

By examining CD4 T cells from pediatric SLE patients, we sought to evaluate the factors involved in the dysregulation of CD154 expression. We corroborated the findings in adults and showed that CD154 was overexpressed in pediatric SLE CD4 T cells at later time points following activation, and this correlated with the presence of nephritis in these children. We also showed that following activation *ex vivo*, lupus CD4 T cells had increased CD154 mRNA levels and transcription rates, and this correlated with increased NF-AT transcriptional activity and with significantly increased NF-AT2 mRNA levels. These results have important implications for the use of transcriptional inhibitors of NF-AT (e.g., tacrolimus and cyclosporine) in the treatment of lupus nephritis.

## PATIENTS AND METHODS

**Patient populations.** Children and young adults ages 13–23 years who had been diagnosed as having pediatric SLE before the age of 16 were recruited from the Pediatric Rheumatology Clinic at the Children's Hospital of Philadelphia (CHOP). Clinical features, laboratory findings, and therapies used were abstracted from the patients' clinical records (Table 1). For each of the 29 SLE patients analyzed, CD4 T cells from a friend of the patient were analyzed in parallel. As closely as possible, the pairs of friends were matched for age (median age 16 years in both groups; range 13–21 years in the patients and 13–23 years in the controls), sex (27 of 29 pairs of subjects matched), and ethnicity (25 of 29 pairs of subjects matched). The mean age of the SLE patients was 16.0 years and that of the controls was 16.8 years. Since they were matched pairs of subjects, Wilcoxon signed rank testing showed the ages to be statistically different ( $P = 0.01$ ), but it is not anticipated that these small differences in age are pathophysiologically significant.

In addition, as a disease control, CD4 T cells from 6 children with juvenile idiopathic arthritis (ages 14–16 years) were studied for CD154 surface expression following T cell activation *ex vivo*. Furthermore, cells from 3 adult patients with SLE (Wake Forest University) were studied in the nuclear run-on assay at time point 0. Approval was obtained from the Institutional Review Board at CHOP and at Wake Forest University.

**Table 1.** Demographic and clinical characteristics of the 29 patients with pediatric lupus\*

Patient/ age/sex	Ethnicity	SLEDAI score	Nephritis	ESR, mm/hour	Medications
1/15/F	African American	16	Yes	48	CS (HD), MMF
2/17/F	African American	0	No	20	CS (HD), CYC (monthly), HCQ
3/21/F	African American	9	No	NA	NA
4/19/F	African American	0	Yes	NA	NA
5/18/F	African American	5	No	NA	CS (MD), HCQ, ASA, CYC (every 3 months)
6/16/F	African American	2	Yes	27	CS (HD), HCQ, CYC (monthly), warfarin sodium
7/13/F	Caucasian	4	No	23	CS (MD), HCQ, ASA
8/17/F	African American	1	Yes	65	CS (HD), CYC (monthly), sertraline, OCP, pentamidine
9/17/F	African American	9	Yes	NA	CS (LD), MMF, sertraline, pentamidine, ondansetron
10/16/F	Caucasian	8	Yes	12	CS (LD), HCQ
11/16/F	American Indian	0	Yes	9	CS (LD), HCQ, ASA
12/14/F	Indian	4	No	5	ASA, AZA, warfarin sodium, cetirizine
13/18/F	Caucasian	10	Yes	NA	CS (LD), HCQ, ASA, ranitidine
14/14/F	Caucasian	6	Yes	NA	CS (MD), HCQ, CYC (monthly), enalapril, pentamidine, isotretinoin acid
15/13/F	African American	16	Yes	70	CS (HD), HCQ, ASA, CYC (monthly), pentamidine
16/16/F	African American	21	Yes	35	CS (HD), HCQ, MTX
17/15/M	Caucasian	4	No	NA	CS (MD), ASA, MMF, methylphenidate, loratadine, albuterol
18/15/F	Caucasian	5	Yes	0	CS (LD), HCQ, CYC (every 3 months)
19/17/F	Caucasian	4	No	4	CS (LD), HCQ, ASA
20/14/F	African American	17	Yes	18	CS (MD), HCQ, ASA, AZA, losartan, enalapril, fluticasone
21/17/F	African American	2	No	10	CS (LD), HCQ
22/16/F	Caucasian	2	Yes	5	CS (LD), HCQ, CYC (every 3 months)
23/15/M	Latino	NA	Yes	2	HCQ, MMF
24/13/F	African American	4	Yes	12	CS (MD), HCQ, ASA, enalapril
25/16/F	Indian	4	No	11	HCQ, AZA, warfarin sodium
26/19/F	Latino	12	No	27	CS (LD), HCQ, tacrolimus (topical), ranitidine
27/15/F	African American	8	No	19	CS (HD), HCQ, ASA, MMF
28/15/F	African American	6	Yes	NA	CS (MD), HCQ, ASA, MTX, fluticasone, salmeterol, ranitidine
29/16/F	Caucasian	NA	No	9	CS (MD), HCQ, MMF

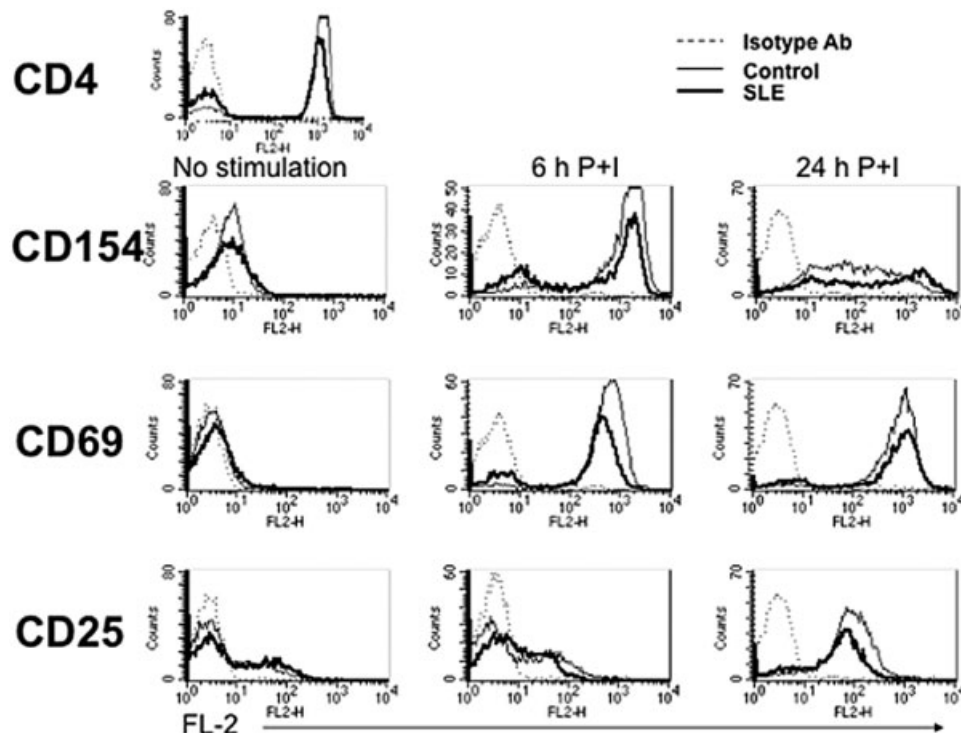
\* Ages are those at the time of the blood draw for the present study. Patients with nephritis were those who had a history of nephritis at any point during their care at Children's Hospital of Philadelphia. SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; ESR = erythrocyte sedimentation rate; CS = corticosteroids (either high dose [HD; equivalent of >25 mg of prednisone/day], medium dose [MD; equivalent of >5 mg but ≤25 mg of prednisone/day], or low dose [LD; equivalent of ≤5 mg of prednisone/day]); MMF = mycophenolate mofetil; CYC = cyclophosphamide (intravenous); HCQ = hydroxychloroquine; NA = not available; ASA = aspirin (low-dose); OCP = oral contraceptive pill; AZA = azathioprine; MTX = methotrexate.

**Peripheral blood CD4 T cell isolation, activation ex vivo, and flow cytometry.** Peripheral blood was obtained via venipuncture by nursing staff in the General Clinical Research Center at CHOP. Primary CD4 T cells were isolated by negative selection, as previously described (19). Populations studied were >90% CD4+, as assessed by flow cytometry. Following CD4 T cell isolation, cells were analyzed ex vivo, or at 6 or 20–24 hours following polyclonal activation with 25 ng/ml of phorbol myristate acetate (PMA; Sigma-Aldrich) and 1.5  $\mu$ M calcium ionophore (ionomycin; Calbiochem), in vitro. For flow cytometry, CD4 T cells were stained with phycoerythrin-conjugated anti-CD4 (Becton Dickinson), anti-CD25 (Becton Dickinson), anti-CD69 (Caltag), anti-CD154 (Ancell), or the immunoglobulin isotype control mAb (Ancell). A total of 10,000 live, cell-gated events were recorded with a FACScan and were analyzed using CellQuest software (Becton Dickinson) as previously described (20).

**Real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis.** Messenger RNA was isolated (TRIzol; Invitrogen) from CD4 T cells ex vivo or was isolated at 6 hours postactivation with PMA and ionomycin in vitro,

and complementary DNA (cDNA) was generated with reverse transcriptase. Message levels for NF-AT1, NF-AT2, and NF-AT4, as well as GAPDH as a control, were determined by SYBR Green incorporation, as detected by real-time RT-PCR (ABI Prism 7000 analyzer; PerkinElmer Life Sciences) using published NF-AT family member-specific primers (21) as previously described (22). Prior to analysis of patient samples, titrated melting (denaturing) curves were generated to optimize amplification conditions, and each amplified product was confirmed to yield a single product of the correct predicted size, based on the particular primer pair, as detected by ethidium bromide incorporation in agarose gels.

**Nuclear run-on assays.** CD154 transcription rates were determined by an avidin–biotin–based nuclear run-on assay as described elsewhere (23). Briefly, nuclei were isolated from peripheral blood CD4 T cells ex vivo, or were isolated at 4–6 or 20–24 hours following stimulation with PMA and ionomycin. Isolation of nuclei was performed as previously described (24). For in vitro RNA synthesis and purification, 1 volume (100  $\mu$ l) of a 2 $\times$  transcription buffer (200 mM KCl, 20 mM Tris HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 4 mM dithiothreitol, 4 mM each of ATP, GTP, and CTP, 200 mM sucrose, and 20% glycerol) was



**Figure 1.** Increased CD154 expression on CD4 T cells from pediatric patients with systemic lupus erythematosus (SLE) 24 hours after activation. CD4 T cells isolated from peripheral blood mononuclear cells obtained from a single, representative pediatric patient with SLE and from his or her age-, sex-, and ethnicity-matched control subject were run in parallel, along with an isotype control antibody (Ab). Flow cytometry was performed on CD4 T cells examined *ex vivo* (left column) and following polyclonal activation for 6 hours (middle column) or 24 hours (right column) with phorbol myristate acetate plus ionomycin (P+I). Analyses of cell surface CD154, CD69, and CD25 are depicted, as well as CD4 expression following negative selection. The y-axes represent the cell numbers, and the x-axes represent fluorescence intensity.

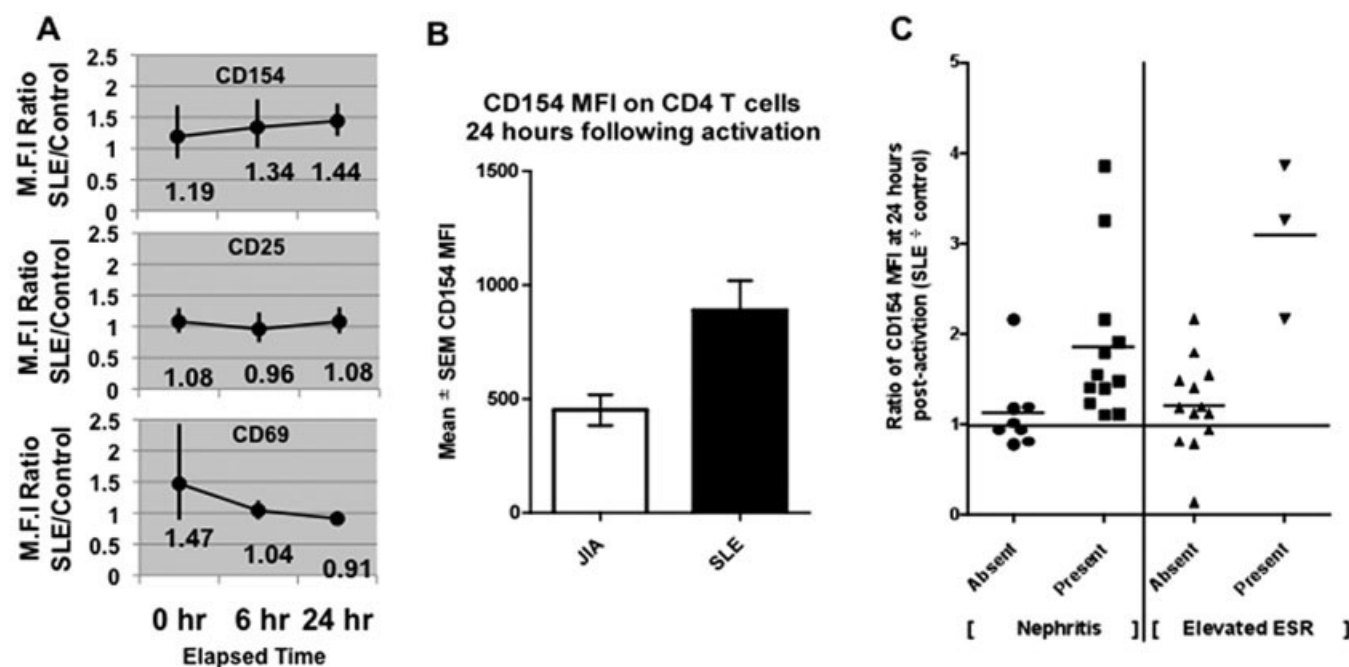
added to 1 volume (100  $\mu$ l) of nuclei on ice. Next, 8  $\mu$ l of biotin-16-UTP (from 10 mM tetralithium salt, catalog no. 1388908; Roche Molecular Biochemicals) was added, and the mixture was incubated for 30 minutes at 29°C. The reaction was stopped by adding 6  $\mu$ l of 250 mM CaCl<sub>2</sub> and 6  $\mu$ l of RNase-free DNase I (10 units/ $\mu$ l; Roche Molecular Biochemicals), and incubating for 10 minutes at 29°C. RNA was purified as above and was resuspended in 50  $\mu$ l of diethylpyrocarbonate (DEPC)-treated water.

Newly synthesized RNA containing biotin-conjugated UTP was extracted by first incubating with streptavidin-conjugated magnetic beads (Dyna Dynabeads, catalog no. 112.05; Invitrogen), which had previously been washed per the manufacturer's instructions and suspended in binding buffer, for 20 minutes at 42°C and then for 2 hours at room temperature. Beads were separated with a magnetic device (Dyna), washed, and suspended in DEPC-treated water. Then, cDNA was prepared from the beads using an Applied Biosystems transcription reagents kit, which contains TaqMan Gold enzyme (catalog no. N808-0234), according to the manufacturer's instructions for 1-cycle synthesis of RNA to cDNA, using a traditional PCR engine (program was 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes, and 4°C indefinitely).

Real-time RT-PCR was performed in triplicate samples to quantify the newly synthesized RNA (now cDNA) to obtain fold differences between patients and matched controls, using the latter as calibrators. The amplification primers for human CD154 have previously been reported (20), and those for human GAPDH (endogenous control to normalize the CD154 RNA) were 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse). Detection and quantification of the amplified real-time RT-PCR products by SYBR Green incorporation were performed as previously described (20).

**Transient transfection and reporter gene analyses.** Freshly isolated peripheral blood CD4 T cells were transiently transfected with 5  $\mu$ g of various firefly luciferase reporter plasmids using an Amaxa nucleofection system as described elsewhere (4). The activator protein 1 (AP-1)-, NF-AT-, and NF- $\kappa$ B-responsive reporter plasmids have been previously described (25). Following transfection, the cells were rested for 2 hours and then stimulated *in vitro* for 6 hours with PMA and ionomycin prior to luciferase detection as described previously (26). Transcription factor-responsive reporter plasmid activity was corrected for transfection efficiency between patient and





**Figure 2.** Increased CD154 expression on CD4 T cells from pediatric patients with systemic lupus erythematosus (SLE) 24 hours after activation is higher than that in paired matched controls and is associated with nephritis and an elevated erythrocyte sedimentation rate (ESR). **A**, Ratios (values in 29 SLE patients divided by those in 29 paired matched controls run in parallel) of CD154, CD25, and CD69 expression on CD4 T cells examined ex vivo and after 6 hours and 24 hours of T cell activation. Values are the mean and 95% confidence interval for the ratio of the mean fluorescence intensity (MFI) in SLE patients to the MFI in controls. Numbers at each data point are the actual mean values represented. **B**, CD154 expression on CD4 T cells obtained from 29 pediatric SLE patients and 6 juvenile idiopathic arthritis (JIA) disease control patients after 24 hours of T cell activation. Values are the mean  $\pm$  SEM MFI. **C**, Ratios (values in SLE patients divided by those in paired matched controls run in parallel) of CD154 expression on CD4 T cells examined after 24 hours of T cell activation in relation to the presence or absence of nephritis or an elevated ESR in samples obtained closest to the time of the analysis. Each data point represents a single patient-to-control MFI ratio; horizontal lines show the mean. Horizontal line at a ratio of 1 indicates no difference between patient and matched control.

matched control samples using the activity of the pCMV-Luc plasmid as a reference control as previously detailed (25).

**Statistical analysis.** Based on the study by Koshy et al (8), we calculated a required sample size of 20 SLE patients and 20 matched controls to have the power ( $\alpha = 0.05$ ) to detect a difference in CD154 expression between the populations at a confidence level of 0.90. To account for interassay variation in flow cytometry analyses, the ratio of SLE values to control values was calculated for each matched concurrent pair at each time point. Average mean fluorescence intensity (MFI) ratios and 95% confidence intervals (95% CIs) were calculated using the geometric mean. Comparisons of raw values and SLE-to-control ratios were made using the Wilcoxon rank sum test. Statistical analyses were calculated using GraphPad Prism 5 software.

## RESULTS

**Increased cell surface expression of CD154 on activated CD4 T cells from pediatric SLE patients as compared with closely matched controls.** CD4 T cells from SLE patients and from age-, sex-, and ethnicity-matched controls were examined side-by-side for CD154

expression and for CD25 and CD69 activation controls by flow cytometry ex vivo and after stimulation with PMA plus ionomycin for 6 hours and 24 hours. Comparison of an individual SLE patient to his or her matched control subject (see Figure 1) showed that the MFI of CD154 at 24 hours poststimulation was, on average, 1.44 times (95% CI 1.19–1.72) higher in the SLE patient than in the control subject (Figure 2A). A significant difference was also seen at 6 hours poststimulation, with SLE T cell expression being 1.34 times (95% CI 1.01–1.79) that of the control. No significant difference was seen at 0 hours (Figure 2A).

In contrast to CD154 expression, no significant difference in CD25 and CD69 expression between patients and controls was seen at any of the time points tested (Figure 2A), which is similar to previously reported findings in adult SLE patients (8,9). The increase in CD154 expression on SLE CD4 T cells did have some disease specificity, in that CD154 expression after 24 hours of stimulation in the JIA disease control group

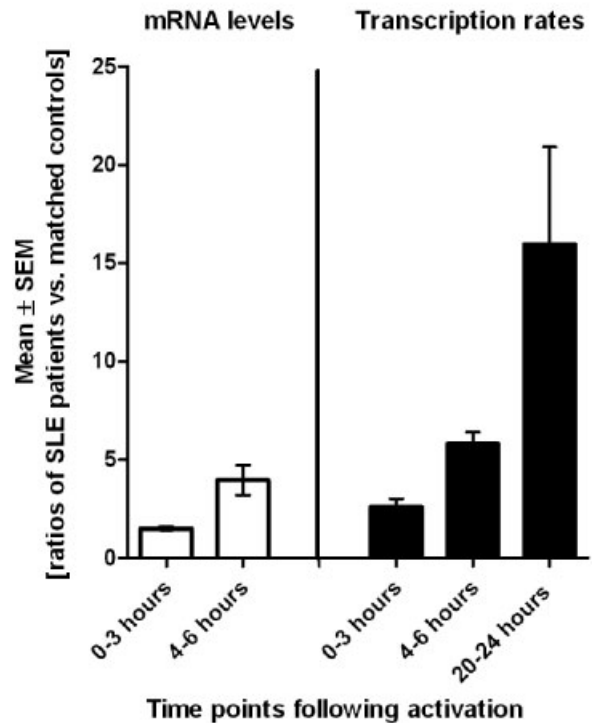
was significantly less than that in the SLE group (median 418 versus 736;  $P = 0.02$  by Wilcoxon's rank sum test) (Figure 2B).

**Association of a history of SLE nephritis with increased CD154 expression on CD4 T cells at 24 hours.** The strong association of increased CD154 expression on SLE CD4 T cells following 24 hours of activation was analyzed for clinical associations in the SLE cohort. Demographic, laboratory, and clinical data (Table 1) that showed no association with CD154 levels included the prednisone dose, whether or not a patient was receiving specific medications (hydroxychloroquine, any disease-modifying antirheumatic drug, or specifically, cyclophosphamide), the Systemic Lupus Erythematosus Disease Activity Index score (27), the C3 and C4 levels, the absolute lymphocyte count, and history of central nervous system manifestations of SLE.

However, the CD154 levels were significantly higher at 24 hours in the 12 SLE patients who had a history of biopsy-proven SLE glomerulonephritis at some point during their disease course, as compared with their respective controls, than in the 9 SLE patients who did not have a history of nephritis, as compared with their respective controls ( $P = 0.01$ ) (Figure 2C). Similarly, in the 3 SLE patients who had an elevated erythrocyte sedimentation rate (ESR;  $>20$  mm/hour) at the blood draw closest to the date of their CD154 measurement (generally within 1 month), the CD154 levels at 24 hours were significantly higher, as compared with their respective controls, than in the 13 SLE patients who had a normal ESR ( $P = 0.0004$ ). Thus, elevated CD154 expression on SLE CD4 T cells following 24 hours of activation was strongly correlated with the presence of glomerulonephritis and an elevated ESR.

**Increased CD154 mRNA levels and transcription rates in SLE CD4 T cells.** To assess the etiology of increased surface expression of CD154 on SLE CD4 T cells, real-time RT-PCR was used to quantify CD154 and GAPDH message levels in 4 SLE patients and their matched controls. After correcting for the starting RNA levels by GAPDH, increased CD154 mRNA levels were seen in the 4 SLE patients as compared with their individual matched control subjects (Figure 3). This was seen both ex vivo and at 6 hours following T cell stimulation with PMA plus ionomycin. In these same patients, nuclear run-on assays were used to quantify transcription rates at 6 hours and at 20–24 hours postactivation. Increased CD154 transcription rates were seen in SLE patients as compared with their individual controls at all time points examined, particularly at the later

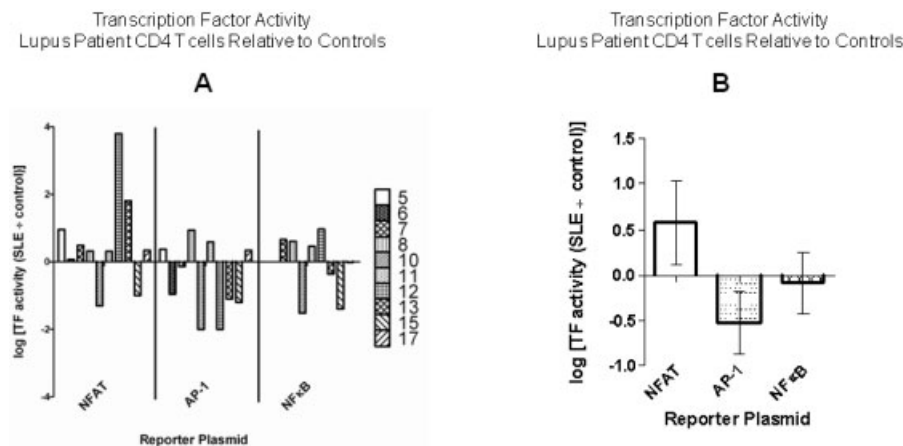
### CD154 mRNA Levels and Transcription Rates



**Figure 3.** Increased CD154 mRNA levels and transcription rates in CD4 T cells from pediatric patients with systemic lupus erythematosus (SLE). Ratios (values in SLE patients divided by those in paired matched controls run in parallel) of CD154 mRNA levels and transcription rates (relative to GAPDH control) in CD4 T cells analyzed ex vivo (0–3 hours) and after 4–6 hours and 20–24 hours (transcription rate only) of T cell activation are shown. Values are the mean  $\pm$  SEM of 4 pairs of SLE patients and controls, except for the ex vivo transcription rate, for which 3 pairs of SLE patients and controls were used.

20–24-hour time point (Figure 3). Thus, the increased CD154 cell surface expression on SLE CD4 T cells correlated with notably elevated rates of CD154 transcription.

**Increased activity and levels of NF-AT, particularly NF-AT2, in SLE CD4 T cells.** Using luciferase reporter plasmids and polyclonal in vitro stimulation of CD4 T cells obtained from SLE patients and matched controls, the transcriptional activity of NF-AT, AP-1, and NF- $\kappa$ B was measured and then corrected for transfection efficiency. Each SLE patient was compared with his or her matched control, and the log of the ratio was plotted, such that increased transcription factor activity in the lupus patient relative to that in the matched control was  $>0$  (above the line) and decreased tran-



**Figure 4.** Increased nuclear factor of activated T cell (NF-AT) transcriptional activity in CD4 T cells from pediatric patients with systemic lupus erythematosus (SLE). **A**, Log of the ratios (values in SLE patients divided by those in paired matched controls run in parallel and corrected for transfection efficiency) of transcription factor (TF) activities in polyclonally activated CD4 T cells in 10 matched pairs of subjects, analyzing NF-AT, activator protein 1 (AP-1), and NF- $\kappa$ B activities. Increased activity in the SLE patient relative to the paired matched control subject plots above the horizontal line ( $>0$ ), and decreased activity falls below the line ( $<0$ ). **B**, Ratios (values in SLE patients divided by those in paired matched controls run in parallel) of the transcription factor activities of NF-AT, AP-1, and NF- $\kappa$ B in the 10 matched pairs of SLE patients and controls shown in A. Values are the mean  $\pm$  SEM.

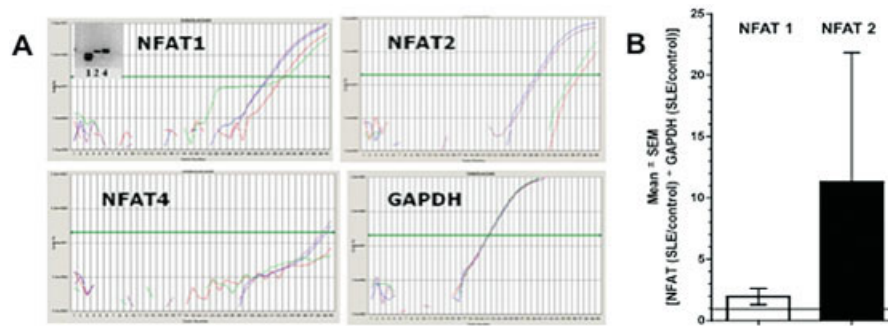
scription factor activity in the lupus patient relative to that in the matched control was  $<0$  (below the line) (Figure 4A). For most paired comparisons, there was a clear trend toward increased NF-AT activity in CD4 T cells from the SLE patients relative to their controls ( $P = 0.057$ ) (Figure 4B). In contrast, AP-1 activity appeared to be mostly decreased (below the line) in CD4 T cells from SLE patients relative to their controls, and NF- $\kappa$ B activity was similar on average (Figure 4B). Thus, there was a relative increase in NF-AT activity in most SLE patients as compared with their matched controls.

Using real-time RT-PCR and probes specific for NF-AT1, NF-AT2, and NF-AT4, we sought to further characterize the increased NF-AT activity in SLE CD4 T cells versus controls in 5 matched pairs of subjects, an example of which is shown in Figure 5A. Both NF-AT1 and NF-AT2 levels were increased, but NF-AT2 levels were markedly higher than the levels in their controls (Figure 5B). NF-AT4 levels were, for the most part, undetectable, which is consistent with NF-AT4 being present primarily in thymocytes (28). Therefore, NF-AT1 levels were increased in SLE CD4 T cells relative to their controls, a finding similar to that of previous studies showing increased NF-AT1 levels in the nucleus of SLE CD4 T cells (17,18). Interestingly, NF-AT2 levels

were substantially elevated in SLE CD4 T cells, which likely reflects increased NF-AT activity, since NF-AT2 transcription is positively regulated by NF-AT engagement of the NF-AT2 transcriptional promoter (29,30).

## DISCUSSION

We present herein the first evidence in children with SLE, as compared with their age-, sex-, and ethnicity-matched controls, that CD154 is overexpressed on activated CD4 T cells (Figures 1, 2A, and 2B), findings similar to those in studies of adults with SLE (8,9,31). This increased expression of CD154 was specific, since there was no difference in the expression of the activation markers CD25 and CD69 (Figure 2A), which again, is similar to the findings in adults with SLE (8,9). These increased CD154 levels corresponded to increased levels of CD154 mRNA and increased rates of transcription, especially at late time points (24 hours) following CD4 T cell activation (Figure 3). Although this does not formally rule out a role for potential increased CD154 mRNA stability in SLE (32), the increased CD154 transcription rates at late time points postactivation were markedly elevated and strongly suggest that increased CD154 transcription at later time points postac-



**Figure 5.** Increased levels of nuclear factor of activated T cells (NF-AT) in CD4 T cells from pediatric patients with systemic lupus erythematosus (SLE). **A**, Real-time reverse transcription–polymerase chain reaction (RT-PCR) was used to quantify NF-AT1, NF-AT2, NF-AT4, and GAPDH (control) mRNA levels (analyzed in duplicate) in CD4 T cells from a representative SLE patient (blue and purple curves) and a paired matched control subject (green and red curves). **Inset**, The predicted sizes of the PCR products for each specific primer pair used to detect the NF-AT1, NF-AT2, and NF-AT4 message were confirmed by agarose gel electrophoresis (lanes 1, 2, and 4, respectively). **B**, Ratios (values in SLE patients divided by those in paired matched controls run in parallel) of NF-AT1 and NF-AT2 message levels (corrected for loading by the control GAPDH message levels) are shown. Values are the mean  $\pm$  SEM of 5 matched pairs of SLE patients and controls. Horizontal line at a ratio of 1 indicates no difference between patients and controls.

tivation contributes to the increased CD154 expression on SLE CD4 T cells.

CD154 transcription is normally highly dependent upon NF-AT activity following T cell activation (14), and relative to the controls, there was a clear trend toward increased NF-AT activity in CD4 T cells from most of the SLE patients studied (Figure 4). In contrast, AP-1 activity was decreased, and NF- $\kappa$ B activity was similar, on average (Figure 4). Thus, there was a relative increase in NF-AT activity for most SLE patients relative to their matched controls. This is not surprising, given the known increased flux of intracellular calcium seen in SLE T cells (16) and the dependence of NF-AT on prolonged calcium signaling to activate calcineurin, which in turn, dephosphorylates NF-AT and helps shuttle it to the nucleus, where it binds target genes such as CD154 (33). In addition, other investigators have shown increased NF-AT1 levels and DNA binding activity in SLE T cells (17,18).

We also identified increased NF-AT1 levels in SLE patients, but the levels of NF-AT2 were more dramatically elevated in the patients as compared with the controls (Figure 5). NF-AT1 is expressed constitutively in resting T cell cytoplasm (25,34), but the inducible isoform of NF-AT2 is up-regulated by NF-AT itself (29,30). Following T cell activation, there is a relative switch in abundance from NF-AT1 to NF-AT2, and it has been suggested that NF-AT2 is the NF-AT family member that is essential for effector T cell development

and function (29). Conversely, NF-AT2 levels are decreased in FoxP3-expressing regulatory CD4 T cells, and this is because the FoxP3 transcription factor directly binds to and inhibits the NF-AT2 transcriptional promoter (35). The increased NF-AT2 levels in CD4 T cells from SLE patients may thus represent a more mature/effector T cell phenotype. Since NF-AT2 quickly becomes the predominant NF-AT family member in activated T cells, it may be the most likely NF-AT family member involved in the later wave of CD154 transcription.

In contrast to elevated NF-AT activity, our finding of decreased AP-1 activity and similar or lower NF- $\kappa$ B activity relative to their activities in controls is consistent with the findings in adults with SLE (36,37), and it is known that these transcription factors are necessary for IL-2 production, which is decreased in SLE T cells (38). The AP-1 deficiency is thought to be due to the binding of the transcriptional repressor CREM, which is expressed in increased amounts in SLE T cells, to the promoter of *c-fos* and the suppression of its transcription (38). Normally, c-Fos combines with c-Jun to produce the dimeric transcription factor AP-1, which combines with NF-AT on the IL-2 promoter, thus contributing to transcriptional activation. The decreased NF- $\kappa$ B activity likely stems from a p65 subunit deficiency, which is of unclear cause (38). However, what does seem to be more evident is that calcium flux and associated NF-AT activity are increased in CD4 T cells



from lupus patients and likely contribute to the overexpression of CD154 and the pathogenesis of SLE (16).

The interaction of CD154 with CD40 appears to play a particular role in the pathogenesis of SLE nephritis. In addition to the studies demonstrating improvement in hematuria (11) and proteinuria (10) in patients with SLE glomerulonephritis treated with anti-CD154 monoclonal antibody, CD40 expression was shown to be up-regulated in renal cells from patients with class III and class IV nephritis (39). CD154-activated monocytes were shown to stimulate the glomerular inflammatory response (40). Moreover, the findings of multiple mouse experiments are supportive of the role of CD154 in models of SLE nephritis (5,7,41). Potential mechanisms for the role of CD154 in SLE nephritis include chronic stimulation of CD40-expressing renal parenchymal cells (e.g., endothelial, mesangial, and distal tubular cells) (39). Alternatively, prolonged CD154 expression and stimulation of B cells likely contributes to increased autoantibody production (e.g., anti-dsDNA) that may contribute to the renal lesion.

In the present study, we demonstrated that patients who, at some point during their disease course, developed SLE nephritis had significantly higher CD154 levels at 24 hours than patients who did not, as compared with their respective controls (Figure 2C). It is unclear whether the higher CD154 levels signify a more active generalized disease state that, in turn, leads to nephritis or whether the higher CD154 expression signifies a specific propensity to develop nephritis, independently of other disease manifestations. Along these lines, other investigators have shown that CD134 expression on CD4 T cells from SLE patients is associated with nephritis (42). Nevertheless, the finding that the development of central nervous system lupus, which is also a potentially severe disease manifestation, did not influence CD154 expression suggests that increased CD154 expression does not simply signify a more active generalized disease state. Thus, there may be a role for specifically targeting CD154 in the treatment of lupus nephritis. Because a monoclonal antibody approach to CD154, although effective, has been complicated by unanticipated clotting abnormalities (43), other means of targeting dysregulated CD154 expression are in order (44).

Cyclosporin A (CSA) blocks the phosphatase activity of calcineurin and therefore prevents the nuclear translocation of NF-AT. Since CD154 overexpression plays a crucial role in the pathogenesis of SLE, our demonstration that NF-AT mediates this overexpression in children renders CSA an attractive potential agent in

the treatment of lupus. To date, the published experience with this agent in childhood SLE consists of 2 prospective studies (45,46) and an open, randomized comparison of CSA versus corticosteroids plus cyclophosphamide (47), all of which showed varying degrees of improvement in disease manifestations, particularly proteinuria. The lack of a controlled clinical trial of this medication in lupus likely reflects its adverse effects profile, which consists of nephrotoxicity and reversible hypertension. In vitro and ex vivo experiments investigating the effects of CSA on CD4 T cell CD154 expression in lupus have been inconclusive (12,17,18). We argue that the presence of an established therapeutic agent that acts on a demonstrated substrate of SLE pathogenesis strongly warrants a controlled clinical trial of the efficacy of CSA in SLE, particularly in those with nephritis.

Tacrolimus is a newer, more potent medication that also acts on the calcineurin/NF-AT pathway (33) and therefore is potentially efficacious in SLE. In topical form, it has demonstrated efficacy as a treatment for cutaneous manifestations of lupus (48), and a small open-label trial of oral tacrolimus in patients with pediatric-onset lupus nephritis demonstrated efficacy in almost all the patients studied (49). In addition, Roehrl and colleagues (50) identified a small organic molecule that selectively interferes with the interaction between calcineurin and NF-AT, and unlike CSA or tacrolimus, does not block all downstream signaling of calcineurin. Such agents with greater specificity for the aberrant pathways in autoimmune disease have the potential to be as efficacious or more efficacious, with fewer adverse effects than current agents that are less discriminating in their targets.

In summary, like adults with SLE (8,9,31), pediatric patients with SLE also demonstrate increased CD154 expression on CD4 T cells, particularly at later time points postactivation. This increase in CD154 expression is associated with the presence of nephritis, increased CD154 transcription, and increased NF-AT levels and transcriptional activity. Therefore, an argument can be made for exploring a role of NF-AT/calcineurin inhibitors, particularly novel agents with increased benefit/side effects ratios, in the treatment of pediatric SLE nephritis.

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### AUTHOR CONTRIBUTIONS

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. Cron 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.

**Study conception and design.** Genin, Cron.

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### REFERENCES

- Van Kooten C, Banchereau J. CD40-CD40 ligand. *J Leukoc Biol* 2000;67:2-17.
- Roy M, Waldschmidt T, Aruffo A, Ledbetter JA, Noelle RJ. The regulation of the expression of gp39, the CD40 ligand, on normal and cloned CD4<sup>+</sup> T cells. *J Immunol* 1993;151:2497-510.
- Brorson KA, Beverly B, Kang SM, Lenardo M, Schwartz RH. Transcriptional regulation of cytokine genes in nontransformed T cells: apparent constitutive signals in run-on assays can be caused by repeat sequences. *J Immunol* 1991;147:3601-9.
- Cron RQ. CD154 transcriptional regulation in primary human CD4 T cells. *Immunol Res* 2003;27:185-202.
- Daikh DI, Finck BK, Linsley PS, Hollenbaugh D, Wofsy D. Long-term inhibition of murine lupus by brief simultaneous blockade of the B7/CD28 and CD40/gp39 costimulation pathways. *J Immunol* 1997;159:3104-8.
- Datta SK, Kalled SL. CD40-CD40 ligand interaction in autoimmune disease [review]. *Arthritis Rheum* 1997;40:1735-45.
- Kalled SL, Cutler AH, Datta SK, Thomas DW. Anti-CD40 ligand antibody treatment of SNF1 mice with established nephritis: preservation of kidney function. *J Immunol* 1998;160:2158-65.
- Koshy M, Berger D, Crow MK. Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes. *J Clin Invest* 1996;98:826-37.
- Desai-Mehta A, Lu L, Ramsey-Goldman R, Datta SK. Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. *J Clin Invest* 1996;97:2063-73.
- Grammer AC, Slota R, Fischer R, Gur H, Girschick H, Yarboro C, et al. Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154-CD40 interactions [published erratum appears in *J Clin Invest* 2007;117:835]. *J Clin Invest* 2003;112:1506-20.
- Boumpas DT, Furie R, Manzi S, Illei GG, Wallace DJ, Balow JE, et al, on behalf of the BG9588 Lupus Nephritis Trial Group. A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with proliferative lupus glomerulonephritis. *Arthritis Rheum* 2003;48:719-27.
- Yi Y, McNerney M, Datta SK. Regulatory defects in Cbl and mitogen-activated protein kinase (extracellular signal-related kinase) pathways cause persistent hyperexpression of CD40 ligand in human lupus T cells. *J Immunol* 2000;165:6627-34.
- Cedeno S, Cifarelli DF, Blasini AM, Paris M, Placeres F, Alonso G, et al. Defective activity of ERK-1 and ERK-2 mitogen-activated protein kinases in peripheral blood T lymphocytes from patients with systemic lupus erythematosus: potential role of altered coupling of Ras guanine nucleotide exchange factor hSos to adapter protein Grb2 in lupus T cells. *Clin Immunol* 2003;106:41-9.
- Schubert LA, King G, Cron RQ, Lewis DB, Aruffo A, Hollenbaugh D. The human gp39 promoter: two distinct nuclear factors of activated T cell protein-binding elements contribute independently to transcriptional activation. *J Biol Chem* 1995;270:29624-7.
- Tsytyskova AV, Tsitsikov EN, Geha RS. The CD40L promoter contains nuclear factor of activated T cells-binding motifs which require AP-1 binding for activation of transcription. *J Biol Chem* 1996;271:3763-70.
- Tsokos GC. Calcium signaling in systemic lupus erythematosus lymphocytes and its therapeutic exploitation [editorial]. *Arthritis Rheum* 2008;58:1216-9.
- Fujii Y, Fujii K, Iwata S, Suzuki K, Azuma T, Saito K, et al. Abnormal intracellular distribution of NFAT1 in T lymphocytes from patients with systemic lupus erythematosus and characteristic clinical features. *Clin Immunol* 2006;119:297-306.
- Kyttaris VC, Wang Y, Juang YT, Weinstein A, Tsokos GC. Increased levels of NF-ATc2 differentially regulate CD154 and IL-2 genes in T cells from patients with systemic lupus erythematosus. *J Immunol* 2007;178:1960-6.
- Cron RQ. HIV-1, NFAT, and cyclosporin: immunosuppression for the immunosuppressed? *DNA Cell Biol* 2001;20:761-7.
- Jullien P, Cron RQ, Dabbagh K, Cleary A, Chen L, Tran P, et al. Decreased CD154 expression by neonatal CD4<sup>+</sup> T cells is due to limitations in both proximal and distal events of T cell activation. *Int Immunol* 2003;15:1461-72.
- Jinquan T, Quan S, Jacobi HH, Reimert CM, Millner A, Hansen JB, et al. Cutting edge: expression of the NF of activated T cells in eosinophils: regulation by IL-4 and IL-5. *J Immunol* 1999;163:21-4.
- Schubert LA, Cron RQ, Cleary AM, Brunner M, Song A, Lu LS, et al. A T cell-specific enhancer of the human CD40 ligand gene. *J Biol Chem* 2002;277:7386-95.
- Patrone G, Puppo F, Cusano R, Scaranari M, Ceccherini I, Puliti A, et al. Nuclear run-on assay using biotin labeling, magnetic bead capture and analysis by fluorescence-based RT-PCR. *Biotechniques* 2000;29:1012-4, 1016-7.
- Brunner M, Zhang M, Genin A, Ho IC, Cron RQ. A T-cell-specific CD154 transcriptional enhancer located just upstream of the promoter. *Genes Immun* 2008;9:640-9.
- Cron RQ, Bort SJ, Wang Y, Brunvand MW, Lewis DB. T cell priming enhances IL-4 gene expression by increasing nuclear factor of activated T cells. *J Immunol* 1999;162:860-70.
- Cron RQ, Bandyopadhyay R, Genin A, Brunner M, Kersh GJ, Yin J, et al. Early growth response-1 is required for CD154 transcription. *J Immunol* 2006;176:811-8.
- Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang DH, and the Committee on Prognosis Studies in SLE. Derivation of the SLEDAI: a disease activity index for lupus patients. *Arthritis Rheum* 1992;35:630-40.
- Amasaki Y, Masuda ES, Imamura R, Arai K, Arai N. Distinct NFAT family proteins are involved in the nuclear NFAT-DNA binding complexes from human thymocyte subsets. *J Immunol* 1998;160:2324-33.
- Serfling E, Chuvpilo S, Liu J, Hofer T, Palmethofer A. NFATc1 autoregulation: a crucial step for cell-fate determination. *Trends Immunol* 2006;27:461-9.
- Zhou B, Cron RQ, Wu B, Genin A, Wang Z, Liu S, et al. Regulation of the murine Nfatc1 gene by NFATc2. *J Biol Chem* 2002;277:10704-11.
- Mishra N, Brown DR, Olorenshaw IM, Kammer GM. Trichostatin A reverses skewed expression of CD154, interleukin-10, and

- interferon- $\gamma$  gene and protein expression in lupus T cells. *Proc Natl Acad Sci U S A* 2001;98:2628–33.
32. Hamilton BJ, Genin A, Cron RQ, Rigby WF. Delineation of a novel pathway that regulates CD154 (CD40 ligand) expression. *Mol Cell Biol* 2003;23:510–25.
  33. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 1997;15:707–47.
  34. Lyakh L, Ghosh P, Rice NR. Expression of NFAT-family proteins in normal human T cells. *Mol Cell Biol* 1997;17:2475–84.
  35. Torgerson TR, Genin A, Chen C, Zhang M, Zhou B, Anover-Sombke S, et al. FOXP3 inhibits activation-induced NFAT2 expression in T cells thereby limiting effector cytokine expression. *J Immunol* 2009;183:907–15.
  36. Kyttaris VC, Juang YT, Tenbrock K, Weinstein A, Tsokos GC. Cyclic adenosine 5'-monophosphate response element modulator is responsible for the decreased expression of c-fos and activator protein-1 binding in T cells from patients with systemic lupus erythematosus. *J Immunol* 2004;173:3557–63.
  37. Wong HK, Kammer GM, Dennis G, Tsokos GC. Abnormal NF- $\kappa$ B activity in T lymphocytes from patients with systemic lupus erythematosus is associated with decreased p65-RelA protein expression. *J Immunol* 1999;163:1682–9.
  38. Katsiari CG, Tsokos GC. Transcriptional repression of interleukin-2 in human systemic lupus erythematosus. *Autoimmun Rev* 2006;5:118–21.
  39. Yellin MJ, D'Agati V, Parkinson G, Han AS, Szema A, Baum D, et al. Immunohistologic analysis of renal CD40 and CD40L expression in lupus nephritis and other glomerulonephritides. *Arthritis Rheum* 1997;40:124–34.
  40. Kuroiwa T, Lee EG, Danning CL, Illei GG, McInnes IB, Boumpas DT. CD40 ligand-activated human monocytes amplify glomerular inflammatory responses through soluble and cell-to-cell contact-dependent mechanisms. *J Immunol* 1999;163:2168–75.
  41. Mohan C, Shi Y, Laman JD, Datta SK. Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis. *J Immunol* 1995;154:1470–80.
  42. Patschan S, Dolff S, Kribben A, Durig J, Patschan D, Wilde B, et al. CD134 expression on CD4+ T cells is associated with nephritis and disease activity in patients with systemic lupus erythematosus. *Clin Exp Immunol* 2006;145:235–42.
  43. Toubi E, Shoenfeld Y. The role of CD40-CD154 interactions in autoimmunity and the benefit of disrupting this pathway. *Autoimmunity* 2004;37:457–64.
  44. Cron RQ. CD154 and lupus. *Pediatr Rheumatol Online J* 2003;1:172–81.
  45. Feutren G, Querin S, Noel LH, Chatenoud L, Beaurain G, Tron F, et al. Effects of cyclosporine in severe systemic lupus erythematosus. *J Pediatr* 1987;111:1063–8.
  46. Baca V, Catalan T, Villasis-Keever M, Ramon G, Morales AM, Rodriguez-Leyva F. Effect of low-dose cyclosporine A in the treatment of refractory proteinuria in childhood-onset lupus nephritis. *Lupus* 2006;15:490–5.
  47. Fu LW, Yang LY, Chen WP, Lin CY. Clinical efficacy of cyclosporin A Neoral in the treatment of paediatric lupus nephritis with heavy proteinuria. *Br J Rheumatol* 1998;37:217–21.
  48. Tzung TY, Liu YS, Chang HW. Tacrolimus vs. clobetasol propionate in the treatment of facial cutaneous lupus erythematosus: a randomized, double-blind, bilateral comparison study. *Br J Dermatol* 2007;156:191–2.
  49. Tanaka H, Oki E, Tsugawa K, Nonaka K, Suzuki K, Ito E. Effective treatment of young patients with pediatric-onset, long-standing lupus nephritis with tacrolimus given as a single daily dose: an open-label pilot study. *Lupus* 2007;16:896–900.
  50. Roehrl MH, Kang S, Aramburu J, Wagner G, Rao A, Hogan PG. Selective inhibition of calcineurin-NFAT signaling by blocking protein-protein interaction with small organic molecules. *Proc Natl Acad Sci U S A* 2004;101:7554–9.