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Prolonged CD154 Expression on Pediatric Lupus CD4 T Cells Correlates with Increased CD154 Transcription, Increased NFAT Activity, and Glomerulonephritis

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Abstract

Objective—To assess CD154 expression in pediatric lupus and explore a transcriptional mechanism explaining dysregulated CD154 expression.

Methods—Cell surface CD154 expression was examined, pre- and post-activation, on peripheral blood CD4 T cells from 29 children with lupus and matched controls by flow cytometry. CD154 expression was correlated with clinical features, laboratory parameters, and treatments received. Increased CD154 expression on lupus CD4 T cells was correlated with CD154 message and transcription rates by real-time RT-PCR and nuclear run-on assays, respectively. NFAT transcriptional activity and NFAT mRNA levels in lupus CD4 T cells were explored by reporter gene analysis and real-time RT-PCR, respectively.

Results—CD154 surface protein levels were increased 1.44-fold on lupus CD4 T cells compared to controls at one day post-activation *ex vivo*. This increase correlated clinically with the presence of nephritis and elevated erythrocyte sedimentation rate. Increased CD154 protein also correlated with increased CD154 mRNA levels and rates of CD154 transcription, particularly at later time-points post-T cell activation. Reporter gene analyses revealed a trend for increased NFAT, but decreased AP-1 and similar NFκB, activity in lupus CD4 T cell compared to controls. Moreover, NFAT1 and, in particular, NFAT2 mRNA levels were notably increased in lupus CD4 T cells compared to controls.

Conclusion—Following activation, cell surface CD154 is increased on pediatric lupus CD4 T cells compared to controls, and this correlates with the presence of nephritis, increased CD154

transcription rates, and NFAT activity. These results suggest that NFAT/calcineurin inhibitors, such as tacrolimus and cyclosporine, may be beneficial in treating lupus nephritis.

The interaction between CD154 and CD40 is important for B cell development, antibody production by B cells, germinal center formation, IL-12 production, CD8 T cell effector function, and optimal T cell-dependent antibody responses (1). As might be expected from its role of driving 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 over-expression in systemic lupus erythematosus (SLE) (4).

The role of CD154 over-expression 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 human SLE. Over-expression 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. Lipsky and colleagues reported SLE patients treated with anti-CD154 mAb showed disappearance of CD38-expressing antibody-secreting cells, not found in normal individuals, and significant improvement in anti-double-stranded DNA (anti-dsDNA) levels, proteinuria, and disease activity scores (10). 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 significant reduction in anti-dsDNA antibodies, increase in serum C3, and 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 for CD154 over-expression on CD4 T cells contributing to the pathogenesis of SLE, it remains unclear why, or at what level, this increased and prolonged expression occurs. Examining lupus CD4 T cell lines exposed to anergy-inducing stimulation, Datta *et al.* found prolonged expression of strongly phosphorylated extracellular signal-regulated kinase (ERK), a member of the MAPK family, and specifically inhibiting a kinase of ERK blocked the early and prolonged hyper-expression of CD154 (12). In contrast, Cedeno and colleagues found decreased enzymatic activity of ERK-1 and ERK-2 in resting SLE T cells, and suggested that there are upstream signaling defects leading to MAPK activation in lupus T cells (13). 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 (NFAT), in CD154 mRNA production (14,15), and it is possible that the over-expression of CD154 on T cells in lupus is NFAT-mediated. Along these lines, increased calcium flux, critical to NFAT signaling, in lupus T cells has been well established (16). Examining this possibility, Fujii *et al.* recently observed skewed NFAT1 activation (dephosphorylated) in patients with active SLE nephritis and pleuritis (17). Moreover, a recent article reported increased nuclear NFAT1 levels and NFAT binding to the CD154 promoter in SLE T cells, suggesting increased CD154 transcription contributes to CD154 over-expression 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 corroborate the findings in adults that CD154 is over-expressed in pediatric SLE CD4 T cells at later time points post-activation, and this correlates with the presence of nephritis in these children. We also show that following activation *ex vivo* lupus CD4 T cells have increased CD154 mRNA levels and

transcription rates, and this correlates with increased NFAT transcriptional activity and significantly increased NFAT2 mRNA levels. These results have important implications for the use of transcriptional inhibitors of NFAT (e.g., tacrolimus and cyclosporine) in the treatment of lupus nephritis.

PATIENTS AND METHODS

Patient populations. Children and young adults, ages 13–23 years, diagnosed with 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 results, and therapies utilized were abstracted from the clinical record (Table 1). For each SLE patient analyzed, CD4 T cells from a friend of the patient were analyzed at the same time, side-by-side. As close as possible, the friends were matched for age [median age of 16 years for both the SLE group (range, 13–21 years) and the controls (range, 13–23 years)], sex (27 of 29 pairs matched), and ethnicity (25 of 29 pairs matched). Statistically, the mean ages for the SLE group and the controls were 16.0 and 16.8 years, respectively. Since they were matched pairs, the Wilcoxon signed rank test showed the ages were 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 six children (ages 14–16 years) with juvenile idiopathic arthritis were studied for CD154 surface expression following T cell activation *ex vivo*. Lastly, 3 adult SLE patients (Wake Forest University) were studied for the nuclear run-on assay at time point 0 (Figure 3). Institutional Review Board approval was obtained from CHOP and Wake Forest University, respectively.

Peripheral blood CD4 T cell isolation, activation *ex vivo* and flow cytometric (FCM) analysis

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 FCM (Figure 1). Following CD4 T cell isolation, cells were analyzed *ex vivo*, or 6, or 20–24 hours following polyclonal activation with phorbol ester (PMA, 25 ng/ml; Sigma-Aldrich, St. Louis, MO) and calcium ionophore (ionomycin, 1.5 μ M; Calbiochem, San Diego, CA). For FCM, CD4 T cells were stained with phyco-erythrin-conjugated anti-CD4 (Becton-Dickinson, San Jose, CA), anti-CD25 (Becton-Dickinson), anti-CD69 (Caltag, Burlingame, CA), anti-CD154 (Ancell, Bayport, MN), or the immunoglobulin isotype control mAb (Ancell). Ten-thousand live cell-gated events were recorded with a FACScan and analyzed using CellQuest software (Becton-Dickinson) as previously described (20).

Real-time RT-PCR analysis

mRNA was isolated (Trizol; Invitrogen, Carlsbad, CA) from CD4 T cells *ex vivo*, or at 6 hours post-activation with PMA and ionomycin *in vitro*, and cDNA was generated with reverse transcriptase. Message levels for NFAT1, NFAT2, and NFAT4, as well as GAPDH as a control, were determined by SYBR green incorporation as detected by real-time PCR (ABI Prism 7000, Perkin-Elmer Life Sciences, Waltham, MA) using published NFAT 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 (see insert in Figure 5A).

Nuclear run-on assays

CD154 transcription rates were determined by an avidin-biotin-based nuclear run-on assay as described (23). In summary, nuclei were isolated from peripheral blood CD4 T cells *ex vivo*, or 4–6 or 20–24 hours following stimulation with PMA and ionomycin. Nuclei isolation was performed as previously described (24). For *in vitro* RNA synthesis and purification, one volume (100 μ l) of a 2x transcription buffer [200 mM KCl, 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 4 mM dithiothreitol (DTT), 4 mM each of ATP, GTP and CTP, 200 mM sucrose, and 20% glycerol] was added to one volume (100 μ l) of nuclei on ice. Next, 8 μ l of biotin-16-UTP (from 10 mM tetralithium salt: #1388908, Roche Molecular Biochemicals, Indianapolis, IN) was added, and the mixture was incubated for 30 minutes at 29°C. The reaction was stopped by adding 6 μ l of 250 mM CaCl₂ and 6 μ l of RNase-free DNase I (10 U/ μ l; Roche Molecular Biochemicals), and incubating for 10 minutes at 29°C. RNA was purified as above and was re-suspended in 50 μ l of diethylpyrocarbonate (DEPC)-treated water.

Newly synthesized RNA, containing biotin-conjugated UTP, was extracted by first incubating with streptavidin-conjugated magnetic beads (Dynabeads: #112.05, Dynal®, Invitrogen), previously washed per manufacturer's instructions and suspended in binding buffer, for 20 minutes at 42°C and 2 hours at room temperature. Beads were separated with a magnetic device (Dynal), washed, and suspended in DEPC-treated water. cDNA was made from the bead using an Applied Biosystems Transcription Reagents Kit (cat # N808-0234) which contains Taqman Gold enzyme following manufacturers instructions for one cycle synthesis of RNA to cDNA using a traditional PCR engine (program was 25°C for 10 min, 48°C for 30 min, 95°C for 5 min, and 4°C indefinitely).

Real-time 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 hCD154 are previously reported (20), and those for hGAPDH (endogenous control to normalize the CD154 RNA) are: forward-5'-GAAGGTGAAGGTCGGAGTC-3' and reverse-5'-GAAGATGGTGATGGGATTTC-3'. Detection and quantification of amplified real-time PCR products by SYBR green incorporation was as previously described (20).

Transient transfection and reporter gene analyses

Freshly-isolated peripheral blood CD4 T cells were transiently transfected with 5 μ g of various firefly luciferase reporter plasmids by Amaxa nucleofection (Gaithersburg, MD) as described (4). The AP-1-, NFAT-, and NF κ B-responsive reporter plasmids have been previously reported (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 (26). Transcription factor responsive reporter plasmid activity was corrected for transfection efficiency between patient and matched control sample using the pCMV-Luc plasmid activity as a reference control as previously detailed (25).

Statistical analyses

Based on Koshy et al. (8), we calculated a requirement of a 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 with a confidence level of 0.90. To account for inter-assay variation in FCM analyses, the ratio of SLE/control values was calculated for each matched concurrent pair at each time point. Average MFI ratios and 95% confidence intervals were calculated using the geometric mean. Comparisons of raw values and SLE/control ratios were made using the Wilcoxon rank-sum test. Statistical analyses were calculated using GraphPad Prism 5 software (La Jolla, CA).

RESULTS

Activated CD4 T cells from pediatric SLE patients have increased cell surface expression of CD154 versus closely-matched controls

CD4 T cells from both SLE patients and age-, ethnicity-, and sex-matched controls, were examined side-by-side for CD154 expression, and CD25 and CD69 activation controls, by flow cytometry *ex vivo* and after stimulation with PMA+ionomycin for 6 and 24 hours. When comparing an individual SLE patient to his/her matched control (example in Figure 1), CD154 mean fluorescence intensity (MFI) at 24 hours post-stimulation was, on average, 1.44x (95% confidence interval 1.19–1.72) higher in the SLE patient versus control (Figure 2A). A significant difference was also seen at 6 hours post-stimulation, with SLE T cell expression being 1.34x (95% CI 1.01–1.79) that of an individual control. No significant difference was seen at 0 hours (Figure 2A). In contrast to CD154 expression, there was no significant difference between patients and controls seen at any time points for CD25 and CD69 expression (Figure 2A) similar to what has been seen in adult SLE patients (6,8). The increase in CD154 expression on the SLE CD4 T cells did have some disease specificity in that CD154 expression after 24 hours of stimulation in the JIA group was significantly less than SLE group (median 418 vs 736; Wilcoxon rank-sum, $p = 0.02$; Figure 2B).

A history of SLE nephritis is associated 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 among the SLE cohort. Demographic, laboratory, and clinical data (Table 1) that showed no association with CD154 levels included prednisone dose, whether or not a patient was receiving specific medications (hydroxychloroquine, any disease-modifying anti-rheumatic drug, or specifically cyclosporine), SLEDAI (SLE disease activity index) score, C3, C4, absolute lymphocyte count, or history of central nervous systems manifestations of SLE. However, for the 12 patients who had a history of biopsy-proven SLE glomerulonephritis at some point throughout their disease course, there were significantly higher CD154 levels at 24 hours compared to their respective controls than did those 9 SLE subjects without a history of nephritis (Figure 2C, $p=.01$). Similarly, for the 3 SLE subjects who had an elevated sedimentation rate (>20 mm/hr) at the blood draw closest to their date (generally within one month) of their CD154 measurement, there were significantly higher CD154 levels at 24 hours compared to their respective controls than did those 13 SLE subjects with a normal erythrocyte sedimentation rate (ESR) ($p=.0004$). Thus, elevated CD154 expression on SLE CD4 T cells following 24 hours of activation was strongly correlated with the presence of glomerulonephritis and with elevated ESR.

SLE CD4 T cells have increased CD154 mRNA levels and transcription rates

To assess the etiology of increased CD154 surface expression on SLE CD4 T cells, real-time PCR (RT-PCR) was used to quantify CD154 and GAPDH message levels in 4 patients and matched controls. After correcting for starting RNA by GAPDH, increased CD154 mRNA levels were seen when comparing a SLE patient to his/her individual control (Figure 3). This was seen both *ex vivo* and at 6 hours post T cell stimulation with PMA+ionomycin. In these same patients, nuclear run-on assays were used to quantify transcription rates at 6 and 20–24 hours post-activation. Increased CD154 transcription rates were seen in SLE patients, when compared to their individual controls, at all time points, particularly at the later 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 NFAT activity and increased NFAT levels, in particular NFAT2, are seen in SLE CD4 T cells

Using luciferase reporter plasmids and polyclonally stimulating CD4 T cells *in vitro* from SLE patients and matched controls, transcriptional activity for NFAT, AP-1, and NFκB were measured and corrected for transfection efficiency. Each SLE patient was compared to his/her control, and the log of the ratio was plotted such that increased transcription factor activity in the lupus patient relative to the matched control was greater than 0 (above the line) and less than 0 (below the line) when the SLE transcription factor activity was less than the matched control (Figure 4A). There was a clear trend for most paired comparisons demonstrating increased NFAT activity in the SLE CD4 T cells relative to controls (Figure 4B, $p = 0.057$). By contrast, AP-1 activity appeared to be mostly decreased (below the line) in SLE CD4 T cells relative to controls, and NFκB activity was similar on average (Figure 4B). Thus, there was a relative increase in NFAT activity for most SLE patients compared to controls.

Using real-time RT-PCR and probes specific for NFAT1, NFAT2, and NFAT4, we sought to further characterize increased NFAT activity in SLE CD4 T cells versus controls in 5 pairs (example in Figure 5A). Both NFAT1 and NFAT2 levels were increased, but those of NFAT2 were markedly higher than that of controls (Figure 5B). NFAT4 levels were, for the most part, undetectable, consistent with NFAT4 being primarily present in thymocytes (27). Therefore, NFAT1 levels were increased in SLE CD4 T cells relative to controls, similar to prior studies showing increased NFAT1 in the nucleus (17, 18) of SLE CD4 T cells. Interestingly, NFAT2 levels were substantially elevated in SLE CD4 T cells likely reflecting increased NFAT activity since NFAT2 transcription is positively regulated by NFAT engagement of the NFAT2 transcriptional promoter (28, 29).

DISCUSSION

We present the first evidence in children with SLE, as compared to controls, that CD154 is over-expressed on activated CD4 T cells (Figure 1, Figure 2A, Figure 2B), similar to results seen in adult studies (8,9,30). This increased CD154 expression was specific, as there was no difference in expression of activation markers, CD25 and CD69 (Figure 2A), similar to what was shown in adults with SLE (8,9). These increased CD154 levels correspond to increased CD154 mRNA levels and rates of transcription, especially at late (24 hours) time points following CD4 T cell activation (Figure 3). Although this does not formally rule out a role for potential increased CD154 mRNA stability in SLE (31), the increased CD154 transcription rates at late time points post-activation are markedly elevated and strongly suggest increased CD154 transcription at later time points post-activation contribute to increased CD154 expression on SLE CD4 T cells.

CD154 transcription is normally highly dependent upon NFAT activity following T cell activation (14), and, relative to controls, there was a clear trend towards increased NFAT activity in CD4 T cells from most SLE patients (Figure 4). In contrast, AP-1 activity was decreased, and NFκB activity was similar on average (Figure 4). Thus, there was a relative increase in NFAT activity for most SLE patients relative to controls. This is not surprising given the known increased flux of intracellular calcium seen in SLE T cells (16), and the dependence of NFAT on prolonged calcium signaling to activate calcineurin, which, in turn, dephosphorylates NFAT and helps shuttle it to the nucleus where it binds target genes like CD154 (32). In addition, others have shown increased NFAT1 levels and DNA binding activity in SLE T cells (17,18).

We also identified increased NFAT1 levels in SLE patients, but the levels of NFAT2 were more dramatically elevated in SLE patients compared to controls (Figure 5). NFAT1 is

found constitutively in resting T cell cytoplasm (25,33), but the inducible isoform of NFAT2 is upregulated by NFAT itself (28,29). Following T cell activation, there is a relative switch in abundance from NFAT1 to NFAT2, and NFAT2 has been suggested to be the NFAT family member essential for effector T cell development and function (28). Conversely, NFAT2 levels are decreased in FOXP3-expressing regulatory CD4 T cells, and this is due to the FOXP3 transcription factor directly binding to and inhibiting the NFAT2 transcriptional promoter (34). The increased NFAT2 levels in CD4 T cells from SLE patients may thus represent a more mature/effector T cell phenotype. As NFAT2 quickly becomes the predominant NFAT family member in activated T cells, it may be the most likely NFAT family member involved in the later wave of CD154 transcription.

In contrast to elevated NFAT activity, our finding of decreased AP-1 and similar or lower NFκB activity, relative to controls, is consistent with similar findings (35,36) in adults, and it is known that these transcription factors are necessary for interleukin-2 (IL-2) production, which is decreased in SLE T cells (37). The AP-1 deficiency is thought to be due to the transcriptional repressor CREM, which is expressed in increased amounts in SLE T cells, binding to the promoter of *c-fos* and suppressing its transcription (37). Normally, *c-fos* combines with *c-jun* to produce the dimeric transcription factor, AP-1, which combines with NFAT on the IL-2 promoter, thusly contributing to transcriptional activation. The decreased NFκB activity likely stems from a p65 subunit deficiency, which is of unclear etiology (37). However, what does seem to be more evident is that calcium flux and associated NFAT activity are increased in lupus CD4 T cells and likely contribute to CD154 over-expression and SLE pathogenesis (16).

The CD154-CD40 interaction appears to have 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 IV nephritis (38). CD154-activated monocytes were shown to stimulate the glomerular inflammatory response (39). Moreover, multiple mouse experiments are supportive of the role of CD154 in models of SLE nephritis (5,7,40). 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) (38). Alternatively, prolonged CD154 expression and stimulation of B cells likely contributes to increased autoantibody production (e.g., anti-double stranded DNA) that may contribute to the renal lesion.

Here, we demonstrate 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, when compared to their respective controls (Figure 2C). It is unclear whether the higher CD154 levels signify a more active generalized disease state which, in turn, leads to nephritis, or higher CD154 expression signifies a specific propensity to develop nephritis, independent of other disease manifestations. Along these lines, others have shown that CD134 expression on SLE CD4 T cells is associated with nephritis (41). Nevertheless, the finding that the development of CNS 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 for treatment of lupus nephritis. Because a monoclonal antibody approach to CD154, although effective, has been complicated by unanticipated clotting abnormalities (42), other means of targeting dysregulated CD154 expression are in order (43).

Cyclosporin A (CsA) blocks the phosphatase activity of calcineurin, and, therefore, prevents the nuclear translocation of NFAT. As CD154 over-expression has a crucial role in the pathogenesis of SLE, our demonstration that NFAT mediates this over-expression 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 two prospective studies (44,45) and an open-randomized comparison of CsA versus corticosteroids plus cyclophosphamide (46), 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 effect profile, which consists of nephrotoxicity and reversible hypertension. *In vitro* and *ex vivo* experiments investigating CsA's effect on CD4 T cell CD154 expression in lupus have been inconclusive (12,17,18). We argue that the presence of an established therapeutic agent which acts on a demonstrated substrate of SLE pathogenesis strongly warrants a controlled clinical trial of CsA's efficacy in SLE, particularly those with nephritis.

Tacrolimus is a newer, more potent, medication which also acts on the calcineurin-NFAT pathway (32) and, therefore, is potentially efficacious in SLE. In topical form, it has demonstrated efficacy as a treatment for cutaneous manifestations of lupus (47), and a small open-label trial of oral tacrolimus in patients with pediatric-onset lupus nephritis demonstrated efficacy in almost all the studied patients (48). In addition, Hogan, Wagner, and colleagues (49) have identified a small organic molecule which selectively interferes with the interaction between calcineurin and NFAT, 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, or more, efficacious, with fewer adverse effects than current agents which are less discriminating in their targets.

In summary, like adults with SLE (8,9,30), pediatric SLE patients also demonstrate increased CD154 expression on CD4 T cells, particularly at later time points post activation. This increase in CD154 expression is associated with the presence of nephritis, increased CD154 transcription, and increased NFAT levels and transcriptional activity. Therefore, an argument can be made to explore a role for NFAT/calcineurin inhibitors, particularly novel agents with increased benefit to side-effect ratios, in the treatment of pediatric SLE nephritis.

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Figure 1. Increased CD154 expression on SLE CD4 T cells 24 hours after activation

CD4 T cells were isolated from PBMC from a single (as a representative example) SLE patient (bold lines) and side-by-side matched control individual (thin lines). Flow cytometry was performed on CD4 T cells *ex vivo* (left column), and following activation for 6 (middle column) or 24 (right column) hours post-polyclonal activation with PMA+ionomycin. Analyses of cell surface CD154 (top row), CD69 (middle row), and CD25 (bottom row) are depicted. CD4 expression, following negative selection, is shown in the upper left hand panel. For all panels, cell number is on the Y-axis, and fluorescence intensity is on the X-axis. Isotype control antibody staining is depicted with a dotted line.

Figure 2. Increased CD154 expression on SLE CD4 T cells 24 hours after activation is higher than controls, and is associated with nephritis and elevated ESR

A. The means and 95% confidence intervals are shown for the ratios (SLE patient values divided by the side-by-side controls, n=29) of CD154 (top graph), CD25 (middle graph), and CD69 (bottom graph) expression on CD4 T cells *ex vivo* (left means), and 6 (middle means) and 24 (right means) hours after T cell activation. **B.** The mean $\pm$ SEM values of CD154 MFI expression on CD4 T cells after 24 hours of activation are shown for the SLE (n=29) and JIA disease control (n=6) groups. **C.** The individual matched SLE to control ratios for CD154 expression at 24 hours post activation are plotted in relation to the presence or absence of nephritis (left graph), or whether or not the SLE patient ESR is elevated closest to the time of the analysis (right graph).

CD154 mRNA Levels and Transcription Rates

Figure 3. Increased CD154 mRNA levels and transcription rates in SLE CD4 T cells

Means $\pm$ SEM of the ratios (SLE divided by matched controls) of CD154 mRNA levels and transcription rates (relative to GAPDH control) in CD4 T cells analyzed *ex vivo* (0–3 hours), and 4–6 and 20–24 (transcription rate only) hours following T cell activation. Sample sizes of $n=4$, except for the *ex vivo* time point of the transcription rate ($n=3$, adult SLE patients and controls).

Figure 4. Increased NFAT transcriptional activity in SLE CD4 T cells

A. The log of the ratios (SLE divided by matched control, corrected for transfection efficiency) of transcription factor activities in polyclonally activated CD4 T cells is depicted for the individual matched pairs (n=10) analyzing NFAT (left), AP-1 (middle), and NFκB (right) activities, such that increased activity in the lupus patient relative to the control plots above the line (>0), and decreased activity falls below the line (<0). **B.** The means $\pm$ SEM of the ratios (from the 10 matched groups) is plotted for the individual transcription factor activities analyzed.

Figure 5. Increased NFAT levels in SLE CD4 T cells

A. Real-time RT-PCR was used to quantify NFAT1, NFAT2, NFAT4, and GAPDH (control) mRNA levels (analyzed in duplicate) from CD4 T cells of a representative patient (blue and purple curves) and matched control (green and red curves). The predicted sizes of the PCR products for each specific primer pair used to detect NFAT1, NFAT2, and NFAT4 message were confirmed by agarose gel electrophoresis (upper left insert). **B.** The mean $\pm$ SEM of ratios of SLE patient divided by matched control values for NFAT1 and NFAT2 message levels (corrected for loading by the control GAPDH message levels) are plotted (n=5).

Table 1

Lupus patient demographics and clinical characteristics

Pt #	Age ¹	Sex	Ethnicity ²	SLEDAI ¹	Nephritis ⁴	ESR ¹	Medications ^{1,3}
1	15	F	AA	16	Y	48	HD-CS, Mmf
2	17	F	AA	0	N	20	HD-CS, Cyc (q1m), Hcq
3	21	F	AA	9	N	N/A	N/A
4	19	F	AA	0	Y	N/A	N/A
5	18	F	AA	5	N	N/A	MD-CS, Hcq, Asa, Cyc (q3m)
6	16	F	AA	2	Y	27	HD-CS, Hcq, Cyc (q1m), coumadin
7	13	F	C	4	N	23	MD-CS, Hcq, Asa
8	17	F	AA	1	Y	65	HD-CS, Cyc (q1m), sertraline, OCP, pentamidine
9	17	F	AA	9	Y	N/A	LD-CS, Mmf, sertraline, pentamidine, ondansetron
10	16	F	C	8	Y	12	LD-CS, Hcq
11	16	F	I	0	Y	9	LD-CS, Hcq, Asa
12	14	F	EA	4	N	5	Asa, azathioprine, coumadin, cetirizine
13	18	F	C	10	Y	N/A	LD-CS, Hcq, Asa, ranitidine
14	14	F	C	6	Y	N/A	MD-CS, Hcq, Cyc (q1m), enalapril, pentamidine, isoretinic acid
15	13	F	AA	16	Y	70	HD-CS, Hcq, Asa, Cyc (q1m), pentamidine
16	16	F	AA	21	Y	35	HD-CS, Hcq, Mtx
17	15	M	C	4	N	N/A	MD-CS, Asa, Mmf, methylphenidate, loratadine, albuterol
18	15	F	C	5	Y	0	LD-CS, Hcq, Cyc (q3m)
19	17	F	C	4	N	4	LD-CS, Hcq, Asa
20	14	F	AA	17	Y	18	MD-CS, Hcq, Asa, azathioprine, losartan, enalapril, fluticasone
21	17	F	AA	2	N	10	LD-CS, Hcq
22	16	F	C	2	Y	5	LD-CS, Hcq, Cyc (q3m)
23	15	M	L	N/A	Y	2	Hcq, Mmf
24	13	F	AA	4	Y	12	MD-CS, Hcq, Asa, enalapril
25	16	F	EA	4	N	11	Hcq, azathioprine, coumadin
26	19	F	L	12	N	27	LD-CS, Hcq, tacrolimus (topical), ranitidine
27	15	F	AA	8	N	19	HD-CS, Hcq, Asa, Mmf
28	15	F	AA	6	Y	N/A	MD-CS, Hcq, Asa, Mtx, fluticasone, salmeterol, ranitidine

Pt #	Age ¹	Sex	Ethnicity ²	SLEDAI ¹	Nephritis ⁴	ESR ¹	Medications ^{1,3}
29	16	F	C	N/A	N	9	MD-CS, Hcq, Mmf

¹ At time of study blood draw; N/A = not available

² AA=African-American; C=Caucasian, I=Native Indian, EA=East Asian, L=Latino

³ LD-CS=equivalent of ≤5 mg prednisone daily; MD-CS=equivalent of >5 mg but ≤25 mg prednisone daily; HD-CS= equivalent of >25 mg prednisone daily; Mmf=mycophenylate mofetil; Cyc=IV cyclophosphamide (interval); Hcq=hydroxychloroquine; Asa=low-dose aspirin; Mtx=methotrexate

⁴ Y=a history of nephritis at any point while being followed at our institution