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CD154 expression and mRNA stability of activated CD4-positive T cells in patients with systemic lupus erythematosus

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Abstract The expression of CD154 (CD40 ligand) on activated CD4+ T cells is known to be transient and tightly regulated for antigen-specific immune responses, and is increased and prolonged among patients with systemic lupus erythematosus (SLE). We investigated the regulation of CD154 expression by determining the protein and mRNA expression with PMA and ionomycin stimulation in CD4+ T cells, and confirmed their increase and prolongation in SLE T cells. Treatment with actinomycin D, a transcription inhibitor, after PMA and ionomycin stimulation was performed, and the findings revealed that the stability of CD154 mRNA increased significantly in activated SLE T cells compared with that of controls. However, alternations or abnormal sequences were not identified in the 3′ untranslated region, including AU-rich elements and CU-rich sequences, while their partial involvement in the post-transcriptional regulation of CD154 mRNA stability has been reported. With 96 h culture in vitro, the destabilization of CD154 mRNA was demonstrated, resulting in a corresponding decrease and normalization of surface expression on activated SLE T cells. We speculate that the CD154 expression on T cells from SLE patients may be increased and prolonged, with mRNA stabilization being related to a continuous stimulation in vivo.

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by various autoantibodies and pathogenic immune complexes containing DNA and antidouble-stranded DNA antibody, which are deposited in the glomeruli, the dermo–epidermal junction of skin lesions, blood vessel walls, and the choroid plexus of SLE patients.[1–3]

CD154, a type II glycoprotein belonging to the TNF-α family, is expressed predominantly on activated CD4+ T cells. Transient expression of CD154 may allow only appropriate antigen-specific B cell activation via CD40 expressed on B cells following immunoglobulin class switching and germinal center formation.[3,4] An increased percentage of peripheral lymphocytes expressing CD154 is reported in patients with SLE.[5,6] The infiltration of CD154-positive mononuclear cells has also been demonstrated in kidney sections of class III or V lupus glomerulonephritis. Furthermore, the blocking of CD154 with monoclonal antibodies (MAb) has been demonstrated to interfere with ongoing lupus nephritis and prolong survival in murine models.[1,7] These findings indicate that excess or inappropriate expression of CD154 plays a pivotal role in immune responses in promoting the pathogenesis of SLE.

Several regulatory mechanisms exist in CD154 expression. In posttranslational regulation, cell-surface CD154 is reduced by receptor-mediated endocytosis as a result of binding with CD40[8] or through the release of soluble CD154, which is a proteolytic cleavage product of CD154.[9,10] Ligation of T cell receptor (TCR) initiates rapid augmentation of CD154 gene transcription, and the transient CD154 gene expression gradually decreases in a similar manner to the protein expression.[11,12] The 3′ untranslated region (3′UTR) of CD154 mRNA contains five AU-rich elements, which is a potential sequence motif

affecting mRNA stability as well as that in several transiently expressed genes, including TNF-α, by selective binding of RNA-binding proteins during T cell activation.[13,14] Moreover, other studies have revealed distinct proteins bound to unique sites lacking AU-rich elements in the 3′UTR, e.g., a CU-rich sequence, and increased CD154 mRNA stability.[15,16] These studies suggest the possibility that inadequate posttranscriptional regulation contributes to an increase in the surface expression and mRNA stability of CD154 in lymphocytes from patients with SLE.

To investigate the difference in CD154 regulation during T cell activation between normal and SLE T cells, we examined the surface and mRNA expression of CD154 in CD4+ T cells stimulated with PMA and ionomycin, and analyzed the posttranscriptional regulation of the mRNA stability.

Materials and methods

Patients and controls

Peripheral blood samples were obtained from the 28 Japanese SLE patients enrolled in this study. There were 3 males and 25 females, with an average age of 36.2 years (range 17–45 years, who all fulfilled), the American College of Rheumatology revised criteria for the classification of SLE.[17] We also enrolled 18 age-matched healthy volunteers, 2 males and 16 females, with an average age of 34.4 years (range 22–43 years) as controls. CD4+ T cells from five female controls and the patients with active SLE were used to analyze surface and mRNA expression of CD154. Disease activity was determined using the SLE disease activity index (SLEDAI) scoring system,[18] and the disease of patients with a SLEDAI score ≥ 5 was defined as active. This study was reviewed and approved by the institution's review board, and informed consent was obtained from all subjects enrolled in the study.

Cell preparation and culture conditions

Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll–Hypaque centrifugation (Lymphoprep, AXIS-SHIELD PoC AS, Oslo, Norway), and CD4+ T cells were isolated from total PBMCs by positive selection on CD4-conjugated magnetic beads according to the manufacturer's instructions (Dynal AS, Oslo, Norway). Cells were cultured with RPMI 1640 medium (Sigma Chemical, St. Louis, MO, USA), containing 10% fetal calf serum, 2.05 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen Corp., Carlsbed, CA, USA), in 96 round wells at 37°C and 5% CO_2. Cells were stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin. Since the half-life of CD154 mRNA at 8 and 16 h stimulation with PMA and ionomycin is reported to range from 4.3 to 5.2 h,[11] we conducted some experiments where activated cells were washed with phosphate-buffered solution (PBS) and then incubated with 10 μg/ml actinomycin D (Act D, Sigma Chemical), a transcriptional inhibitor, for 4 h to evaluate the difference in the stability of CD154 mRNA between SLE patients and controls.

Flow cytometric staining

CD4+ T cells at a concentration of 1×10^6 and stimulated with PMA + ionomycin for different lengths of time were incubated with a saturating concentration of FITC-conjugated antihuman CD154 Mab (TRAP1, PharMingen, San Diego, CA, USA) at 4°C for 30 min. After three washes with PBS, cell fluorescence levels were analyzed with a FACScan flow cytometer (BD Bioscience, San Jose, CA, USA).

RNA isolation and Northern blot analysis

Total RNA was isolated from CD4+ T cells at concentrations of 2–8 $\times 10^6$ using an RNeasy kit (QIAGEN, Hilden, Germany). RNA (3.0–5.0 μg) was run in 1.2% agarose/formaldehide gels and transferred to nylon membranes. Membranes were prehybridized at 42°C for 6 h. The CD154 CDNA, isolated from a HeLa cell line transfected with an expression vector encoding the full-length human CD154[10] was used as the probe for Northern blot analysis. Hybridization was carried out with a random primer ^{32}P-labeled CD154 probe (Roche Dignostics, Mannheim, Germany), at 42°C for 40 h, and then washed twice at 42°C for 10 min with 1 × SSPE + 0.1% SDS, followed by two washes at 65°C for 20 min with 0.5 × SSPE + 0.1% SDS, and two more washes at 65°C for 20 min with 0.1 × SSPE + 0.1% SDS. Membranes were exposed and scanned by densitometry using an image analyzer BAStation (Fuji Photo Film Co., Tokyo, Japan), and values were normalized to the signals of the control β_2-microglobulin (β_2M) probe.

Reverse transcriptase–polymerase chain reaction (RT–PCR) and sequence analysis of the 3′ untranslated region of CD154 CDNA

PBMCs were washed once with PBS and used immediately for reverse transcriptase – polymerase chain reaction (RT–PCR). RT–PCR was performed as follows: 1 μg total RNA in a total volume of 20 μl was reverse-transcribed into CDNA using oligo-dT primer and M-MuLV reverse transcriptase (Roche Diagnostics), and amplification was performed using 2.5 μl CDNA in a total volume of 50 μl containing 60 mmol/l Tris-HCl (pH 9.5, at 25°C), 25 mmol/l ammonium sulfate, 3.5 mmol/l $MgCl_2$, 0.5 μmol/l of each oligonucleotide primer, 2.5 μmol/l dNTP, and 2.5 U Platinum Taq DNA polymerase (Invitrogen Corp.).

Primer sequences used for CD154 amplification were as follows: sense, 5′-ATGATCGAAACATACAACCAAA-3′; antisense, 5′-TCAGAGTTTGAGTAAGCCAAAG-3′. The size was 846 base pairs.[19] Primer sequences for the control probe β_2M were: sense, 5′-ACCCCCACTGAAAA AGATGA-3′; antisense, 5′-ATCTTCAAACCTCCAT GATG-3′. The PCR products were analyzed electro-

phoretically in 1.2% agarose gels prepared with Tris-borate EDTA buffer, stained with 10 mg/ml ethidium bromide, and photographed.

For direct sequence analysis, PBMCs were stimulated with PMA + ionomycin for 6 h before RT–PCR. Three oligonucleotide primers were selected for CD154 3′ UTR, sense-1, 5′-ATACAGCACAGCGGTTAAGC-3′, antisense-1, 5′-AGGCCATAGGAACCCAGAGT-3′, 405 bp, sense-2, 5′-CACCCTCTCGGACAGTTATT-3′, antisense-2, 5′-GTGTTAGAAAGGGGGATTGA-3′, 152 bp, sense-3, 5′-ACACACACACAGAGTCAGGC-3′, and antisense-3, 5′-CAAGTTCACTCTGGAAACAA-3′, 181 bp, to cover the AU-rich elements and CU-rich sequence of the 3′ UTR of CD154 mRNA. Amplifications were performed with 30 cycles of denaturation (93°C, 1 min), annealing (55°C, 2 min), and extension (72°C, 2 min). The PCR products were purified and subjected to direct sequencing using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Fostercity, CA, USA) according to the manufacturer's instructions.

Statistical analysis

The difference between the values for patients with SLE and those for controls were determined by the use of two-way repeated-measures ANOVA and Scheffe's test, or Mann–Whitney's *U*-test with STAT-View software (SAS Institute, NC, USA).

Results

Detection of CD154 mRNA in PBMCs from patients with active SLE

The expression of CD154 mRNA in freshly isolated PBMCs was examined by RT–PCR. CD154 mRNA was detected in the nine patients with active SLE who were examined, but not in the controls (Fig. 1).

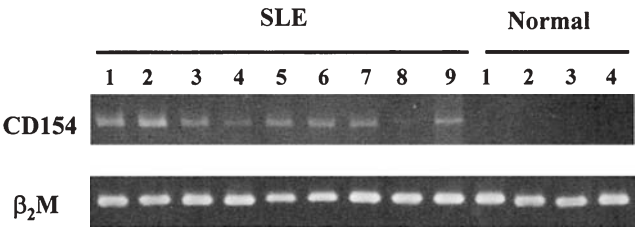

Fig. 1. Detection of CD154 mRNA from peripheral blood mononuclear cells (PBMCs) of active systemic lupus erythematosus (*SLE*) patients. RNA was extracted from freshly isolated PBMCs and used for reverse transcriptase – polymerase chain reaction (RT–PCR) analysis for CD154 and β_2M. The data were obtained from nine patients with active SLE and four control subjects

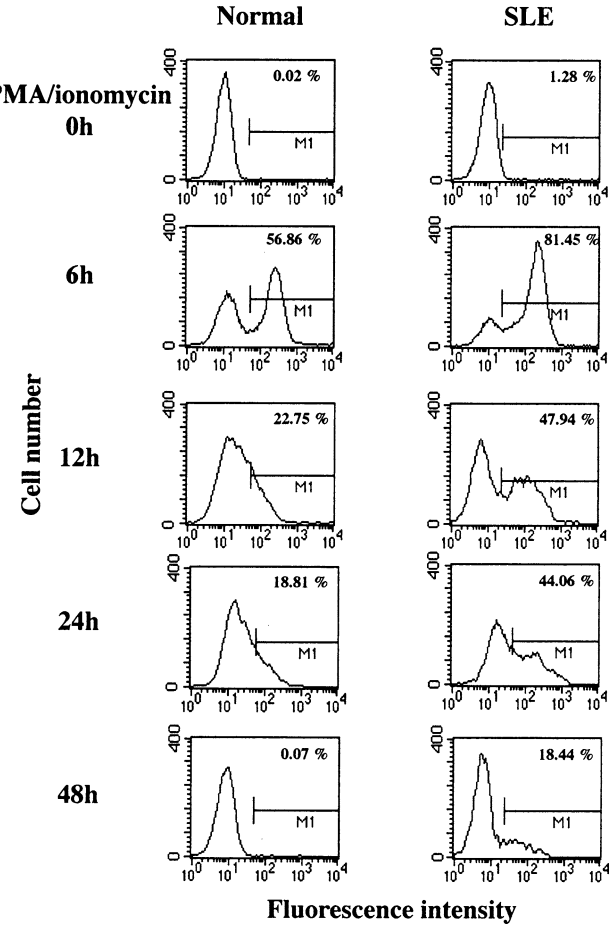

Fig. 2. Kinetics of CD154 expression on activated CD4+ T cells. CD4+ T cells from control subjects ($n = 5$) and SLE patients ($n = 5$) were incubated with phorbol myristate acetate (*PMA*) + ionomycin for 0–48 h, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD154 monoclonal antibodies (Mab). Representative data are shown

Surface expression of CD154 on activated CD4+ T cells

To assess surface CD154 expression in response to mitogen stimulation, CD4+ T cells from SLE patients and healthy controls were stimulated with PMA + ionomycin for various time periods and analyzed with flow cytometry. As shown in Fig. 2, the maximum expression was observed at 6 h of stimulation in both control and SLE CD4+ T cells, but the percentage (mean ± SD) of CD154+ cells was statistically higher in SLE patients ($n = 5$, 74.21 ± 8.53%) than in controls ($n = 5$, 48.45 ± 10.31%) ($P < 0.01$ by two-way repeated-measures ANOVA with Scheff's test). Moreover, prolonged CD154 expression was observed even after 48 h stimulation in the SLE T cells, while a sharp decease in expression to the baseline level was recorded for the normal T cells. As previously described,[5,6,11] increased and prolonged expression was observed in T cells from all five SLE patients examined, but not in control T cells. The expression patterns of CD154 in T cells were statistically different between SLE patients and healthy controls ($P < 0.01$ by two-way repeated-measures ANOVA with Scheff's test). Northern blot analysis revealed that the levels of CD154

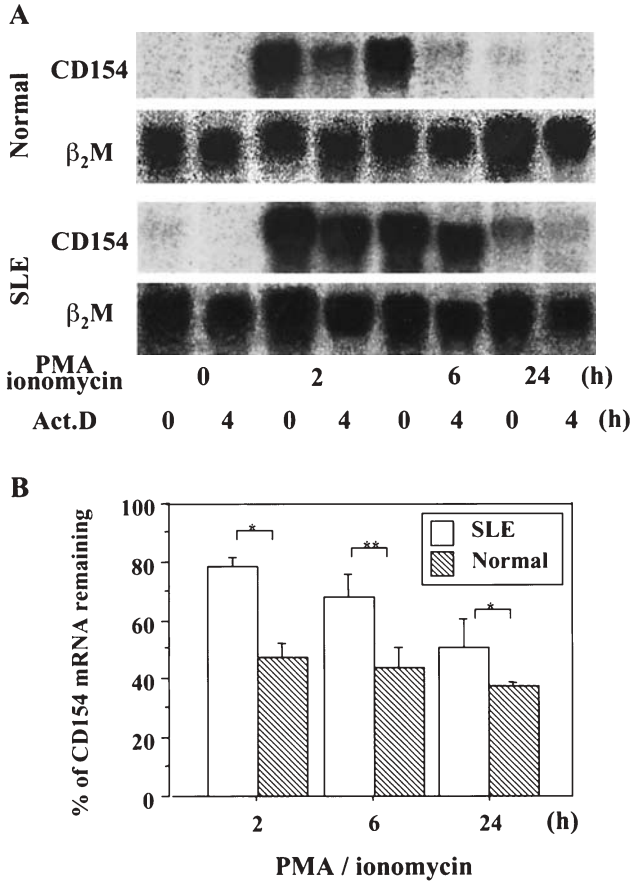

Fig. 3. CD154 mRNA stability of activated CD4+ T cells. CD4+ T cells from control subjects and SLE patients were stimulated with PMA + ionomycin at the time-periods indicated, and then incubated with Act D for 4 h to terminate the transcription before RNA extraction. The amount of mRNA was determined with Northern blot analysis with hybridization of probes for CD154 and β_2M. **A** Representative result of phosphoimaging. **B** The phosphoimage of mRNA was quantified with a densitometer, and then the density of the CD154 mRNA in each lane was standardized with that of β_2M in the corresponding lane. The quantifiable data at 2, 6, and 24 h of stimulation are indicated. The *columns* represent the mean percentage ($n = 5$ for both SLE and control subjects) of the density remaining after Act D treatment, and the *bars* indicate the standard deviation (SD). The percentage of the remaining CD154 mRNA was significantly higher in SLE patients than in the controls at 2, 6, and 24 h after stimulation. $*P < 0.01$, $**P < 0.05$, by Mann–Whitney's *U*-test

mRNA corresponded to its surface expression (data not shown).

Stability of CD154 mRNA in patients with SLE

The turnover of CD154 mRNA is known to be rapid.[12] To investigate whether mRNA stability is related to the increased and prolonged mRNA expression of CD154 in SLE patients, we examined mRNA stability with Act D treatment for 4 h after PMA + ionomycin stimulation. As shown in Fig. 3A, levels of CD154 mRNA were higher in SLE T cells activated for 2–24 h, and the detection was still confirmed after Act D treatment at each stimulation time period, whereas the levels apparently decreased in the controls. The percentage of CD154 mRNA remaining, representing mRNA stability, was significantly higher in SLE patients than in the controls at 2, 6, and 24 h of stimulation (Fig. 3B, $P < 0.01$, 0.05, and 0.01, respectively, by Mann–Whitney's *U*-test). PMA + ionomycin stimulation has been reported to increase mRNA stability. However, these results revealed that CD154 mRNA degradation was significantly delayed in SLE T cells compared with those of the controls. This indicated that there exists a possibility that the stabilization of CD154 mRNA in SLE T cells contributed to the increased and prolonged expression of CD154 in SLE T cells.

3′ UTR sequence of CD154 CDNA in activated CD4+ T cells

TNF-α gene expression is partially regulated at the post-transcriptional level and, in particular, AU-rich elements in 3′ UTR play an important role in the mRNA turnover.[14] CD154 mRNA, which has considerable sequence homology with TNF-α, is reported to have five AU-rich elements also, and other regions like a CU-rich sequence, which are involved in mRNA stability, in the 3′UTR.[16] We analyzed the 3′ UTR sequence of CD154 CDNA, including those sequences in PMA + ionomycin-activated PBMCs from 28 SLE patients (17 active and 11 inactive) and 18 controls, by direct sequence. However, our results revealed no insertions, defects, or abnormal sequences in five AU-rich elements and a CU-rich sequence of CD154 3′ UTR CDNA from SLE (both active and inactive) patients and controls (data not shown).

Change of CD154 expression and mRNA stability after in vitro culture

It is suggested that continuous activation of T cells in vivo results in various T cell abnormalities in SLE patients.[2,3] To examine whether the kinetics of CD154 expression changes after culture in vitro, we incubated CD4+ T cells for 96 h in the absence of mitogen, and then stimulated the cells with PMA + ionomycin. Culturing for 96 h prior to stimulation resulted in significantly decreased and shortened CD154 expression on SLE T cells ($P < 0.01$ by repeated-measures ANOVA with Scheff's test), which were similar to those of normal T cells (Fig. 4). In normal T cells, no significant change in CD154 expression was demonstrated between those with and without 96 h pre-stimulation culturing. We further evaluated whether the change in CD154 mRNA stability affected the protein expression on SLE T cells in the same condition. Our results revealed that CD154 mRNA in activated SLE T cells was significantly destabilized with preculturing as compared with those without preculturing, and this was similar for the controls (Fig. 5).

Next, we examined the influence of serum factors on CD154 surface expression. SLE and normal CD4+ T cells were precultured in 100% culture medium (described above), or with culture medium consisting of 50% sera from

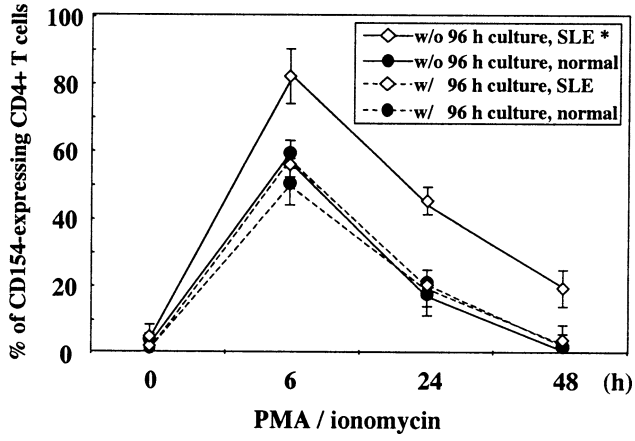

Fig. 4. Kinetics of CD154 surface expression on activated CD4+ T cells after 96 h culture in vitro. CD4+ T cells from SLE patients ($n = 5$) and control subjects ($n = 5$) were stimulated with PMA + ionomycin immediately after separation, or cultured in mitogen-free medium for 96 h followed by stimulation with PMA + ionomycin for the durations indicated. The percentage of CD154 + cells was determined with flow cytometry and compared between freshly isolated and 96-h precultured CD4+ T cells. The *symbols* and *bars* indicate the mean percentage of CD154+ cells and the SD of the mean, respectively. Culturing for 96 h prior to stimulation resulted in a significant decrease and shortened CD154 expression on SLE T cells ($P < 0.01$ by repeated-measures ANOVA with Scheff's test). *w/o*, without; *w/,* with; *star*, $P < 0.01$

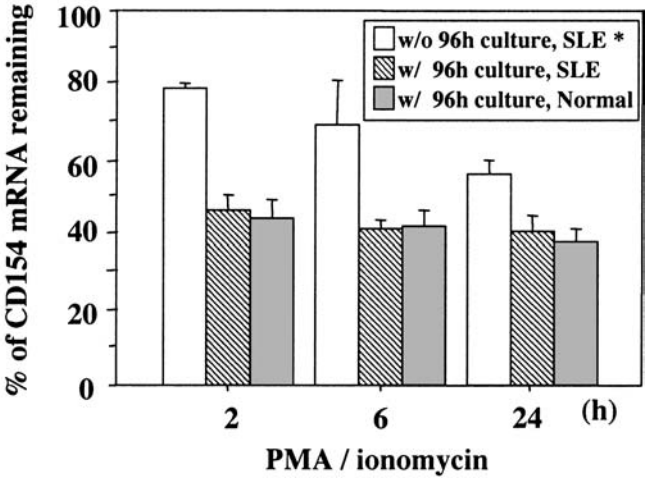

Fig. 5. Decreased CD154 mRNA stability of lupus CD4+ T cells after in vitro culture. CD4+ T cells from SLE and control individuals ($n = 3$ for both SLE and controls) were cultured in the absence of mitogen for 96 h, and then activated under identical conditions as in Fig. 4. for 2, 6, and 24 h, followed by incubation with Act D for 4 h. The CD154 mRNA was quantified as described in Fig. 3. The percentage of CD154 mRNA remaining after Act D treatment was compared between CD4+ T cells with and without the 96-h pre-stimulation culture. The symbols indicate the mean percentage of CD154 mRNA remaining and the SD of the mean, respectively. Preculture of SLE T cells without stimulation for 96 h decreased CD154 mRNA stability to similar levels to those of control individuals. *w/o*, without; *w/,* with; *star*, $P < 0.01$

active SLE patients and 50% culture medium, for 96 h and then stimulated with PMA + ionomycin. The influence of SLE sera was not demonstrated on CD154 expression in this study (date not shown).

Discussion

The interaction between CD40 and its ligand CD154 is pivotal for the development and maintenance of both humoral and cellular immune systems. The expression of CD154 on CD4+ T cells is transient and tightly regulated to mediate antigen-specific immune responses, while the constitutive presence of CD40 is observed in a wide spectrum of target cells.[3]

As previously described, CD154 mRNA is detected in freshly isolated PBMCs from patients with active SLE, but not from controls (see Fig. 1), and surface expression has also been demonstrated.[5,11] Moreover, a high concentration of soluble CD154 in SLE plasma has been demonstrated, and this may contribute to the production of autoantibodies and consequent inflammation in patients with SLE.[10] These findings indicate that some SLE T cells express CD154 constitutively.

We showed that CD154 mRNA of SLE patients was more stable than that of controls in this study (see Fig. 3). Recent studies have demonstrated that certain nucleotide sequence motifs, e.g., AU-rich elements and CU-rich regions, in the 3′ UTR provide binding sites for proteins to promote the decay of CD154 and several other mRNAs.[15,16,20–22] Although SLE susceptibility is not linked to the x chromosome where CD154 is located,[23] it is still possible that abnormal sequences of mRNA contribute to mRNA stability in a similar way to the TCR ζ chain reported to be deleted in the 3′ UTR by alternative splicing.[24–26] Therefore, we analyzed the nucleotide sequence of 3′ UTR of CD154 mRNA in PMA + ionomycin-activated PBMCs, but no abnormal or unique sequences were recognized in SLE patients when compared with the controls.

In SLE patients, increased expression of HLA-DP, an early activation marker of T cells, has been reported to decrease during 4 days of culture in a mitogen-free medium in vitro.[27] Moreover, impaired production of IL-2 is reported to be recovered by in vitro culture.[27] These findings indicate that some of the T cell abnormalities in SLE patients can be altered when these cells are in the resting condition. On the other hand, it is reported that the hyperexpression of CD154 on SLE T and B cells persisted during culture of SLE PBMCs in vitro.[6] Therefore, we cultured SLE CD4+ T cells alone in vitro for 96 h regardless of continuous T–B interaction and extracelluler stimulation, and then stimulated these cells with PMA + ionomycin. Protein and mRNA expression of CD154 in response to these stimuli was revealed to decrease to normal levels, and furthermore, mRNA stability also decreased to levels similar to those of the controls (see Figs. 4 and 5).

Several cytokines increase CD154 expression, and in particular IL-12 and IL-15 are reported to be increased in SLE patients.[28–30] Furthermore, stromal cell-derived factor-1 and osteopontin are also reported to augment CD154 expression.[31,32] To examine the influence of these serum factors, we determined CD154 expression on activated CD4+ T cells after culturing for 96 h in the presence of 50% sera from active SLE patients prior to PMA + ionomycin

stimulation. There was no difference in CD154 expression between those in the presence or absence of the SLE sera (data not shown), suggesting that the addition of SLE serum only was insufficient to continue CD154 hyperexpression on SLE T cells.

Distinct components of intracellular signaling molecules and transcriptional factors may be involved in the prolonged and increased expression of CD154 in SLE T cells. The TCR signal pathway has three main streams, Ca^{2+}/calcinurin/nuclear factor of activated T cells (NF–AT), Ras/mitogen-activated protein kinase (MAPK), and protein kinase C/nuclear factor-κB pathways.[33–35] Increased calcium influx has been observed after TCR ligation in SLE T cells compared with normal T cells.[36] Translocation of NF–AT from cytosol to nuclear factor, and binding to the CD154 promotor region to induce gene transcription, requires a calcium-dependent calcinurin pathway. Cyclosporin A, an inhibitor of this pathway, failed to inhibit CD154 expression on activated SLE T cell lines with TCR ligation when added at the late phase of activation.[37,38] Furthermore, the Cbl and MAPK, which activate transcriptional factor AP-1, were believed to be candidate molecules regulating the late phase of the activation, including mRNA stability, and to be defected in SLE T cells.[38] However, T cell signaling in vivo may be more complicated, since other accessory molecules are also involved in T–B cell interactions. It was shown that T cell surface molecules CD4, CD28, LFA-3, and ICOS augment CD154 expression.[39–42]

In SLE patients, increased calcium influx after TCR ligation, and abnormalities in the phosphorylation of TCR ζ and Cbl, have been reported.[24–26,38] PMA + ionomycin induce a similar activation pattern of T cells mediated through ligation of both TCR and other co-stimulatory molecules.[37] However, PMA + ionomycin directly stimulates the downstream abnormal signal transduction pathways PKC, Ras, and Ca^{2+} influx.[35,43] We demonstrated that increased expression and mRNA stability of CD154 in SLE CD4+ T cells decreased to normal levels with 96h prestimulation culture. These data indicated that CD154 hyperexpression on SLE T cells could not be due to intrinsic abnormalities in the downstream signal transduction pathways of TCR ligation.

The mechanism of inappropriate CD154 expression in SLE patients in vivo is still ambiguous. The continuous stimulation of T cells with abundant autoantigen-presenting cells may deliver a distinct form of signal transduction cascade, and increase and prolong the expression of CD154 in SLE T cells.

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