
S N Liossis, … , G J Dennis, G C Tsokos


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Altered Pattern of TCR/CD3–mediated Protein-tyrosyl Phosphorylation in T Cells from Patients with Systemic Lupus Erythematosus

Deficient Expression of the T Cell Receptor Zeta Chain

Stamatis-Nick C. Liossis,*† Xuan Z. Ding,* Greg J. Dennis,§ and George C. Tsokos*‡
*Department of Clinical Physiology, Walter Reed Army Institute of Research, Washington, DC 20307-5100; †Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; and §Rheumatology Service, Walter Reed Army Medical Center, Washington, DC 20307

Abstract

Cellular immunity aberrations in patients with SLE are underscored by the abnormal early Ag receptor–mediated lymphocyte signal transduction pathway. To further characterize the T cell receptor (TCR)/CD3–initiated signaling defects, we studied 22 patients with SLE, 12 patients with other systemic rheumatic diseases, and 14 normal donors. The early (1 min) TCR/CD3–mediated tyrosine phosphorylation of cellular proteins with a molecular size between 36 and 64 kD was increased in 15 of 21 SLE patients, compared to normal or disease control subjects. The deficiency or absence of a band with a molecular size of ~ 16 kD in the immunoblots of SLE patients led us to investigate the expression of the TCRζ chain. In immunoblots using anti-ζ antibodies we found that 10 of 22 lupus patients tested lacked the expression of TCRζ, which was always present in control subjects (P < 0.001). Flow cytometric studies using permeabilized cells confirmed the deficiency or absence of the TCRζ chain in lupus T cells. Using Northern blots we found that for eight patients tested, the TCRζ mRNA was missing in three, decreased in three, and apparently normal in two patients (P < 0.003), but was always present in control subjects. Reverse transcriptase-PCR verified Northern blot results. We conclude that TCRζ chain expression is either decreased or absent in the majority of patients with SLE, but not in patients with other systemic rheumatic diseases, regardless of disease activity, treatment status, or clinical manifestations. The previously described increases in TCR-initiated Ca2+ responses and the herein described increases in TCR-induced protein tyrosine phosphorylation and deficient TCRζ expression may represent intrinsic defects modulating lupus T cell function. (J. Clin. Invest. 1998. 101:1448–1457.) Key words: human autoimmunity • lymphocyte signaling • tyrosine phosphorylation • antigen receptor • signal transduction

Introduction

T lymphocytes use a cell surface multisubunit structure, the T cell receptor (TCR)/CD3 complex, as an antigen-specific recognition site. The clonotypic TCRα/β (or γ/δ) chains are responsible for specific antigen binding, but they are unable to transmit any intracellular signals due to the very short cytoplasmic stretches they possess. Signal transduction is carried out by a nonpolymorphic T lineage-specific heterooligomer, the CD3 molecule, which is noncovalently associated with the Ag-binding TCR chains, as well as with the TCRζ chain dimer. The extended cytoplasmic domains of the CD3-γ, -δ, -ε, and TCRζ polypeptides contain the signal transducing module immunoreceptor tyrosine-based activation motif (ITAM) in one (for each CD3 chain) or three (for each TCRζ) copies, to a total of 10 ITAMs per TCR/CD3 complex. The ability of tyrosine residues within an ITAM to undergo reversible phosphorylation is the essence of the early signal transduction pathway. Events that closely mimic the physiologic cascade initiated by Ag occur when an anti-CD3 antibody is used to ligate the TCR/CD3 complex. The subsequent immediate phosphorylation and activation of several cytoplasmic protein tyrosine kinases (PTK) and of their substrates leads to intracytoplasmic events, such as the mobilization of intracellular Ca2+, activation of key enzymes, gene transcription, cytokine synthesis, and release, proliferation, activation, or apoptosis (1, 2).

T cells from patients with SLE display multiple, frequently opposing abnormalities, including decreased cytotoxic cell function, increased helper activity, abnormal cytokine production, and the presence of unusual T cell subpopulations (3, 4). Work from this laboratory has shown that in SLE patients the TCR/CD3 signal transduction machinery is hyperactive in a disease-specific mode (5). In fresh peripheral T cells and in T cell lines, cross-linking of the TCR/CD3 complex led to a disease-specific increase in the intracytoplasmic concentration of free Ca2+ [Ca2+]i responses that originated from the intracellular Ca2+ stores. Interestingly, this finding did not characterize any particular T cell subpopulation. Recently, we have demonstrated that Ag receptor–mediated (surface IgM and IgD) signaling in B cells from lupus patients also leads to a disease-specific increase in [Ca2+]i responses that is correlated with significant increases of tyrosyl phosphorylation of cellular proteins with an apparent molecular size between 36 and 64 kD (6).

The purpose of this study was to investigate whether an abnormality in the antigen-receptor–initiated early protein ty-
rosine phosphorylation is part of the dysregulated signal transduction pathway in lupus T cells.

**Methods**

**Patients and controls.** 22 patients fulfilling the American College of Rheumatology classification criteria for lupus (7) (mean age ± SD = 47 ± 15 yr, range 21–77 yr, 20 female and 2 male) were studied. 14 patients were on prednisone, 13 on hydroxychloroquine, 6 were receiving cytotoxic medications (1 on cyclophosphamide, 4 on azathioprine, and 1 on methotrexate), and 2 patients were receiving no treatment. 8 of the patients were Caucasian, 13 African American, and 1 Hispanic. Patients on prednisone were studied at least 24 h after their last dose.

12 additional patients (disease-control group) with other rheumatic diseases (7 with RA, 1 with primary Sjögren’s syndrome, 1 with osteoarthritis, 1 with psoriatic arthritis, 1 with polymyalgia rheumatica [PMR], and 1 with Takayasu’s arteritis, mean age ± SD = 53 ± 15 yr, range 25–77 yr, 11 women and 1 man). Six were receiving prednisone and eight were on methotrexate. 14 normal volunteers (normal-control group) were also analyzed. Disease activity for the SLE patients was determined using the SLEDAI scoring system (8). The protocol of the study was approved by the Health Use Committee of the Walter Reed Army Medical Center, and written informed consent was obtained from all participating patients.

**Cells and antibodies.** Heparinized peripheral venous blood was obtained from the study subjects. PBMCs enriched in T cells were obtained as described previously (6). The percentage of T cells in the obtained subpopulation in all cases was >90% as determined by using an Epics Elite (Coulter, Hialeah, FL) flow cytometer. Cells were rested overnight in RPMI 1640 supplemented with 2 mM glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 25 mM Heps, and 10% heat-inactivated FCS (pH 7.4). The murine anti-CD3 mAb OKT3 (Ortho Biotech, Inc., Raritan, NJ) was used for cell stimulation. The antiphosphotyrosine mAb 4G10 (horseradish peroxidase [HRPO] conjugated) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), the murine anti-TCRα mAb (clone 6B10.2) was from Santa Cruz Biotechnology (Santa Cruz, CA), and the rabbit polyclonal anti–human TCRα–TCRβ (anti-TCRα) was a generous gift from Dr. L.E. Samelson (National Institutes of Health, Bethesda, MD). The goat anti–mouse antiserum M-6149 was purchased from Sigma Chemical Co. (St. Louis, MO).

**Immunoblotting and immunoprecipitation.** Five million PBMCs enriched in T cells were stimulated with 10 μg/ml OKT3 for 1 or 2 min at 37°C. To stop the reaction, 1 ml of ice-cold stop buffer was added (10 mM Tris, 50 mM NaCl, 50 mM NaF, 0.1 mM NaVO₄, 5 mM EDTA, 30 mM sodium pyrophosphate, 1 mM PMSF, and 2 μg/ml aprotinin), and whole cell lysates were obtained in a lysis buffer) of precleared cell lysate obtained as above was incubated overnight at 4°C on a rotator. Agarose-bound immune complexes were collected, washed three times (each with 500 μl of stop buffer, lysis buffer, and Tris wash buffer), and the pellet was boiled for 5 min with 50 μl of Laemml sample buffer. Agarose was discarded, the supernatants were brought to a final concentration of 5% with 2-mercaptoethanol, and incubated at 37°C for 1 h. Electrophoretic protein fractionation of equal sample volumes (25 μl of sample per lane) was followed by transfer to membranes and immunoblotting with a murine anti–human TCRα mAb (6B10.2). Detection was performed as described above. Densitometric measurements were performed using a DC 40 camera and analyzed with the Digital Science 1D program (Eastman Kodak Co., Rochester, NY).

**Flow cytometry.** The expression of cell surface markers and of TCRα was examined in partially permeabilized, mildly fixed PBMCs using a FACSScan® (Becton-Dickinson, Mountain View, CA) flow cytometer as described previously (9). Briefly, 10⁶ PBMCs were washed three times in ice-cold PBS, and mildly fixed in 0.01% parafformaldehyde for 20 min on ice. Digitonin (Sigma Chemical Co.) was added at 10 μg/ml final, for 5 min on ice, and the efficiency of cell permeabilization was confirmed with the trypan blue exclusion method under a light microscope (always >99%). Cells were incubated with 2 μg of the murine anti–human TCRα 6B10.2 mAb (or the MOPC 21 isotypic IgG1 mAb control [Sigma Chemical Co.]) for 30 min on ice, after having been incubated previously for 30 min on ice with an excess (50×) concentration of purified human IgG (Cappel; ICN Pharmaceuticals, Aurora, OH) to block nonspecific Fc binding sites. Then, cells were washed twice with ice-cold PBS 0.05% Tween, and further incubated with 4 μg of goat anti–mouse FITC-conjugated antibodies (Becton-Dickinson) for 30 min on ice. After washing three times with ice-cold PBS/1%, FBS/0.1% NaN₃, cells were incubated with excess mouse IgG (50×) for 30 min (S25 Mouse Serum: Chemicon, Temecula, CA) on ice to block the remaining anti–mouse binding sites. Cells were washed again, incubated with the proper murine mAb against cell surface markers, and fixed in 1% paraformaldehyde in PBS before analysis. All the latter mAbs were phycoerythrin (PE) conjugated. The anti-CD3-PE, anti-CD4-PE, anti-CD8-PE (Becton-Dickinson), and anti-CD45RO-PE (Caltag Laboratories, Burlingame, CA) were used for identification of T cell subsets. The murine anti-CD16-PE mAb was used to identify natural killer (NK) cells, and the anti-CD14-PE and anti-CD20-PE mAb (Becton-Dickinson) were used as markers for TCRα-negative cells (monocytes and B cells) and as monitors of efficient Fc blocking.

Fixed nonpermeabilized cell samples were also stained, either with anti-CD3-PE alone or with anti-CD3-PE and anti-CD7-FITC, to measure the intensity of CD7 expression. The detection of expression of CD3 expression on T cells in this system represents CD3 chain expression, according to the specificity of the anti-CD3-PE mAb used. Results were analyzed using the CellQuest (Becton Dickinson, Rutherford, NJ) analysis software.

**Flow cytometric [Ca²⁺]i measurement.** The OKT3-induced (after goat anti–mouse cross-linking) changes of [Ca²⁺]i in negatively selected and sorted CD19+ B cells were determined as described previously (5). A helium-cadmium air-cooled UV laser-equipped Epics Elite (Coulter Corp.) flow cytometer was used, and results were analyzed using the Multi-Time analysis software (Phoenix Flow Systems, San Diego, CA).

**Proliferation assays.** Plastic tissue culture plates (96-well, round bottom) (Falcon Labware, Cockeysville, MD) were coated with OKT3 (0.1, 1.0, 10.0, and 100 ng/ml) and the intensity of CD3 expression on T cells in this system represents CD3 chain expression, according to the specificity of the anti-CD3-PE mAb used. Results were analyzed using the CellQuest (Becton Dickinson, Rutherford, NJ) analysis software.

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last 18 h. Incubation for 3 d at 10.0 μg/ml of OKT3 was optional. Cells were harvested using a semiautomated cell harvester (Skatron Instruments, Sterling, VA) on glass fiber filters and radioactivity was measured using a 1205 Betaplate liquid scintillation counter (LKB Wallac, Turku, Finland). For triplicate wells, one SD was always <10% of the mean. Results are expressed as the mean ± SEM of 4 pm. The effect of equal concentrations of the IgG2a isotypic control mAb (again in triplicate wells) was always negligible.

Reverse transcriptase (RT)-PCR. Total RNA was extracted using the RNA SATA™ method (TEL-TEST B, Inc., Friendswood, TX) from PBMCs enriched in T cells from lupus or disease-control patients and normal volunteers. Two pairs of primers specific for human TCR and β-actin were designed and synthesized according to their sequences (10, 11). The primers were as follows: TCRβ, 5'-CAAGATGAGTGGAAGGCGC-3' and 5'-AATCCCTGCGGTGTTAGCGA-3'; and β-actin, 5'-CATGGGTCAAGAGATT-CCT-3' and 5'-AGCTGCTAGCCTCTTCAG-3'. Each primer (1 μM) and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) in 80 μl of PCR buffer containing 3 mM MgCl₂ and 400 μM dNTP were added to the RNA extract, and 30 PCR cycles were run at 95°C for 1 min, then at 54°C for 1.5 min, and finally at 72°C for 1.5 min.

Northern blotting. 10 μg of RNA from each sample was denatured, electrophoretically separated in 1% agarose, 7% formaldehyde gels, and transferred onto nitrocellulose membranes. cDNA probes encoded for TCRβ and β-actin were synthesized with RT-PCR and terminally labeled with [32P]dCTP (3,000 Ci/mmol) (Amersham) to a specific activity of 2.5-5 x 10⁸ cpm/µg DNA. Hybridization (overnight at 42°C) and exposure of the blots either to Kodak X-Omat film (71%) when compared to the lysates of cells from normal individuals (n = 8) and from patients (n = 11) with other systemic rheumatic diseases (Fig. 1, A, D, and E). The mean densitometric intensity of 1 min OKT3-stimulated cells was signifi-

Figure 1. Increased TCR/CD3–mediated protein tyrosine phosphorylation in T cells from patients with SLE. T cell–enriched PBMCs from lupus patients, individuals with other systemic rheumatic diseases, or normal volunteers were treated with 10 μg/ml OKT3 isotypic control mAb, or medium at 37°C for 1 min. Whole cell lysates were obtained from unstimulated resting cells and at 1 min after stimulation as described previously (6). 20 μg of protein from each lysate was separated electrophoretically on 10% polyacrylamide/SDS gels and analyzed for the overall amount of protein tyrosine phosphorylation. We did not observe the appearance of new bands in the lysates obtained from unstimulated cells from lupus patients. In addition, we did not record significant differences in the densitometric readings of the basal unstimulated levels of protein tyrosine phosphorylation bands between lupus patients and healthy volunteers (P < 0.8), lupus patients and disease-control patients (P < 0.7), or normal- and disease-control individuals (P < 0.9) (Fig. 1, A and B). However, after incubation with OKT3 for 1 min, the intensity, determined by densitometry, of bands located between the 64- and the 36-kD markers was obviously increased in T cells from 15 of the 21 SLE patients (71%) when compared to the lysates of cells from normal individuals (n = 8) and from patients (n = 11) with other systemic rheumatic diseases (Fig. 1, C, D, and E). The mean densitometric intensity of 1 min OKT3–stimulated cells was signifi-

36-64-kD area (densities obtained from resting cell lysates are collectively shown). The decrease or absence of a band located between the 16- and 30-kD markers in cell lysates obtained from either resting or OKT3-stimulated T cells from some patients with SLE should be noted.
cantly higher in lysates of cells from SLE patients compared to cells from normal volunteers ($P < 0.02$) or from patients with other systemic rheumatic diseases ($P < 0.015$) and are collectively presented in Fig. 1 F. Differences between normal volunteers and disease-control patients were not significant ($P < 0.08$). In some experiments we cross-linked the OKT3 mAb with a goat anti-mouse antiserum, but we did not notice any further enhancement of the phosphorylation pattern. Also, the use of an isotypic control murine mAb instead of OKT3 failed to induce protein tyrosyl phosphorylation above that observed with medium alone. We did not observe any correlation between the densitometric intensity of the bands with disease activity, specific clinical feature(s), or treatment status. These observations are highly similar to previously reported findings regarding the Ag receptor–initiated protein tyrosyl phosphorylation in lupus B cells in terms of band localization, disease specificity, and disease activity independence (6).

The time–response curve of the anti-CD3–mediated protein tyrosine phosphorylation is markedly altered in SLE patients. We conducted time course experiments to investigate whether the anti-CD3–mediated protein tyrosine phosphorylation in lupus T cells displayed a different pattern. We incubated lupus ($n = 6$) and normal ($n = 3$) T cells as described above for 1 and 2 min with OKT3. As shown in Fig. 2 A, the densitometric intensity of the 36–64-kD area from OKT3-stimulated normal T cells was further increased at 2 min. In contrast, the 2-min densitometric intensity of lysates from OKT3-stimulated lupus T cells was significantly lower than that detected for the first minute after stimulation (Fig. 2 B). Interestingly, in two lupus patients (of six studied) the OKT3-induced protein tyrosine phosphorylation at 2 min was lower than that obtained before stimulation. The densitometric intensities of all the subjects studied are collectively presented in Fig. 2 C. The difference of the means of the densitometric readings obtained at the 2-min time point between normal individuals and SLE patients were significant ($P < 0.034$).

**Diminished expression of the TCRζ chain in lupus patients using immunobLOTS, immunoprecipitations, and flow cytometry.** In contrast to the increased tyrosyl phosphorylation pattern that was observed for the 36–64-kD area, the intensity of a band located between the 16- and 30-kD markers was significantly decreased (at all time points) in the cell lysates from 17 of 22 lupus patients (77%) when compared to normal and disease-control individuals, and in some lysates from lupus patients the band was undetectable. The characteristics of this band, i.e., molecular size between 16 and 30 kD, tyrosyl phosphorylation in the resting state, and TCR/CD3–induced further tyrosyl phosphorylation (Fig. 3 A), led us to consider the TCRζ chain as a possible candidate. To investigate whether the TCRζ protein in lupus T cells was in fact decreased or missing, or present but simply resistant to early tyrosyl phosphorylation, we conducted immunoblotting and immunoprecipitation experiments. T cell–enriched PBMC (5×10⁶) from lupus patients, normal and disease-control individuals were lysed, and equal amounts of protein (30 μg) were separated electrophoretically and analyzed for content of TCRζ protein using two different anti-ζ antibodies. Using a murine anti-human TCRζ chain mAb, no bands corresponding to the TCRζ chain could be identified in 10 of 22 lupus patients studied, while the ζ protein was always present in cell lysates from normal ($n = 12$) and 12 disease-control individuals (seven patients with RA, one with PMR, one with primary Sjögren’s syndrome, one with Takayasu arteritis, one with osteoarthritis, and one with psoriatic arthritis) as shown in Fig. 3 B. Application of the Fisher’s exact test (2×3 table) revealed a statistically significant difference ($P = 0.001$). We next used the polyclonal 387 anti-TCRζ antibody to distinguish whether the whole protein or just a portion which includes the epitope detected by the murine 6B10.2 mAb (amino acids 36–54) was conformationally modified or missing. Immunoblots using the 387 antiserum showed that the ζ chain protein was again either obviously decreased or undetectable in 6 of 9 SLE patients examined, when compared to the disease-control patients ($n = 7$) and normal individuals ($n = 4$), where it was always present in comparable amounts. One representative immunoblot using the rabbit 387 antiserum (one patient with SLE and two different patients with RA) is shown in Fig. 3 C. To further document our findings, 500 μg of total protein from lysates obtained from three SLE patients, one normal and two disease-control individuals, was immunoprecipitated using the 387 rabbit anti-human TCRζ antibody, and subsequently the TCRζ chain was detected in immunoblots using the murine 6B10.2 mAb. Preliminary experiments showed that both these antibodies could detect not only the unphosphorylated, but the phosphorylated form(s) of TCRζ as well (data not shown). The TCRζ chain could be immunoprecipitated from the cell lysates obtained from normal and disease-control individuals. In the lysates from three SLE patients (Fig. 3 D), the immunoprecipitable amounts of TCRζ were decreased in one, comparable to nor-

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**Figure 2.** Time curve of the anti-CD3 mAb-mediated lupus T cell protein tyrosine phosphorylation. T cell–enriched PBMCs from normal donors and lupus patients were stimulated for 1 or 2 min with the OKT3 mAb. The electrophoretically separated cell lysate proteins were immunoblotted with the 4G10 antiphosphotyrosine mAb. Representative immunoblots from T cell lysates from (A) one healthy volunteer and (B) one SLE patient are shown. (C) Mean±SEM ($n = 3$ for normals and 6 for SLE patients) ratios of the densitometric intensities of the 36–64-kD area obtained for stimulated (1 or 2 min) over the densities obtained from resting cells.
mal in another, and undetectable in a third patient. In lupus patients displaying absence of the full-length $\zeta$ chain, there was no other product with a smaller size (indicative of possible cleavage) detected further down in our gels.

To further establish the deficiency (or absence) of the TCR$\zeta$ chain in whole lupus T cells and to characterize its expression in T cell subsets, we conducted flow cytometry experiments. Digitonin-permeabilized cells were incubated with the

Figure 3. Deficient TCR$\zeta$ protein expression in lysates from T cells of SLE patients. Five million resting T cell–enriched PBMCs from lupus patients, disease-control patients, and normal volunteers were lysed and an equal amount of protein (30 $\mu$g) from these whole-cell lysates was analyzed electrophoretically, transferred to nitrocellulose membranes, and then immunoblotted either with (A) the 4G10 antiphosphotyrosine mAb, (B) the murine 6B10.2 anti–human TCR$\zeta$ mAb, or (C) the 387 rabbit anti–human TCR$\zeta$ polyclonal antibody. (D) T cell–enriched PBMC lysate protein (500 $\mu$g) from normal (NL), lupus (L), RA, and PMR patients was immunoprecipitated with the 387 antibody and the amount of $\zeta$ chain was detected by immunoblotting with the 6B10.2 mAb.

Figure 4. Detection of the TCR$\zeta$ chain in T cells from patients with SLE using flow cytometry. Patients with undetectable $\zeta$ chain in immunoblots were also $\zeta$ chain negative by flow cytometric analysis of digitonin-permeabilized PBMCs. Cells were indirectly stained for TCR$\zeta$ chain expression using a murine anti-$\zeta$ mAb and FITC-conjugated goat anti–mouse antibody ($x$ axis). Double staining was performed for CD3$^+$, CD4$^+$, CD8$^+$, CD45RO$^+$, and CD16$^+$ cells using PE-conjugated mAbs ($y$ axis). CD20$^+$ cells (B cells) were also stained as negative control cells and as indicators of efficient Fc blockade (analyzed in Methods). A representative experiment of a TCR$\zeta^-$ lupus patient and a normal volunteer is shown A. All $\zeta$-bearing cell populations clearly stained positively for the normal donor and negatively in the patient with SLE. Another patient with decreased expression of TCR$\zeta$ in immunoblots is shown in B. While the CD4$^+$ cells are largely unaffected and not obviously different than the respective normal control, the CD3$^+$ and CD16$^+$ cells were predominantly TCR$\zeta$ negative. The histograms in C represent staining with anti-CD3$^+$ and TCR$\zeta^+$ of a $\zeta$-negative lupus patient and a healthy individual. Expression of the CD3$^+$ chain (left) was similar in the normal and the lupus individuals (dotted lines). Negative control is shown as the shadowed histogram. Expression of the $\zeta$ chain (right) for the lupus patient (dotted line) is similar to that of the negative control (solid line) in the $\zeta$-negative lupus patient. TCR$\zeta$ chain expression in the normal donor is shown as the shadowed histogram.
6B10.2 mAb, followed by FITC-conjugated goat-anti-mouse
Ab, and PE-conjugated anti-CD3, anti-CD4, anti-CD8, anti-
CD45RO, and anti-CD16 antibodies as described in Methods.
Cells bearing Fc receptors but not TCRζ were also PE stained
(anti-CD14, anti-CD20, or both) as negative control cells. The
relevant isotypic control mAb was always used as an internal control. Three representative experiments are
shown here, including seven different patients with SLE (lanes 1–7), three different normal donors (NL), two different RA patients, and one with PMR. Northern blot analysis of TCRζ mRNA and β-actin
mRNA was used as an internal control (as described in Methods)
from different lupus patients (L-2, -4, -5, -7, -10, and -11), disease controls (RA, PMR, osteoarthritis [OA], and psoriatic arthritis [PsA]), and normal controls (NL) are shown in B.

Absent or decreased mRNA for TCRζ in lupus patients but not in control individuals. The TCRζ chain can be selectively
degraded upon T cell activation (12) or in other conditions by
posttranslational processes which are incompletely understood
(13, 14). To investigate whether the decreased amounts of
TCRζ chain detected in lupus T cells was the result of post-
translational degradation, or the outcome of abnormalities at
the mRNA level, we extracted total RNA from PBMCs en-
riched in T cells and conducted RT-PCR using oligomer prim-
ers (described in Methods) that amplify the full-length mRNA
of the TCRζ chain. Results (Fig. 5 A) obtained for eight lupus
patients revealed that in three patients there was no detectable
full-length mRNA band for the TCRζ, while this band was always
present in the PBMC extracts from disease-control pa-
tients (n = 6 in total, [three RA patients, one with PMR, one
with Sjögren’s syndrome, and one with Takayasu’s arteritis])
and in all normal individuals (n = 3). An internal control cor-
responding to the mRNA of a housekeeping gene (β-actin)
was always included and detected. Northern blots performed
for the same controls and patients showed that of the eight
SLE patients studied, the TCRζ mRNA was undetectable in
three, decreased but detectable in three, and no different than
normal in two patients as shown in Fig. 5 B. Northern blot
quantitative analysis of the TCRζ (when present) and β-actin
mRNA was performed either densitometrically or with the use
of a PhosphorImager. Application of the Fisher’s exact test
(3 × 2 table) revealed a statistically significant difference (P <
0.0023).

Associations of TCRζ chain deficiency with early and late
TCR/CD3-mediated T cell functions. The ζ chain of the TCR
is a molecule involved in the TCR/CD3–initiated signal trans-
duction biochemistry. To assess the consequences of TCRζ
deficiency, we studied the anti-CD3–induced changes in [Ca2+],
an early event in the signal transduction pathway, and the
anti-CD3–induced cell proliferation (a late event).

The OKT3-induced (after cross-linking by goat anti–mouse
antibody) peak [Ca2+]i ratios were studied in negatively selected and Indo-1–loaded T cells from eight patients with SLE
(four were TCRζ+ and four were TCRζ− as determined by
Western immunoblots) and were compared to the responses of
T cells obtained from four normal individuals (normal controls)
as described previously (5). Baseline values of [Ca2+]i ratios
were comparable for unstimulated T cells from lupus pa-
tients and from normal volunteers. All eight lupus patients
responded in a supraphysiological manner, which is in agree-
ment with previously published data (5). Analysis of the
[Ca2+]i responses according to the presence or absence of
TCRζ yielded significant differences. Specifically, absence of
TCRζ was associated with [Ca2+]i responses which were higher
than normal (peak ratio of [Ca2+]i: 16.1 ± 0.65 vs. 14.1 ± 0.26,
P < 0.026), but were significantly lower than the [Ca2+]i
responses of patients who were TCRζ+ (16.1 ± 0.65 vs. 19.2 ± 0.71,
P < 0.019) and were characterized as low supranormal. Pa-
tients who were TCRζ− responded in a high supranormal man-
ner. Their responses were significantly higher both from the
TCRζ+ patients (P < 0.019) and from the normal controls
(P < 0.0005). Thus, it is clear that SLE patients display higher
than normal [Ca2+]i responses and that they could be stratified
as high or low hyperresponders according to the presence or
absence of the TCRζ chain (Fig. 6 A).

We also studied the OKT3-induced proliferation profile of
TCRζ positive and negative SLE patients. For the proliferat-

Figure 5. Detection of TCRζ chain mRNA. RT-PCR (A). Two prim-
ers were constructed for the ζ chain and the products were analyzed
electrophoretically in 1% agarose gels prepared with Tris-borate
EDTA buffer, stained with 20 μg ethidium bromide, and photo-
graphed. The mRNA of a housekeeping gene (β-actin) was always
used as an internal control. Three representative experiments are
shown here, including seven different patients with SLE (lanes 1–7),
three different normal donors (NL), two different RA patients, and
one with PMR. Northern blot analysis of TCRζ mRNA and β-actin
mRNA was used as an internal control (as described in Methods)
from different lupus patients (L-2, -4, -5, -7, -10, and -11), disease controls (RA, PMR, osteoarthritis [OA], and psoriatic arthritis [PsA]), and normal controls (NL) are shown in B.
tion assays PBMCs were used. To obtain comparable results, the percentage of CD3+ cells was determined previously by flow cytometry, and only those patients and controls with similar CD3+ subpopulations were used. Time- and dose-response experiments determined the optimal OKT3 dose and incubation time (10 μg/ml OKT3, and incubation for 3 d). Eight patients with SLE and six normal volunteers were studied. The proliferative responses of PBMCs obtained from normal subjects were not different from the responses of lupus patients who were TCRz1+ (P = 0.35) or TCRz2− (P = 0.87). The difference in the responses between TCRz1+ and TCRz2− patients was, similarly, not statistically significant (P = 0.28) (Fig. 6 B).

The increased TCR/CD3–induced protein tyrosine phosphorylation and the absence of TCRz in T cells from the same SLE patients persist over time despite changes in disease activity. Even though the pattern of TCR/CD3–mediated early protein tyrosine phosphorylation was found to be independent of disease activity, one could still argue that this aberration may correlate, in an individual SLE patient, with changes in disease activity, and/or the appearance or disappearance of specific clinical manifestations. To address this question, four unselected lupus patients were restudied 3–9 mo after their initial evaluation. In all four patients the SLEDAI had changed. Nevertheless, the increased Ag receptor–mediated overall protein tyrosine phosphorylation at 1 min did not parallel changes in disease activity. Patients A and D had significant changes in their disease activity (a decrease of 75% and an increase of 172%, respectively, in their SLEDAI) while the densitometrically measured ratio (1:0 min) of tyrosine phosphorylation was not substantially altered. On the other hand, for patients B and C, who displayed no major changes in their SLEDAI, the ratio (1:0 min) was substantially either increased or decreased, respectively (Fig. 7). These experiments further confirm that the anti-CD3 mAb–induced early overall protein tyrosine phosphorylation pattern does not correlate with changes in disease activity, thus, implying a permanent intrinsic defect in the early Ag receptor–initiated signaling machinery. Importantly, patient D had detectable and patients A, B, and C had undetectable bands corresponding to the TCRz chain as determined by Western immunoblotting in both evaluations. Also, lack of TCRz chain expression found by Western immunoblots during the first visit was in agreement with the results of flow cytometric TCRz chain detection in permeabilized cells during a later (after 12–14 mo) visit. These data imply that the pattern of z chain expression is time- and disease activity–independent.

Patients with SLE are usually receiving treatment with agents which alter the function of the immune system. To exclude a potential drug effect on the TCRz chain deficiency, our lupus patients were analyzed in comparison with RA patients from our disease-control group who suffered from systemic autoimmune rheumatic diseases and received similar agents. As depicted in Table I, the z chain deficiency in patients with SLE cannot be attributed to a drug effect.

**Discussion**

We conducted this study to investigate possible aberrations in the early events of the signal transduction pathway in lupus T cells. Since disease-specific abnormalities in the TCR/CD3–mediated [Ca2+]i responses have been demonstrated already...
Table I. Expression of the TCRζ Chain in T Cells from Patients with SLE and RA Who Were Treated with Immunosuppressive Agents

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>TCRζ chain (+)</th>
<th>TCRζ chain (–)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE patients on prednisone (14)</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>RA patients on prednisone (4)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>SLE patients on cytotoxics* (6)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>RA patients on cytotoxics† (7)</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

*Four were on azathioprine, one was on cyclophosphamide, and one was on methotrexate. †All seven were on methotrexate.

(5), we explored biochemical events which precede the Ag receptor–induced Ca\(^{2+}\) fluxes. The earliest known events in the Ag receptor–initiated signaling is the activation of the src and the ZAP-70/Syk family of PTKs, that results in reversible tyrosyl phosphorylation of multiple protein substrates (2), the majority of which remain unknown. We used the OKT3 mAb to trigger the signaling cascade, and we evaluated the pattern of early tyrosine phosphorylation in primary T cells from patients with SLE, patients with other systemic rheumatic diseases, and normal individuals.

Although no additional bands (corresponding to tyrosyl phosphorylated proteins) were noted in the antiphosphotyrosine immunoblots of lysates of unstimulated T cells from patients with SLE, lysates from OKT3-stimulated lupus T cells, but not from disease-control or normal-control T cells, displayed altered quantitative and time course responses. Specifically, at the first minute of treatment with anti-CD3 mAb, the intensity of bands with apparent molecular sizes between 64 and 36 kD was significantly enhanced in lupus T cells compared to control individuals, in a disease-specific and disease activity–unrelated mode. This abnormality persisted in four patients who were reevaluated after several months, when disease activity and clinical manifestations had changed. While normal T cells exhibit gradually rising levels of protein tyrosine phosphorylation over a 2-min period in a near-linear mode, the lupus T cells respond to the same OKT3-induced stimulus in a burst-like fashion, achieving supranormal amounts of tyrosyl phosphorylated cellular proteins quite early (by the first minute) and return to a close-to-resting state by the second minute.

This earlier and higher than normal response of T cells from SLE patients is in agreement with the earlier and higher than normal Ca\(^{2+}\) fluxes which follow TCR/CD3 ligation (5). Our current data are also similar to those obtained from the study of Ag receptor–initiated early signaling events in lupus B cells (6), suggesting a possibly unifying pathology in the signal transduction of SLE lymphocytes. In a previously published study investigating the induction of tyrosyl phosphorylated proteins after anti-CD3 mAb cross-linking, T cells from SLE patients were reportedly refractory to stimulation compared to T cells from normal donors (15). Nevertheless, this anti-CD3 antibody effect was studied at a relatively late point (3 min), and earlier points were omitted. In our study we show that even earlier (at 2 min) the lupus T cells have produced a fast supraphysiological response, and have already returned to a close-to-resting state.

In contrast to the obvious and consistent OKT3-induced hyperphosphorylation of proteins of 36–64 kD, we noticed that a band located between 16 and 30 kD, which was consistently present in lysates from normal T cells, was frequently decreased in the antiphosphotyrosine immunoblots of lupus T cells. This substrate (when present) displayed decreased intensity in the resting state, but it was undergoing tyrosyl phosphorylation after stimulation with OKT3. Immunoblotting and immunoprecipitation experiments identified this protein to be the TCRζ chain. The possibility of detecting only one epitope (linear or conformational) of this protein was largely eliminated by using two different (a murine monoclonal and a rabbit polyclonal) antibodies. The expression of the ζ chain was either deficient or diminished in 77% of the studied SLE patient population, while it was always sufficiently present in all normal donors and patients with other systemic rheumatic diseases that were also studied. Further confirmation of the TCRζ chain deficiency was provided by flow cytometry experiments of digitonin-permeabilized cells. The TCRζ chain deficiency in lupus T cells can be considered chain-specific, since our data (and a previously reported study) did not find differences in the expression of CD3ε polypeptide on lupus T cells, regardless of the presence or absence of the ζ chain (5). TCRζ was absent from cell subsets (CD3ε, CD4ε, CD8ε, CD45ROε, and CD16ε) from patients who lacked the TCRζ chain by immunoblotting. For those patients with obviously normal TCRζ expression, no difference in all subsets could be detected by flow cytometry. In patients who had decreased TCRζ expression, the CD4ε cells were not obviously affected, while the CD8ε and CD16ε cell subsets were, to a greater extent, ζ chain deficient. This relatively selective involvement has been previously described in ζ-knockout mice (16, 17) where the CD8ε T cell subset was more severely affected by the absence of the ζ polypeptide as compared to the CD4ε subset. Functionally, this defect may contribute to the multiplicity of immune cell abnormalities which predominantly involve CD8ε and NK cells in lupus.

Selective degradation of a previously expressed TCRζ after T cell activation has also been reported (12), as well as the lack of TCRζ chain in tumor-infiltrating lymphocytes (13, 14) under the influence of poorly understood (but reversible) post-translational mechanisms. In the latter case, FcεRIγ may substitute for the missing TCRζ. The absence or diminished expression in T cells from some SLE patients reported herein is not the result of posttranslational modifications since, in lupus patients, the mRNA for the TCRζ was undetectable or severely decreased either by Northern blots or (more importantly) by RT-PCR. None of the normal or the disease-control individuals studied displayed a similar abnormality.

The TCRζ forms a homodimer which is part of the TCR/CD3 complex. Different functions have been attributed to the ζ chain including roles in signaling in mature T cells, in proper assembly and transfer from the cytoplasm to the cell surface of the complete TCR/CD3 heterooligomer, and in normal development and selection of immature thymocytes (16–18). The long cytoplasmic domain of each ζ chain contains three ITAM modules which participate in signaling. After these ITAMs become tyrosyl phosphorylated (by members of the src family of PTK), they form anchoring sites for the tandem SH2-domains of ZAP-70, a PTK important for further signal propagation (19). Data originating from the study of transformed T cells suggest that in the resting state the TCRζ is found in a non-phosphorylated form and becomes rapidly phosphorylated after TCR/CD3 ligation. This is not true for the normal peripheral T cells, where TCRζ is constantly phosphorylated (increasing...
further after TCR/CD3 ligation) and ZAP-70 is constantly bound on it (20). In a majority of T cells (5–10%), the ζ chain may be found as part of a heterodimer with the highly homologous TCRζ chain (with no known physiological role), which is an alternatively spliced product of the ζ gene (21) and has two ITAMs. There are T cell subsets which normally do not contain any TCRζ and its place is taken by another homologous protein, the γ chain of the high-affinity receptor type I for the Fc fragment of IgE (FcεRIγ) (22–24), which contains a single ITAM. FcεRIγ has also been identified as a signal transducing molecule in receptors for the Fc fragment of IgG as well (25), where TCRζ can also be found (26, 27).

To assess the functional consequences of TCRζ deficiency in lupus T cells we studied the anti-CD3–induced [Ca²⁺]i responses and the anti-CD3–induced proliferation in comparison with T cells from healthy volunteers. TCRζ status significantly affected the [Ca²⁺]i responses. The constantly supra-physiological OKT3-induced [Ca²⁺]i responses of lupus T cells could be clearly stratified as high or low supranormal in correlation with the presence or absence of the TCRζ chain. While this early signal transduction event was clearly correlated to the ζ chain status, the same was not true for the OKT3-induced cell proliferation. This late event was not affected by the ζ chain status. The AgR-mediated proliferative behavior of lupus T cells is unclear, with different groups reporting different results (for review see reference 28). It is possible that other mediators participating in this relatively late cellular function may overcome or compensate for the absence of the ζ chain. Also, the sensitivity of this method may not allow for the detection of differences in the two patient groups. Other indicators of cellular activation may be more sensitive to the ζ chain defect, an issue which further studies should address.

The TCRζ chain is important for the proper assembly and transfer to the cell membrane of the whole TCR/CD3 complex. Experiments in ζ-knockout mice have shown that the development of mature T cells and the expression of the TCRζ/β complex on the surface of such T cells are greatly impaired. Nevertheless, the expression of TCRζ is not an absolute requirement for proper development of T cells. Mature single-positive TCRζ/β cells appear in the periphery of ζ-knockout mice, and the development and function of non-TCRζ/β cells is totally unaffected. Moreover, even when small (ITAM-less) fragments of the ζ chain are expressed only, instead of the full-length chain, functional TCR/CD3 complexes are sufficiently expressed on the cell surface. It was recently reported that ζ-knockout mice transfected with the FcεRIγ gene have normal T cell development and function (29). In murine knockout models that express neither TCRζ nor TCRζ and FcεRIγ, mature T cells were still produced (for review see reference 30). The above data imply that the TCRζ chain may not be absolutely required either for the signal transduction cascade initiated by the TCR/CD3 complex in mature or immature T cells, or for the proper assembly and transfer to the surface of the TCR/CD3 polypeptides. Thus, TCRζ-deficient T cells can initiate and propagate the signal transduction cascade, provided that they express on their cell surface sufficient amounts of TCR/CD3. In such a situation, signaling can be transduced by using the ITAMs of the other invariant chains of the CD3 complex (31, 32). In fact, signal transducing molecules such as ZAP-70 have been shown to functionally bind to these non-ζ ITAMs (33). It has also been reported that Syk instead of ZAP-70 is alternatively and preferentially recruited to the polypeptide (if any) which may substitute for the missing TCRζ chain (i.e., the FcεRIγ) and propagates the generated signals further downstream (34). Even though Ag receptor signaling takes place in the absence of ζ, the outcome is different. The pattern of protein tyrosyl phosphorylation which is observed under such settings is altered, and the production of IL-2 is decreased (31). This outcome is similar to the presently described abnormal pattern of OKT3-mediated tyrosine phosphorylations in lupus T cells, and with the known deficient IL-2 production in lupus (35). The absence of the ζ chain in NK cells may also influence their function, which is also decreased in lupus patients (36, 37).

One aspect of T cell physiology which is influenced significantly by the absence of the ζ chain is the positive and negative selection of the immature thymus-dependent cells. The amplification effects of the three ITAMs of the ζ chain in signaling are considered important and are supported by experimental data stemming from the study of these selection processes. Absence of the ζ chain decreases both positive and negative selection. This may apply to SLE as well. The decreased negative selection may enhance an autoimmune repertoire, and the decreased positive selection may be the basis for the decreased thymus-dependent immune responses known in lupus T cells in vitro and in vivo. Further support for the former hypothesis comes from a recent study reporting an enhanced autoimmune potential for the T cells produced by ζ-knockout mice (38).

The TCR/CD3–induced signaling in mature ζ-less T cells has been studied in the setting of tumor-infiltrating lymphocytes by proliferation assays and was found decreased (39). Nevertheless, the ζ chain deficiency in the above cells was accompanied by decreased expression of src family members which may offer an explanation to this phenomenon. On the other hand, while the levels or the activities of src-PTK in lupus T cells are unknown, the T cells of SLE patients display a decreased counterregulatory mechanism for signal transduction that may contribute to the enhanced Ag receptor signaling events. This consists of the decreased activity of cAMP-dependent protein kinase A type I (40). This enzyme negatively affects TCR/CD3–mediated signaling by decreasing the Ca²⁺ fluxes, and presumably also the anti-CD3 mAb-induced protein tyrosyl phosphorylation. In mature T cells the TCRζ chain is particularly effective in triggering activation-induced cell death (41). This particular mode of TCR/CD3-mediated apoptotic cell death is reportedly decreased in peripheral T cells of SLE patients in a disease-specific fashion (42), a phenomenon which may be explained by a deficient expression of the ζ chain in these cells.

Thus, abnormalities in the expression of the TCRζ protein may consist of a common denominator in signal dysregulation and in the dysfunction of ζ chain–bearing immune cells in SLE. Our TCRζ mRNA findings imply that this specific mRNA was either absent or abnormal, or finally, extremely unstable and selectively degraded. Further studies are necessary to investigate these possibilities. They also raise the possibility that the underlying abnormality may lie at the DNA level. Analysis of the gene for the TCRζ chain, which is located on chromosome 1, in lupus patients and their families should be the next step to further characterize the observed defect. Genetic factors are well known contributors for SLE, and a possible link with a locus on human chromosome 1 has recently been claimed (43).

In this study, we report for the first time that the TCRζ protein is either absent or expressed at greatly decreased levels in...
the majority of patients with SLE. This abnormality is disease specific, disease activity independent, treatment unrelated, and does not represent posttranslational modifications. Because these aberrations were limited to lupus T cells, we conclude that they may represent intrinsic defects of potential pathogenic significance for the disease.

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References


Altered Signaling In Lupus T Cells and Deficient TCRζ Chain Expression