The Journal of Immunology

Increased Caspase-3 Expression and Activity Contribute to Reduced CD3 ζ Expression in Systemic Lupus Erythematosus T Cells

Sandeep Krishnan, Juliann G. Kiang, Carolyn U. Fisher, Madhusoodana P. Nambiar, Hang T. Nguyen, Vasileios C. Kyttaris, Bhabadeb Chowdhury, Violeta Rus and George C. Tsokos

J Immunol 2005; 175:3417-3423; ;
doi: 10.4049/jimmunol.175.5.3417
http://www.jimmunol.org/content/175/5/3417

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Increased Caspase-3 Expression and Activity Contribute to Reduced CD3ζ Expression in Systemic Lupus Erythematosus T Cells[1]

Sandeep Krishnan,* Juliann G. Kiang,*‡§ Carolyn U. Fisher,* Madhusoodana P. Nambiar,†‡ Hang T. Nguyen,*¶ Vasileios C. Kyttaris,*‖ Bhabadeb Chowdhury,* Violeta Rus,# and George C. Tsokos[2]*‡¶

T cells isolated from patients with systemic lupus erythematosus (SLE) express low levels of CD3ζ-chain, a critical molecule involved in TCR-mediated signaling, but the involved mechanisms are not fully understood. In this study we examined caspase-3 as a candidate for cleaving CD3ζ in SLE T cells. We demonstrate that SLE T cells display increased expression and activity of caspase-3. Treatment of SLE T cells with the caspase-3 inhibitor Z-Asp-Glu-Val-Asp-FMK reduced proteolysis of CD3ζ and enhanced its expression. In addition, Z-Asp-Glu-Val-Asp-FMK treatment increased the association of CD3ζ with lipid rafts and simultaneously reversed the abnormal lipid raft preclustering, heightened TCR-induced calcium responses, and reduced the expression of FcRγ-chain exclusively in SLE T cells. We conclude that caspase-3 inhibitors can normalize SLE T cell function by limiting the excessive digestion of CD3ζ-chain and suggest that such molecules can be considered in the treatment of this disease. ***The Journal of Immunology,*** **2005, 175: 3417–3423.**

Systemic lupus erythematosus (SLE)[3] T cells are characterized by abnormal TCR-mediated signaling responses that include a lowered excitation threshold and heightened intracellular calcium responses (1–4). Compared with normal T cells, human SLE T cells have decreased levels of CD3ζ, the function of which is conducted by an FcR γ-chain that is up-regulated in SLE T cells and functionally associates with the TCR (1–3, 5). This rewiring of the TCR has been proposed to be responsible for the aberrant TCR/CD3-mediated signaling in SLE T cells (6). Several mechanisms have been shown to contribute to reduced CD3ζ expression in SLE, including defects in CD3ζ mRNA transcription (7), production of alternatively spliced forms of CD3ζ mRNA (8), and increased lysosomal degradation (2). However, whether additional mechanisms also contribute to this pathology remains unclear.

Recently, it has emerged that apoptotic caspases, such as caspase-3, are involved in several nonapoptotic cellular processes, such as T cell proliferation and cell cycle regulation (9–14). Selective substrate cleavage mediated by caspases is believed to be responsible for these events (reviewed in Refs. 13 and 15). CD3ζ bears several caspase-cleaving sites in its cytoplasmic domain (16). Caspase-3 has been shown to cleave in vitro translated CD3ζ as well as intracellular CD3ζ in Jurkat cells (16, 17) and has been shown to be associated with low levels of CD3ζ in chronic diseases, such as gastric cancer (18). Recently, it was proposed that caspase-3 may be involved in CD3ζ digestion in several diseases (19). In light of these observations and considering that caspase-3 activity might be high in SLE T cells because of their increased susceptibility to spontaneous apoptosis (20), we hypothesized that caspase-3 might be involved in cleaving CD3ζ in SLE T cells. In this study we tested this hypothesis by determining the levels of expression and activity of caspase-3 in SLE T cells and exploring the direct effect of inhibition of caspase-3 activity on CD3ζ expression. We report that SLE T cells display higher levels and activity of caspase-3 than normal T cells. Moreover, reversal of heightened caspase-3 activity increased the amounts of CD3ζ protein and dampened the abnormal TCR-induced calcium responses in SLE T cells.

Materials and Methods

Patient samples and T cell isolation

Twenty-nine SLE patients (27 women and two men) with an SLE disease activity index (SLEDAI) ranging from 0 to 16, 26 healthy volunteers (age, 19–71 years), and six Sjogren's syndrome patients were included in this study. The SLEDAI scores were calculated as originally described (21). The study protocol and isolation of T cells have been described previously (22). The study protocol was approved by the health use committees of Walter Reed Army Institute of Research, Walter Reed Army Medical Center, and University of Maryland. Written informed consent was obtained from all participating patients and volunteers.

Caspase-3 expression, activity, and intracellular ATP measurement

After specific treatment, cells (5×10^6) in Na^+ Hanks' solution containing 1 mM DTT, 1 mM PMSF, 5 mM EDTA, 1 mM $NaOV_4$, 1 mM sodium fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin were sonicated, and

*Department of Cellular Injury and †Department of Biochemistry, Walter Reed Army Institute of Research, Silver Spring, MD 20910; ‡Department of Medicine and §Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814; ¶Rheumatology Service, Walter Reed Army Medical Center, Washington, D.C. 20307; ‖Section of Rheumatology, Washington Hospital Center, Washington, D.C. 20010; and #Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201

Received for publication January 18, 2005. Accepted for publication June 13, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Public Health Service Grant RO1AI42269. The opinions expressed herein are the private ones of the authors and do not represent those of the Department of Defense or the Department of the Army.

[2] Address correspondence and reprint requests to Dr. George C. Tsokos, Department of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500. E-mail address: gtsokos@usuhs.mil

[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CHX, cycloheximide; CT-B, cholera toxin B; MFI, mean fluorescence intensity; pNA, pNitroaniline; SLEDAI, SLE disease activity index.

0022-1767/05/$02.00

the lysates were assayed for cellular caspase-3 expression, activity, and ATP content. Caspase-3 expression and activity were measured using a commercial assay kit (BIOMOL). The data are presented in picomoles of pNitroaniline (pNA) minute per microgram of protein. Cellular ATP levels were determined using the ATP Bioluminescence Assay kit HS II (Roche). Luminescence was measured with a TD-20/20 luminometer (Turner Designs). Data were normalized to total protein, and the cellular ATP level was expressed as picomoles per microgram of protein.

Cell culture, flow cytometry, and confocal microscopy

Treatment of cells with cycloheximide (CHX) has been described previously (23). Briefly, cells (5×10^6) were treated with 10 μg/ml CHX for 10 min at 37°C, washed, and cultured in plain RPMI 1640 medium supplemented with 10% FBS at 37°C for 0, 3, 4, or 6 h as indicated. For caspase-3 inhibition experiments, cells were cultured in the presence of equal volumes of 50 μM Z-Asp-Glu-Val-Asp-FMK (DEVD; Calbiochem) or DMSO vehicle (Sigma-Aldrich) for 8 h as described previously (16). In some experiments the pan-caspase inhibitor Z-Val-Ala-Asp-FMK (VAD; Calbiochem) was used instead of DEVD. CD3ζ staining was performed with anti-CD3ζ (Santa Cruz Biotechnology), followed by secondary donkey anti-mouse FITC as described previously (22), and was analyzed by FACS. For confocal microscopy, 0.5×10^6 cells were adhered on polylysine-coated glass slides for 1 h at room temperature and immediately fixed with 4% paraformaldehyde solution. For intracellular staining, cells were permeabilized with a buffer containing 0.05% (w/v) saponin in RPMI 1640 medium. Cells were stained with FITC-conjugated cholera toxin-B (CT-B; Sigma-Aldrich) for 30 min. Cells were washed, air-dried, and mounted using Gel/Mount (Biomeda), and coverslips were applied. Samples were analyzed with a laser scanning confocal fluorescence microscope (1X70; Olympus) with Lasersharp-2000 software (Bio-Rad).

Lipid raft isolation and Western blotting

Normal or SLE T cells (10×10^6) were treated with caspase-3 inhibitors as described above, and lipid raft fractions were isolated as described previously (22). Proteins from raft fractions were concentrated using a standard TCA precipitation protocol, then suspended in denaturing sample buffer (24). After resolution on a 4–12% bis-Tris NuPage gel (Invitrogen Life Technologies), proteins were transferred onto polyvinylidene difluoride membranes, then incubated with specific Abs against CD3ζ (clone 6B10.2) and linker for activation of T cells (Santa Cruz Biotechnology). HRP-coupled Abs (Santa Cruz Biotechnology) were used as secondary Abs, and detection was performed with ECL (Amersham Biosciences).

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response analysis

These assays were performed as described previously (22). Five million cells were washed with RPMI 1640 and incubated with 1 μg/ml indo AM (Molecular Probes) for 30 min at 37°C, washed with RPMI 1640, and kept on ice. Cells were analyzed using an EPICS Altra (Coulter) flow cytometer equipped with a high power dual wavelength (365 and 488 nm argon laser). Samples were run, and at 30 s, either OKT3 (10 μg/ml) or the isotype control $mIgG_{2a}$ was added, followed by goat anti-mouse cross-linker at 1 min, and the ratio of the fluorescence that is directly proportional to free cytosolic Ca^{2+}, was recorded for a period of 400 s as described previously (1, 25). In some cases, 0.5 μg/ml ionomycin (Sigma-Aldrich) was used to stimulate cells.

Results

CD3ζ undergoes a higher degree of proteolysis in SLE than normal T cells

We first determined whether CD3ζ underwent more extensive proteolysis in SLE T cells compared with normal T cells. We treated normal and SLE T cells with 10 μg/ml CHX and explored whether blocking protein translation would result in greater decreases in CD3ζ expression in SLE T cells. Western blot analysis of lysates derived from cells rested for 3 and 6 h after CHX treatment revealed that in SLE T cells, a profound reduction in CD3ζ expression occurred at both 3 and 6 h of culture (Fig. 1*A*, *top panel*). However, a concomitant reduction in the expression of CD3ζ levels was not observed in normal T cells even up to 6 h of culture (Fig. 1*A*). Because T cells from a minority of SLE patients demonstrate normal amounts of CD3ζ expression, it was important to assess whether the observed decrease in the expression of CD3ζ after treatment with CHX could be found in this subset of patients as well. As shown in Fig. 1*A*, *bottom panel*, SLE T cells containing normal amounts of CD3ζ also demonstrated higher degrees of CD3ζ proteolysis than normal T cells, suggesting that basal levels of CD3ζ do not affect the increased rate of CD3ζ proteolysis in SLE T cells. T cells from patients with Sjogren's syndrome, however, did not display enhanced kinetics of CD3ζ degradation after treatment with CHX (Fig. 1*B*). However, the levels of TCR-β or β-actin expression did not vary significantly among normal, SLE, and Sjogren's T cells (Fig. 1), suggesting that heightened proteolytic digestion of CD3ζ is a unique feature of SLE T cells.

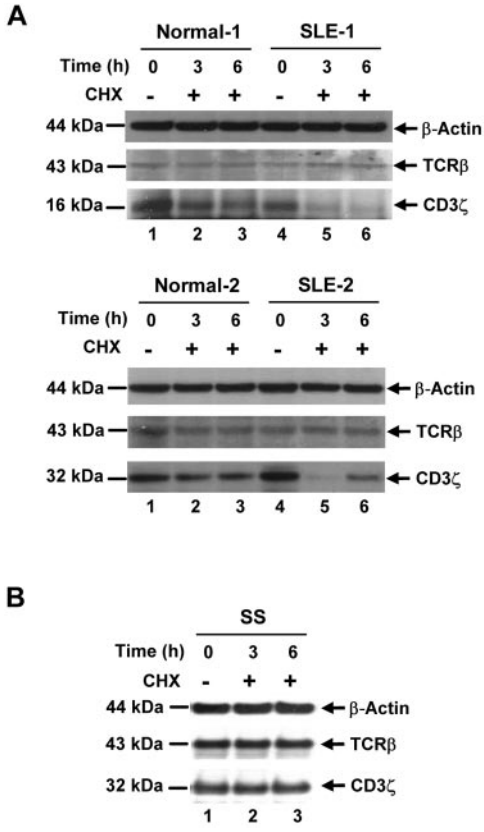

FIGURE 1. CD3ζ undergoes excessive proteolysis in SLE T cells. Normal and SLE T cells (*A*) and Sjogren T cells (*B*) 5×10^6/sample were treated with 10 μg/ml CHX for 10 min at 37°C, washed, and cultured in plain RPMI 1640 medium supplemented with 10% FBS at 37°C for 0, 3, or 6 h as indicated. Whole cell lysates from each group were loaded (10 μg/lane) and resolved on 4–12% bis-Tris NuPage gels under reducing (*A*, *top panel*, and *B*) or nonreducing (*A*, *bottom panel*) conditions, blotted onto polyvinylidene difluoride membranes, and probed with anti-actin (1/1000), anti-CD3ζ (1/1000), and anti-TCR-β (1/1000). SLE, $n = 5$; normal, $n = 5$; Sjogren's, $n = 2$. SS, Sjogren's T cells.

Caspase-3 activity is increased in SLE T cells

We hypothesized a possible contribution of caspase-3 in mediating cleavage of CD3ζ in SLE T cells. Accordingly, we first compared the basal protein levels and activity of caspase-3 between normal and SLE T cells. We observed that SLE T cells expressed 1.69-fold higher (normal, $n = 17$; SLE, $n = 17$; $p = 0.0467$) amounts of caspase-3 protein than normal T cells (Fig. 2*A*). The levels of caspase-3 activity were also significantly higher in SLE than in normal T cells (2.87-fold higher; normal, $n = 17$; SLE, $n = 17$; $p = 0.0107$; Fig. 2*B*). We also observed that increased caspase-3 activity levels in SLE T cells did not significantly correlate with SLE disease activity ($r = -0.52$; $p = 0.101$) or treatment status. Sjogren's T cells, used as a disease control in these experiments,

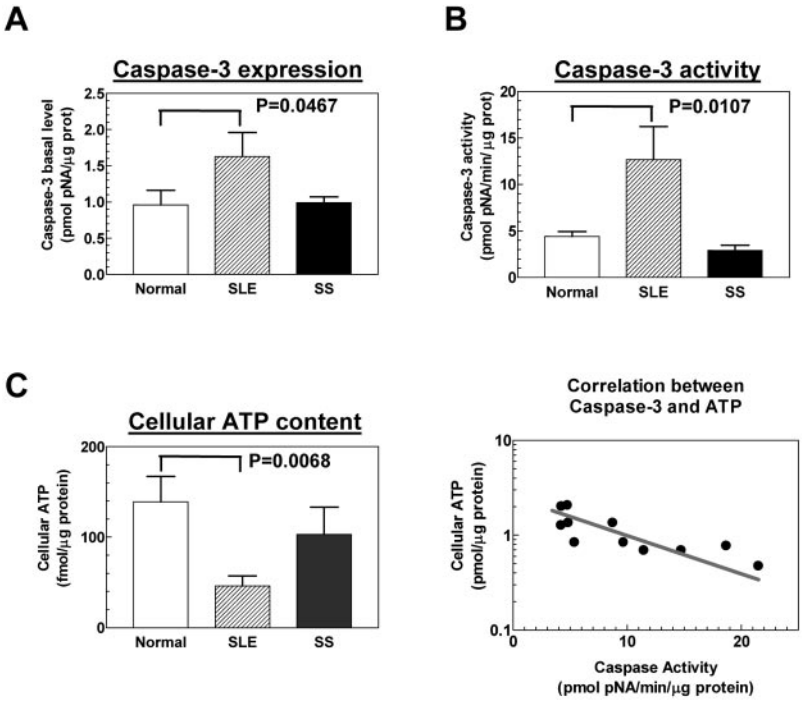

FIGURE 2. SLE T cells demonstrate increased caspase-3 expression and activity compared with normal T cells. Cells (5×10^6) in PBS buffer containing 1 mM DTT, 1 mM PMSF, 5 mM EDTA, 1 mM $NaOV_4$, 1 mM sodium fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin were sonicated, and the lysates were assayed for cellular caspase-3 expression (*A*) and activity (*B*) using a commercial assay kit. The data are presented in picomoles of pNA per minute per microgram of protein and represent values from 17 normal, 17 SLE, and four Sjogren's patient samples. *C*, Intracellular ATP content was estimated in both normal and SLE T cells using the ATP Bioluminescence Assay Kit HS II. Luminescence was measured with a TD-20/20 luminometer. Data were normalized to total protein, and the cellular ATP level was expressed as picomoles per microgram of protein. A correlation curve of caspase-3 activity vs intracellular ATP content is shown in the *right panel*. SS, Sjogren's T cells.

demonstrated caspase-3 levels and activity similar to those observed in normal T cells (Fig. 2, *A* and *B*), suggesting that the increased expression and activity of caspase-3 may not be common to other rheumatic autoimmune diseases.

It has been reported previously that proteolytic fragments derived from caspase-3-mediated cleavage are cleared up by the proteasome complex, a process that consumes intracellular ATP (26). Thus, if caspase-3 is actively involved in cleavage of proteins in SLE, intracellular ATP stores would be expected to be decreased in SLE T cells. Indeed, PBL from SLE patients have been previously shown to contain lower amounts of ATP than those from normal individuals (27). In this study we specifically tested whether T cells from SLE patients would display lower amounts of ATP content than normal T cells. We observed a statistically significant reduction in the intracellular ATP content in SLE T cells compared with normal cells (Fig. 2*C*, *left panel*; normal, $n = 15$; SLE, $n = 16$; $p = 0.0068$). This decrease in intracellular ATP also correlated with high levels of caspase-3 activity ($p = 0.0105$; $r = -0.73$; Fig. 2*C*, *right panel*). Predictably and consistent with their normal amounts of expression and activity of caspase-3, Sjogren's T cells demonstrated intracellular ATP stores comparable to those of normal T cells (Fig. 2*C*). Taken together, these data demonstrate that caspase-3 activity is increased in SLE T cells and suggest that caspase-3-mediated proteolysis of intracellular proteins could be enhanced in SLE T cells.

Role of caspase-3 in cleaving CD3ζ in SLE T cells

Next, we explored the role of caspase-3 in mediating CD3ζ cleavage by inhibiting caspase-3 activity in SLE T cells. To inhibit caspase-3 activity, we cultured cells in the presence of various concentrations (25–150 μM) of the caspase-3 inhibitor DEVD for 8 h and observed profound inhibition of caspase-3 activity with 50 μM and higher concentrations of DEVD, consistent with previous reports (Fig. 3*A*) (16), without inducing alterations in the basal expression of caspase-3 (data not shown). Using a trypan blue exclusion method, we determined that concentrations >100 μM were toxic to T cells (data not shown). Thus, we chose a nontoxic concentration of 50 μM DEVD for additional experiments. We first tracked CD3ζ expression in SLE T cells pretreated with CHX and cultured for 4 h in the presence or the absence of DEVD. We observed that DEVD, but not the DMSO vehicle, prevented extensive proteolysis of CD3ζ in SLE T cells (Fig. 3B), thus demonstrating a role for caspase-3 in cleaving CD3ζ in SLE. As expected, treatment of either SLE or normal T cells with DEVD resulted in no significant alterations to the expression of TCR-β or β-actin. Flow cytometric analysis of cells treated with either DEVD or DMSO revealed that although inhibition of caspase-3 activity resulted in minimal alterations in the expression of CD3ζ in normal T cells, SLE T cells consistently demonstrated a 37.7% increase ($p = 0.005$) in CD3ζ protein levels (Fig. 3*C*). These findings were observed in SLE patients with either reduced or normal basal amounts of CD3ζ (Fig. 3*C*; SLE-1 and SLE-2, respectively) and were independent of the disease activity and treatment status of the patients. Sjogren's T cells did not demonstrate appreciable differences in the expression of CD3ζ after treatment with DMSO or DEVD (Fig. 3*C*). We also noted that the DEVD-mediated increase in protein expression was limited to the CD3 ζ-chain in SLE T cells, because the expression of CD3ε and TCR-β remained unaltered in normal, SLE, and Sjogren's T cells after DEVD treatment (Fig. 3*D* and data not shown). Moreover, real-time quantitative PCR analysis of CD3ζ mRNA expression revealed no differences in mRNA production between SLE and normal T cells after DEVD treatment (data not shown), thus demonstrating that the DEVD treatment-mediated increase in CD3ζ expression is a post-transcriptional event. This finding is consistent with our claim that reversal of caspase-3-mediated proteolysis is responsible for replenishing CD3ζ in DEVD-treated SLE T cells.

Inhibition of caspase-3 activity alters lipid raft dynamics in SLE T cells

CD3ζ has been shown to associate with lipid rafts in both normal and SLE T cells (22, 28, 29). In this study we asked whether the DEVD-mediated increase in CD3ζ protein is reflected in the lipid raft compartment of SLE T cells. Western blot analysis of CD3ζ revealed that treatment with DEVD exclusively enhanced the amount of CD3ζ associated with lipid rafts in SLE T cells (Fig.

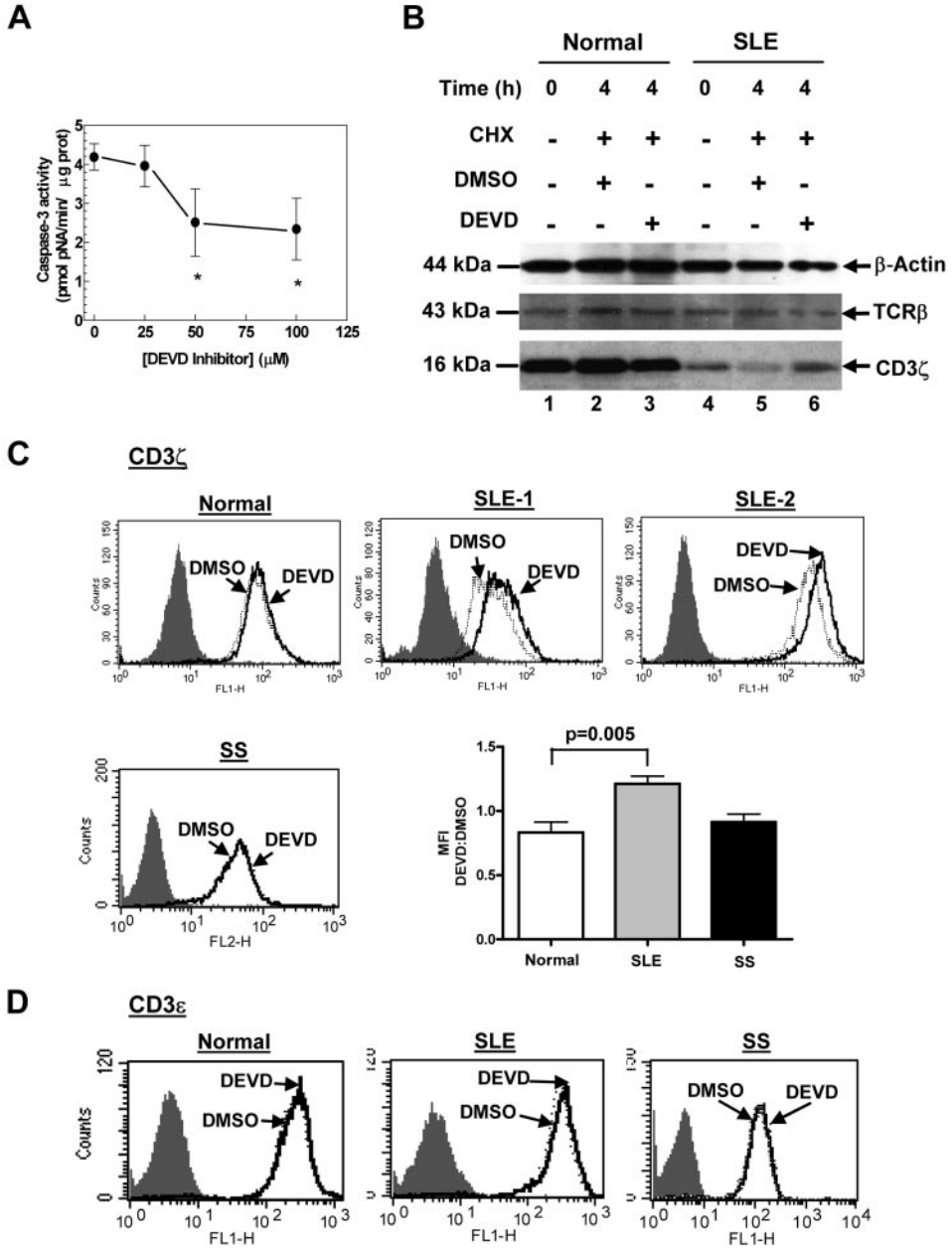

FIGURE 3. Involvement of caspase-3 in cleaving CD3ζ in SLE T cells. *A*, SLE T cells were cultured for 8 h at 37°C in the presence of various concentrations of DEVD as indicated. Cells were sonicated, and the lysates were assayed for cellular caspase-3 activity. ∗, $p < 0.05$. *B*, Cells were pretreated with 10 μg/ml CHX for 10 min at 37°C, washed, and subsequently treated with either 50 μM DEVD or equal volumes of DMSO for the indicated time periods at 37°C. Cells were lysed, 10 μg of protein/lane was loaded and run on a 4–12% bis-Tris NuPage gel under reducing conditions, and the blot was probed with anti-actin, anti-CD3ζ, and anti-TCR-β. *C*, Normal, SLE, or Sjogren T cells were cultured in RPMI 1640 medium (supplemented with 10% FBS) with DEVD or DMSO for 8 h. Cells were harvested, fixed with 0.75% paraformaldehyde, and stained after permeabilization with 0.05% (w/v) saponin, anti-CD3ζ (1/1000), or isotype control mouse IgG, followed by FITC-conjugated anti-mouse IgG, and staining was detected by FACS. A representative experiment is shown. Cumulative data are shown in the *right panel* (normal, $n = 5$; SLE, $n = 14$; Sjogren's, $n = 3$). Values on the *y*-axis represent ratios of the MFI of CD3ζ staining of DMSO- and DEVD-treated cells within each group. *D*, Normal, SLE, or Sjogren T cells treated with DMSO or DEVD were stained for surface expression of CD3ϵ using anti-CD3ϵ-FITC (clone UCHTI; $n = 3$ for each group). SS, Sjogren's T cells.

4*A*). We had previously reported that a majority of SLE T cells (>50%) demonstrate preclustering of membrane rafts, as detected by visualization of CT-B binding to the lipid raft marker, GM1 (22, 30). Moreover, replenishment of CD3ζ into SLE T cells induced dissolution of these preformed lipid raft clusters (25). In this study we explored whether restoration of CD3ζ in SLE T cells by DEVD would likewise alter the lipid raft distribution pattern. We observed that although treatment of SLE T cells with DMSO did not affect the preclustered morphology of lipid raft distribution, treatment with DEVD dramatically decreased the number of cells (16–28%) that displayed a preclustered raft phenotype, unlike normal T cells, which consistently maintained a homogenous membrane raft distribution (Fig. 4*B*, *top* and *bottom panels*). The pattern of lipid raft distribution in Sjogren's T cells remained unaltered after treatment with either DMSO or DEVD and appeared similar to that observed in normal T cells (Fig. 4*B*, *top* and *bottom panels*). Taken together, these results demonstrate that DEVD treatment of SLE T cells enhances the CD3ζ content of lipid rafts and raise the possibility of resultant alterations in lipid raft-mediated signaling.

Inhibition of caspase-3 activity reverses TCR-induced calcium responses

TCR-mediated calcium responses are heightened in SLE T cells (1). Because lipid rafts actively serve as platforms for orchestrating TCR-induced intracellular calcium responses (28, 31), we analyzed the effect of DEVD-mediated replenishment of CD3ζ on the TCR-mediated calcium response in SLE T cells. We observed that previous treatment of cells with DEVD resulted in a statistically significant ($p = 0.027$) decrease in the anti-CD3-induced calcium responses in SLE, but not normal T cells (Fig. 5*A*). However, previous treatment of SLE T cells with DEVD failed to lower calcium responses elicited by the TCR-bypassing reagent, ionomycin (Fig. 5*B*), suggesting that the observed differences in anti-CD3-mediated calcium responses after treatment of SLE T cells with DEVD are directed through the TCR. These observations

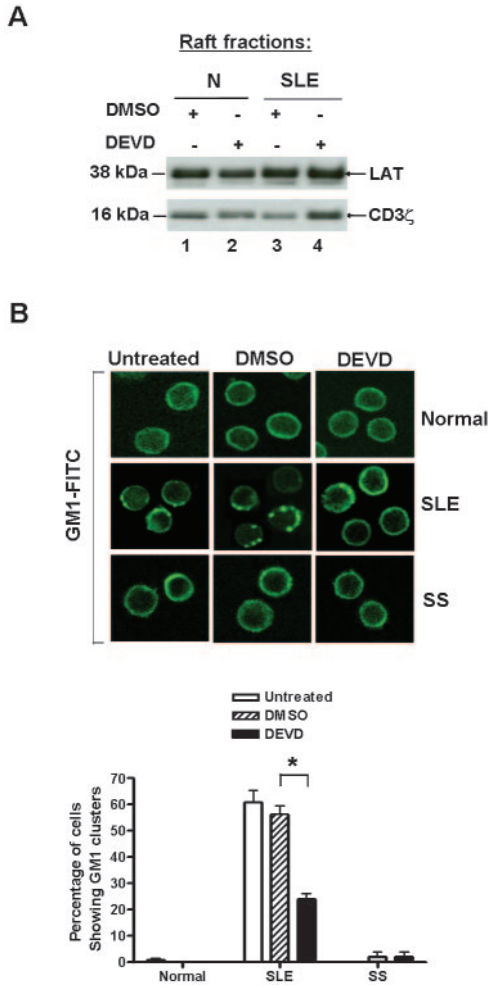

FIGURE 4. Inhibition of caspase-3 activity alters lipid raft dynamics in SLE T cells. *A*, Normal and SLE T cells (10×10^6) were lysed in a lysis buffer containing 1% Brij 58 after culture for 8 h at 37°C with 50 μM DEVD or equal volumes of DMSO, and raft fractions were extracted by discontinuous sucrose density gradient ultracentrifugation as described previously (22). Proteins of each fraction was concentrated by standard TCA precipitation, resolved on 4–12% bis-Tris NuPage under reducing conditions, and transferred to a polyvinylidene difluoride membrane. The blot was probed with anti-linker for activation of T cells (LAT; 1/1000; Santa Cruz Biotechnology) and anti-CD3ζ (1/1000) Abs. *B*, Normal, SLE, or Sjogren's T cells were left untreated or were treated with either DMSO or DEVD and subsequently adhered to polylysine-coated slides. Cells were fixed and stained with choleratoxin-B-FITC (green) and analyzed by a laser scanning confocal microscope. Twenty-five cells were counted per field, and the percentage of positive cells was calculated. A representative field is shown for each sample. Magnification, $\times$100 (oil objective). The *bottom panel* represents statistical analysis of the results from two to five experiments. SS, Sjogren's T cells. *, $P < 0.05$.

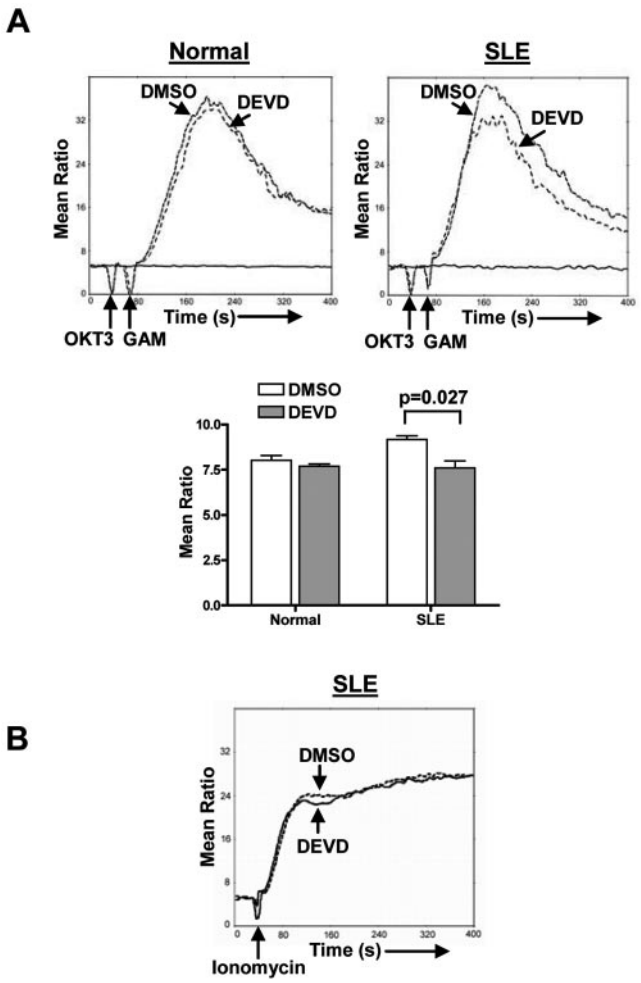

FIGURE 5. Inhibition of caspase-3 activity reverses heightened TCR-induced calcium responses in SLE T cells. *A*, $[Ca^{2+}]_i$ measurement was performed in normal and SLE T cells pretreated with DMSO or DEVD for 8 h and subsequently loaded with indo AM. Cells were stimulated with OKT3 (1/100) and goat anti-mouse IgG at 30 and 60 s, respectively, and $[Ca^{2+}]_i$ was measured for 400 s with and EPICS Altra flow cytometer. A representative of five separate experiments is shown. Statistical analysis is presented in the *bottom panel*. *B*, $[Ca^{2+}]_i$ measurement was also performed in SLE T cells pretreated with DMSO or DEVD after treatment with 0.5 μg/ml ionomycin at 30 s. The mean ratio is the mean fluorescence ratio corresponding to $[Ca^{2+}]_i$.

suggest that restoration of CD3ζ expression by DEVD dampens the abnormally heightened TCR-mediated calcium responses in SLE T cells.

Previously, we had reported that reintroduction of CD3ζ-chain into SLE T cells results in down-regulation of FcRγ-chain expression and thus offered a possible explanation for the reduced TCR-induced calcium responses in CD3ζ-transfected cells (25). In this study we explored whether the DEVD-mediated increase in the expression of CD3ζ resulted in concomitant down-regulation of FcRγ, by treating normal and SLE T cells with either DMSO or DEVD. We observed that treatment with DMSO did not result in discernable alterations in the expression of CD3ζ or FcRγ, whereas treatment with DEVD resulted in an increase in the expression of CD3ζ while simultaneously decreasing the expression of FcRγ chain specifically in SLE T cells (Fig. 6). These observations are consistent with our previous findings after transfection of CD3ζ into SLE T cells and offer a possible mechanism for the observed decrease in TCR-induced calcium signaling after treatment with DEVD (25).

Additionally, we observed that treatment of SLE T cells with the pan-caspase inhibitor VAD induced higher amounts of CD3ζ expression and caused a greater decrease in the expression of FcRγ compared with DEVD (Fig. 6). These findings raise the possibility that in addition to caspase-3, other caspases (for example, caspase-7) might also be involved in cleaving CD3ζ in SLE T cells (16).

Discussion

In this study we demonstrate that SLE T cells display high levels of basal caspase-3 activity compared with normal T cells, and by

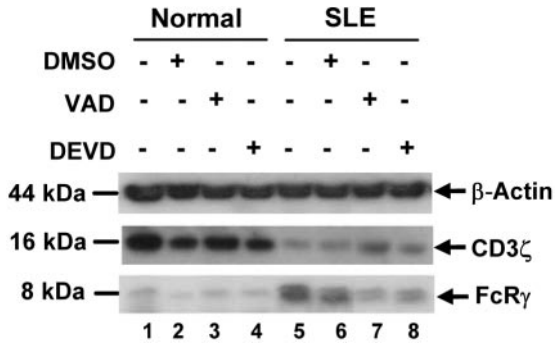

FIGURE 6. Inhibition of caspase-3 activity reciprocally regulates the expression of CD3ζ and FcRγ in SLE T cells. Normal and SLE T cells (5×10^6/sample) were treated with 50 μM DEVD, 50 μM VAD, or equal volumes of DMSO for 8 h at 37°C; washed; and lysed. Whole cell lysates from each group were loaded (5 μg/lane) and resolved on 4–12% bis-Tris NuPage gels under reducing conditions, blotted onto polyvinylidene difluoride membranes, and probed with anti-actin (1/1000), anti-CD3ζ (1/1000), and anti-FcRγ (1/1000; Upstate Biotechnology).

reversing this heightened caspase-3 activity, we demonstrate significant restoration of the amounts of CD3ζ expression in SLE T cells. In addition, caspase-3 inhibitor-induced restoration of CD3ζ corrected the abnormal TCR-mediated calcium signaling in SLE T cells. These findings were observed in SLE T cells, but not control T cells. Thus, we conclude that caspase-3-mediated cleavage of CD3ζ is an important mechanism contributing to the diminished expression of CD3ζ in SLE T cells.

Reduced levels of CD3ζ have been observed in T cells from a wide array of chronic diseases, such as chronic infections, autoimmune diseases (including SLE), and cancer (reviewed in Ref. 6). In SLE T cells, diminished CD3ζ stems from abnormalities at multiple levels, such as transcription, translation, and protein expression (1–3). Restoration of CD3 ζ-chain levels in SLE T cells by gene transfer results in normalization of the CD3-mediated signaling responses and increased production of IL-2 (25), testifying to the importance of CD3ζ in coordinating TCR signaling. Therefore, complete understanding of the mechanisms responsible for the decreased expression of the CD3 ζ-chain in SLE T cells may offer possibilities for therapeutic interventions, more practical than gene transfer, aimed at the correction of levels of the CD3 ζ-chain and restoration of normal T cell function in SLE.

Our observations that 1) proteolysis of CD3ζ occurred more dramatically in SLE; 2) treatment with DEVD protected significant amounts of CD3ζ from cleavage; and 3) DEVD treatment restored CD3ζ levels in SLE all strongly suggest a role for caspase-3 in cleaving CD3ζ in SLE. Our observation that DEVD failed to alter CD3ζ mRNA expression (data not shown) shows that caspase-3 does not enhance transcription of the CD3 ζ-chain and is consistent with its expected effect at the protein level. Another notable feature is that the caspase-3-mediated proteolysis described in our study appears to be specific to CD3ζ, because it spared other members of the TCR/CD3 complex, such as CD3ϵ and TCR chains (Fig. 3*D* and data not shown). This finding is consistent with previous observations that the expressions of CD3ϵ and TCR remain unaltered in SLE T cells (1, 5).

What is the significance of DEVD-mediated increased association of CD3ζ with lipid rafts in SLE T cells? In normal T cells, some amount of CD3ζ is constitutively associated with lipid rafts, although in SLE T cells, there is a marked reduction in this fraction of CD3ζ (22, 28, 29). This finding might have far-reaching consequences on downstream TCR-mediated signaling. Although proximal signaling appears to be accelerated, IL-2 production is hampered in SLE T cells, demonstrating the ineffectiveness of TCR signaling in eliciting a meaningful functional response (32). We had shown previously that although F-actin polymerization is accelerated in SLE T cells, it is not optimally sustained and undergoes rapid depolymerization compared with normal T cells (22). Rapid dephosphorylation of Vav was postulated to be a factor contributing to this phenomenon (22). Previous studies have shown that actin polymerization events leading to sustained downstream signaling are mediated at least in part by phosphorylation of Vav associated with CD3ζ (33). Thus, restoration of optimum amounts of CD3ζ by caspase-3 inhibitors can be conceived to normalize at least in part the abnormal kinetics of TCR-induced F-actin polymerization and thereby restore sustained downstream signaling in SLE T cells. Additional studies are necessary to evaluate this possibility.

Another point of note is the reversal of preclustering of lipid rafts and lowering of abnormally heightened calcium signaling in SLE T cells, followed by DEVD-induced CD3ζ replenishment. These findings mimic our previous observations that transfection of CD3ζ into SLE T cells reversed lipid raft preclustering and also reduced TCR-induced calcium fluxes (25) and emphasize the importance of optimum CD3ζ expression in T cells for maintaining normal TCR-mediated responses.

It is not clear what triggers higher basal activity of caspase-3 in SLE T cells. Whether it is activated as a result of increased susceptibility of SLE T cells to the apoptotic process (20) or is specifically turned on to regulate the expression of multiple intracellular proteins are possibilities that need to be addressed. In addition to caspase-3, caspase-7 has been implicated in cleavage of CD3 ζ-chain (16). Whether caspase-7 is also involved in CD3ζ proteolysis in SLE remains unclear. However, our observation that treatment of SLE T cells with the pan-caspase inhibitor, VAD, induces greater amounts of CD3ζ expression compared with the caspase-3 inhibitor, DEVD, suggests that factors in addition to caspase-3 might play a role in cleaving CD3ζ, with caspase-7 being one of them. Another important question that remains unanswered is why up-regulation of FcRγ occurs in SLE T cells. Our previous studies have shown that reintroduction of CD3ζ induced down-regulation of FcRγ (25). Our present finding that up-regulation of CD3ζ by inhibition of caspase-3 (and possibly other caspases) resulted in simultaneous down-regulation of FcRγ mirrors our previous observations. In other T cell systems, such as in effector CD4 T cells and tumor infiltration lymphocytes, up-regulation of FcRγ is associated with down-regulation of CD3ζ expression (34, 35). On the basis of these observations, we propose that the regulation of expression of CD3ζ and FcRγ in T cells is reciprocally linked.

We thus show that along with decreased gene transcription, defective mRNA splicing, and lysosomal degradation, caspase-3-mediated proteolysis of CD3ζ protein represents an additional distinct mechanism controlling diminished expression of CD3ζ in SLE T cells. The extent to which each of the above mechanisms contributes to decreased CD3ζ expression in each SLE patient is presumably variable. To summarize, we have provided the first evidence of caspase-3-mediated cleavage as a mechanism exploited by the disease process in mediating pathological degradation of CD3ζ in SLE T cells. Because replenishment of CD3ζ levels in SLE T cells results in restoration of the defective IL-2 production (25), our data provide sufficient rationale for the use of caspase inhibitors in the treatment of systemic autoimmune disease (36).

Acknowledgments

We gratefully acknowledge Dr. Michael Zidanic (Department of Medical Audio Visual Services, Walter Reed Army Institute of Research, Silver Spring, MD) and Douglas Smoot (Walter Reed Army Institute of

Research) for help with confocal microscopy and calcium analyses, respectively; Drs. Ashima Saxena and Ramachandra Naik (Department of Biochemistry, Walter Reed Army Institute of Research) for help with sucrose gradient experiments; Dr. Arthur Weinstein (Section of Rheumatology, Washington Hospital Center, Washington, DC) and his team for recruiting SLE patients for the study; and Dr. Donna Farber (Department of Surgery, University of Maryland, Baltimore, MD) for critically reviewing this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Liossis, S. N., X. Z. Ding, G. J. Dennis, and G. C. Tsokos. 1998. 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 ζ chain. *J. Clin. Invest.* 101: 1448–1457.
2. Brundula, V., L. J. Rivas, A. M. Blasini, M. Paris, S. Salazar, I. L. Stekman, and M. A. Rodriguez. 1999. Diminished levels of T cell receptor ζ chains in peripheral blood T lymphocytes from patients with systemic lupus erythematosus. *Arthritis Rheum.* 42: 1908–1916.
3. Pang, M., Y. Setoyama, K. Tsuzaka, K. Yoshimoto, K. Amano, T. Abe, and T. Takeuchi. 2002. Defective expression and tyrosine phosphorylation of the T cell receptor ζ chain in peripheral blood T cells from systemic lupus erythematosus patients. *Clin. Exp. Immunol.* 129: 160–168.
4. Vratsanos, G. S., S. Jung, Y. M. Park, and J. Craft. 2001. $CD4^+$ T cells from lupus-prone mice are hyperresponsive to T cell receptor engagement with low and high affinity peptide antigens: a model to explain spontaneous T cell activation in lupus. *J. Exp. Med.* 193: 329–337.
5. Enyedy, E. J., M. P. Nambiar, S. N. Liossis, G. Dennis, G. M. Kammer, and G. C. Tsokos. 2001. Fcϵ receptor type I chain replaces the deficient T cell receptor ζ chain in T cells of patients with systemic lupus erythematosus. *Arthritis Rheum.* 44: 1114–1121.
6. Krishnan, S., D. L. Farber, and G. C. Tsokos. 2003. T cell rewiring in differentiation and disease. *J. Immunol.* 171: 3325–3331.
7. Juang, Y. T., K. Tenbrock, M. P. Nambiar, M. F. Gourley, and G. C. Tsokos. 2002. Defective production of functional 98-kDa form of Elf-1 is responsible for the decreased expression of TCR ζ-chain in patients with systemic lupus erythematosus. *J. Immunol.* 169: 6048–6055.
8. Tsuzaka, K., I. Fukuhara, Y. Setoyama, K. Yoshimoto, K. Suzuki, T. Abe, and T. Takeuchi. 2003. TCRζ mRNA with an alternatively spliced 3′-untranslated region detected in systemic lupus erythematosus patients leads to the down-regulation of TCRζ and TCR/CD3 complex. *J. Immunol.* 171: 2496–2503.
9. Alam, A., L. Y. Cohen, S. Aouad, and R. P. Sekaly. 1999. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J. Exp. Med.* 190: 1879–1890.
10. Kennedy, N. J., T. Kataoka, J. Tschopp, and R. C. Budd. 1999. Caspase activation is required for T cell proliferation. *J. Exp. Med.* 190: 1891–1896.
11. Chun, H. J., L. Zheng, M. Ahmad, J. Wang, C. K. Speirs, R. M. Siegel, J. K. Dale, J. Puck, J. Davis, C. G. Hall, et al. 2002. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature* 419: 395–399.
12. Los, M., C. Stroh, R. U. Janicke, I. H. Engels, and K. Schulze-Osthoff. 2001. Caspases: more than just killers? *Trends Immunol.* 22: 31–34.
13. Fischer, U., R. U. Janicke, and K. Schulze-Osthoff. 2003. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ.* 10: 76–100.
14. Zhou, B. B., H. Li, J. Yuan, and M. W. Kirschner. 1998. Caspase-dependent activation of cyclin-dependent kinases during Fas-induced apoptosis in Jurkat cells. *Proc. Natl. Acad. Sci. USA* 95: 6785–6790.
15. Schwerk, C., and K. Schulze-Osthoff. 2003. Non-apoptotic functions of caspases in cellular proliferation and differentiation. *Biochem. Pharmacol.* 66: 1453–1458.
16. Gastman, B. R., D. E. Johnson, T. L. Whiteside, and H. Rabinowich. 1999. Caspase-mediated degradation of T-cell receptor ζ-chain. *Cancer Res.* 59: 1422–1427.
17. Menne, C., J. P. Lauritsen, J. Dietrich, J. Kastrup, A. K. Wegener, P. S. Andersen, N. Odum, and C. Geisler. 2001. T-cell receptor downregulation by ceramide-induced caspase activation and cleavage of the ζ chain. *Scand. J. Immunol.* 53: 176–183.
18. Takahashi, A., K. Kono, H. Amemiya, H. Iizuka, H. Fujii, and Y. Matsumoto. 2001. Elevated caspase-3 activity in peripheral blood T cells coexists with increased degree of T-cell apoptosis and down-regulation of TCRζ molecules in patients with gastric cancer. *Clin. Cancer Res.* 7: 74–80.
19. Baniyash, M. 2004. TCR ζ-chain downregulation: curtailing an excessive inflammatory immune response. *Nat. Rev. Immunol.* 4: 675–687.
20. Emlen, W., J. Niebur, and R. Kadera. 1994. Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. *J. Immunol.* 152: 3685–3692.
21. Bombardier, C., D. D. Gladman, M. B. Urowitz, D. Caron, and C. H. Chang. 1992. Derivation of the SLEDAI: a disease activity index for lupus patients: the Committee on Prognosis Studies in SLE. *Arthritis Rheum.* 35: 630–640.
22. Krishnan, S., M. P. Nambiar, V. G. Warke, C. U. Fisher, J. Mitchell, N. Delaney, and G. C. Tsokos. 2004. Alterations in lipid raft composition and dynamics contribute to abnormal T cell responses in systemic lupus erythematosus. *J. Immunol.* 172: 7821–7831.
23. Oki, S., A. Chiba, T. Yamamura, and S. Miyake. 2004. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J. Clin. Invest.* 113: 1631–1640.
24. Dupre, L., A. Aiuti, S. Trifari, S. Martino, P. Saracco, C. Bordignon, and M. G. Roncarolo. 2002. Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. *Immunity* 17: 157–166.
25. Nambiar, M. P., C. U. Fisher, V. G. Warke, S. Krishnan, J. P. Mitchell, N. Delaney, and G. C. Tsokos. 2003. Reconstitution of deficient T cell receptor ζ chain restores T cell signaling and augments T cell receptor/CD3-induced interleukin-2 production in patients with systemic lupus erythematosus. *Arthritis Rheum.* 48: 1948–1955.
26. Du, J., X. Wang, C. Miereles, J. L. Bailey, R. Debigare, B. Zheng, S. R. Price, and W. E. Mitch. 2004. Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J. Clin. Invest.* 113: 115–123.
27. Gergely, P., Jr., C. Grossman, B. Niland, F. Puskas, H. Neupane, F. Allam, K. Banki, P. E. Phillips, and A. Perl. 2002. Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus. *Arthritis Rheum.* 46: 175–190.
28. Xavier, R., T. Brennan, Q. Li, C. McCormack, and B. Seed. 1998. Membrane compartmentation is required for efficient T cell activation. *Immunity* 8: 723–732.
29. Moran, M., and M. C. Miceli. 1998. Engagement of GPI-linked CD48 contributes to TCR signals and cytoskeletal reorganization: a role for lipid rafts in T cell activation. *Immunity* 9: 787–796.
30. Nambiar, M. P., E. J. Enyedy, C. U. Fisher, S. Krishnan, V. G. Warke, W. R. Gilliland, R. J. Oglesby, and G. C. Tsokos. 2002. Abnormal expression of various molecular forms and distribution of T cell receptor ζ chain in patients with systemic lupus erythematosus. *Arthritis Rheum.* 46: 163–174.
31. Janes, P. W., S. C. Ley, and A. I. Magee. 1999. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J. Cell Biol.* 147: 447–461.
32. Solomou, E. E., Y. T. Juang, M. F. Gourley, G. M. Kammer, and G. C. Tsokos. 2001. Molecular basis of deficient IL-2 production in T cells from patients with systemic lupus erythematosus. *J. Immunol.* 166: 4216–4222.
33. Valensin, S., S. R. Paccani, C. Ulivieri, D. Mercati, S. Pacini, L. Patrussi, T. Hirst, P. Lupetti, and C. T. Baldari. 2002. F-actin dynamics control segregation of the TCR signaling cascade to clustered lipid rafts. *Eur. J. Immunol.* 32: 435–446.
34. Krishnan, S., V. G. Warke, M. P. Nambiar, G. C. Tsokos, and D. L. Farber. 2003. The FcR subunit and Syk kinase replace the CD3ζ-chain and ZAP-70 kinase in the TCR signaling complex of human effector CD4 T cells. *J. Immunol.* 170: 4189–4195.
35. Mizoguchi, H., J. J. O'Shea, D. L. Longo, C. M. Loeffler, D. W. McVicar, and A. C. Ochoa. 1992. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* 258: 1795–1798.
36. Seery, J. P., V. Cattell, and F. M. Watt. 2001. Cutting edge: amelioration of kidney disease in a transgenic mouse model of lupus nephritis by administration of the caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-(β-*o*-methyl)-fluoromethylketone. *J. Immunol.* 167: 2452–2455.