Journal of Cellular Biochemistry 85:459–469 (2002)

FAST TRACK

Effect of Trichostatin A on Human T Cells Resembles Signaling Abnormalities in T Cells of Patients With Systemic Lupus Erythematosus: A New Mechanism for TCR ζ Chain Deficiency and Abnormal Signaling

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Abstract Trichostatin A (TSA) is a potent reversible inhibitor of histone deacetylase, and it has been reported to have variable effects on the expression of a number of genes. In this report, we show that TSA suppresses the expression of the T cell receptor ζ chain gene, whereas, it upregulates the expression if its homologous gene Fcε receptor I γ chain. These effects are associated with decreased intracytoplasmic-free calcium responses and altered tyrosine phosphorylation pattern of cytosolic proteins. Along with these effects, we report that TSA suppresses the expression of the interleukin-2 gene. The effects of TSA on human T cells are predominantly immunosuppressive and reminiscent of the signaling aberrations that have been described in patients with systemic lupus erythematosus. J. Cell. Biochem. 85: 459–469, 2002. Published 2002 Wiley-Liss, Inc.†

Key words: T lymphocytes; T cell signaling; chromatin remodeling; Trichostatin; systemic lupus erythematosus

The dynamic process of reversible histone acetylation that occurs at the ε-amino group of conserved lysine residues near the amino terminus of core histones, mediates changes in nucleosome conformation, and is important in the regulation of gene expression [Grunstein, 1997]. Alteration in the tertiary structure of DNA affects transcription by modulating the accessibility of transcription factors to their target DNA segments. Nucleosomal integrity is regulated by the acetylation status of core histones. In hypoacetylated state, nucleosomes remain tightly compact hindering the accessibility of the promoter to the transcription machinery and ultimately blocking transcription. On the other hand, nucleosomes are relaxed by acetylation of core histones that enhances the accessibility of the promoter to transcription factors and RNA polymerase, thereby, promoting transcriptional initiation [Pazin et al., 1998]. The acetylation status of histones is governed by the balance of activities of histone acetyl transferase (HAT) and histone deacetylase (HDAC). Accumulating evidence suggests that inhibition of HDAC results in the modulation of around 2% of cellular genes that include upregulation as well as repression of gene expression [Huang et al., 1997; Richon et al., 2000].

Recently, it has been shown that HDAC inhibitor trichostatin A (TSA) reverses skewed expression of CD154 (CD40L), interleukin-10, and interferon-γ gene and protein expression in lupus T cells, and is therefore being considered as a candidate drug for the treatment of this

Grant sponsor: NIH; Grant number: RO1 AI42269; Grant sponsor: STOC of the Medical Research and Material Command.

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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Received 27 December 2001; Accepted 30 January 2002

DOI 10.1002/jcb.10160

autoimmune disease [Mishra et al., 2001]. In addition to these genes, abnormal expression of several other genes and subsequent alterations in signaling has been well documented in lupus T cells. We have identified a decrease in the expression of TCR ζ chain mRNA and protein in a vast majority of racially diverse SLE patients [Liossis et al., 1998a; Nambiar et al., 2001c,d]. Defective expression of the ζ chain at both protein and mRNA levels has been reported by other laboratories [Takeuchi et al., 1998; Brundula et al., 1999]. Investigators from this and other laboratories also showed that cross-linking of the TCR/CD3 complex of SLE T cells leads to a disease-specific increase in $[Ca^{2+}]_i$ responses [Vassilopoulos et al., 1995] and tyrosine phosphorylation of cytosolic protein substrates [Blasini et al., 1998; Liossis et al., 1998a]. These findings prompted us to explore the possibility that TSA may modulate the expression of TCR ζ chain in human T lymphocytes. Moreover, the effect of TSA on the expression of TCR ζ chain and T cell signaling including antigen-receptor-mediated phosphorylation of cellular protein substrates or Ca^{2+} responses in human T cells, has not been pursued previously. Since, the signaling function of TCR complex is critically dependent on the expression of ζ chain, it is important to study the effect of TSA on TCR/CD3-mediated signaling in human T lymphocytes.

Although the majority of the genes that respond to HDAC inhibition increase their expression, in this communication, we show that TCR ζ chain expression is repressed following treatment with TSA. The loss of ζ chain expression is readily reversible following removal of the drug. In contrast, TSA enhanced the expression of a ζ chain family member, FcεRIγ chain. Consistent with the decrease in the expression of ζ chain, TSA inhibit TCR/CD3 mediated early signaling and IL-2 expression reflecting an immunosuppressive function on human T cells.

MATERIALS AND METHODS

Cells and Antibodies

Monocyte and macrophage depleted peripheral blood mononuclear cells (PBMCs) were obtained by elutriation of the leukopheresed blood samples of normal donors of 18–38 years age. T-lymphocytes were isolated from the PBMCs by negative depletion of non-T cells using a cocktail of hapten-conjugated Abs and magnetic separation (Miltenyi Biotec, Auburn, CA) as described previously [Nambiar et al., 2001a]. The purity of T-cells in the isolated population usually remained $>97\%$ by FACS analysis after CD3 ε staining. The TCR ζ chain mAb, 6B10.2, recognizing the amino acids 31–45 of the polypeptide (N-terminal mAb) anti-CD3 ε and ZAP70 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The C-terminal TCR ζ chain mAb recognizing the amino acids from 145 to 161 (C-terminal mAb) [Hall et al., 1993] was from BD Pharmingen (San Diego, CA). The anti-CD3 mAb (OKT3) was from Ortho Biotech, Inc., (Raritan, NJ). Horseradish peroxidase (HRP) conjugated anti-phosphotyrosine mAb, 4G10, and FcεRIγ Ab was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). TSA and other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

TSA Treatment

Cells were suspended at a concentration of 5×10^6 cells/5 ml in RPMI medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin in tissue culture flasks. Indicated concentrations of TSA were added and the cells were incubated at 37°C for various time intervals and harvested for the study. Control T lymphocytes were incubated with the vehicle DMSO at 37°C in the medium.

Immunoblotting of ζ Chain and Other Signaling Proteins

Five million control or TSA-treated T cells were lysed in 1% Nonidet P-40 (NP-40) (Sigma) lysis buffer containing multiple protease and phosphatase inhibitors as described previously [Liossis et al., 1998a]. The detergent-soluble protein (20 μg) was separated on a 4–12% Nu-PAGE gel (Invitrogen Novex, Carlsbad, CA), transferred to PVDF membranes (Millipore, Bedford, MA) and immunoblotted with ζ chain Ab and developed using the ECL kit from Amersham Pharmacia Biotech (Piscataway, NJ). Membranes were stripped in Immunopure (Pierce, Chicago, IL) solution and reprobed with control and other Abs of interest. The detergent-insoluble membrane pellet was solubilized by mechanical agitation in the presence of 4% SDS. The mixture was boiled for 20 min in sample buffer containing 4% SDS, electrophoretically

separated, and immunoblotted with various Abs as described above.

RT-PCR Analysis

RNA was isolated using the RNeasy mini kit (Qiagen, Santa Clarita, CA) and cDNA was synthesized using oligo-dT primer and AMV reverse transcriptase. The primers for PCR were synthesized by Sigma-Genosys (The Woodlands, TX) and are as follows: TCR ζ chain forward 5′-AGC CTC TGC CTC CCA GCC TCT TTC TGA G-3′ and reverse 5′-TCA GTG GCT GAG AAG AGT GAA CCG GGT TG-3′; CD3 ε chain, forward 5′-AAG TAA CAG TCC CAT GAA ACA AAG-3′ and reverse-5′ TTC TCC AGA GGG TCA GAT GCG TCT CTG A-3′; ZAP 70, forward 5′-GAC GTG GCC ATC AAG GTG CTG AAG CAG-3′ and reverse 5′-GCG CTG CTC CAC GGT CAG GAA GTC G-3′. Reverse transcription products were amplified using Taq polymerase and the PCR products (12 μl) were electrophoresed on agarose gels and stained with ethidium bromide as described previously [Nambiar et al., 2001d].

Flow Cytometry

The expression of ζ chain was examined in permeabilized and mildly fixed T cells using a FACScan flowcytometer (Becton-Dickinson, Mountain View, CA) as described previously [Enyedy et al., 2001].

T Cell Activation and Anti-Phosphotyrosine Immunoblotting

TSA-treated or untreated T cells were stimulated with 10 μg/ml OKT3 for 1 and 2 min at 37°C and the cells were pelleted and lysed in 1% NP-40 (Sigma) as described previously [Liossis et al., 1998a]. The lysate proteins (20 μg/lane) were analyzed in 12% SDS–PAGE gel, and immunoblotted with the anti-phosphotyrosine-HRP conjugated 4G10 mAb (1:1,500) and detected using ECL kit.

Measurement of Free Cytoplasmic Ca^{2+} Concentration

Free cytoplasmic Ca^{2+} concentrations were estimated in INDO-1 (Molecular Probes, Eugene, OR) (1 μg/ml) loaded cells as previously described [Nambiar et al., 2001b]. In each run, first the cells were run unstimulated to record the baseline fluorescence ratio, which represents the resting $[Ca^{2+}]_i$ levels. After 40 s, either antibody, OKT3 (10 μg/ml) or the isotype control, mIgG2a, was added to the tube and the ratio of the fluorescence, which is directly proportional to the free cytosolic Ca^{2+}, was recorded for a total of 10 min. The recorded signals were statistically analyzed using MultiTime (version 3, Phoenix Flow Systems, San Diego, CA).

T Cell Activation and IL-2 Assay

T cells were activated via TCR/CD3 complex with 10 μg/ml anti-CD3 mAb plus 2.5 μg/ml anti-CD28 mAb for 24 h. Supernatants were collected and IL-2 was measured by Quantikine ELISA kit (R&D Systems, Minneapolis, MN). RT-PCR analysis of IL-2 was done using the forward primer 5′-CAC TAC TCA CAT TAA CCT CAA CTC CTG-3′ and reverse primer 5′-GGG GAA GCA CTT AAT TAT CAA GTT AGT G-3′ as described above.

RESULTS

TSA Inhibits TCR ζ Chain Protein Expression in Human T Lymphocytes

In light of the observation that TSA reversed skewed expression of few genes in systemic lupus erythematosus (SLE) T cells [Mishra et al., 2001], we sought to study the effect of TSA on TCR ζ chain expression in normal human T lymphocytes. As shown in Figure 1A, the expression of ζ chain was significantly decreased in human T cells treated with 100 and 1,000 ng/ml TSA for 18 h compared to the untreated control, whereas, the expression of CD3 ε chain and the downstream signaling intermediate ZAP-70 was virtually unaffected by exposure to TSA (Fig. 1A). Densitometry and normalization of the data with β-actin showed that on an average, there was more than a 70% decrease in the level of expression of TCR ζ chain in 100 ng/ml and 75% decrease in 1,000 ng/ml of TSA-treated cells compared to control (Fig. 1B). Immunoblotting with the C-terminal ζ chain mAb that recognizes the phosphorylated and ubiquitinated forms of the ζ chain [Nambiar et al., 2002] showed that in TSA-treated samples, the level of the phosphorylated p21 and 23 kDa forms and the 38 kDa major ubiquitinated form of the ζ chain were also decreased in a dose-dependant manner indicating that the TSA-mediated decrease is neither the outcome of increased phosphorylation nor ubiquitin-mediated degradation of ζ chain in human T cells (Fig. 1A).

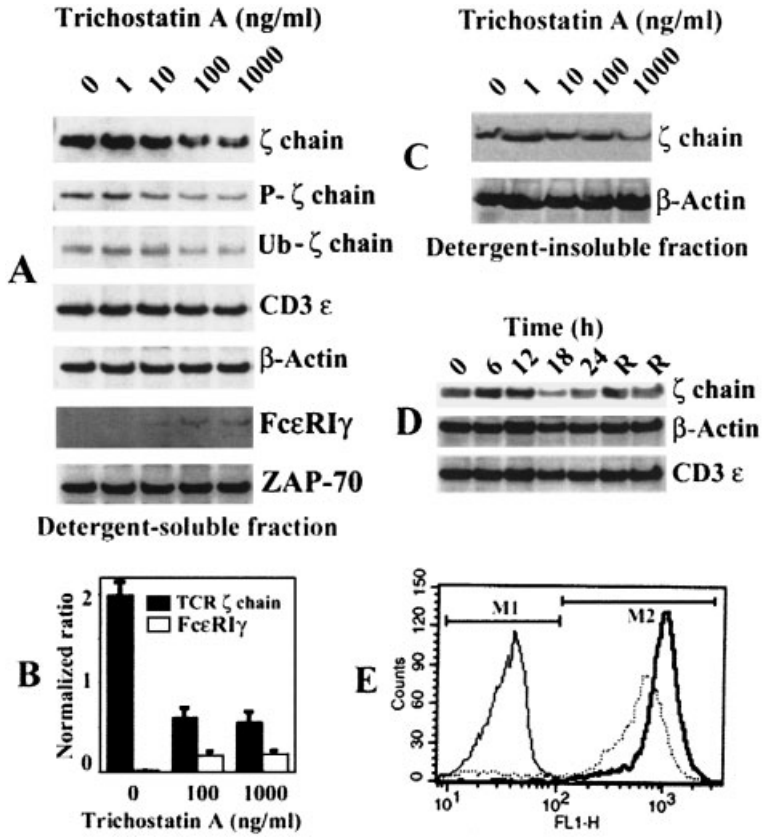

Fig. 1. TSA suppresses TCR ζ chain expression in human T lymphocytes. **A**: Purified T cells were treated with 1, 10, 100, and 1,000 ng/ml or without TSA, for 18 h at 37°C. Cells were lysed with NP-40 lysis buffer containing various protease inhibitors and 15 μg of protein from the detergent-soluble fractions was analyzed on 4–12% Nu-PAGE gel under reducing condition, transferred, and immunoblotted with ζ chain mAb 6B10.2, and developed using a chemiluminescence kit. The membrane was stripped and sequentially reprobed with ζ chain C-terminal mAb that recognizes the tyrosine phosphorylated and ubiquitinated forms of the ζ chain, CD3 ε, β-actin, FcεRIγ, and ZAP-70 Abs. The ubiquitinated form of TCR ζ chain was confirmed by immunoblotting with anti-ubiquitin antibody. **B**: Immunoblots were analyzed by densitometry and the levels of expression of ζ chain were normalized to β-actin and plotted against TSA (mean ± SEM (n = 3)). **C**: The detergent-insoluble membrane fraction was solubilized in 4% SDS by mechanical agitation and boiling for 20 min, electrophoretically separated, and immunoblotted with ζ chain mAb 6B10.2 and other control Abs. A representative of three different experiments is shown (**D**). Cells were treated with or without100 ng/ml TSA for 0, 6, 12, 18, and 24 h at 37°C, lysed, and proteins were separated and immunoblotted with ζ chain mAb 6B10.2 and control Abs as above. The **lane R** represents cells treated with 100 ng/ml TSA for 15 h, and then the medium was removed, washed, and incubated for 9 h in fresh medium without TSA. **E**: Flow cytometric analysis of ζ chain expression in TSA-treated human T lymphocytes. Magnetically separated T cells (5×10^6) were treated with or without 100 ng/ml TSA for 18 h at 37°C. Cells were mildly fixed, permeabilized, and stained with anti-TCR ζ chain mAb 6B10.2 and FITC-conjugated goat anti-mouse secondary Ab (thin line, isotype control; thick line, cells incubated without TSA; and dotted line, cells incubated with 100 ng/ml TSA for 18 h). The staining of ζ chain was significantly decreased in TSA-treated cells and the mean fluorescence intensity of cells incubated without TSA was 638; and cells incubated with 100 ng/ml of TSA was 330. The experiment was repeated four times with very similar results.

In non-activated T cells, 10–40% of the TCR ζ chain is linked to the cytoskeleton [Rozdzial et al., 1995; Caplan and Baniyash, 1996] and activation of T cells translocates the soluble ζ chain to the insoluble membrane fraction [Kosugi et al., 1999]. To ensure that the decrease in the level of ζ chain in the detergent-soluble fraction of TSA-treated cells is not due to its increased association with the membrane fraction, we analyzed the levels of ζ chain in the detergent-insoluble membrane fraction. The pellet obtained after cell lysis was solubilized in sample buffer containing 4% SDS by mechanical agitation, electrophoretically separated and immunoblotted. Similar to the decreased expression of TCR ζ chain in the soluble fraction, the level of ζ chain was also decreased in the detergent-insoluble membrane fraction in TSA-treated cells (Fig. 1C), suggesting that the overall ζ chain protein expression was decreased rather than a translocation or shift in the equilibrium to membrane.

Time Course Study and Reversal of Inhibition of ζ Chain Expression by TSA

To determine the time kinetics of the TSA-mediated decrease in ζ chain expression, T cells were treated with 100 ng/ml of TSA for 0, 6, 12, 18, 24 h, and the ζ chain expression was determined by Western blotting (Fig. 1D). Normalized data against β-actin show that the level of ζ chain was significantly decreased at 12 h following TSA-treatment, and this loss was optimal at 18 h followed by a slight recovery at 24 h (Fig. 1D). The increase in the level of ζ chain at 24 h may be the consequence of breakdown of the TSA compound. Since TSA reversibly inhibits HDAC, we treated cells for 15 h with 100 ng/ml of TSA followed by washing and incubation in fresh medium without TSA for another 9 h to determine whether the effect of TSA on ζ chain expression is reversible. The results show that ζ chain expression returned to normal levels upon removal of the drug, suggesting that the repression of ζ chain expression by TSA is reversible (Fig. 1D).

The effect of TSA on the expression of ζ chain in human T lymphocytes was also studied by flow cytometry. The cells were treated with or without TSA for 18 h, mildly fixed, permeabilized, and stained for ζ chain. As shown in Figure 1E, after 18 h of treatment with 100 ng/ml TSA, ζ chain expression was significantly decreased compared to the untreated cells incubated at 37°C. The mean fluorescence intensity of the untreated control and 100 ng/ml TSA-treated sample were 638 and 330 (arbitrary units), respectively. Thus, the FACS analysis data were consistent with the decreased expression of ζ chain in TSA-treated cells, as was previously revealed by the immunoblotting experiments.

We have reported that in lupus T cells, the deficient TCR ζ chain is replaced by FcεRIγ chain, a member of the ζ chain family of proteins [Enyedy et al., 2001]. There is also ample evidence that the FcεRIγ chain can functionally replace the deficient ζ chain and facilitate TCR/CD3 complex-mediated signaling [Mizoguchi et al., 1992; Qian et al., 1993; Khattri et al., 1996]. Investigation of whether the deficient ζ chain in TSA-treated cells is compensated by FcεRIγ chain expression, showed that the expression of FcεRIγ was increased in a dose-dependent manner in TSA-treated cells (Fig. 1A). Optimal upregulation of FcεRIγ expression was found at 100 ng/ml of TSA.

TSA Inhibits ζ Chain mRNA Expression in Human T Cells

Because TSA exerts its effect by inhibiting HDAC that leads to hyperacetylation of the chromatin and modulation of transcription, we studied the effect of TSA on the level of ζ chain mRNA by semi-quantitative RT-PCR analysis. Agarose gel electrophoresis of the RT-PCR products showed a significant decrease in the level of mRNA in 100 and 1,000 ng/ml TSA-treated cells compared to control (Fig. 2A). Densitometric analyses of the data show that nearly 60% inhibition of ζ chain mRNA synthesis occurred at 100 ng/ml of TSA and more than 80% inhibition was found at 1,000 ng/ml TSA (Fig. 2B). Kinetic studies revealed a significant loss of ζ chain mRNA at 12 h following 100 ng/ml of TSA treatment, and this loss was sustained up to 24 h (Fig. 2C). As seen for ζ chain protein expression, mRNA expression returned to normal levels upon removal of the drug following 15 h treatment with 100 ng/ml TSA and recovery in TSA-free medium for 9 h, suggesting that the repression of ζ chain message by TSA is reversible. To rule out the possibility that TSA destabilizes the ζ chain message, mRNA half-life experiments were performed to assess the change in mRNA stability following TSA treatment. Cells incubated with 100 ng/ml TSA for 12 h and then treated with actinomycin D (5 μg/ml) did not degrade ζ chain message to any appreciable extent over the 12 h time course examined (data not shown). These results indicate that ζ chain expression was negatively regulated by histone deacetylation and TSA virtually does not influence the stability of ζ chain mRNA in human T lymphocytes.

Next, we tested the effect of TSA on the expression of ζ chain transcription factor Elf-1. TCR ζ chain promoter has two binding sites for transcription factor Elf-1 [Rellahan et al., 1998]. Immunoblotting experiments showed that TSA treatment significantly downregulated the expression of active 98 kDa form of the Elf-1 in the detergent-soluble fraction of T cells (Fig. 2D). The localization of the active 98 kDa form of Elf-1 in the nuclear fraction was also decreased in cells exposed to TSA (Fig. 2D). Densitometric analysis indicated that the inhibition of Elf-1 expression reached optimal levels (55%) at 100 ng/ml of TSA and was very similar

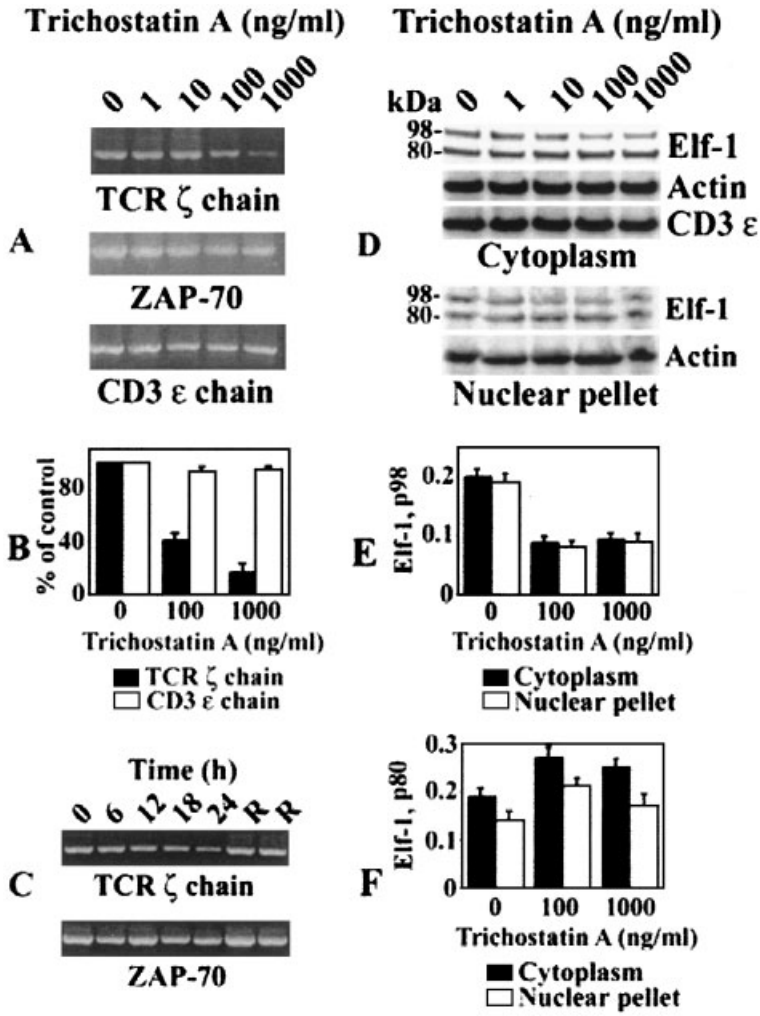

Fig. 2. Inhibiton of TCR ζ chain mRNA expression in TSA-treated cells. **A**: Total RNA was isolated from 5×10^6 T lymphocytes treated with or without TSA for 18 h, reverse transcribed, and ζ chain was amplified by specific primers. The PCR products (15 µl) were electrophoresed in 1.2% agarose gel and visualized by ethidium bromide staining. **B**: Gels were analyzed by densitometry and the level of expression of ζ chain was normalized to β-actin and plotted against TSA (mean ± SEM (n = 4). **C**: Cells were treated with or without 100 ng/ml TSA for 0, 6, 12, 18, and 24 h at 37°C, and RT-PCR analysis of ζ chain and ZAP-70 was carried out as above. The **lane R** represents cells treated with 100 ng/ml TSA for 15 h and then the medium was removed, washed, and incubated for 9 h in fresh medium without TSA. **D**: Cytoplasmic and nuclear extracts of cells treated with or without TSA was immunoblotted with ζ chain transcription factor Elf-1 Ab. **E**, **F**: Densitometric analysis and plotting the normalized data against β-actin showed inhibition of p98 kDa form of the Elf-1 expression in TSA-treated cells (n = 3). Inversely, TSA upregulated the expression of p80 kDa form of Elf-1 in human T cells.

in the cytoplasmic and nuclear fractions (Fig. 2E). In contrast to the inhibition of 98 kDa form, the level of p80 kDa form of the Elf-1 was found to be increased in TSA-treated cells (Fig. 2F). Collectively, these data suggest that TSA inhibits ζ chain mRNA expression by decreasing the expression of active 98 kDa form of Elf-1, which is the main transcription factor of the TCR ζ chain gene. The decrease in the level of p98 kDa form is probably a failure in the posttranslational modification of the native p80 kDa form of the Elf-1 in TSA-treated cells.

Effect of TSA on TCR/CD3-Mediated Tyrosine Phosphorylation of Cellular Protein Substrates and $[Ca^{2+}]_i$ Response in Human T Cells

TCR ζ chain is critical for the assembly, transport, and surface expression of T cell receptor complex and T cell signaling. The first step in the signal transduction after TCR/CD3 engagement is the tyrosine phosphorylation of ζ chain followed by the tyrosine phosphorylation of ZAP 70, and other downstream cellular signaling protein substrates. To demonstrate the

consequences of inhibition of ζ chain expression in TSA-treated cells, we analyzed the TCR/CD3-mediated tyrosine phosphorylation of cellular protein substrates. Cells were preincubated with or without 100 ng/ml TSA for 18 h and then stimulated with 10 μg/ml of OKT3 for 1 and 2 min, followed by immunoblotting with anti-phosphotyrosine Ab. As shown in Figure 3A, TSA-treated cells displayed an increase in the TCR/CD3-mediated tyrosine phosphorylation of the cellular protein substrates at 1 min followed by decrease at 2 min compared to untreated control. A similar pattern of tyrosine phosphorylation with faster kinetics has been reported in lupus T cells [Liossis et al., 1998a].

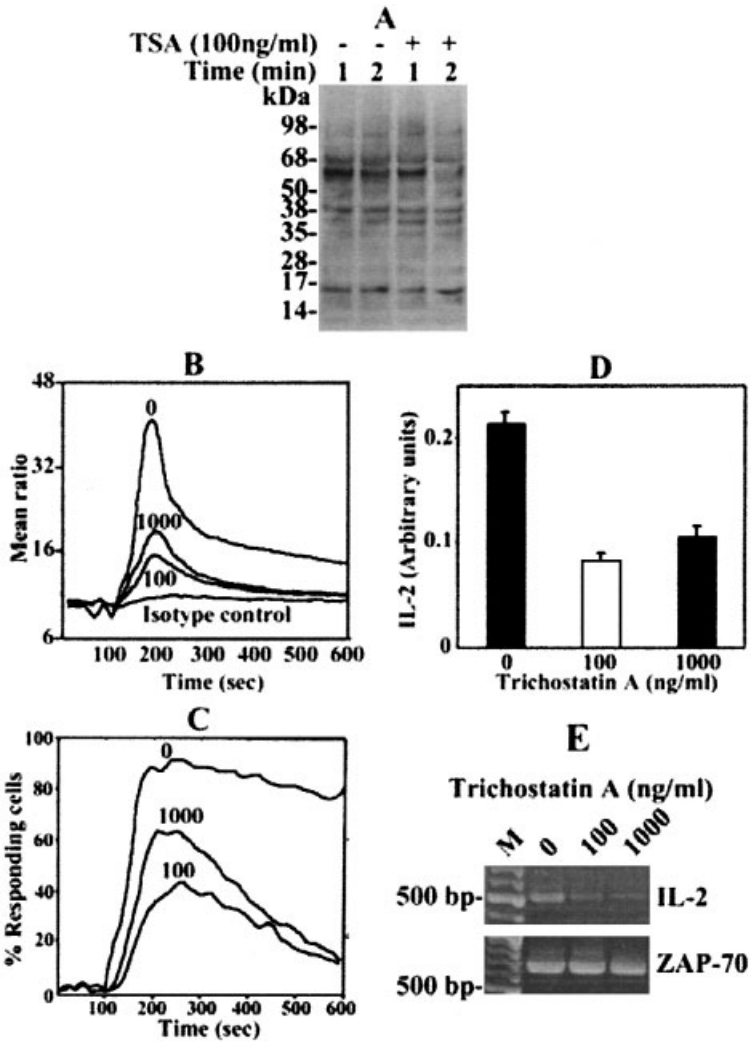

Fig. 3. Trichostatn A inhibits TCR/CD3-mediated tyrosine phosphorylation of cellular protein substrates, $[Ca^{2+}]_i$ response, and interleukin-2 expression in TSA-treated cells. **A**: Magnetically purified T cells were treated with or without TSA at 37°C for 18 h. Cells were stimulated through TCR/CD3 with 10 μg/ml of OKT3 for 1 and 2 min and lysed. Proteins (20 μg) were analyzed on Nu-PAGE under reducing conditions and immunoblotted with horse-radish peroxidase conjugated anti-phosphotyrosine antibody, 4G10. **B**: Cells were treated with or without TSA for 18 h and loaded with INDO-1 for 20 min at 37°C. $[Ca^{2+}]_i$ responses of the cells were analyzed using an Epics Altra flow-cytometer as described in Materials and Methods. In each run, first the cells were unstimulated to record the baseline fluorescence ratio, which represents the resting $[Ca^{2+}]_i$ levels. After 40 s, 10 μg/ml OKT3 Ab or the isotype control mIgG2a (base line) was added to the tube followed by cross-linker goat anti-mouse Ab and recorded for a total of 10 min. **C**: Results are represented as percent of responding cells. A positive responding cell is one whose $[Ca^{2+}]_i$ is increased by two standard deviations above the mean background levels. The concentration of TSA is indicated above the curves. Representative of five different experiments with very similar results is shown. **D**: Cells were treated with or without TSA for 18 h and then stimulated with 10 mg/ml OKT3 and 2.5 mg /ml anti-CD28 for 24 h. The supernatant was collected, and the IL-2 activity was measured by ELISA. The data is represented as mean ± SEM (n = 4). **E**: Semi-quantitative RT-PCR analysis of IL-2 message in TSA-treated and untreated cells.

We next asked whether the TSA-mediated suppression of ζ chain expression in human T cells is associated with similar changes in the TCR/CD3 induced $[Ca^{2+}]_i$ response. To address this question, we monitored the $[Ca^{2+}]_i$ response in T cells treated with or without 100 or 1,000 ng/ml TSA after TCR/CD3 stimulation with OKT3 (10 μg/ml) for 10 min. The data show that treatment with 100 ng/ml TSA strongly inhibited the TCR/CD3-induced $[Ca^{2+}]_i$ response in T cells (Fig. 3B). Interestingly, the inhibition of TCR/CD3 induced $[Ca^{2+}]_i$ response was slightly recovered in 1,000 ng/ml TSA-treated cells, probably due to the differential effect of TSA on the expression of a large set of genes that interplay and are necessary to induce the $[Ca^{2+}]_i$ response. Nevertheless, these results demonstrate that the TCR/CD3 mediated early signaling cascade is downregulated in TSA-treated human T cells.

TSA Inhibits TCR/CD3-Induced IL-2 Production in Human T Lymphocytes

Increase in the intracellular Ca^{2+} after T cell activation gives rise to sequential activation of sets of genes that in turn initiate proliferation, differentiation, and immunological functions. To determine whether treatment with TSA inhibits the expression of cytokines, we studied the effect of TSA on IL-2 expression. T cells were treated with 0, 100, and 1,000 ng/ml TSA for 18 h, followed by activation with 10 μg/ml of anti-CD3 and 2.5 μg/ml anti-CD28 mAbs for 24 h, and the IL-2 level in the culture supernatant was assayed by ELISA. As shown in Figure 3D, the amount of IL-2 production was significantly decreased in TSA-treated cells. Although the maximal repression was at 100 ng/ml TSA, a further increase in the concentration of TSA resulted in slight recovery of IL-2 inhibition similar to that seen for the TCR/CD3 induced $[Ca^{2+}]_i$ response (Fig. 3D). Consistent with the decrease in IL-2 activity, semi-quantitative RT-PCR analysis showed that the level of IL-2 mRNA was significantly decreased in TSA-treated cells (Fig. 3E). Collectively, these results suggest that TSA inhibits the TCR/CD3 mediated stimulation of IL-2 synthesis in human T lymphocytes.

DISCUSSION

The present findings demonstrate that HDAC inhibitor TSA negatively regulates the expression of TCR ζ chain and subsequent antigen receptor-mediated T cell signaling in human T cells in a dose-dependent manner. This effect was reversible as ζ chain expression returned to normal levels upon removal of the drug. The inhibition of ζ chain expression was neither due to an increase in the posttranslational modifications of ζ chain, nor due to translocation and compartmentalization to membranes. The amount of TSA needed to inhibit ζ chain expression corresponded well with the reported effective concentrations of this anti-fungal agent [Yoshida et al., 1990]. Hyperacetylation of histones and other proteins following TSA treatment opens chromatin structure that favors access to transcription factors and promotes a general increase in gene expression [Luo and Dean, 1999]. However, TSA treatment does not always follow conventional logic, and has been reported to have contrasting effects on gene expression although the reason for such a selective effect on gene expression is presently not known. Besides, the herein observed effect on TCR ζ chain, paradoxical effects of TSA on the expression of few other genes like, PU.1 [Laribee and Klemsz, 2001], CD154 and IL-10 [Mishra et al., 2001a], cyclin A and B1 [Nair et al., 2001], and Cdk-1 [Inokoshi et al., 1999] have also been reported.

To date, the precise mechanism of modulation of gene expression following the inhibition of HDACs by TSA is poorly understood. Our study suggests a straightforward explanation for the TSA-induced gene repression based on the ability of TSA to inhibit the expression of active 98 kDa form of Elf-1, the key transcription factor regulating ζ chain expression. The inverse expression patterns of the 98 and 80 kDa forms with no effect on the global level of Elf-1in TSA-treated cells led us to hypothesize that TSA may act directly on Elf-1 by blocking the posttranslational modifications, or it may act indirectly by modulating the glycosylation and phosphorylation enzymes that are required for the modification of Elf-1. Although a decrease in the levels of Elf-1 could contribute to the downregulation of ζ chain expression, other direct possibilities also need to be considered. For example, inhibition of HDAC activity may influence nucleosomal positioning that in turn regulates chromatin structure. Movement of the nucleosome on ζ chain locus might mask the *cis*- and *trans*- acting sites resulting in the loss of ζ chain transcription. The promoter region of

TCR ζ chain includes two distinct basal promoter elements, which independently initiate TCR ζ gene transcription and a positive and negative *cis*- acting element [Rellahan et al., 1998]. There are several potential binding sites for the T cell-specific transcription factor in the TCR ζ chain promoter, including three GATA-3 binding sites and two canonical Elf-1 transcription factor-binding sites. Exploring the effect of TSA on the transcriptional activity of ζ chain in Jurkat cell lines transfected with TCR ζ chain promoter-luciferase gene constructs (−306 to +58) showed that the luciferase activity was significantly enhanced in TSA-treated cells (data not shown). An increase in the length of the promoter (−1468) did not further change the upregulation of promoter activity in 100 ng/ml TSA-treated cells (data not shown). A similar increase in the promoter activity following treatment with 100 ng/ml of TSA was observed in cells transfected with a mutant ζ chain promoter that has been mutated at both the Elf-1 binding sites. These results suggest that transcriptional regulation of transfected ζ chain promoter-luciferase constructs by TSA is different from that of chromosomal locus. Upregulation rather than inhibition of the PU.1 mRNA by TSA was reported in a null cell line in which PU.1 was re-expressed via retroviral transduction [Laribee and Klemsz, 2001]. Preliminary experiments using cycloheximide indicate that TSA inhibits ζ chain expression albeit to a lesser extent in the absence of protein synthesis suggesting a combination of direct and indirect role of TSA in ζ chain repression.

Expression of FcεRIγ, in lieu of ζ chain, has been reported in murine large granular lymphocytes. T lymphocytes from tumor-bearing mice expressed TCR that completely lacked ζ chain, and this was replaced by FcεRIγ [Mizoguchi et al., 1992]. Also, TCR ζ-deficient mice have been shown to express FcεRIγ as part of the TCR-γδ complex [Khattri et al., 1996]. In parallel, TCR ζ chain can substitute for FcεRIγ chain in the assembly and surface expression of the FcεRI receptor. In lupus, the deficient ζ chain is replaced by increased FcεRIγ expression [Enyedy et al., 2001]. In addition to these pathophysiologic processes, upregulation of FcεRIγ expression in TSA-treated cells suggests that loss of ζ chain expression rapidly triggers a compensatory mechanism that increases FcεRIγ expression and takes over T cell signaling to keep the cell functioning. In support of this, we have recently found that the overexpressed FcεRIγ associates with TCR and participates in T cell signaling leading to TCR/CD3-mediated hyper $[Ca^{2+}]_i$ response [Nambiar et al., unpublished experiments].

TSA was found here to alter CD3-induced protein tyrosine phosphorylation in an interesting manner—increasing it during the first minute and suppressing it later. This observation is difficult to interpret, but it is reminiscent of the phosphorylation pattern that we observed in primary T cells from SLE patients [Liossis et al., 1998b]. The effect of TSA on the CD3-induced $[Ca^{2+}]_i$ is interesting in that its suppression is not extended when the drug is used at the concentration of 1,000 ng/ml. This reversal of the suppression indicates first, the drug is not toxic to the cells and second, the regulation of $[Ca^{2+}]_i$ is more complex. The observed suppressive effect on IL-2 extends previous observations made in the Jurkat T cell line [Takahashi et al., 1996]. The effect on IL-2 may be secondary to the suppressive effect on the TCR ζ chain expression or it may represent the direct effect of TSA on the acetylation of histones connected to the transcription of the IL-2 gene.

The implications of this study are far-reaching. There is a striking similarity between the effect of TSA described here and the pattern of gene expression reported in SLE T cells. Our laboratory has shown that in majority of lupus patients, the expression of ζ chain is deficient at both protein and mRNA level [Liossis et al., 1998a], with the concomitant replacement of the deficient ζ chain by FcεRIγ [Enyedy et al., 2001b]. Lupus T cells also showed decreased expression of the active 98 kDa form of the ζ chain transcription factor, Elf-1 and simultaneous upregulation of the 80 kDa inactive form of Elf-1. Similarly, lupus T cells showed TCR/CD3-mediated hyper-tyrosine phosphorylation of cellular substrates that peaked at 1 min. Finally, the IL-2 activity and mRNA expression after T cell activation is significantly downregulated in SLE T cells. In the context of these close similarities, it is tempting to speculate that a genetic imbalance in histone acetylation/deacetylation might underlie ζ chain deficiency and related defect in the biological activities in SLE T cells. Also, in support of this notion, chromosomal instability has been well documented in SLE patients [Emerit and Michelson, 1980]. The immunosuppressive effects of TSA

described here may be useful in treating patients receiving tissue transplants.

In conclusion, we have shown that TSA represses TCR ζ chain protein and mRNA expression in a time- and dose-dependent fashion and is reversible upon removal of the drug. In contrast, TSA upregulates the expression of FcεRIγ chain in human T lymphocytes. The suppression of ζ chain expression results in the inhibition of TCR/CD3-induced early signaling as well as IL-2 gene expression. In this regard, TSA may not be used in diseases where the TCR ζ chain is decreased, such as, lupus, HIV, rheumatoid arthritis, cancer, tuberculosis, etc., while its use in situations where T cell hyporesponsiveness is sought, such as transplantation should be explored. The fact that TSA effects on T cells closely mimic the molecular abnormalities of T cell signaling identified in SLE, may prove useful in understanding pathogenesis of this autoimmune disorder.

REFERENCES

Blasini AM, Brundula V, Paris M, Rivas L, Salazar S, Stekman IL, Rodriguez MA. 1998. Protein tyrosine kinase activity in T lymphocytes from patients with systemic lupus erythematosus. J Autoimmun 11:387–393.

Brundula V, Rivas LJ, Blasini AM, Paris M, Salazar S, Stekman IL, Rodriguez MA. 1999. Diminished levels of T cell receptor zeta chains in peripheral blood T lymphocytes from patients with systemic lupus erythematosus. Arthritis Rheum 42:1908–1916.

Caplan S, Baniyash M. 1996. Normal T cells express two T cell antigen receptor populations, one of which is linked to the cytoskeleton via zeta chain and displays a unique activation-dependent phosphorylation pattern. J Biol Chem 271:20705–20712.

Emerit I, Michelson AM. 1980. Chromosome instability in human and murine autoimmune disease: anticlastogenic effect of superoxide dismutase. Acta Physiol Scand 492 (Suppl):59–65.

Enyedy EJ, Nambiar MP, Liossis SN, Dennis G, Kammer GM, Tsokos GC. 2001. Fc epsilon receptor type I gamma chain replaces the deficient T cell receptor zeta chain in T cells of patients with systemic lupus erythematosus. Arthritis Rheum 44:1114–1121.

Grunstein M. 1997. Histone acetylation in chromatin structure and transcription. Nature 389:349–352.

Hall CG, Sancho J, Terhorst C. 1993. Reconstitution of T cell receptor zeta-mediated calcium mobilization in nonlymphoid cells. Science 261:915–918.

Huang N, Katz JP, Martin DR, Wu GD. 1997. Inhibition of IL-8 gene expression in Caco-2 cells by compounds which induce histone hyperacetylation. Cytokine 9:27–36.

Inokoshi J, Katagiri M, Arima S, Tanaka H, Hayashi M, Kim YB, Furumai R, Yoshida M, Horinouchi S, Omura S. 1999. Neuronal differentiation of neuro 2a cells by inhibitors of cell cycle progression, trichostatin A and butyrolactone I. Biochem Biophys Res Commun 256: 372–376.

Khattri R, Sperling AI, Qian D, Fitch FW, Shores EW, Love PE, Bluestone JA. 1996. TCR-gamma delta cells in CD3 zeta-deficient mice contain Fc epsilon RI gamma in the receptor complex but are specifically unresponsive to antigen. J Immunol 157:2320–2327.

Kosugi A, Saitoh S, Noda S, Yasuda K, Hayashi F, Ogata M, Hamaoka T. 1999. Translocation of tyrosine-phosphorylated TCRzeta chain to glycolipid-enriched membrane domains upon T cell activation. Int Immunol 11:1395–1401.

Laribee RN, Klemsz MJ. 2001. Loss of pu.1 expression following inhibition of histone deacetylases. J Immunol 167:5160–5166.

Liossis SN, Ding DZ, Dennis GJ, Tsokos GC. 1998a. 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. J Clin Invest 101:1448–1457.

Liossis SN, Hoffman RW, Tsokos GC. 1998b. Abnormal early TCR/CD3-mediated signaling events of a snRNP-autoreactive lupus T cell clone. Clin Immunol Immunopathol 88:305–310.

Luo RX, Dean DC. 1999. Chromatin remodeling and transcriptional regulation. J Natl Cancer Inst 91:1288–1294.

Mishra N, Brown DR, Olorenshaw IM, Kammer GM. 2001. Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. Proc Natl Acad Sci USA 98:2628–2633.

Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC. 1992. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. Science 258:1795–1798.

Nair AR, Boersma LJ, Schiltz L, Chaudry A, Muschel RJ. 2001. Paradoxical effects of trichostatin A: inhibition of NF-Y-associated histone acetyltransferase activity, phosphorylation of hGCN5 and downregulation of cyclin A and B1 mRNA. Cancer Lett 166:55–64.

Nambiar MP, Enyedy EJ, Fisher CU, Warke VG, Juang YT, Tsokos GC. 2001a. Dexamethasone modulates TCR zeta chain expression and antigen receptor-mediated early signaling events in human T lymphocytes. Cell Immunol 208:62–71.

Nambiar MP, Enyedy EJ, Fisher CU, Warke VG, Tsokos GC. 2001b. High dose of dexamethasone upregulates TCR/CD3-induced calcium response independent of TCR zeta chain expression in human T lymphocytes. J Cell Biochem 83:401–413.

Nambiar MP, Enyedy EJ, Warke VG, Krishnan S, Dennis G, Kammer GM, Tsokos GC. 2001c. Polymorphisms/mutations of TCR-zeta-chain promoter and 3′ untranslated region and selective expression of TCR zeta-chain with an alternatively spliced 3′ untranslated region in patients with systemic lupus erythematosus. J Autoimmun 16:133–142.

Nambiar MP, Enyedy EJ, Warke VG, Krishnan S, Dennis G, Wong HK, Kammer GM, Tsokos GC. 2001d. T cell signaling abnormalities in systemic lupus erythematosus are associated with increased mutations/polymorphisms and splice variants of T cell receptor zeta chain messenger RNA. Arthritis Rheum 44:1336–1350.

Nambiar MP, Enyedy EJ, Fisher CU, Krishnan S, Warke VG, Gilliland W, Oglesby R, Tsokos GC. 2002. Abnormal expression of various molecular forms and distribution of TCR ζ chain in T cells of patients with systemic lupus erythematosus. Arthritis Rheum 46:163–174.

Pazin MJ, Hermann JW, Kadonaga JT. 1998. Promoter structure and transcriptional activation with chromatin templates assembled in vitro. A single Gal4-VP16 dimer binds to chromatin or to DNA with comparable affinity. J Biol Chem 273:34653–34660.

Qian D, Sperling AI, Lancki DW, Tatsumi Y, Barrett TA, Bluestone JA, Fitch FW. 1993. The gamma chain of the high-affinity receptor for IgE is a major functional subunit of the T-cell antigen receptor complex in gamma delta T lymphocytes. Proc Natl Acad Sci USA 90:11875–11879.

Rellahan BL, Jensen JP, Howcroft TK, Singer DS, Bonvini E, Weissman AM. 1998. Elf-1 regulates basal expression from the T cell antigen receptor zeta-chain gene promoter. J Immunol 160:2794–2801.

Richon VM, Sandhoff TW, Rifkind RA, Marks PA. 2000. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 97:10014–10019.

Rozdzial MM, Malissen B, Finkel TH. 1995. Tyrosine-phosphorylated T cell receptor zeta chain associates with the actin cytoskeleton upon activation of mature T lymphocytes. Immunity 3:623–633.

Takahashi I, Miyaji H, Yoshida T, Sato S, Mizukami T. 1996. Selective inhibition of IL-2 gene expression by trichostatin A, a potent inhibitor of mammalian histone deacetylase. J Antibiot (Tokyo) 49:453–457.

Takeuchi T, Tsuzaka K, Pang M, Amano K, Koide J, Abe T. 1998. TCR zeta chain lacking exon 7 in two patients with systemic lupus erythematosus. Int Immunol 10:911–921.

Vassilopoulos D, Kovacs B, Tsokos GC. 1995. TCR/CD3 complex-mediated signal transduction pathway in T cells and T cell lines from patients with systemic lupus erythematosus. J Immunol 155:2269–2281.

Yoshida M, Kijima M, Akita M, Beppu T. 1990. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 265:17174–17179.