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Research Article

Immunology

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# Protein phosphatase 2A is a negative regulator of IL-2 production in patients with systemic lupus erythematosus

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**Decreased IL-2 production in systemic lupus erythematosus (SLE) represents a central component of the disease immunopathology. We report that the message, protein, and enzymatic activity of the catalytic subunit of protein phosphatase 2A (PP2Ac), but not PP1, are increased in patients with SLE regardless of disease activity and treatment and in a disease-specific manner. Treatment of SLE T cells with PP2Ac-siRNA decreased the protein levels and activity of PP2Ac in a specific manner and increased the levels of phosphorylated cAMP response element-binding protein and its binding to the *IL2* and *c-fos* promoters, as well as increased activator protein 1 activity, causing normalization of IL-2 production. Our data document increased activity of PP2A as a novel SLE disease-specific abnormality and define a distinct mechanism whereby it represses IL-2 production. We propose the use of PP2Ac-siRNA as a novel tool to correct T cell IL-2 production in SLE patients.**

## Introduction

T cells from patients (1) and mice (2) with systemic lupus erythematosus (SLE) produce decreased amounts of IL-2. IL-2 is essential for both the promotion and the suppression of the immune response (3). Decreased amounts of IL-2 contribute to increased susceptibility to infections (4), decreased activation-induced cell death (5), and the subsequent extended survival of autoreactive lymphocytes (6). IL-2 is also considered to be vital for the development and function of regulatory cells (3), which are presumed to be important in the control of the systemic autoimmune response typified in SLE (7).

In normal T cells the production of IL-2 is controlled at the transcription level (8). A number of transcription factors, including nuclear factor of activated T cells (NFAT), activator protein 1 (AP1), NF- $\kappa$ B, and cAMP response element-binding protein (CREB) bind to well-defined *cis* sites of the proximal promoter (9). In SLE T cells, the activity of the *IL2* promoter is decreased because of decreased NF- $\kappa$ B (10, 11) and AP1 activity (12) and increased binding of the transcriptional repressor CREM to the -180 site (13, 14). In normal T cells, the -180 site is occupied by CREB (15), but following T cell activation, downstream kinases, including PKA, phosphorylate CREB at the Ser133 residue (16). Phosphorylated CREB (pCREB) bound to the -180 site interacts with the transcription coactivators CRE-binding protein (CBP) and p300 and enhances the activity of the *IL2* promoter (17). SLE T cells display increased amounts of cAMP response element modulator (CREM) that binds to the *IL2* promoter and represses its activity (13, 14). Removal of CREM by an antisense approach leads to significant increase in *IL2* promoter activity and IL-2 production (13). CREM increases in nor-

mal T cells following activation and replaces pCREB at the -180 site, and this results in the termination of IL-2 production (15). Therefore, it is possible that the ratio of pCREB/CREM that occupies the -180 site of the *IL2* promoter determines the production of IL-2. In SLE T cells, the documented decreased PKA I activity (18) should be expected to result in decreased pCREB levels. Also, the RII $\beta$  subunit of PKA II, which is abnormally translocated from the cytoplasm to the nucleus (19), binds CREB and limits its binding to the *IL2* promoter (20).

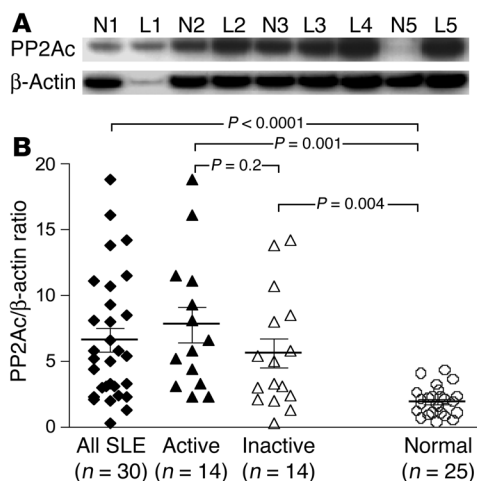
Protein phosphatase 2A (PP2A) is the primary enzyme that leads to dephosphorylation of pCREB in T lymphocytes (16, 21–26), and it has been shown to suppress the production of IL-2 (27–32). Therefore, T cell PP2A levels determine in an inverse manner the levels of pCREB and, consequently, through this pathway PP2A represents a negative regulator of the *IL2* promoter activity, although there are reports indicating that PP2A can increase the production of IL-2 (33–35). PP2A, a Ser/Thr phosphatase ubiquitously expressed in eukaryotic cells, is a heterotrimeric enzyme that consists of a scaffold-type structural subunit (A), a catalytic subunit (C), and one of a diverse array of regulatory subunits (B) (36). The extensive variety of different regulatory subunits enables PP2A to act on a wide range of substrates and, as a result, to monitor several cellular processes, including the cell cycle, gene transcription and translation, and apoptosis (36). PP2A represents a highly conserved molecule through evolution (37, 38), and its importance is furthermore underscored by the fact that mice lacking the gene for the catalytic subunit of PP2A (PP2Ac) do not survive for more than 4–6 days of embryonic life (39). Recent studies have identified PP2A as a tumor suppressor (40) as well as a key molecule in the pathogenesis of disorders such as Alzheimer disease (41) and Oritz BBB/G syndrome (42).

We present data that introduce PP2A as a novel regulator of the *IL2* promoter activity and IL-2 production. We report here that the protein and catalytic activity of PP2A is increased in patients with SLE but not in patients with RA and that it is responsible, at least in part, for the decreased production of IL-2. We demonstrate that we can effectively decrease the levels of PP2A using a proper siRNA and correct the production of IL-2 in SLE T cells.

**Nonstandard abbreviations used:** AP1, activator protein 1; CamKII, CaM-dependent kinase II; ChIP, chromatin immunoprecipitation; CREB, cAMP response element-binding protein; CREM, cAMP response element modulator; DN, dominant negative; OA, okadaic acid; pCREB, phosphorylated CREB; PP2A, protein phosphatase 2A; PP2Ac, catalytic subunit of PP2A; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1** PP2Ac protein levels are increased in SLE T cells. Cellular protein extracts of T cells derived from patients with SLE (L) and normal (N) subjects were examined in parallel for the expression of PP2Ac using Western blots. (A) Immunoblots from a representative experiment are shown.  $\beta$ -Actin was used as control. (B) Scatter diagram showing cumulative data from 30 patients with SLE and 25 normal subjects. Results for active (SLEDAI, 4–14) and inactive (SLEDAI, 0–3) patients are depicted separately. The intensity of the bands was measured by densitometry, and the PP2Ac/ $\beta$ -actin ratio was calculated. All measurements are depicted as well as the mean value for each study group  $\pm$  SEM. *P* values derived from statistical analysis (2-tailed unpaired *t* test with Welch correction) are also shown.

**Results**

*PP2Ac protein levels are increased in SLE T cells.* PP2A mediates the dephosphorylation and thus inactivation of pCREB (16, 21–26), a transcription factor that induces the expression of the *IL2* gene. To examine whether PP2A has a role in the defective production of IL-2 in SLE T cells, we determined the protein levels of PP2Ac in protein extracts from peripheral blood T cells derived from SLE patients ( $n = 30$ ) and normal subjects ( $n = 25$ ) by Western blotting.  $\beta$ -Actin protein levels were used as control, and the ratio of PP2Ac to  $\beta$ -actin was compared between SLE and control T cells. As shown in Figure 1, SLE T cells expressed significantly higher levels of PP2Ac compared with normal T cells (mean PP2Ac/ $\beta$ -actin ratio  $\pm$  SEM:  $6.57 \pm 0.87$  vs.  $1.89 \pm 0.21$ , respectively;  $P < 0.0001$ ). Sixty percent of the SLE patients studied expressed PP2Ac above the maximum levels observed in normal subjects.

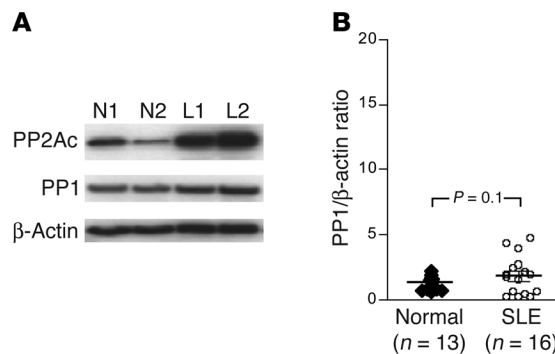
Subsequently, we examined whether increased PP2Ac expression in lupus T cells was related to the activity of the disease. Analysis of PP2Ac levels in T cells from patients with active (SLE disease activity index [SLEDAI], 4–14;  $n = 14$ ) and inactive (SLEDAI, 0–3;  $n = 16$ ) disease revealed comparable levels of the enzyme ( $7.73 \pm 1.37$  vs.  $5.56 \pm 1.08$  respectively;  $P = 0.2$ ), while both groups showed increased expression of PP2Ac compared with normal T cells ( $P = 0.001$  and  $P = 0.004$ , respectively, when compared with patients with active and inactive disease) (Figure 1B). Inspection of the data did not reveal an association between any particular clinical or laboratory manifestation and increased PP2Ac levels. Furthermore, at least 6 months after the initial assessment, 5 patients were reexamined. In 2 patients the disease activity had not changed, whereas in 3 the disease activity had

changed by more than 3 SLEDAI points. Moderate changes in PP2Ac protein levels that were observed in these patients did not parallel the changes in disease activity. In all cases changes in PP2Ac/ $\beta$ -actin protein ratio were less than or equal to 10%, while the direction of the change in the SLEDAI score did not predict the direction of the minor change in PP2Ac protein levels. Likewise, there was no association between the levels of PP2Ac and the treatment that patients were receiving at the time of the study. In particular, the PP2Ac/ $\beta$ -actin ratio did not correlate with the dose of prednisone (Pearson product moment correlation coefficient,  $-0.172$ ;  $P = 0.36$ ).

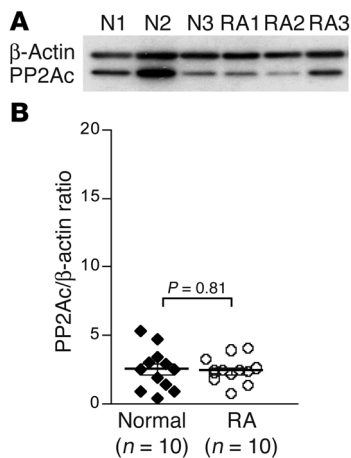
PP2A shares structural and functional characteristics with the Ser/Thr PP1 (43). To examine whether PP2Ac overexpression represents a finding limited to this specific enzyme, a parallel study of the expression of PP2Ac and PP1 was performed in T cells derived from 16 patients with SLE and 13 normal subjects. SLE and control T cells displayed comparable levels of PP1 ( $1.84 \pm 0.37$  vs.  $1.28 \pm 0.14$  respectively;  $P = 0.1$ ), while at the same time PP2Ac expression was again increased in lupus T cells (SLE:  $6.82 \pm 0.94$ ; control:  $2.07 \pm 0.32$ ;  $P = 0.03$ ). A representative experiment and cumulative data are shown in Figure 2.

To address the question whether PP2Ac overexpression is limited to SLE T cells, PP2Ac expression in T cells from 10 patients with RA and 10 normal donors was examined. As shown in Figure 3, PP2Ac levels were similar in normal T cells and T cells from patients with RA (RA:  $2.41 \pm 0.25$ ; normal:  $2.56 \pm 0.39$ ;  $P = 0.81$ ). Therefore, PP2Ac but not PP1 is increased in SLE patients regardless of disease activity and treatment, and the increase appears to occur in a disease-specific manner.

*PP2Ac enzymatic activity is increased in SLE T cells.* Although overexpression of PP2Ac in a transgenic mouse led to upregulation of PP2Ac enzymatic activity (44), mutation of the catalytic site leads to increased expression of PP2A protein (45). Since phosphorylation of PP2Ac inhibits its catalytic activity (46) and SLE T cells display increased TCR/CD3-initiated phosphorylation of cytosolic proteins (47), we considered the increased protein levels of PP2Ac may represent a compensatory response to improperly phosphorylated and therefore inactive PP2Ac (46). Accordingly, we



**Figure 2** SLE T cells express increased levels of PP2A but not PP1. Western blot analysis was used for the parallel study of PP2Ac and PP1 expression in T cells from patients with SLE and normal subjects. (A) Immunoblots depicting PP2Ac, PP1, and  $\beta$ -actin from a representative experiment. (B) Diagram presenting cumulative data from the study of 16 patients with SLE and 13 normal subjects. The intensity of the bands was measured by densitometry. The levels of PP1 are expressed as the PP1/ $\beta$ -actin ratio.

**Figure 3**

T cells from patients with RA do not express increased levels of PP2Ac protein. Western blot analysis of PP2Ac expression in normal subjects and patients with RA. (A) Results from a representative experiment are shown with simultaneous immunoblotting for PP2Ac and  $\beta$ -actin. (B) Cumulative data from 10 RA patients and 10 normal subjects are presented. PP2Ac expression was determined as described for Figure 1.

determined the catalytic activity of the enzyme in T cells derived from 10 patients with SLE and 10 normal subjects. PP2Ac was isolated by immunoprecipitation, and its catalytic activity was assessed after incubation with an appropriate phosphopeptide by measuring the levels of released phosphate ( $\text{PO}_4^{3-}$ ). PP2Ac protein expression was studied in parallel using immunoblotting. The catalytic activity of PP2Ac was 1.7-fold higher in SLE T cells than in normal T cells (mean pmol  $\text{PO}_4^{3-}/\mu\text{g} \pm \text{SEM}$ :  $10.814 \pm 1.658$  vs.  $6.390 \pm 1.213$ , respectively;  $P = 0.01$ ). In contrast, PP2Ac activity in T cells from patients with RA ( $n = 10$ ) was similar to that observed in normal T cells studied in parallel ( $n = 10$ ). Cumulative data are shown in Figure 4A. Parallel assessment of PP2Ac expression in lupus T cells revealed a strong positive correlation between the protein levels and the catalytic activity of the enzyme (Pearson product moment correlation coefficient, 0.9446;  $P = 0.004$ ). A representative experiment is depicted in Figure 4B. As shown in Figure 4C, our assays were not contaminated with exogenous phosphate or nonspecific immunoprecipitates, because in the presence of the Ser/Thr phosphatase inhibitor NaF, no  $\text{PO}_4^{3-}$  was detected, while Western blot analysis of samples subjected to immunoprecipitation with anti-PP2Ac detected only PP2Ac.

In a previous study, *in vitro* treatment of the leukemic cell line HL-60 with methyl-prednisolone led to a substantial increase in PP2A activity without altering the expression of PP2Ac (48). Because among the SLE patients studied, 2 were receiving no treatment while others were receiving either low ( $\leq 7.5$  mg/d; 4 patients) or moderate dose of prednisone ( $> 7.5$  to  $< 40$  mg/d; 4 patients), we examined whether there was a relation between the prednisone dose received and the phosphatase activity. Our results indicate that corticosteroid treatment could not account for the increased activity of PP2Ac in lupus T cells (Figure 4D). Moreover, disease activity did not seem to affect PP2Ac enzymatic activity (Figure 4D). Therefore, increased PP2Ac protein levels in SLE T cells lead to increased activity of the phosphatase.

**Increased PP2Ac mRNA in lupus T cells.** Previous studies have shown that in a given cell system the expression of PP2Ac is strictly regu-

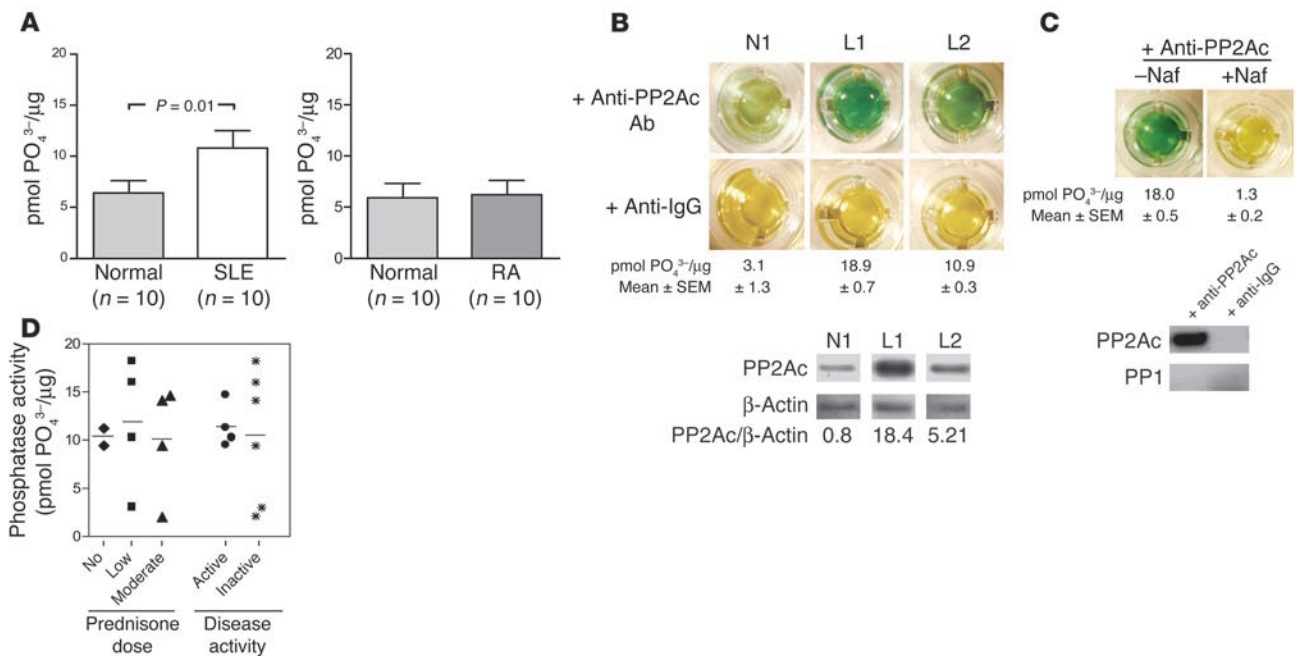
lated by translational (49) as well as transcriptional (28) regulatory mechanisms. To determine whether increased expression of PP2Ac in SLE T cells is associated with enhanced production of the enzyme, total mRNA was isolated from T cells derived from 19 SLE patients and 12 normal subjects. Real-time RT-PCR was used to assess PP2Ac and  $\beta$ -actin mRNA levels, and the ratio of PP2Ac to  $\beta$ -actin mRNA was compared between SLE and control T cells. PP2Ac protein expression was also studied in parallel in 9 SLE patients and 6 normal subjects. PP2Ac mRNA levels were increased in SLE T cells compared with normal T cells (mean PP2Ac/ $\beta$ -actin mRNA ratio:  $2.22 \pm 0.53$  vs.  $0.76 \pm 0.12$ , respectively;  $P = 0.03$ ). Results from a representative experiment and cumulative data are presented in Figure 5. Furthermore, PP2Ac mRNA levels correlated positively with protein levels in the SLE ( $r^2 = 0.79$ ;  $P = 0.001$ ) and normal group ( $r^2 = 0.86$ ;  $P = 0.007$ ), indicating that increased PP2Ac mRNA levels correlate with increased protein levels (Figure 5C).

**Downregulation of PP2Ac using specific small interfering RNA.** To study the role of PP2Ac overexpression in SLE T cells, we first developed small interfering RNAs (siRNAs), to directly and specifically downregulate the expression of the phosphatase in short-term cultures of primary T cells. T cells derived from healthy individuals were transfected with either PP2Ac- or control siRNA and subsequently cultured for 4–48 hours. PP2Ac protein and mRNA levels were assessed with Western blotting and real-time RT-PCR, respectively. Time- and dose-response experiments revealed that transfection of T cells with PP2Ac-siRNA resulted in a transient, siRNA dose-dependent downregulation of PP2Ac mRNA and protein levels (Figure 6, A and B). As shown in Figure 6, higher doses of PP2Ac-siRNA almost abolished PP2Ac expression. Nevertheless, because in some experiments, doses greater than 100 nM had a nonspecific suppressive effect on the levels of proteins other than PP2Ac, doses less than 100 nM were used in subsequent studies. Parallel assessment of T cell survival showed that the lowest PP2Ac protein expression/T cell number ratio was achieved following 24–36 hours of culture. Under these conditions (siRNA dose, 25–50 nM, 24–36 hours culture), we were able to downregulate PP2Ac in a specific manner, as the expression of other cellular proteins (including PP1) remained stable, while experiments using control siRNA did not alter the expression of PP2Ac. Results from a representative experiment and cumulative results from 5 independent experiments are shown in Figure 6, C and D.

**PP2Ac downregulation in lupus T cells leads to increased pCREB expression and increased binding of pCREB to the IL2 promoter.** Because PP2Ac leads to the dephosphorylation of pCREB (16, 23), we examined whether suppression of PP2Ac in lupus T cells could enhance the expression of pCREB. T cells from patients with SLE were transfected with PP2Ac-siRNA using the conditions described above and were subsequently stimulated with PMA/A23187 for 2 hours. The expression of PP2Ac in whole cellular protein extracts and the expression of pCREB in nuclear protein extracts were assessed in parallel by immunoblotting.  $\beta$ -Actin and heterogeneous ribonucleoprotein (hnRNP) protein levels were used as controls, respectively. In the presence of PP2Ac-siRNA, the expression of PP2Ac decreased significantly, while at the same time pCREB levels were consistently upregulated. Results from a representative of 5 similar experiments are shown in Figure 7A. Transfection with control siRNA did not affect the expression of pCREB.

T cell activation triggers the binding of pCREB to the IL2 promoter. We therefore asked whether the increased expression of pCREB following PP2Ac downregulation in stimulated lupus T cells





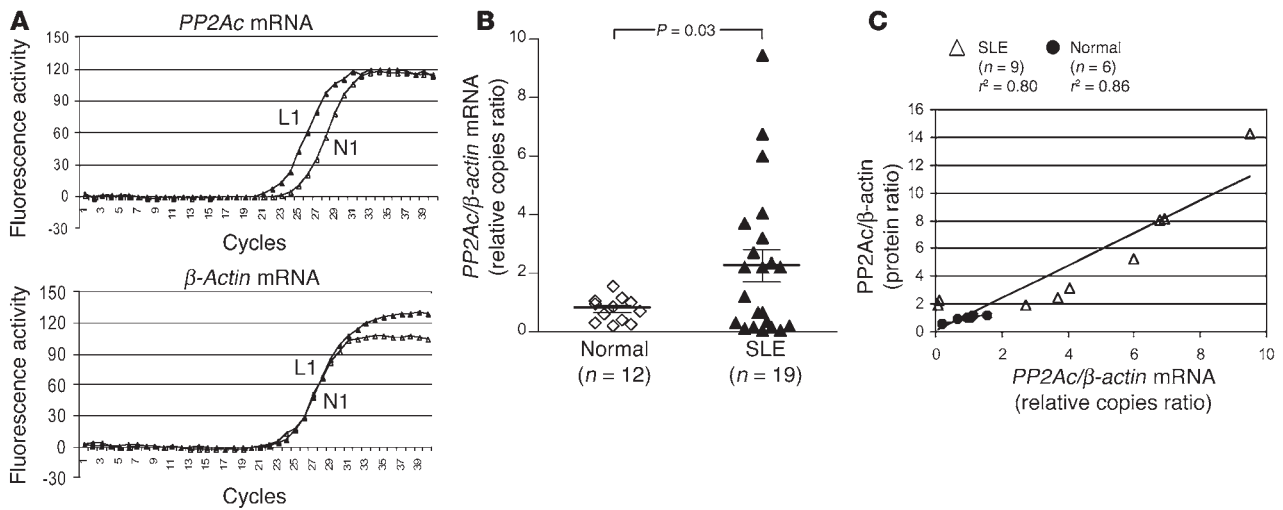
**Figure 4**

PP2Ac enzymatic activity is increased in SLE T cells. PP2Ac enzymatic activity was studied in peripheral blood T cells from patients with SLE or RA and normal subjects as described in Methods and is presented as picomoles  $\text{PO}_4^{3-}/\text{microgram}$  of protein. PP2Ac protein levels were studied in parallel using Western blots. (A) Cumulative data of PP2Ac catalytic activity are presented. Statistical significance is also shown. (B) Results from a representative experiment are shown where PP2Ac enzymatic activity (upper panel: malachite green assay; phosphatase activity values [ $\text{pmol PO}_4^{3-}/\mu\text{g}$  protein, mean  $\pm$  SEM of triplicates] corresponding to each sample are noted below the panel) was assessed in parallel with PP2Ac protein levels (lower panel; PP2Ac/ $\beta$ -actin ratios are noted below the panel). Anti-IgG immunoprecipitates and  $\beta$ -actin protein levels were used as controls for the determination of the activity and the expression of PP2Ac, respectively. (C) Experiments performed to assess nonspecific results due to contamination with residual  $\text{PO}_4^{3-}$  or with nonspecific immunoprecipitates. Enzymatic activity of anti-PP2Ac immunocomplexes was abolished in the presence of the Ser/Thr phosphatase inhibitor NaF (upper panel, representative of 6 experiments). Western blot analysis of the anti-PP2Ac immunoprecipitates detected only PP2Ac protein, whereas when the control anti-IgG Ab was used, no protein was precipitated (lower panel). (D) PP2Ac activity is increased in SLE T cells from patients with active and inactive disease, regardless of prednisone treatment. Results for patients receiving no, low dose ( $\leq 7.5$  mg/d), or moderate dose ( $>7.5$  to  $<40$  mg/d) of prednisone as well as active (SLEDAI, 4–14) and inactive (SLEDAI, 0–3) patients are depicted separately.

results in increased binding of pCREB to the *IL2* promoter. Following transfection, lupus T cells were activated for 1 and 2 hours, using PMA/A23187. We used chromatin immunoprecipitation (ChIP) assays to determine pCREB binding to the *IL2* promoter. Our data showed that in stimulated but not transfected T cells, pCREB binding gradually increased over time, as expected. As shown in Figure 7B, transfection of T cells with PP2Ac-siRNA led to a significant increase in the amount of pCREB bound to the *IL2* promoter. On the contrary, ChIP using an unrelated anti-E47 Ab showed no binding to the *IL2* promoter. Transfection with control siRNA did not affect pCREB binding.

*Downregulation of PP2Ac restores the production of IL-2 in SLE T cells.* Because in activated SLE T cells PP2Ac negatively regulates the binding of pCREB to the *IL2* promoter and because lupus T cells display increased expression of active PP2Ac, we examined whether suppression of PP2Ac could restore the production of IL-2. Five patients with SLE and 5 normal subjects were studied in parallel. T cells were transfected with either PP2Ac- or control siRNA and subsequently were cultured in the presence or absence of PMA/A23187 for 1 and 3 hours. Following 3 hours of culture, supernatants were collected, and the concentration of IL-2 was measured using ELISA. Cells cultured for 1 hour were harvested, and the expression of PP2Ac was assessed in Western blots. Transfection of T

cells with PP2Ac-siRNA resulted in a substantial decrease in PP2Ac expression in both SLE patients and normal subjects (63% and 41% average decrease, respectively), while the levels of PP2Ac in transfected and 1 hour-stimulated T cells from patients with SLE and normal subjects did not differ (mean PP2Ac/ $\beta$ -actin ratio  $\pm$  SEM:  $2.96 \pm 0.97$  vs.  $2.24 \pm 0.36$ ;  $P = 0.3$ ). As shown in Figure 8A, stimulated T cells from patients with SLE produced decreased amounts of IL-2 compared with normal subjects (mean value  $\pm$  SEM:  $362.22 \pm 98.35$  pg/ml vs.  $665.12 \pm 69.77$  pg/ml, respectively;  $P = 0.03$ ). In contrast, SLE T cells that were transfected with PP2Ac-siRNA produced upon stimulation similar amounts of IL-2 compared with normal T cells ( $855.46 \pm 186.5$  pg/ml vs.  $894.18 \pm 75.65$  pg/ml, respectively;  $P = 0.9$ ). Of note, transfection with PP2Ac-siRNA resulted in the significant increase in IL-2 concentration in culture supernatants in both groups ( $P = 0.05$ ). Moreover, PP2Ac-siRNA-transfected T cells that were not stimulated did not upregulate the production of IL-2, while T cells that were transfected with control siRNA and subsequently stimulated produced IL-2 at levels similar to those observed in nontransfected activated T cells (data not shown). In addition, because PP2A structural subunit A (PP2Aa) has been reported to be involved in the regulation of IL-2 through interaction with the cytoplasmic tail of CTLA4 (33), we examined whether PP2Ac-siRNA treatment affected PP2Aa



**Figure 5**

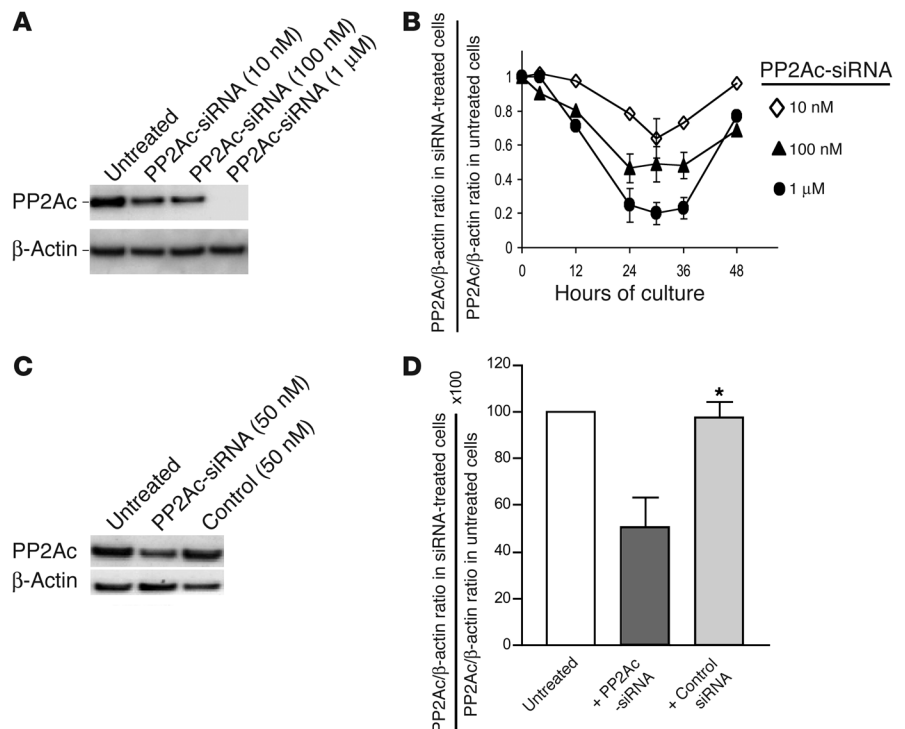
PP2Ac mRNA is increased in lupus T cells and correlates with PP2Ac protein levels. T cell PP2Ac mRNA levels were assessed using real-time RT-PCR as described in Methods. PP2Ac protein levels were determined by Western blotting. (A) Results from a representative experiment with 1 SLE (L1) patient and 1 normal subject (N1) studied in parallel. PP2Ac (upper panel) and β-actin (lower panel) mRNA levels were assessed simultaneously (PP2Ac mRNA, L1 threshold cycle [Ct]: 24.34, N1 Ct: 26.88; β-actin mRNA, L1 Ct: 26.25, N1 Ct: 26.07). (B) Cumulative data of PP2Ac-mRNA levels from 12 normal subjects and 19 patients with SLE. All values are depicted as well as the mean value ± SEM. (C) Levels of PP2Ac mRNA are plotted against PP2Ac protein levels. Results from linear regression analysis are also shown.

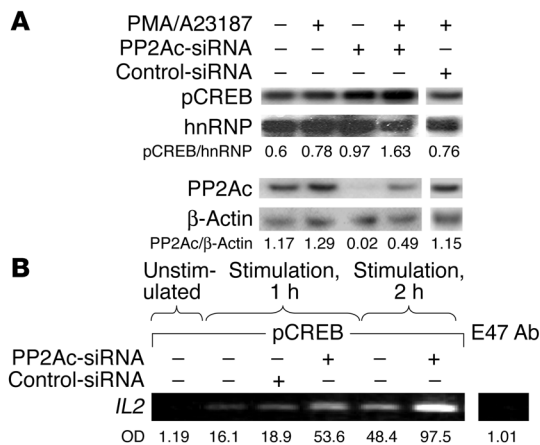
expression. As shown in Figure 8B, T cells treated with different doses of PP2Ac-siRNA downregulated the expression of PP2Ac but not of PP2Aa. These findings confirm that increased IL-2 production following siRNA-induced downregulation of PP2Ac was not a nonspecific, siRNA-related event. Our data indicate that the increased levels of active PP2Ac found in T cells from patients with SLE impair the production of IL-2.

To verify the negative role of PP2A in the regulation of IL-2 production, we used additional approaches to modulate its enzymatic activity. First, we used okadaic acid (OA) in stimulated T cell cultures. OA interrupts the interaction between the catalytic (C) and the structural (A) subunit of PP2A and thus abrogates the activity of the phosphatase (50). Although OA is a potent inhibitor of phosphatases, in low doses it is considered to selectively target PP2A

**Figure 6**

Downregulation of PP2Ac expression using RNA interference. Primary T cells were transfected with siRNAs that target PP2Ac. Protein levels of PP2Ac, assessed with Western blots, were examined in time- and dose-response experiments. Cells were also transfected with an irrelevant nonsilencing siRNA to control for nonspecific effects. (A) Representative experiment showing the effect of different doses of PP2Ac-siRNA on PP2Ac protein levels in normal T cells cultured for 30 hours. (B) Time/dose-response curves of PP2Ac downregulation following transfection with PP2Ac-siRNA. (C) Expression of PP2Ac in T cells transfected with either PP2Ac-siRNA or control siRNA. Results from a representative experiment are shown. (D) Cumulative data from 5 independent experiments showing the degree of PP2Ac downregulation following PP2Ac-siRNA and as compared with cells that were either untreated or transfected with control siRNA. \*P = 0.05 when compared with PP2Ac-siRNA-treated T cells.





**Figure 7**

Suppression of PP2Ac in SLE T cells enhances pCREB expression and pCREB binding to the *IL2* promoter. T cells from patients with SLE were transfected with either PP2Ac- or control siRNA. Following 30 hours of culture, cells were stimulated for 1–2 hours with PMA/A23187. **(A)** Western blot analysis of pCREB and PP2Ac expression in nuclear and total cellular protein extracts, respectively. Heterogeneous ribonucleoprotein (hnRNP) and β-actin were used as controls (pCREB/hnRNP and PP2Ac/β-actin ratios are noted below the corresponding panels). Results from a representative of 5 similar experiments are shown. **(B)** ChIP assay performed as described in Methods. Anti-pCREB-immunoprecipitated DNA, derived from cells manipulated as described above, were subjected to PCR using primers specific for the -180 binding site of the *IL2* promoter (OD values corresponding to each sample are noted below the panel). Results from a representative of 3 similar experiments are shown.

rather than other Ser/Thr phosphatases (50). We found that in the presence of low doses of OA (5 nM), the production of IL-2 by stimulated human T cells was substantially increased (Figure 8C).

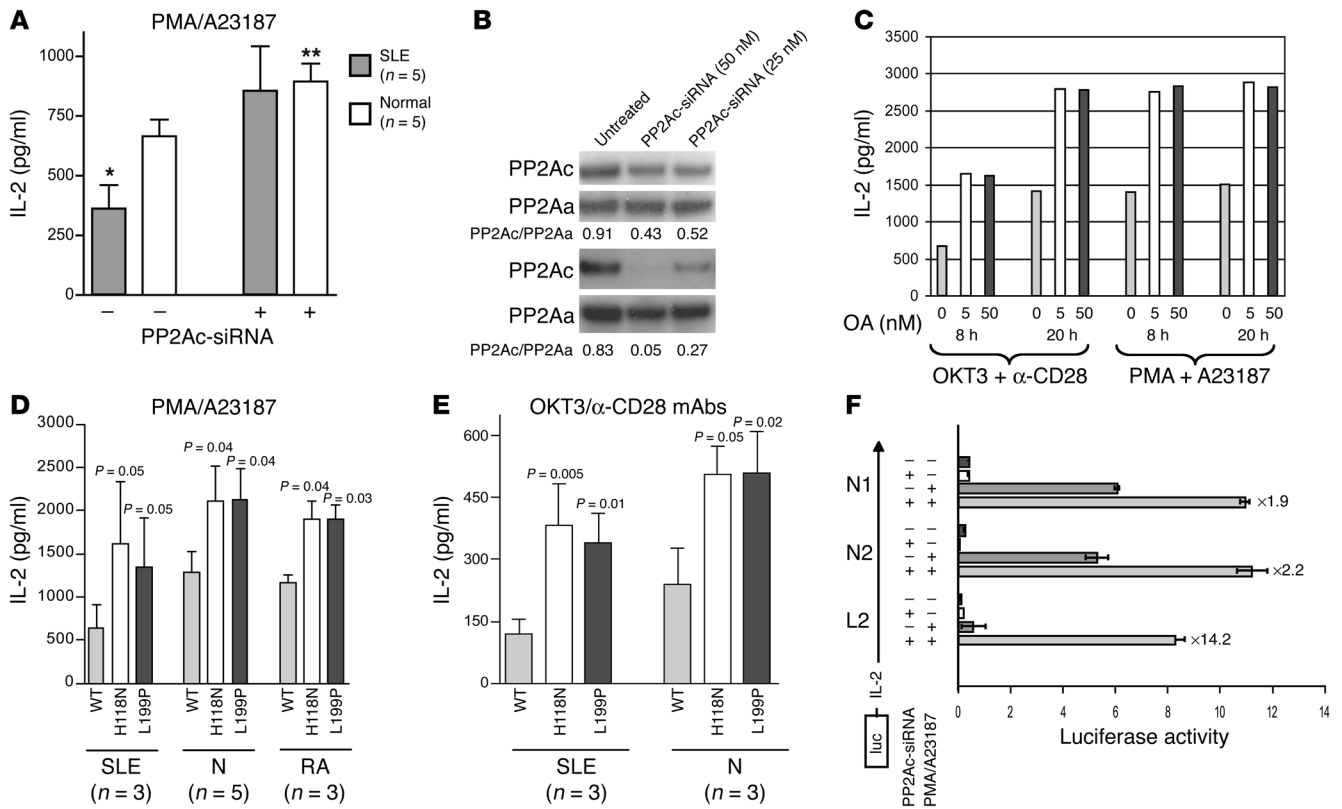
Second, in order to inhibit PP2Ac activity using an additional specific approach, T cells were transfected with either H118N or L199P, 2 different PP2Ac mutant plasmids previously shown to encode dominant negative (DN) forms of PP2Ac (45, 51). T cells were subsequently stimulated with either PMA/A23187 or plate-bound OKT3/anti-CD28 mAbs, and IL-2 production was assessed in culture supernatants. Six patients with SLE, 8 healthy subjects, and 3 patients with RA were studied. As shown in Figure 8, D and E, in all study groups, transfection of either PP2Ac mutant resulted in a significant increase in IL-2 production compared with that observed in T cells transfected with the wild-type PP2Ac. It should be noted that downregulation of PP2Ac with OA or transfection with inactive forms of PP2Ac resulted in increased production of IL-2 in T cells stimulated either with OKT3/anti-CD28 (Figure 8, C and E) or PMA/A23128, an activation method that bypasses the early TCR/CD3-initiated signaling events (Figure 8, C and D). While T cells from healthy individuals and patients with RA upregulated the production of IL-2 in a similar manner (1.7- and 1.6-fold increase, respectively; Figure 8D), transfection of SLE T cells with PP2Ac mutant forms resulted in a higher degree of IL-2 upregulation (2.26-fold increase; Figure 8D). Overall, these results confirm that downregulation of PP2Ac results in increased IL-2 production.

Increased binding of pCREB to the *IL2* promoter in the presence of reduced amounts of PP2Ac suggests that the upregulation of IL-2 is due to increased transcription. To determine the effect of PP2Ac on the activity of *IL2* promoter, T cells were transfected with a plasmid encoding an *IL2* promoter luciferase construct or a corresponding empty vector. A β-galactosidase plasmid was cotransfected to control for transfection efficiency. At the same time, cells were subjected to transfection with either PP2Ac- or control siRNA. Following 24 hours of culture, cells were stimulated for an additional 1 hour with PMA/A23187. As shown in Figure 8F, in the absence of PP2Ac-siRNA, the activity of the *IL2* promoter in lupus T cells was impaired compared with that in normal T cells. Nevertheless, when PP2Ac-siRNA was cotransfected, SLE T cells displayed a dramatic upregulation of *IL2* promoter activity to levels comparable to those of normal T cells.

*PP2Ac-siRNA increases AP1 activity in SLE T cells by increasing the binding of pCREB to the c-fos promoter.* The AP1 family of transcription factors consists of hetero- and homodimers of fos and jun proteins,

which are expressed during the early phase of T cell activation (52). AP1, especially the c-fos/c-jun heterodimer, binds to the *IL2* promoter and enhances the production of IL-2 (8, 53). We have recently reported that c-fos upregulation following T cell stimulation is impaired in SLE, leading to decreased AP1 binding activity (12). Because pCREB binds to the *c-fos* promoter and enhances the production of c-fos (54), we postulated that increased levels of pCREB following PP2Ac downregulation should upregulate c-fos and normalize AP1 binding activity in SLE T cells. T cells from patients with SLE ( $n = 3$ ) and normal subjects ( $n = 3$ ) were transfected with PP2Ac-siRNA, and after 24 hours culture they were stimulated for 2 hours. Nuclear extracts were used to assess AP1 binding by EMSA. An AP1 consensus oligonucleotide labeled with <sup>32</sup>P was used (12), and AP1 binding was assessed by densitometry. The AP1 construct used binds specifically AP1, which consists of the c-fos/c-jun heterodimer (12). In stimulated but not transfected T cells, AP1 binding was significantly lower in SLE patients (mean AP1 binding in densitometry units ± SEM: 24,219 ± 932 in SLE vs. 34,840 ± 2,136 in normal subjects;  $P = 0.03$ ) in accordance with our previous results (12). In contrast, when SLE T cells were treated with PP2Ac-siRNA, AP1 binding increased significantly (33,679 ± 914 [densitometry units];  $P = 0.009$  when compared with nontransfected SLE T cells) and reached levels comparable to those observed in stimulated normal T cells. Results from a representative experiment and cumulative data are shown in Figure 9, A and B, respectively. AP1 activity was not affected when cells were transfected with control siRNA. Our data show that increased levels of PP2Ac in SLE T cells suppress the activity of AP1.

To verify that increased AP1 activity following downregulation of PP2Ac is the result of increased pCREB levels, we studied the effect of PP2Ac suppression on pCREB binding to the *c-fos* promoter using ChIP assays. T cells were transfected with either PP2Ac- or control siRNA and then stimulated for 1–3 hours. As shown in Figure 9C, PP2Ac suppression caused increased pCREB expression and in parallel increased binding of pCREB to the *c-fos* promoter (Figure 9D). Binding of pCREB to the *IL2* promoter was also increased, as expected (Figure 9D). Because following stimulation, upregulated c-fos binds to the *IL2* promoter to induce the transcription of *IL2* gene (8, 53), we examined in the same experiments the binding of c-fos to the *IL2* promoter. We found that at 3 hours of stimulation, a small amount of c-fos remained bound to the *IL2* promoter, as expected. In contrast, when PP2Ac was suppressed, c-fos binding was significantly increased (Figure 9D). In agreement with these



**Figure 8**

Downregulation of PP2Ac restores IL-2 production and *IL2* promoter activity in SLE T cells. (A) T cells were transfected with either PP2Ac- or control siRNA. Cells were then stimulated with PMA/A23187 for 3 hours. IL-2 was measured as described in Methods. \**P* = 0.03 and 0.05 when compared with nontransfected normal T cells and with transfected lupus T cells, respectively; \*\**P* = 0.05 compared with nontransfected normal T cells. (B) PP2Ac-siRNA treatment downregulates PP2Ac but not PP2Aa expression. Results from 2 independent experiments with normal T cells are shown (PP2Ac/PP2Aa ratios are noted below the panels). (C) T cells were treated as indicated, and IL-2 was measured. Results from 1 of 2 similar experiments are shown. (D) T cells were transfected with plasmids encoding either the wild-type PP2Ac or DN PP2Ac mutants (H118N or L199P). Twenty hours following transfection, cells were stimulated with PMA/A23187 for 6 hours. *P* values represent the result of paired *t* test analysis. (E) Results from the same experiment as shown in D, except that T cells were stimulated with OKT3/anti-CD3 mAbs for 18 hours. (F) T cells were cotransfected with (a) a plasmid encoding an *IL2* promoter luciferase construct or an empty vector, (b) a  $\beta$ -galactosidase plasmid, and (c) either PP2Ac- or control siRNA. Following 24 hours of culture, cells were stimulated for 1 hour with PMA/A23187. *IL2* promoter activity was assessed as the luciferase activity. Numbers show the fold increase in *IL2* promoter activity when compared with stimulated cells in the absence of PP2Ac-siRNA. Results from 1 of 2 similar experiments are presented.

results, when T cells were transfected with the DN PP2Ac plasmid H118N, pCREB and c-fos binding to the *IL2* promoter increased (Figure 9E). Our data indicate that downregulation of PP2Ac increases AP1 activity in SLE T cells by increasing the binding of pCREB to the *c-fos* promoter. At the same time, c-fos binding to the *IL2* promoter is increased, suggesting that increased AP1 activity in SLE T cells following the suppression of PP2Ac contributes in the induction of IL-2 production described above.

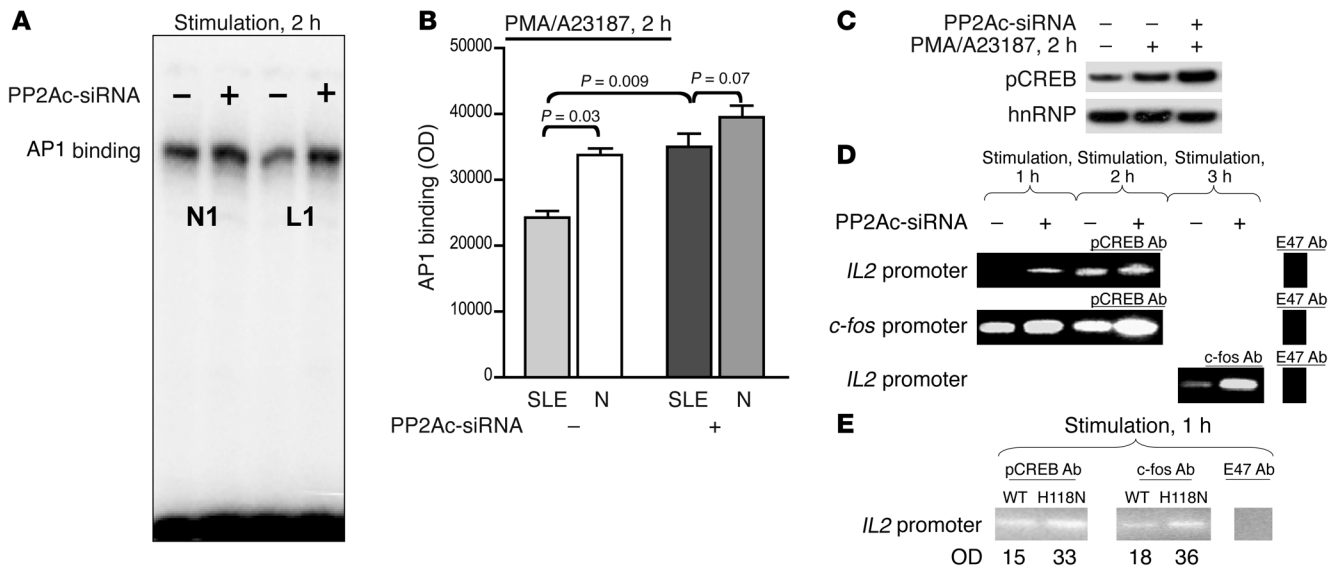
*SLE sera do not upregulate PP2Ac expression in normal T cells.* We have recently reported that SLE serum IgG can suppress the production of IL-2 in normal T cells (55). Because PP2A is regulated by a number of different signaling pathways initiated by extracellular stimuli (56), we considered whether increased expression of PP2Ac in SLE T cells represents the effect of SLE sera. Normal T cells were cultured in medium supplemented with sera derived from normal subjects (*n* = 9) or from patients with SLE (*n* = 9) as previously described (55), and PP2Ac expression was examined by Western blotting. PP2Ac expression in the lysates of T cells incubated with

SLE or normal sera was comparable (*P* = 0.46) (data not shown). In addition, we found that when freshly isolated SLE T cells (*n* = 4) were rested overnight in the absence of SLE serum, PP2Ac expression did not change (data not shown). These results reveal that serum factors present in the sera of SLE patients are not responsible for PP2Ac overexpression in SLE T cells.

**Discussion**

The present study demonstrates that the message, protein, and catalytic activity of PP2Ac are increased in human SLE T cells. Furthermore, it shows that specific downregulation of PP2Ac in SLE T cells with PP2Ac-siRNA results in increased binding of pCREB to the *IL2* promoter and increased AP1 binding activity, causing normalization of IL-2 production. PP2Ac protein levels and activity are upregulated in SLE T cells from patients with active and inactive disease, regardless of the type of treatment. The expression of PP1, a closely related phosphatase, is not altered, suggesting that SLE T cells do not have a generalized phosphatase abnormality. These facts, along with the





**Figure 9** PP2Ac-siRNA increases AP1 activity in SLE T cells by increasing the binding of pCREB to the *c-fos* promoter. (A and B) PP2Ac-siRNA increases AP1 activity in SLE T cells. An AP1 binding [<sup>32</sup>P]-radiolabeled oligonucleotide was used in EMSA, where AP1 binding activity was assessed in nuclear extracts derived from cells that were transfected in the presence or absence of PP2Ac-siRNA and subsequently stimulated for 2 hours. Results from a representative experiment with 1 SLE patient and 1 normal subject are shown (A). (B) Mean ± SEM of AP1 binding from 3 patients with SLE and 3 normal subjects examined in parallel are displayed. AP1 binding was quantitated by densitometry of autoradiographed bands. P values are also shown. (C and D) PP2Ac-siRNA-mediated pCREB upregulation parallels increased binding of pCREB to the *c-fos* promoter and increased binding of both pCREB and *c-fos* to the *IL2* promoter. Normal T cells were transfected with either PP2Ac- or control siRNA and then stimulated with PMA/A23187 for 1–3 hours. pCREB expression was assessed with Western blots (C), and in vivo DNA binding of pCREB and *c-fos* was examined using ChIP assays (D) as described in Methods. Anti-E47 immunoprecipitates were used as negative control. Results from a representative of 3 similar experiments are shown. (E) T cells were transfected with wild-type PP2Ac or H118N and then stimulated with PMA/A23187 for 1 hour. DNA binding of pCREB and *c-fos* to the *IL2* promoter was examined with ChIP assays (OD values corresponding to each sample are noted below the panel). Results from 1 of 2 similar experiments is shown.

observation that RA T cells do not express increased levels of PP2Ac and that SLE sera cannot induce the expression of the enzyme in normal T cells, suggest that PP2Ac overexpression represents an intrinsic SLE T cell abnormality and not a secondary phenomenon related to disease activity, treatment, or extracellular factors.

Increased PP2Ac protein correlated with increased expression of PP2Ac mRNA, suggesting either increased transcription of the PP2Ac gene or increased stability of the corresponding mRNA. Increased *IL-6* mRNA stability has been reported previously in patients with SLE (57). In living cells, PP2A is active only as a heterotrimeric molecule (36); the association of the catalytic subunit with the structural and regulatory subunits is a decisive factor for the activity of the enzyme (58). The parallel study of PP2Ac enzymatic activity and PP2Ac protein levels suggests that increased activity of PP2Ac in SLE T cells derives from increased protein levels of the enzyme.

PP2A has been previously shown to suppress (27–32) or increase the expression of *IL-2* (33–35). This discrepancy is probably due to different experimental settings and/or methods used to determine PP2A function. The role of PP2A in the regulation of *IL-2* production was examined using either OA or polyomavirus middle tumor (middle T) antigen, which bind to PP2A and abrogate its activity (50, 59). Because OA affects other phosphatases such as PP1, PP4, PP5, and PP6 (50), and because middle T antigen also binds to a variety of other signaling molecules such as proteins containing SH2 domains or the src family of protein kinases (59), these studies have not provided conclusive results. In one study, though, where PP2A activity in Jurkat T cells was specifically targeted with a DN mutant of PP2A,

the anti-CD28 antibody-initiated production of *IL-2* was significantly enhanced (28). Nonetheless, it is possible that in response to different stimuli, diverse PP2A holoenzymes may form that affect *IL-2* regulation differentially (58). In this study, we reduced PP2Ac levels in peripheral blood T cells using a PP2Ac-siRNA in a specific manner. Nevertheless, because the use of siRNA has been criticized as having unpredicted effects on the recipient cells, the effect of DN forms of PP2Ac in the production of *IL-2* was also tested. Overall, using 3 different approaches to inhibit PP2Ac function — that is, PP2Ac-siRNA, PP2Ac DN constructs, and OA — we have confirmed the role of PP2A as a suppressor of *IL-2* production.

We demonstrate that in SLE T cells, overexpression of active PP2Ac suppresses the expression of *IL-2* through the downregulation of pCREB expression. We have recently proposed a 2-step model for the transcriptional regulation of *IL-2* by CREB and CREM (15). During the first step, T cell stimulation results in phosphorylation of CREB, which in turn recruits p300 and CBP and binds to the -180 site on the *IL2* promoter. Other factors like AP1 and NF-κB, which also recruit CBP and p300, bind to the *IL2* promoter and together with pCREB form an enhanceosome that associates with the RNA polymerase II complex and activates the transcription of *IL-2* mRNA and the production of protein (8, 60–62). In the second step of the T cell activation, *CREM* mRNA is induced, and the produced CREM protein binds to the *IL2* promoter, gradually replacing pCREB and terminating *IL-2* production (15, 63). It appears that in SLE T cells, increased PP2Ac activity leads to defective formation of pCREB and impaired transcriptional activation



of the *IL2* promoter. In addition, decreased levels of pCREB permit CREM to occupy their common -180 binding site on the *IL2* promoter and directly suppress *IL-2* production (15, 63). Furthermore, decreased pCREB affects *IL2* gene transcription through the downregulation of *c-fos*, which results in lower AP1 activity. This is in agreement with our previous studies, which showed impaired AP1 activity in SLE T cells (12). PP2A could also affect AP1 activity by altering the phosphorylation status of *c-fos* and *c-jun* (64).

Although PP2A may directly dephosphorylate pCREB (16), it is also likely that it affects CREB phosphorylation because it counteracts the function of kinases that lead to the formation of pCREB, such as PKA, PKC, ERK1, and CaM-dependent kinase II (CaMKII) and CaMKIV (65). Consequently, increased PP2A may contribute to the defective function of PKA, PKC, and ERK1 in SLE T cells (66–68), leading to impaired activation of CREB. We have recently reported that SLE T cells display increased activity of CaMKIV (55). CaMKIV phosphorylates and activates both CREB and CREM. We showed that in SLE T cells, CaMKIV upregulates CREM and suppresses *IL-2*. The reason(s) why increased CaMKIV preferentially promotes the function of CREM in SLE T cells is unclear, as is the role of PP2A in regulating CaMKIV. It should be noted, though, that while PP2A directly dephosphorylates and inactivates CaMKIV (23), at the same time it inactivates PKA, PKC, and CaMK II (65), which also regulate CaMKIV function in a negative manner (69–71). The specific targets of the PP2A holoenzymes that are formed probably determine the net result of the multiple interactions of PP2A in SLE T cells. Altered kinetics of these interactions may also have a profound impact on rewiring the activation process in SLE T cells.

Similar to PP2A, a small number of tyrosine phosphatases have been implicated in the negative regulation of *IL-2* production (72–80). Among these phosphatases, only PTEN and SHP-1, which were also shown to reduce *IL-2* production (76, 78), have been linked to autoimmune phenomena. Specifically, mice carrying *PTEN* gene mutations present impaired Fas-mediated apoptosis and develop autoimmune disease (81), while SHP-1-deficient mice produce pathogenic autoantibodies and suffer from lupus-like glomerulonephritis (76). In addition, autoimmune phenomena are documented in patients with Cowden syndrome, a disease caused by germline *PTEN* mutations (82). Nevertheless, no abnormalities of the expression and function of these phosphatases have been demonstrated in patients with SLE. On the other hand, CD45, a transmembrane tyrosine phosphatase, which has been implicated in the molecular aberrations in SLE (83, 84), exerts diverse effects on *IL-2* production (75, 85–87). In particular, T cell lines that do not express cell surface CD45 cannot generate TCR signals (86) and thus cannot produce *IL-2*, while coligation of CD45 with the antigen receptor inhibits *IL-2* production (87). The role of CD45 in *IL-2* regulation in SLE T cells has not been studied.

We have studied the role of PP2Ac in the regulation of *IL-2* in SLE T cells. Nevertheless, increased expression of PP2Ac may have broader consequences in the function of SLE T cells. The available information on PP2A function comes from studies that describe the effects of PP2A suppression. The opposite approach, that is, the stable overexpression of PP2A, has been proven to be largely unsuccessful due to the stringent control of PP2A at both the transcriptional and the translational levels (49, 88). In addition, paradigms of PP2A dysregulation in human disease identify abnormalities related to impaired function of the enzyme (41, 42). SLE is the first disease where increased PP2A expression and function are documented. Our data are in agreement with previous reports

showing that T cells derived from the lupus-prone MRL/lpr mice lymphoid tissues display increased activity of PP2A (89, 90). Only recently, a transgenic mouse was generated to express high levels of PP2Ac in the myocardial tissue (44). A similar approach targeting T lymphocytes could provide valuable information for the role of PP2A in the immune response. PP2A is recognized as a major protein kinase phosphatase (65). In particular, PP2A has been shown to modulate the activities of ERK/MAPKs, CaMKs, JNK, PKA, -B, and -C, as well as I $\kappa$ B kinases resulting in the regulation of the activity of numerous transcription factors such as AP1, NF- $\kappa$ B, CREB, Sp-1, and STAT3 and -6 (64). Notably, the specific effects of PP2A vary considerably depending on the target molecule and the cell type examined. In the same context, the broader role of PP2Ac overexpression in SLE T cells should be defined along with the characterization of the holoenzymes that PP2Ac forms in SLE T cells as well as the identification of the respective targets.

In summary, our data document increased activity of PP2Ac as a novel SLE disease-specific abnormality and defines a distinct mechanism whereby it represses *IL-2* production. We propose that PP2A-siRNA may represent a novel tool to correct T cell *IL-2* production and limit disease activity.

## Methods

**Study subjects.** Forty-four female patients with SLE, 24–71 years old, fulfilling at least 4 of the 11 American College of Rheumatology classification criteria for SLE, were studied. Thirty-two patients were on hydroxychloroquine, 29 on prednisone (1–60 mg/day), 6 on azathioprine, 7 on mycophenolate mofetil, 2 on methotrexate, and 1 on cyclophosphamide, and 2 patients were receiving no treatment. SLEDAI ranged between 0 and 14. Fifteen patients with RA, aged 37–80, were also evaluated. Patients receiving prednisone were examined at least 24 hours following their last dose. Fifty-three healthy female subjects, 21–64 years old, were analyzed in parallel. The institutional review boards of the institutions involved (Washington Hospital Center, Walter Reed Army Institute of Research, and Uniformed Services University of the Health Sciences) approved the study protocol, and informed consent was obtained from all study subjects.

**Antibodies.** Antibodies against PP2Ac, PP2Aa,  $\beta$ -actin, PP1, CREB, pCREB, and *c-fos* were purchased from Upstate. Anti-rabbit HRP, anti-goat HRP, anti-E47, and normal mouse IgG Abs were purchased from Santa Cruz Biotechnology Inc. Rabbit anti-hnRNP serum was used in Western blots, as previously described (91). OKT3/anti-CD3 Ab was purchased from Ortho-McNeil Pharmaceuticals Inc. The anti-CD28 Ab was purchased from BD Biosciences.

**T cells.** T cells were purified from peripheral venous blood using a rosette T cell purification kit (StemCell Technologies) as previously described (12). Purified cells were greater than 98% CD3 positive, as flow cytometry analysis disclosed. Cells were then either directly lysed to extract protein and RNA or cultured. Sera were also collected and frozen at  $-70^{\circ}\text{C}$  until used. Sera were heat inactivated at  $56^{\circ}\text{C}$  for 30 minutes and centrifuged before use.

**Cell cultures.** T cells ( $1 \times 10^6$  to  $2.5 \times 10^6$ ) were cultured in RPMI 1640 medium, 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (full RPMI) at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , in 24- or 6-well plates. In some experiments, sera from lupus or healthy subjects were added to the medium. In some cases, cells were harvested after an overnight rest. In a few experiments, T cells were directly stimulated in cultures containing OA (5 or 50 nM). In the majority of experiments, transfection of cells was performed prior to plating as described below. After 4–48 hours of culture, transfected cells were either collected or stimulated for an additional 1–18 hours. T cells were stimulated with either PMA (10 ng/ml) and calcium ionophore A23187 (0.5  $\mu\text{g}/\text{ml}$ ) or OKT3/anti-CD3 Ab (5  $\mu\text{g}/\text{ml}$ ) and anti-CD28 Ab (1  $\mu\text{g}/\text{ml}$ ). Cells were used for protein



extraction, ChIP, or luciferase assays. Culture supernatants were collected and kept at  $-20^{\circ}\text{C}$  for the quantification of IL-2.

**IL-2 ELISA.** The concentration of IL-2 in culture supernatants was determined using the Quantikine Immunoassay kit (R&D Systems) according to the manufacturer's instructions.

**siRNA, plasmids, and T lymphocyte transfection.** Duplexes of 21-nucleotide siRNA with two 3'-TT ends were synthesized by QIAGEN. Combinations of 3 siRNA targeting different positions within the  $\beta$  isoform of human PP2Ac mRNA (PP2Ac-siRNA) were used. The sense strands were: AUGUGCAAGAGGUUCGUUG, UGUCUGCGAAAGUAUGGGA, and UUGGUGUCAUGAUCGAAU. A nonsilencing siRNA was included as control (control siRNA) (sense strand: AATTCTCCGAACGTGTCACGT). Transfection was carried out by electroporation using the Nucleofection System (Amaxa), according to the manufacturer's protocols. Final siRNA concentrations ranged from 10 to 1,000 nM. Electroporation was also used to transfect plasmids encoding the wild-type PP2Ac and the PP2Ac mutants H118N and L199P (kindly provided by B.A. Hemmings, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland). Two micrograms of each plasmid were used per transfection ( $3 \times 10^6$  to  $5 \times 10^6$  cells). Following transfection, cells were cultured as described above. In some experiments, a plasmid encoding an IL2 promoter luciferase construct or a corresponding empty vector and a plasmid encoding  $\beta$ -galactosidase were cotransfected (5:1 ratio) in the presence or absence of siRNA. The total amount of plasmids used in each sample was 5  $\mu\text{g}$ . Following 24 hours culture and subsequent 1-hour stimulation, the activity of the IL2 promoter was examined using a luciferase assay.

**Luciferase assay.** Cytoplasmic extracts were prepared using a luciferase assay kit (Promega), and luminescence was measured immediately for 30 seconds using a TD20/20 luminometer (Promega). The luciferase activity was normalized using the  $\beta$ -galactosidase readings.

**ChIP assay.** ChIP assays were performed as previously described (15). Three to 5 million T cells were used per investigated Ab. The DNA was amplified with primers flanking the IL2 promoter, including the -180 site (forward sequence, 5'-ATGCTCACGAGATTAGGACACG-3'; reverse sequence, 5'-TGTAACACTGTGGGGGT-3'), or with primers flanking the *c-fos* promoter, including the -57 site (forward, 5'-ATGCTCACGAGATTAGGACACG-3'; reverse, 5'-GCTGCAGATGCGGTTGGAGT-3'). PCR products were separated on a 1.0% agarose gel, and the OD of each band was quantitated using Quantity One software (Bio-Rad Laboratories).

**EMSA.** Nuclear extracts (3  $\mu\text{g}$ ) were incubated with a [ $^{32}\text{P}$ ]-radiolabeled AP1 dsDNA probe and 1  $\mu\text{g}$  of poly(dI/dC) in the binding buffer for 15 minutes at room temperature. The reaction mixture was then subjected to separation in 6% nondenaturing gel (Invitrogen Life Technologies). The dried gel was then autoradiographed. The AP1 consensus-binding sequence was: forward, 5'-CGCTTGATGACTCAGCCGGAA-3'; reverse, 5'-TTCCGGCTGAGTCATCAAGCG-3'.

**Protein purification.** To prepare whole-cell protein lysates, cells ( $3 \times 10^6$  to  $5 \times 10^6$ ) were incubated on ice with 200–400  $\mu\text{l}$  lysis buffer (10 mM Tris, pH 7.9), 50 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 supplemented with freshly added proteases inhibitors [1 mM 4-(2-aminoethyl) butylsulfonyl fluoride, 2 mM aprotinin, 1 mM leupeptin, 5 mM NaF, and 1 mM  $\text{Na}_3\text{VO}_4$ ] and DTT (1 mM), for 40 minutes. Following 5 minutes centrifugation at 20,000 g, supernatants were stored at  $-80^{\circ}\text{C}$ . Protein extracts used in phosphatase activity assays were prepared following the same procedure, with the lysis buffer adjusted to pH 7.0 and without the addition of the Ser/Thr phosphatase inhibitor NaF. Cytoplasmic and nuclear protein extracts were prepared as previously described (12). Protein concentration was measured using the Bradford reagent (Bio-Rad Laboratories) and standardized with BSA.

**Western blotting.** Cellular proteins were separated in 4–12% BisTris-NuPAGE gels according to the manufacturer's protocols (Invitrogen

Corp.). The proteins were transferred onto PVDF and blotted with various antibodies as indicated. Protein bands were detected by enhanced chemiluminescence reagents (Amersham Biosciences), and the density of each band was calculated with Quantity One software.

**Reverse transcription and real-time PCR.** RNA extracted from T cells ( $1 \times 10^6$ ) (RNEasy Mini Kit; QIAGEN) was treated with DNase I (QIAGEN) and quantitated. Total RNA (300 ng) was transcribed in cDNA in a conventional thermocycler using AMV reverse transcriptase and oligo-dT primer (RT-PCR kit; Promega). Real-time PCR was conducted with a Cepheid Smart-Cycler thermocycler by adding SYBR green to the reaction mixture. Primers used (Sigma-Aldrich) were: PP2Ac forward 5'-ATGTGCAAGAGGTTCCGTTGC-3', reverse 5' AGTATGTCTATGGATGGAGA-3'; and  $\beta$ -actin forward 5'-CATGGGTCAGAAGGATTCT-3', reverse 5'-AGCTGGTAGCTCTTCTCA-3'. Real-time PCR products were quantitated as previously described (12). Additional details are provided in the Supplemental Figure (available online with this article; doi:10.1172/JCI24895DS1).

**PP2Ac enzymatic activity determination.** PP2A enzymatic activity was assessed following PP2Ac immunoprecipitation using a malachite green-based phosphatase assay (PP2A Immunoprecipitation Phosphatase Assay Kit; Upstate). To preclear protein extracts, protein A agarose slurry was added, followed by rotation at  $4^{\circ}\text{C}$  for 1 hour. Samples were centrifuged to discard the beads, and 100  $\mu\text{g}$  of total cellular protein was incubated with either protein A agarose slurry alone or in the presence of an anti-PP2Ac Ab (clone 1D6; Upstate) or a control normal mouse IgG Ab (Santa Cruz Biotechnology Inc.) at  $4^{\circ}\text{C}$  with constant rocking for 2 hours. Both Abs were used at 4  $\mu\text{g}$ /sample. Agarose-bound immune complexes were collected and, following intense washing with 700  $\mu\text{l}$  TBS (7 times) and 500  $\mu\text{l}$  optimized Ser/Thr buffer (final wash), were resuspended in 20  $\mu\text{l}$  Ser/Thr buffer. An appropriate phosphopeptide (amino acid sequence: K-R-pT-I-R-R) was added as a substrate for PP2Ac, and samples were incubated at  $30^{\circ}\text{C}$  in a shaking incubator for 10 minutes. Supernatants (25  $\mu\text{l}$ ) were transferred in 96-well plate, and released phosphate was measured by adding 100  $\mu\text{l}$  malachite green phosphate detection solution. Color developed for 15 minutes before reading the plate at 650 nm. The absorbance of the reactions was corrected by subtracting the absorbance in samples treated with anti-IgG Ab. Phosphate concentrations were calculated from a standard curve created using serial dilutions of a standard phosphate solution (0–2,000 pmol).

**Statistics.** Data are presented as mean value  $\pm$  SEM. Where mentioned, the paired or otherwise the unpaired 2-tailed *t* test with Welch correction, linear regression ( $r^2$ ), and the Pearson product moment correlation coefficient were used for statistical analysis (SPSS software, version 12.0; SPSS Inc.). Statistical significance was defined as  $P < 0.05$ .

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