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## Decreased SAP expression in T cells from patients with SLE contributes to early signaling abnormalities and reduced IL-2 production

Maria P. Karampetsou<sup>\*</sup>, Denis Comte<sup>\*,†</sup>, Katalin Kis-Toth<sup>\*</sup>, Cox Terhorst<sup>‡</sup>, Vasileios C. Kyttaris<sup>\*</sup>, and George C. Tsokos<sup>\*</sup>

<sup>\*</sup>Division of Rheumatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA <sup>†</sup>Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA <sup>‡</sup>Division of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois, Rue du Bugnon 46, CH 1011 Lausanne, Switzerland

### Abstract

T cells from patients with systemic lupus erythematosus (SLE) display a number of functions including increased early signaling events following engagement of the T cell receptor (TCR). Signaling lymphocytic activation molecule family (SLAMF) cell surface receptors and the X-chromosome-defined signaling lymphocytic activation molecule-associated protein (SAP) adaptor are important in the development of several immunocyte lineages and modulating immune response. Here we present evidence that SAP protein levels are decreased in T cells and in their main subsets isolated from 32 women and 3 men with SLE independently of disease activity. In SLE T cells the SAP protein is also subject to increased degradation by a caspase-3. Forced expression of SAP in SLE T cells simultaneously heightened IL-2 production, calcium (Ca<sup>2+</sup>) responses and tyrosine phosphorylation of a number of proteins. Exposure of normal T cells to SLE serum IgG, known to contain anti-CD3/TCR antibodies, resulted in SAP downregulation. We conclude that SLE T cells display reduced levels of the adaptor protein SAP probably as a result of continuous T cell activation and degradation by caspase-3. Restoration of SAP levels in SLE T cells corrects the overexcitable lupus T cell phenotype.

### Introduction

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease of unknown etiology that mainly affects women of reproductive age. Clinical symptoms may vary from relatively mild to severe life-threatening manifestations involving vital organs including the kidneys, lungs and the central nervous system (CNS) <sup>(1)</sup>. Multiple cellular and molecular aberrations have been claimed to be associated to the immunopathogenesis of the disease <sup>(2)</sup>. A number of loci across the whole genome and especially the long arm of chromosome 1, where also the genes for the signaling lymphocytic activation molecule

family (*SLAMF*) of cell surface receptors reside, have been identified [reviewed in <sup>(3)</sup> and <sup>(4)</sup>]. Associations between *SLAMF* variants and autoimmune diseases, including SLE <sup>(5)</sup> <sup>(6)</sup> and rheumatoid arthritis <sup>(7)</sup>, have been described. Furthermore, *SLAMF* variants have been linked to specific disease manifestations such as neuropsychiatric lupus <sup>(8)</sup> or response to TNF blockade in patients with rheumatoid arthritis <sup>(9)</sup>.

The expression of *SLAMF7* on B cells and of *SLAMF4* on NK and CD8+ T cells is altered in patients with SLE <sup>(10)</sup> <sup>(11)</sup> and *SLAMF3* and *SLAMF6* are also reportedly increased on the cell surface of SLE CD4+ T cells <sup>(12)</sup> <sup>(13)</sup>. The importance of the *SLAMF* cell surface receptors in autoimmunity is further underscored by the finding that the lupus prone congenic mouse strains B6.*Sle1b* [*Sle1b*] and B6.129*chr1b* [129chr1b] develop autoantibodies linked to polymorphisms of *SLAMF1-7* molecules <sup>(14)</sup> <sup>(15)</sup>. *SLAMF3*-deficient mice (129xBALB/c) spontaneously develop autoimmune characteristics including autoantibodies against nuclear antigens, aberrant cytokine production and splenomegaly <sup>(16)</sup> and *SLAMF1*, 5 and 6 serve as negative regulators of humoral immune response <sup>(17)</sup>.

One of the characteristic features of the *SLAMF* members (with the exception of *SLAMF2* and *SLAMF8-9*) is the presence of one or more immunoreceptor tyrosine switch motifs (ITSM), which upon *SLAMF* engagement, interact with high affinity with the signaling lymphocytic activation molecule-associated protein (SAP, SH2D1A). SAP is a highly conserved, non-polymorphic cytoplasmic protein, predominantly expressed in T cells, NK, NKT cells, eosinophils and platelets. SAP has been shown to be requisite for germinal center formation and hence for both normal humoral responses and autoantibody production <sup>(18)</sup> <sup>(19)</sup> <sup>(20)</sup> <sup>(21)</sup>. Although SAP is considered to act as a natural competitor of SH2-containing phosphatases such as SHP-1 and SHP-2 for binding to the same ITSM motifs <sup>(22)</sup>, subsequent work revealed that it interacts with Fyn <sup>(23)</sup> <sup>(24)</sup>, possibly with Lck <sup>(25)</sup>, b-PIX <sup>(26)</sup> and NCK <sup>(27)</sup> and recruits PKC $\theta$  to the immune synapse <sup>(28)</sup>.

Non-transformed T cell lines from SAP-deficient male subjects display an interesting dichotomous CD3/TCR response with elevated [Ca<sup>2+</sup>]<sub>i</sub> response and decreased production of IL-2 both of which were corrected following replenishment of SAP <sup>(29)</sup>. This pattern of response was reminiscent of that observed in SLE T cells <sup>(30)</sup> <sup>(31)</sup> and prompted us to ask whether SAP expression was altered in SLE T cells.

We show that the expression levels of SAP in T cells from patients with active or inactive SLE are decreased. Following forced expression of SAP both [Ca<sup>2+</sup>]<sub>i</sub> response and IL-2 production return to normal. Caspase-3 appears to degrade SAP in SLE T cells. We also show that SLE-derived IgG reduces the levels of SAP in normal T cells. Although the reduction of SAP in the mostly (~90%) female SLE patients appears to be a secondary defect due to continuous T cell activation, restoration of SAP levels by limiting its degradation may warrant clinical attention.

## Patients and methods

### Patients and controls

Patients (n=35) [32 females and 3 males] fulfilling the American College of Rheumatology criteria for lupus were recruited at the Rheumatology Department at Beth Israel Deaconess Medical Center for the study. 29 age- and sex-matched healthy volunteers were evaluated in parallel. Disease activity score for the patients with SLE was measured using the SLEDAI scoring system. SLEDAI scores ranged between 0 and 16. Demographic and clinical information on the patients with SLE that participated in the study is provided on Supplementary Table S1. Informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

### Reagents and antibodies

Murine anti-CD3 clone OKT3 was used for T cell stimulation and was purchased from BioXcell. Affinity purified goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories Inc. Antibodies against SAP (clone 1D12),  $\beta$ -actin, as well as goat anti-rabbit, goat anti-mouse and donkey anti-goat horseradish-peroxidase (HRP)-conjugated secondary antibodies were all purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The anti-phosphotyrosine HRP-conjugated monoclonal antibody (mAb) (clone 4G10) was from Millipore (Temecula, CA). Cycloheximide (CHX) and actinomycin D were purchased from Sigma Aldrich. Pan-caspase inhibitor Z-Val-Ala-Asp-FMK (VAD), caspase-3 inhibitor Z-Asp-Glu-Val-Asp (VEVD) and MG132 proteasome inhibitor were from Enzo LifeSciences (Farmingdale, NY).

### T cell purification

Heparinized venous blood was obtained from the study subjects and primary T cells were isolated by negative selection (RosetteSep, Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's instructions. The percentage of purified T cells was assessed by flow cytometry and was always over 95%. For some experiments total and naïve CD4<sup>+</sup> T cells from normal donors and patients with SLE were further purified from total T cells by negative selection using the CD4<sup>+</sup> T cell Isolation Kit and the naïve CD4<sup>+</sup> T cell Isolation Kit II (both purchased from Miltenyi) according to the manufacturer's instructions. The positive fractions representing CD8<sup>+</sup> T cells and memory/effector CD4<sup>+</sup> T cells respectively were also collected. Cells were either directly lysed in order to extract protein and total RNA or were kept overnight at 37°C in RPMI-1640 medium supplemented with 10% FBS, 100mg/ml streptomycin and 100U/ml penicillin in order to be used for experiments.

Serum samples were also collected and stored at -80 °C from all participants. Sera were heat-inactivated at 56 °C for 30 min and centrifuged before added to cell cultures.

### Cell cultures

T cell cultures were performed at  $2 \times 10^6$  cells/ml density in 24-well plates in complete RPMI-1640 medium. For protein degradation experiments total T cells were cultured in complete medium supplemented with cycloheximide (CHX) at a final concentration 50 $\mu$ g/ml. Cells were collected at 0, 3, 4 and 5 hours after addition of CHX in T cell culture.

Cell lysates were extracted and analyzed with Western immunoblot. For certain experiments T cells were pretreated with proteasome inhibitor MG132 (final concentration 20 $\mu$ M) for 1h before the addition of CHX in the culture medium. For pan-caspase and caspase-3 inhibition experiments, T cells were cultured in complete medium with 50 $\mu$ M VAD, 50 $\mu$ M VEVD or equal volume of vehicle DMSO in the presence of 50 $\mu$ g/ml CHX. In some experiments sera from patients with SLE or from normal donors were added to cell cultures at 1% concentration for 6h. In other experiments sera were fractionated using the ProteoExtract Albumin/IgG removal kit (Calbiochem) according to the manufacturer's instructions and normal T cells were cultured with 1 $\mu$ g/ml of each fraction in complete medium. Purity of each fraction was always assessed by SDS electrophoresis followed by Coomassie staining.

### Transfections

Transient transfections of purified total T cells were performed using the Amaxa Human T cell nucleofector system (Lonza, Cologne, Germany). Briefly, 5–10 $\times$ 10<sup>6</sup> total T cells were rested overnight in complete RPMI 1640 medium and treated with 1 $\mu$ g/ml PHA at a density of 1 $\times$ 10<sup>6</sup>/ml at 37 °C, in 5% CO<sub>2</sub>. Small interfering silencer RNAs against SAP (siSAP) and non-specific control siRNAs (sicontrol) were purchased from Life Technologies. Plasmid DNA encoding SAP (pISH2D1A) and the control pCMV-XL5 vector (pcDNA) were both from Origene. T cells were resuspended in 100 $\mu$ l of nucleofector solution plus 5 $\mu$ g of plasmid (pISH2D1A or pcDNA) or with various concentrations of siRNA (siSAP or sicontrol) and were transfected. 24h after transfection cells were stimulated with 10 $\mu$ g/ml of soluble anti-CD3 antibody in the presence of 20 $\mu$ g/ml goat anti-mouse mAb for various time points. In every case, transfection efficiency was evaluated by western immunoblots and/or real-time PCR analysis.

### Reverse transcription and real-time PCR

Total RNA was extracted from 3–5 $\times$ 10<sup>6</sup> cells using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Santa Clarita, CA). Single stranded complementary DNA (cDNA) was synthesized from 200ng of total RNA by using the RNA into cDNA premix (Clontech). Real time amplification was performed using SYBR Green (LightCycler 480 SYBR Green I Master, Roche) with 40 cycles at 95°C for 15sec and 60°C for 1min. All PCR reactions were performed in triplicates. Primer sequences were as follows: IL-2, forward, 5'-CAAGAAGGCCACAGAACTGA-3' and reverse 5'-TGGTTGCTGTCTCATCAGCAT-3'; IFN $\gamma$ , forward 5'-CTGTTACTGCCAGGACCCAT-3' and reverse 5'-TCTGTCACTCTCCTCTTTCCA-3'; SAP, forward 5'-GTTCTTGGAGTGCTGAGACAG-3' and reverse 5'-GGCAGACATCAGGATCTTCTCTT-3';  $\beta$ -actin, forward 5'-AGAGCTACGAGCTGCCTGAC-3' and reverse 5'-AGCACTGTGTTGGCGTACAG-3', cyclophilin A forward 5'-TTCATCTGCACTGCCAAGAC-3' and reverse 5'-TCGAGTTGTCCACAGTCAGC-3'.

### Protein extraction and immunoblotting

2 $\times$ 10<sup>6</sup> T cells were incubated for 45min in ice-cold lysis buffer (10mM Tris, 50mM NaCl, 5mM EDTA and 1% Triton X-100) containing various protease and phosphatase inhibitors (50mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 30mM sodium pyrophosphate, 1mM PMSF, 2 $\mu$ g/ml leupeptin,

2 $\mu$ g/ml aprotinin). Lysates were then centrifuged at 12.000 g for 15min at 4°C to remove insoluble material. Protein concentration was determined using the Coomassie protein assay reagent (Sigma Aldrich). Proteins (20 $\mu$ g per lane) were separated in 4 to 12% gradient (wt/vol) Bis-Tris gels (Life Technologies) and transferred to nitrocellulose (Life Technologies) or PVDF (Millipore) membrane. Membranes were blocked for 1h with Tris-buffered saline solution containing 0.05% Tween (TBS-T) and 5% non-fat dry milk at room temperature. Following blocking, membranes were incubated overnight at 4°C with the indicated antibody in blocking solution on a shaking surface and were then washed and incubated with the appropriate secondary HRP-conjugated antibody for 1.5h at room temperature. Detection was performed with the Clarity ECL Western Blotting detection reagents (Biorad) and membranes were visualized by the ChemiDoc XRS<sup>+</sup> Molecular Imager (Biorad). Densitometric analysis was performed using the ImageJ software and results are expressed as densitometric ratios of tyrosine phosphorylated proteins or SAP over  $\beta$ -actin  $\pm$  SEM.

### Calcium responses

Five million transfected T cells were stained for CD4 (FITC) and CD8 (PercP), resuspended in RPMI 1640 supplemented with 1% FBS and were loaded with Indo-1 acetoxymethylester (Life Technologies) for 30min at 37°C. Cells were then washed three times with RPMI 1640 at 1500 rpm for 5min and were finally resuspended in RPMI 1640 supplemented with 1% FBS and 1mM CaCl. Data were acquired on an LSR II flow cytometer (BD Biosciences) and were analyzed using FlowJo version 7.6.5 (Tree Star). Samples were allowed to run unstimulated for 60 sec in order to record a baseline fluorescence ratio representing the concentration of intracellular calcium at the resting state. Cells were then stimulated with 10 $\mu$ g/ml anti-CD3 followed by 20 $\mu$ g/ml goat anti-mouse IgG that were added to the tube and the mean fluorescence ratio of violet to blue Indo-1 fluorescence, that is proportionally representative of the free intracytoplasmic calcium concentration, was recorded for a period of 10min. Stimulation with ionomycin (25 $\mu$ g/ml) was used as a positive control at 7 or 9min.

### Quantitative determination of IL-2 and IFN $\gamma$

Cell culture supernatants were collected and kept at -80°C until ready to be analyzed. IL-2 and IFN $\gamma$  productions were measured by ELISA kits (Elisa Deluxe Max Set, Biolegend). The ODs of the wells were determined using a microplate reader set at 450nm, according to the manufacturer's instructions.

### Statistics

The two-tailed unpaired or paired Student's t-test, one-way ANOVA and two-way ANOVA were used for data analysis, as appropriate (\* $p$  0.05, \*\* $p$  0.01, \*\*\* $p$  0.005). Data are presented as mean  $\pm$  SEM.

## Results

### Silencing of SAP in healthy human T cells results in increased early signaling events and aberrant cytokine production

Non-transformed CD4 T cell lines established from SAP-deficient patients display increased free intracytoplasmic calcium responses following anti-CD3 stimulation but decreased IL-2

production<sup>(29)</sup>. We were able to reproduce this functional phenotype in healthy human T cells (n=4) in which SAP was successfully silenced (Fig 1A). Levels of tyrosine phosphorylated proteins after 1 min of TCR-mediated stimulation were higher in SAP-depleted cells compared to T cells transfected with control siRNA (Fig 1B). Furthermore, TCR-triggered free intracytoplasmic calcium responses were higher in total T cells transfected with SAP siRNA (Fig 1C and 1D). This difference was detected in both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets (Fig 1E). Stimulation of normal T cells, in which SAP had been successfully silenced, with an anti-CD3 mAb SAP resulted in significant decrease of IL-2 mRNA expression production and a 2.5 fold increase in IFN $\gamma$  mRNA levels (Fig. 1F, G and H).

### **Expression of SAP is reduced in peripheral blood T cells from patients with SLE**

Because SAP-deficient T cells and SAP-depleted primary human T cells (Fig 1) display increased early signaling events followed by decreased IL-2 production, we asked whether SAP levels are decreased in patients with SLE. First we determined the protein levels of SAP in SLE (n=25) and normal (n=16) T cell lysates by Western blotting. SAP protein levels were significantly decreased in patients with SLE compared to normal donors (Fig 2A and B). SAP levels were decreased in both patients with active (SLEDAI, 4–8, n= 9) and inactive disease (SLEDAI, 0–3, n= 16) (Fig 2C). In the relatively small study sample we were not able to identify an association between reduced T cell SAP content and specific SLE clinical manifestations and/or laboratory findings. SAP mRNA levels were also measured in patients with SLE and normal donors with real-time PCR. Consistent with the decrease in protein levels, we observed a 2.1-fold reduction in SAP mRNA levels in SLE T cells, suggesting a potential transcriptional and/or post-transcriptional defect that may lead to suppressed expression of SAP in SLE (Fig 2D). Three men with SLE were included in the protein (Fig. 2B) and one in mRNA (Fig. 2D) expression analysis and although all measurements were below the recorded mean values they were not the lowest among the SLE samples.

To determine whether all T cell subsets displayed decreased SAP expression we purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells from the peripheral blood of patients with SLE. We observed that the levels of SAP were significantly lower in SLE CD4<sup>+</sup> T cells compared to T cells from normal subjects. We further purified naïve and effector/memory CD4<sup>+</sup> T cells from 5 patients with SLE and found that both naïve and effector/memory SLE CD4<sup>+</sup> T cells express lower levels of SAP compared to those isolated from healthy sample donors (Fig 3A, C and D). In CD8<sup>+</sup> T cells from patients with SLE the expression of SAP displayed a lower but not statistically significant trend (Fig 3B and E). As a minority of SLE patients presented with normal SAP levels, all subsequent mechanistic studies were performed using T cells from those patients who were established to have low SAP levels.

### **Reconstitution of SAP protein levels normalizes early signal transduction events in SLE T cells**

Because SLE T cells display both increased levels of tyrosine phosphorylated proteins following antigenic stimulation as well as heightened calcium responses, we performed experiments to examine whether reconstitution of SAP protein levels in SLE T cells can



reverse the aberrant phenotype. To this end, we transfected purified T cells from normal donors and patients with SLE either with a control vector (pcDNA) or a plasmid expressing SAP (pISH2D1A). Transfected T cells were treated with soluble anti-CD3 (10 $\mu$ g/ml) or control IgG followed by cross-linking at 37 °C for 60 sec. Levels of tyrosine phosphorylated proteins in pcDNA-transfected SLE T cells were significantly higher compared to control T cells following 1min of anti-CD3 stimulation in a manner similar to that described before (31) (32). However, when SAP was re-introduced in SLE T cells, the overall amount of tyrosine phosphorylated proteins following TCR engagement was comparable to the tyrosine phosphorylation levels that were induced by anti-CD3 stimulation in control pcDNA-transfected normal T cells (Fig 4A and 4B). There were no differences at the baseline levels of tyrosine phosphorylated proteins in unstimulated normal T cells or in resting SLE T cells that were transfected with pcDNA or pISH2D1A (Fig 4B). We then asked whether restoration of SAP protein expression in T cells from patients with SLE could ameliorate the aberrant calcium responses that are known to characterize lupus T cells (30). Indeed, when we re-introduced SAP in SLE T cells, the peak ratio of free intracellular Ca<sup>2+</sup> flux following anti-CD3 cross-linking was lower in SAP-transduced T cells and was comparable to the responses that were elicited in normal T cells (Fig 4C and D). Taken together, our data indicate that reduced SAP protein levels contribute to the hyper-responsiveness exhibited by T cells from patients with SLE and that reconstitution of SAP expression in lupus T cells may reverse, at least *in vitro*, the aberrant overexcitable phenotype.

#### **Forced SAP expression in SLE T cells restores anti-CD3-mediated IL-2 production**

It is well established that despite the hyper-responsiveness that is being manifested by lupus T cells, production of IL-2 following antigenic stimulation is decreased in SLE. IL-2 production in patients with X-Linked Proliferative syndrome (XLP), where SAP is either completely absent or non-functional, is also impaired (33) (29). Therefore, we sought to determine whether reconstitution of SAP protein levels in SLE T cells could restore IL-2 production. Accordingly, we isolated total T cells from patients with SLE or from healthy subjects and transfected them either with a pcDNA or pISH2D1A vector (Fig 5A and B). 24h after transfection T cells were stimulated for 6h with soluble anti-CD3 antibody in the presence of a cross-linker. Following TCR-mediated stimulation SAP-transfected T cells from patients with SLE produced higher amounts of IL-2 compared to pcDNA-transfected SLE T cells (Fig 5D and E). When SAP was overexpressed in normal primary T cells, IL-2 production following anti-CD3 stimulation was also significantly enhanced (Fig 5C). Because it has been previously reported that T cells from SAP-deficient patients produce higher amounts of IFN $\gamma$  compared to T cells from healthy donors, we wondered whether decreased SAP levels in SLE T cells is responsible for increased IFN $\gamma$  production. As we previously showed, when SAP was silenced in normal T cells the levels of IFN $\gamma$  mRNA were significantly increased following 6h of TCR-mediated stimulation. However, when SAP was re-introduced in SLE T cells, IFN $\gamma$  production following stimulation with anti-CD3 remained unaltered, suggesting that at least in patients with SLE increased production of IFN $\gamma$  following TCR-mediated stimulation is SAP-independent (Fig 5G and H). No change in IFN $\gamma$  mRNA levels was observed when we overexpressed SAP in normal T cells either (Fig 5F)

### Exposure to SLE IgG results in decreased SAP expression in normal T cells

It has been demonstrated that exposure of normal T cells to serum from patients with SLE results in suppression of IL-2 production and this was attributed to the existence of anti-TCR/CD3 IgG autoantibodies in SLE sera (<sup>34</sup>). Because restoration of SAP protein expression in SLE T cells was able to rescue IL-2 production, we wondered whether downregulation of SAP in lupus T cells represents an effect exerted on T cells by SLE serum. Purified normal T cells were either kept resting in medium supplemented with 1% FBS or were exposed to 1% serum from healthy donors or patients with SLE for 6h. Whole-cell extracts were obtained and SAP content was evaluated with Western immunoblotting. Downregulation of protein levels of SAP was seen in 4 out of 6 normal donors exposed to SLE serum (Fig 6A and B). We then fractionated lupus sera into IgG and non-IgG fractions and normal total T cells were exposed to the two fractions for 6h. Normal T cells exposed to the IgG, but not the non-IgG fraction, of lupus sera displayed reduced SAP protein levels (Fig 6C, D, E and F). Exposure of SLE T cells to serum from healthy donors overnight failed to restore SAP expression to normal levels (Fig 6G and H)

### Caspase 3 degrades SAP protein in T cells from patients with SLE

We investigated the potential mechanisms that may be responsible for the reduced SAP protein and mRNA levels in T cells from patients with SLE. First, we explored whether SAP is subject to a higher rate of protein degradation in SLE T cells. We treated normal and lupus T cells with 50µg/ml cycloheximide (CHX) to block protein synthesis and whole-cell lysates were obtained at 0, 3, 4 and 5h after treatment. Western immunoblot analyses showed that SAP protein levels were diminished by 4h of culture in T cells from patients with SLE. On the contrary, cytoplasmic SAP levels remained constant in normal T cells (Fig 7A and B). Because caspase activity, especially caspase-3, is reportedly increased in patients with SLE (<sup>35</sup>) we asked whether faster SAP protein degradation in SLE T cells is attributed to caspase-mediated cleavage. SLE T cells were cultured with CHX in the presence or absence of a pan-caspase or a caspase-3 inhibitor for 3, 4 and 5h. By inhibiting caspase activity, SAP protein levels were stabilized in SLE T cells (Fig 7C). Conversely, when lupus T cells were incubated with CHX in the presence of the proteasome inhibitor MG132, SAP protein was degraded suggesting that SAP proteolysis is primarily caspase-3 dependent (Fig 7C and D).

Because SAP mRNA levels were significantly reduced in patients with SLE we examined whether SAP mRNA degradation may also be altered in patients with SLE. We purified T cells from patients with SLE and healthy donors and transcription was arrested by adding actinomycin D (50µg/ml). Levels of SAP mRNA were assessed every 30min for a total of 4h. Levels of the housekeeping gene cyclophilin A were additionally analyzed as an internal stable transcript. We did not observe any differences in the rate of the SAP mRNA degradation in lupus and control donors, suggesting that reduced SAP mRNA may be attributed to transcriptional rather than post-transcriptional defects (data not shown).

## Discussion

In the present communication we demonstrate that SAP protein and gene expression is significantly reduced in T cells, and more pronounced, in CD4+ T cell subsets from patients



with SLE. Forced expression of SAP in SLE resulted in correction of the hyper-responsive phenotype that characterizes the lupus T cell and increased production of IL-2 to normal levels. Exposure of normal T cells to lupus IgG caused reduction of SAP levels while in SLE T cells, increased caspase-3 activity appears to account for its degradation.

T cells from SAP-deficient individuals display a similarly dichotomous series of events following stimulation which involves increased  $[Ca^{2+}]_i$  response but limited IL-2 production<sup>(29)</sup>. Similarly, if SAP is silenced in normal T cells, a similar pattern of response was observed (Fig. 1) and this parallels the response of SLE T cells stimulated with an anti-CD3 antibody<sup>(30)</sup>. The increased early signaling events which prevail in SLE T cells have been linked to a rewiring of the CD3 complex with CD3 $\zeta$  been replaced with Fc $\epsilon$ R $\gamma$  and involvement of Syk<sup>(36)</sup>. The experiments which we have presented here imply that decreased SAP levels may also contribute to the “overexcitable” T cell phenotype in SLE T cells. Interestingly, SAP was reported increased in T cells from Chinese patients with SLE and this was linked to decreased expression of miRNA-142-3p/5p<sup>(37)</sup>. Regardless, in our study sample we have included 2 individuals of reported Chinese ancestry in whom the levels of SAP expression were also decreased. Obviously, independent confirmation of the claimed altered SAP expression in SLE T cells is warranted.

Previously<sup>(34)</sup> we had demonstrated that SLE sera containing anti-CD3/TCR antibodies which cause decreased IL-2 production by limiting the transcriptional activity of the *IL-2* promoter due to increased binding of the repressor CREM $\alpha$ . Here we show that lupus sera result in decreased expression of SAP and thereafter to decreased IL-2 production signifying the multiplicity of the molecular abnormalities displayed by SLE T cells and the complexity of its biology. The fact that anti-CD3/TCR antibodies present in lupus sera lead to decreased expression of SAP along with the fact that 90% of the study subjects were women point away from the involvement of genetic causes.

Of clinical relevance is the finding that in SLE T cells SAP is degraded by caspase-3 in a manner similar to that we previously reported for CD3 $\zeta$ <sup>(35)</sup>. Targeted inhibition of caspase-3 in SLE T cells should lead to the restoration of levels of SAP, CD3 $\zeta$  and probably other molecules.

The role of SAP in the expression of autoimmunity and in T cell activation and function is unclear<sup>(38)</sup><sup>(39)</sup><sup>(40)</sup><sup>(25)</sup>. Studies have claimed that SAP activates CD3 $\zeta$  chain and other canonical T cell signaling molecules<sup>(40)</sup>. Because CD3 $\zeta$  is decreased in SLE T cells<sup>(31)</sup>, we have not been able to address directly whether this occurs also in SLE T cells. Yet, when we downregulated SAP in normal T cells the total expression of CD3 $\zeta$  was not different compared to cells that have been transfected with control siRNA (data not shown).

SAP-deficient mice do not spontaneously develop autoimmunity and are resistant to pristane-induced lupus<sup>(41)</sup>. Moreover, a spontaneous mutation in SAP in the MRL/lpr strain led to attenuation of the disease<sup>(42)</sup> and SAP deficiency also ameliorated the lupus phenotype of the Roquin<sup>san/san</sup> mice<sup>(43)</sup>. Sustained expression of SAP has also been shown to be a requisite for the progression of collagen-induced arthritis in the early stages of the disease in mice<sup>(39)</sup>. On the other hand, patients with XLP display defective B cell tolerance

and transitional B cells in the peripheral blood of XLP patients are enriched in autoreactive clones<sup>(44)</sup>. It was also shown that SAP-deficient T cells from patients with XLP appear to be resistant to regulatory T cell-mediated suppression<sup>(44)</sup>. Decreased SAP mRNA transcripts have been reported in PBMCs from patients with rheumatoid arthritis<sup>(45)</sup>.

In summary, this study presents data demonstrating that expression of SAP is decreased in T cells from patients with SLE and contributes to aberrant early signaling events and decreased IL-2 production. Because caspase-3 accounts for the degradation of SAP, in manner similar to that described for CD3  $\zeta$  in SLE T cells, it is proper to consider its pharmaceutical inhibition to restore its expression and correct aberrant cell function<sup>(35)</sup>.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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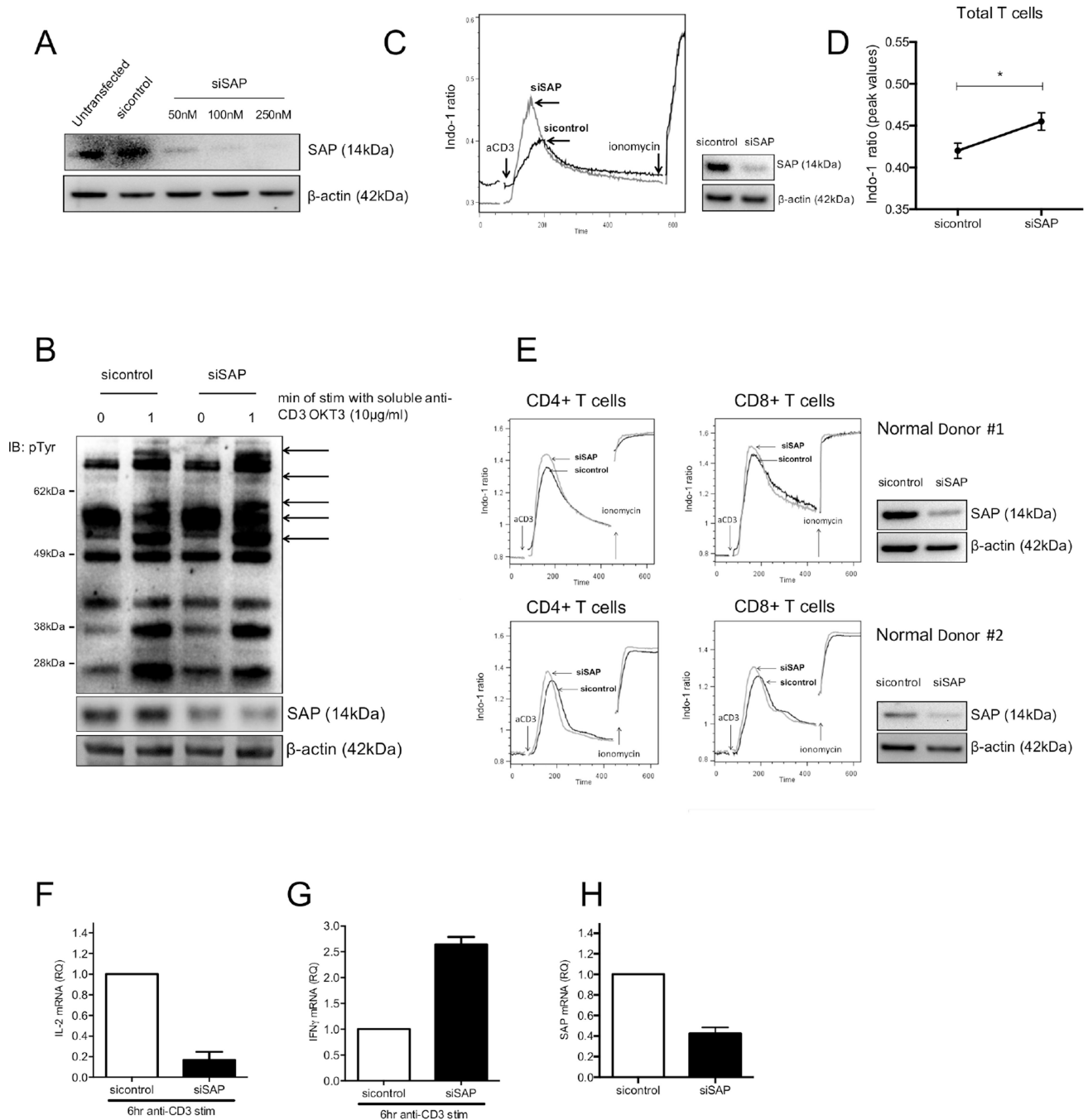
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**Figure 1. Enhanced TCR-mediated early signal transduction events and aberrant cytokine production in SAP-depleted T cells from healthy individuals**

(A) Total T cells from healthy individuals were transfected with various concentrations of small interfering RNA against SAP (siSAP) or control siRNA (sicontrol). Efficacy of transfection was determined by SAP protein levels by Western blotting. (B) Total T cells (n=4) transfected with siSAP or sicontrol were stimulated 24hr post-transfection with anti-CD3 mAb followed by cross-linking for 60sec and levels of pTyr were evaluated by Western blotting. (C) Increased TCR-triggered free intracytoplasmic calcium flux in normal total T cells (n=3). Cumulative results for total T cells shown in (D) and are expressed as the mean



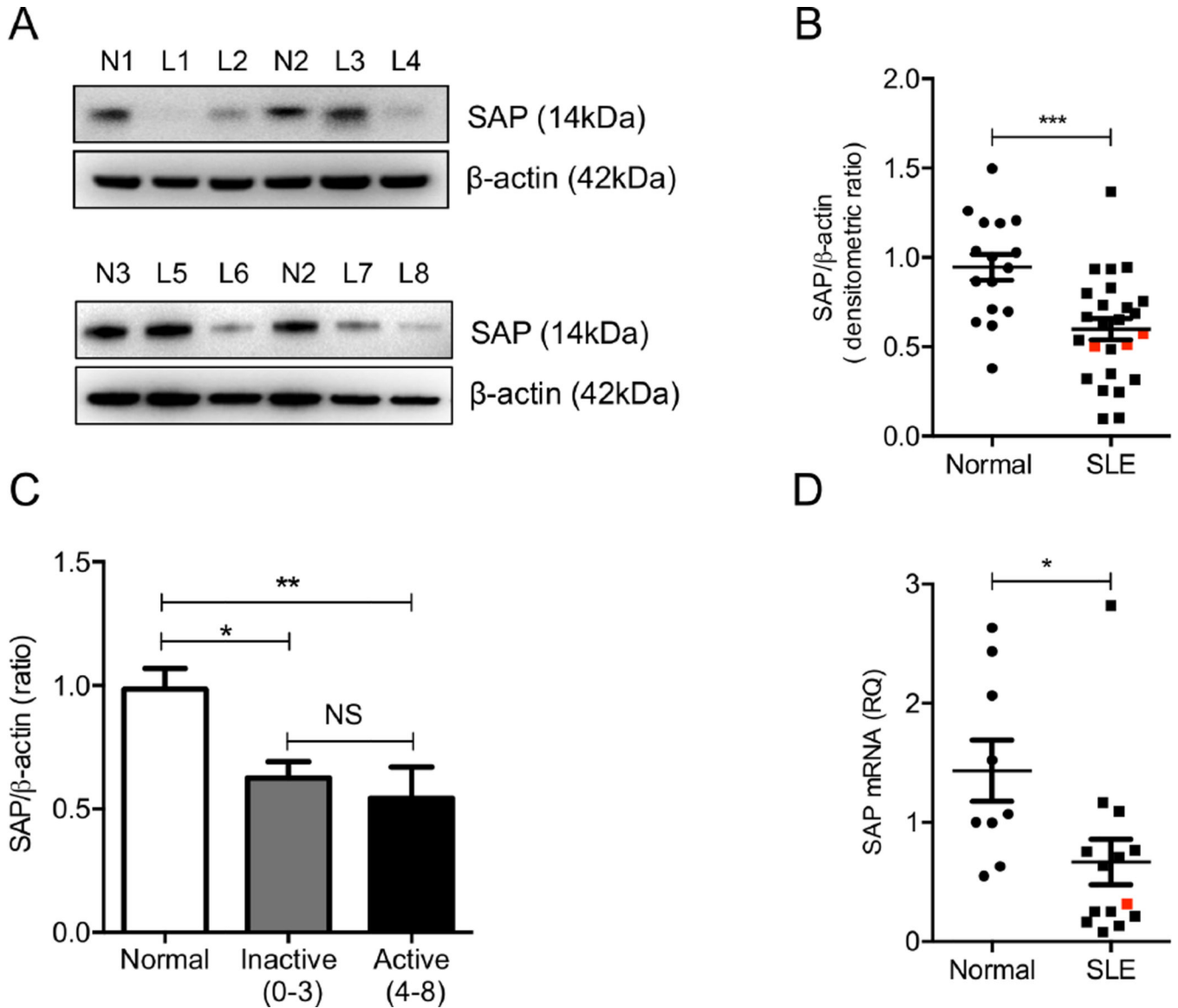
peak value of the Indo-1(violet) to Indo-1 (blue) ratio  $\pm$  SEM. (E) Representative experiments of increased TCR-initiated free cytoplasmic calcium flux in CD4+ and CD8+ T cell subsets following silencing of SAP (n=3). Impaired IL-2 production (F) and elevated IFN $\gamma$  production (G) after 6 hrs of anti-CD3 stimulation in normal T cells following knockdown of SAP. Efficiency of transfection, measured by SAP mRNA levels, is shown in (H).

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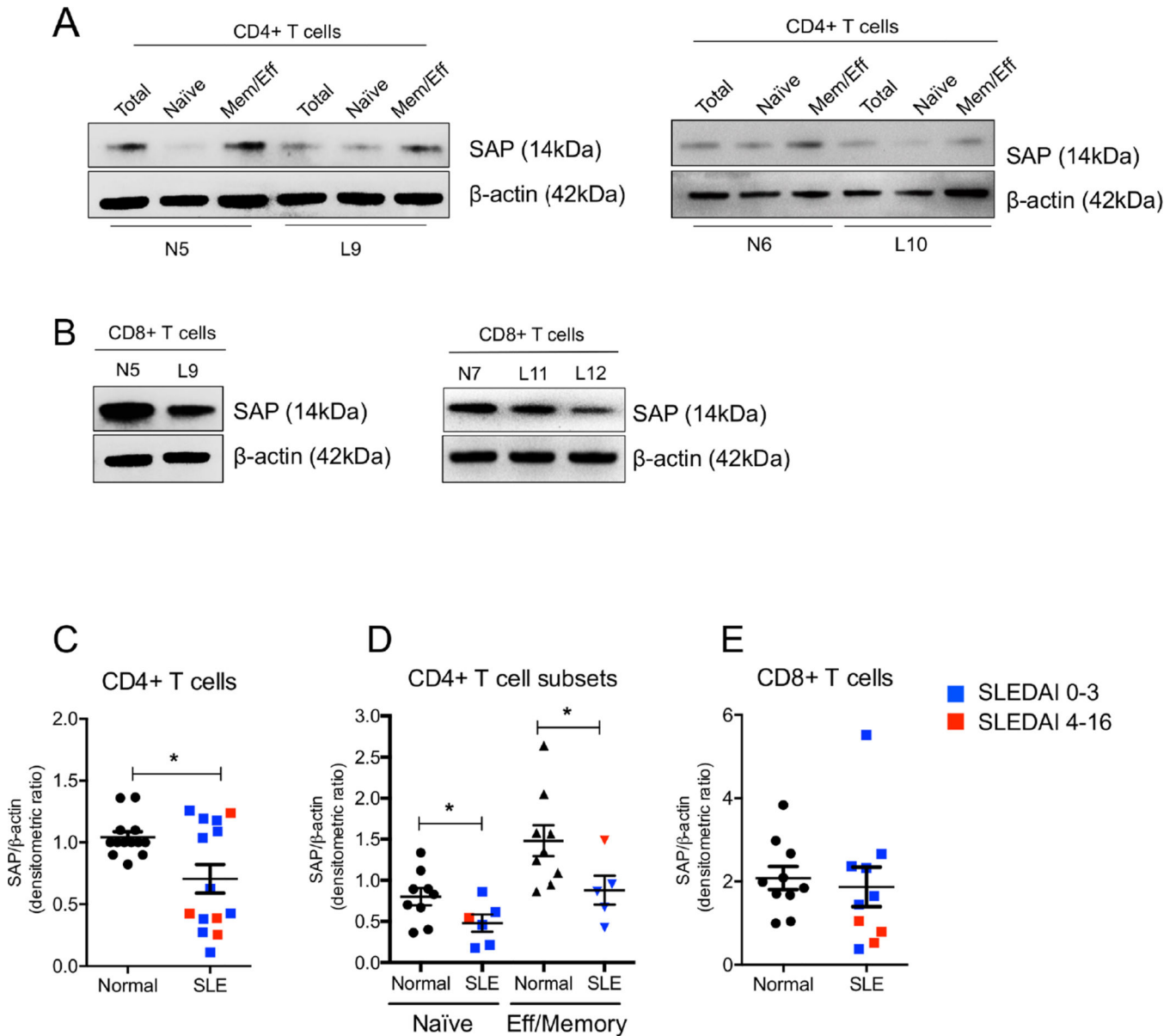
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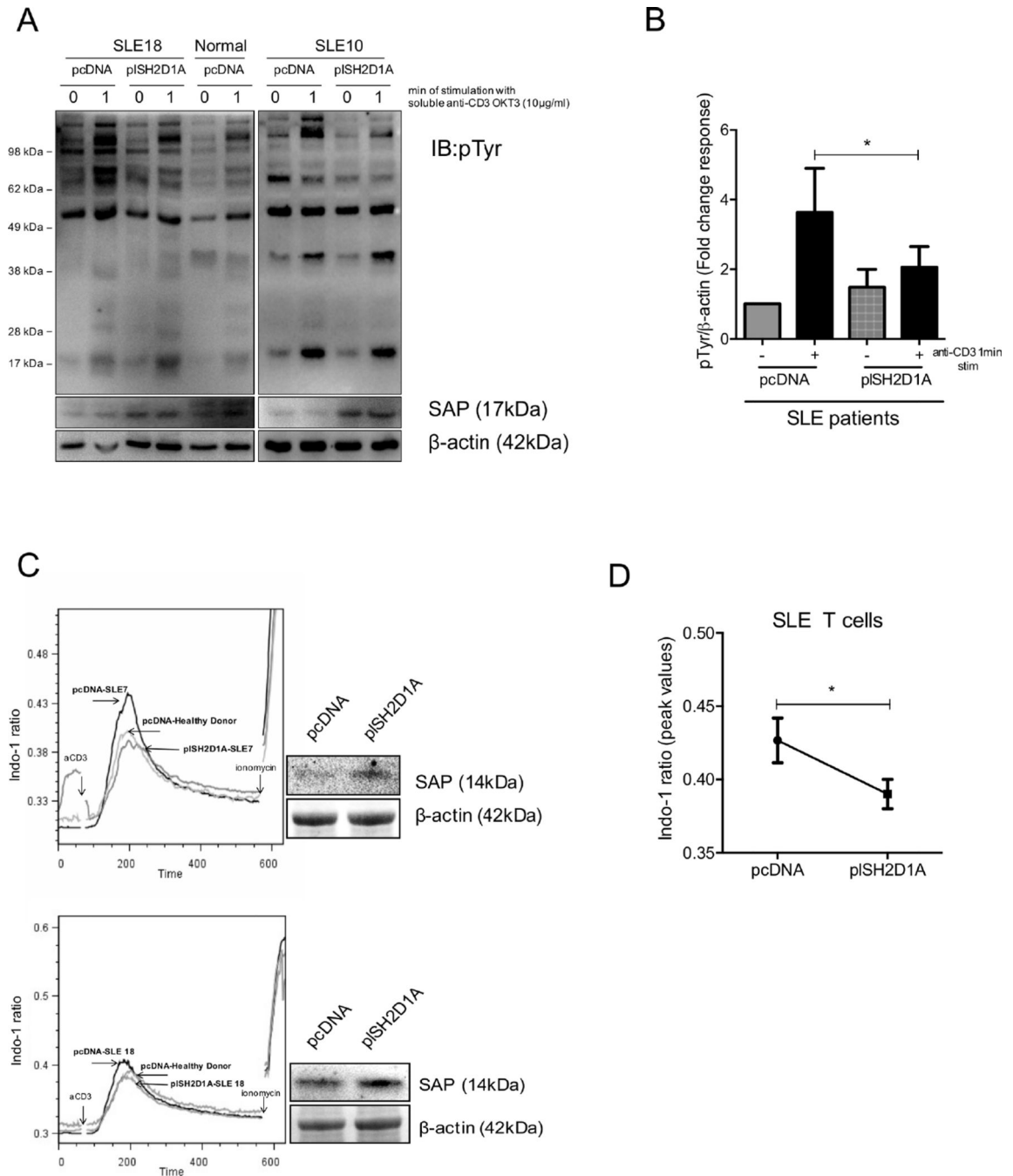


**Figure 2. Levels of SAP in T cells from patients with SLE and from healthy individuals**  
 Protein levels of SAP were assessed in whole-cell lysates of T cells obtained from normal donors (n=16) and patients with SLE (n=25) by Western blotting. Two individual representative experiments are shown in (A) and cumulative results, expressed as the mean densitometric ratio of SAP over  $\beta$ -actin  $\pm$  SEM, are depicted in (B). (C) SAP protein levels are found to be decreased in SLE patients with both inactive and active disease. SAP gene expression was evaluated with qPCR in 9 healthy donors and 14 patients with SLE and results are shown in (D). Red points signify men with SLE. N=Normal Donors and L= SLE patients.



**Figure 3. Expression of SAP in CD4+ T cell subsets and CD8+ T cells in SLE patients and healthy controls**

CD4+ T cells and CD8+ T cells were purified from the peripheral blood of healthy individuals and patients with SLE. Naïve CD4+ T cells and effector-memory CD4+ T cells (Mem/Eff) were further isolated from the CD4+ T cell fraction. SAP protein levels were evaluated by Western blotting. (A) SAP expression in total, naïve and effector/memory CD4+ T cells in normal individuals and patients with SLE. (B) Representative Western blots of SAP expression in healthy and SLE CD8+ T cells. The mean densitometric ratio of SAP over  $\beta$ -actin  $\pm$  SEM is shown for CD4+ T cells (C), CD4+ T cell subsets (D) and CD8+ T cells (E). SLE samples are either depicted as blue squares (SLEDAI: 0–3) or red squares (SLEDAI: 4–16).



**Figure 4. Forced expression of SAP in SLE T cells restores TCR-mediated early signal transduction events**

Purified T cells from normal donors (n=4) and patients with SLE (n=4) were transfected either with a control vector (pcDNA) or a SAP-expressing plasmid (pISH2D1A). (A) Levels of tyrosine phosphorylated proteins (pTyr) were evaluated by Western blotting in transfected cells from one healthy individual and two patients with SLE following stimulation with cross-linked soluble anti-CD3 or control IgG at 37 °C for 60 sec (representative experiment). Cumulative results are shown in (B) and are expressed as the fold change of the mean densitometric ratio of pTyr proteins (36–110kDa area) over β-actin ± SEM. (C)

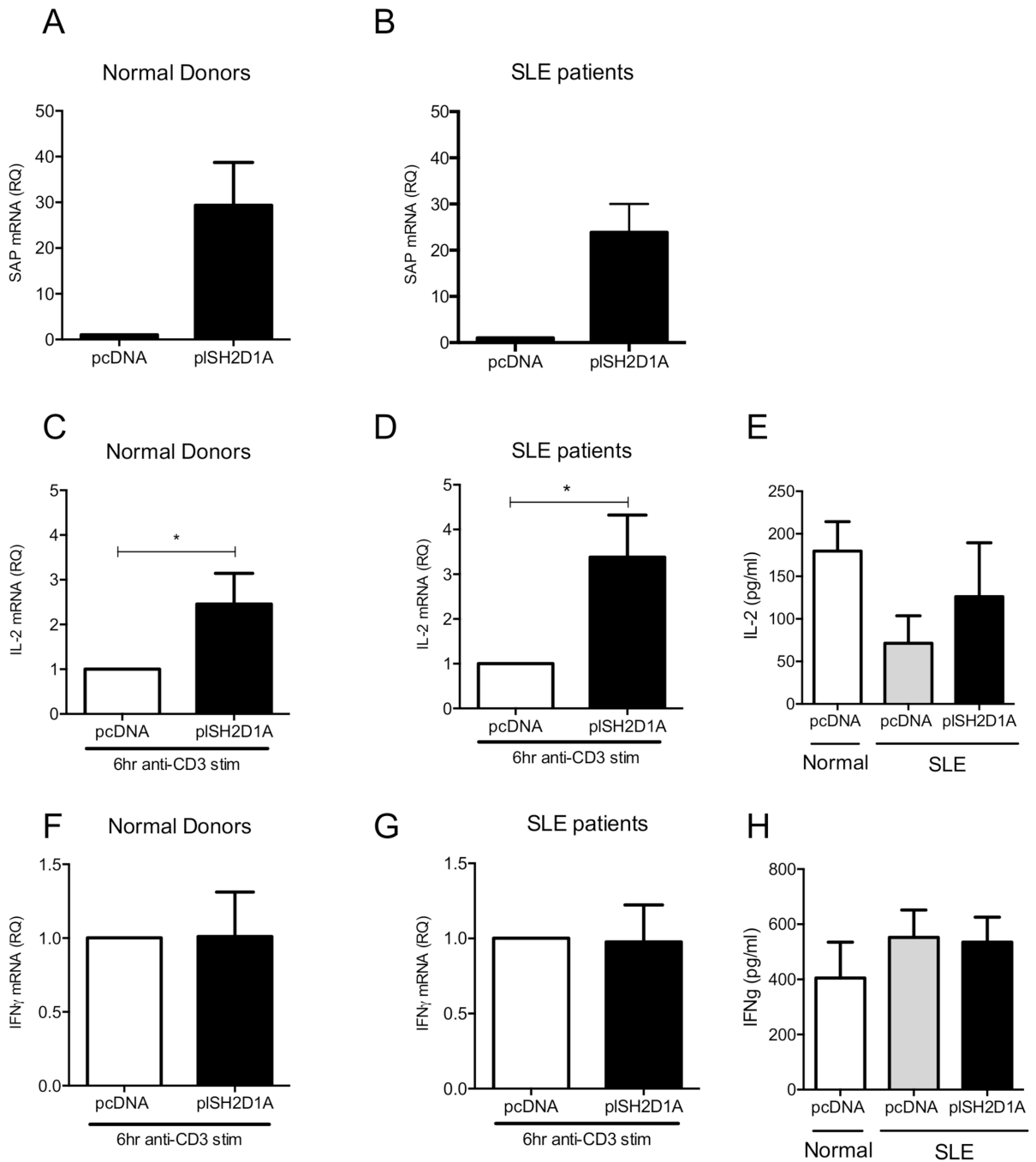
Representative histograms demonstrating the peak intracellular calcium  $[Ca^{2+}]_i$  response in transfected total T cells from normal donors (transfected with control pcDNA) and patients with SLE (transfected with pcDNA or pISH2D1A). Successful transfection for each SLE donor is shown by Western blotting next to each calcium response graph. (D) Results are expressed as the mean peak value of the Indo-1(violet) to Indo-1(blue) ratio  $\pm$  SEM for a total of 4 independent experiments. aCD3= stimulation with anti-CD3 OKT3 mAb.

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**Figure 5. Replenishment of SAP levels in SLE T cells rescues IL-2 production following TCR-mediated stimulation**

Purified total T cells from (A) healthy individuals (n=13) and (B) SLE patients (n=9) and were successfully transfected with control pcDNA or a plasmid expressing SAP (pISH2D1A) and levels of SAP were measured by qPCR to validate transfection efficiency. 24 h post-transfection T cells were stimulated with soluble anti-CD3 mAb in the presence of a cross-linker for 6 h. IL-2 and IFN $\gamma$  mRNA levels were assessed in normal donors (C and F) and patients with SLE (D and G) with qPCR. Production of IL-2 and IFN $\gamma$  was also



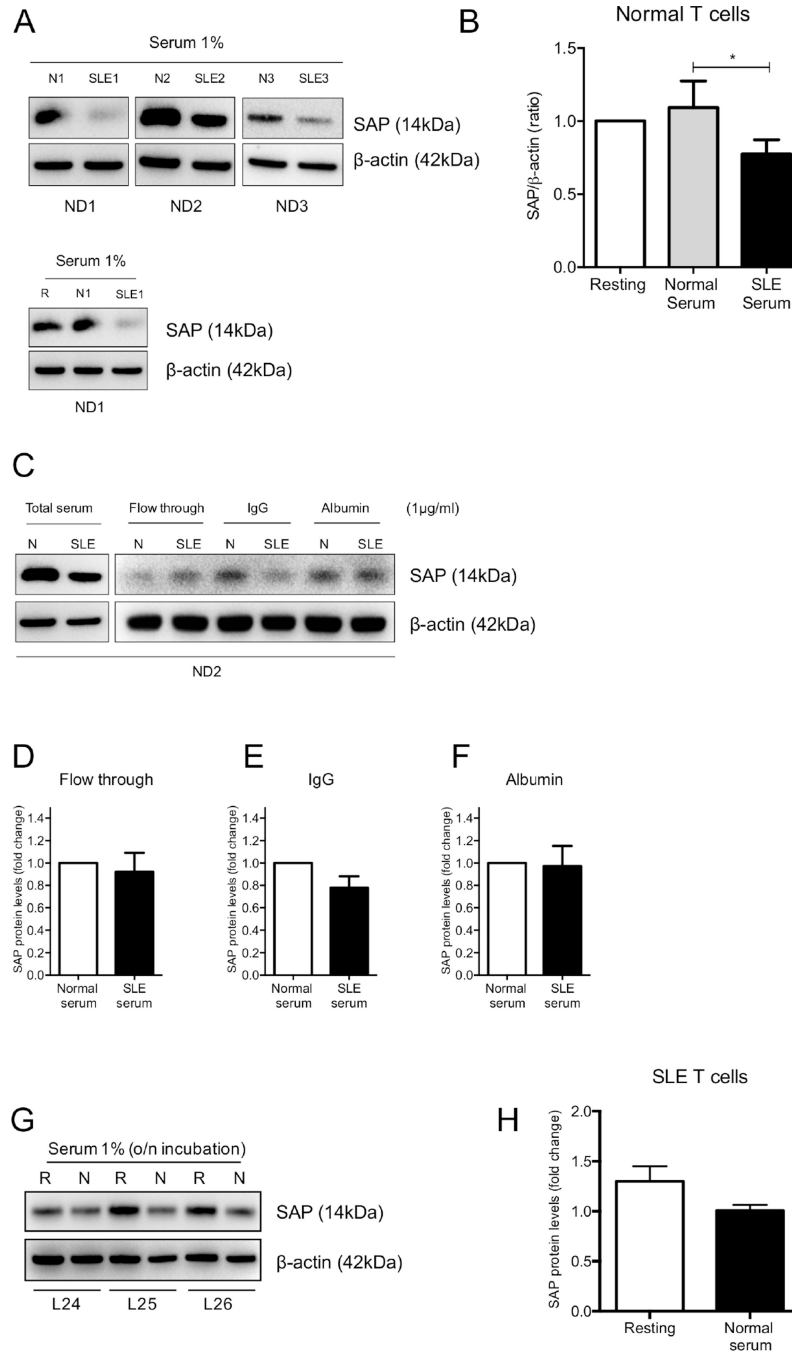
evaluated by ELISA from the supernatants of transfected T cells following 6 h of anti-CD3 stimulation (E and H).

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**Figure 6. Treatment of normal T cells with SLE serum results in SAP downregulation in an IgG-dependent mechanism**

Normal T cells (n=6) were cultured in the presence of 1% serum from patients with SLE, healthy individuals or medium supplemented with 1% FBS for 6 h and SAP protein expression was examined with Western blot. Representative experiments are shown in (A) and cumulative results in (B). Normal T cells were exposed to normal or SLE serum (whole or fractionated) for 6 h and SAP levels were evaluated with Western blot in (C). (D, E and F) Cumulative results of SAP levels following incubation of healthy T cells with fractionated serum from normal donors and patients with SLE are expressed as the fold change of the

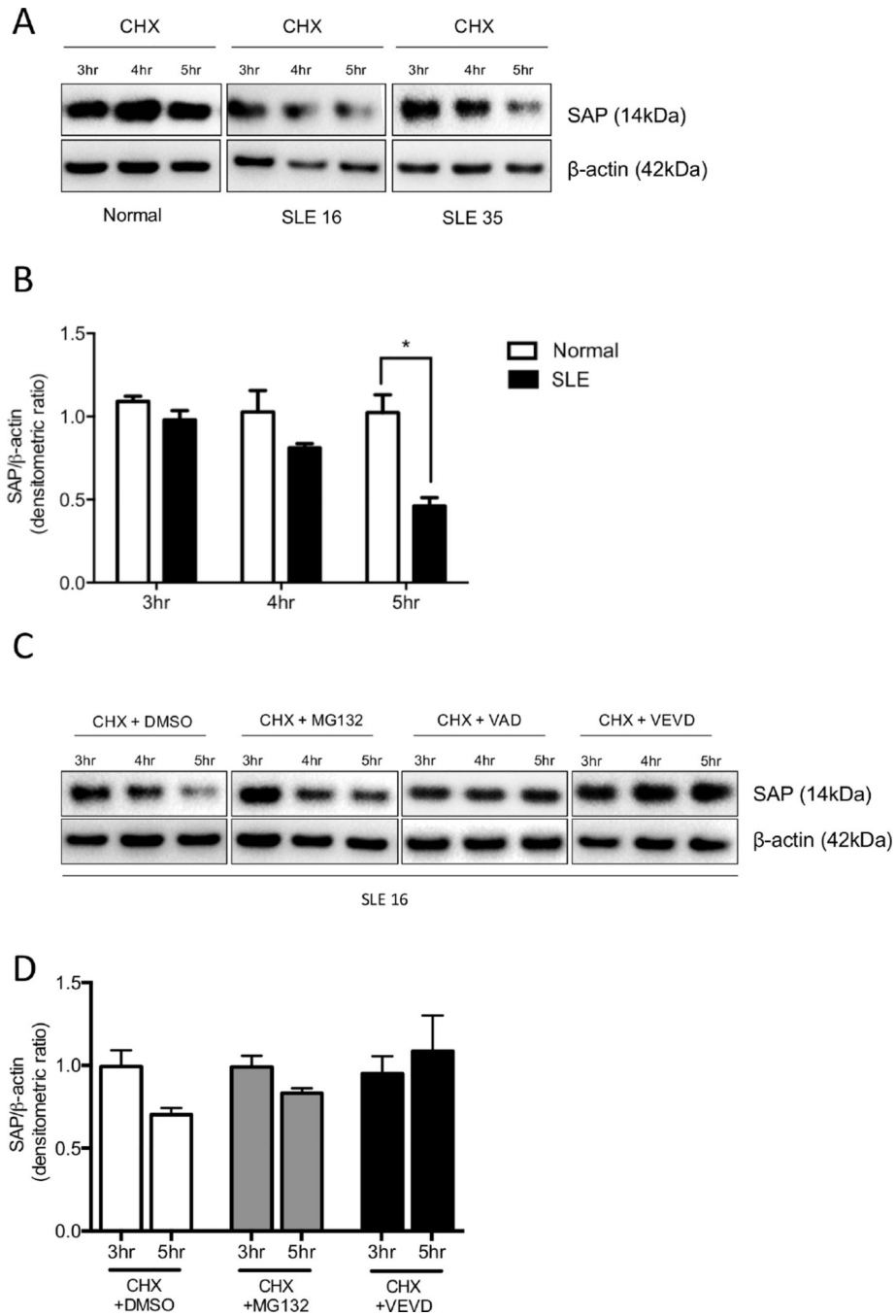
densitometric ratio of SAP over  $\beta$ -actin  $\pm$  SEM. Total T cells from patients with SLE (n=3) were exposed to 1% normal serum overnight. SAP levels were examined with Western blotting (G) and results are depicted in (H), expressed as the mean densitometric ratio of SAP over  $\beta$ -actin  $\pm$  SEM. N=normal serum, SLE=serum from SLE patient, ND=T cells from Normal Donor, L=T cells from SLE patients and R=resting

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**Figure 7. SAP undergoes faster protein degradation in SLE T cells compared to normal T cells** (A) Total T cells from healthy individuals (n=3) and SLE patients (n=5) were cultured in the presence of CHX for up to 5 h to inhibit protein synthesis. Cells were lysed and protein extracts were analyzed for SAP protein levels by Western blotting. Cumulative results are shown in (B) and are expressed as the fold change in densitometric ratio of SAP over  $\beta$ -actin  $\pm$  SEM. (C) Protein synthesis was arrested with CHX and T cells were incubated in the presence of proteasome inhibitor MG132, pan-caspase inhibitor VAD or caspase-3-specific

inhibitor VEVD. SAP levels were evaluated by Western blotting at 3, 4 and 5 h after treatment and cumulative results are shown in (D)

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