Supporting Information

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SI Materials and Methods

Human Subjects. All systemic lupus erythematosus (SLE) patients included in our studies were diagnosed according to the American College of Rheumatology classification criteria and recruited from the Division of Rheumatology at Beth Israel Deaconess Medical Center, Boston, MA and gave written informed consent under protocol 2006-P-0298. Healthy age-, sex-, and ethnicity-matched individuals were chosen as controls. Peripheral venous blood was collected in heparin-lithium tubes, and total human T cells were purified as described before (1). Epidemiologic and clinical information is displayed in *Demographic Information*. Inactive patients were defined by an SLE disease activity index (SLEDAI) between 0 and 3, active patients with an SLEDAI score of 4 and above.

Cell Culture. Peripheral blood mononuclear cells (PBMCs) were enriched for T lymphocytes by precipitation of non-T cells (Rosettesep; Stemcell Technologies), followed by density-gradient centrifugation as reported previously (Lymphoprep; Nycomed) (1). For some experiments, T cells were cultured at a concentration of 1×10^6 cells per mL in RPMI 1640 with 10% FCS in 12-well plates that had or had not been precoated with anti-CD3 and anti-CD28 antibodies (as indicated). Cells were collected after the indicated time points and harvested for semiquantitative real-time PCR (qRT-PCR), flow cytometry, methyl-CpG-DNA immunoprecipitation (MeDIP), or chromatin-immunoprecipitation (ChIP) as indicated.

Cytokine Stimulation and Stat Inhibition. Primary human T cells were cultured at a concentration of 1×10^6 cells per mL in RPMI 1640 with 10% FCS in the absence or presence of Stat3 or Stat5 inhibitors (Stat3 inhibitor VI, CAS 501919–59-1, Stat5 CAS 285986–31-4; EMD Millipore) for 2 h. DMSO was included as a vehicle control for the Stat inhibitors. Stat3- or Stat5-inducing cytokines were added to some wells: 100 ng/mL IL-6 (NCI Preclinical Repository) or 100 ng/mL IL-27 (R&D Systems) were added to primarily induce Stat3; 100 IU/mL IL-2 (NCI Preclinical Repository) or 20 ng/mL IL-15 (Miltenyi Biotec) were added to mainly induce Stat5. After 24 h, cytokine-mediated effects on IL-10 secretion in the absence or presence of Stat inhibitors were assessed with an IL-10 ELISA (Biolegend).

qRT-PCR. Total RNA from controls and SLE T lymphocytes was isolated, using the Qiagen RNeasy Mini Kit (Qiagen). cDNA was generated using a first-strand cDNA synthesis kit (Invitrogen, Life Technologies). For gene expression analyses, real-time PCR was performed using SybrGreen site-specific primers on an ABI StepOnePlus Real-Time PCR System. Results were normalized to 18S. Primer sequences for qRT-PCR, plasmid generation, MeDIP, and ChIP PCR are summarized in *Primers*.

Gene Expression Plasmids and T-Cell Transfection. Expression plasmids for human DNMT3a have been described previously (1). Reporter constructs spanning the proximal 946 bp of the human *IL10* 5′ promoter or a 630 bp-spanning fragment of the fourth intron with enhancer activity were PCR-amplified and cloned into the luciferase vector pGL3-Basic (Promega), using primers with attached restriction sites for MluI and BglII (*Primers*). All plasmid DNA preparations were carried out with DNA purification kits (Qiagen) and sequence-verified (Genewiz). Site-directed mutagenesis at the (−149) Stat-responsive element (SRE) or the intronic I-SRE within the reporter constructs was performed using a DNA oligonucleotide harboring a mutated SRE and *PfuTurbo* DNA polymerase (Stratagene) following the manufacturer's instructions.

Three million human T lymphocytes were transfected with a total amount of 3 μg of the indicated expression plasmids using the Amaxa transfection system (Lonza) or Lipofectamine (Life Technologies) as indicated. After 24 h (RNA, ChIP, and MeDIP analyses) or 120 h (flow cytometry), cells were harvested and assayed.

Luciferase Assays in Human T Cells. One million ex vivo isolated primary human or unstimulated Jurkat T cells were transfected with the indicated amounts of plasmid DNA, using the Amaxa transfection system (Lonza). Effector/reporter transfection experiments were performed at a molar ratio of 3:1. Each reporter experiment included 10 ng of *Renilla* luciferase construct as an internal control. Five hours after transfection, cells were collected and lysed, and luciferase activity was quantified, using the Promega Dual Luciferase Assay System (Promega) following the manufacturer's instructions. Luciferase experiments were repeated at least three times, and values in the bar diagrams are given as mean ± SD.

Methyl-CpG-DNA Immunoprecipitation. The MeDIP assay was carried out following the manufacturer's instructions (Zymo Research). Briefly, genomic DNA from T lymphocytes obtained from 22 SLE patients and healthy control individuals was purified, applying the AllPrep Mini kit (Qiagen), and sheared to fragments of ~200-bp length using DNA shearase (Zymo Research). Subsequently, 160 ng of sheared genomic DNA were used as input for methyl-CpG-DNA immunoprecipitation. Methylated CpG-DNA was recovered and subjected to PCR analysis on an ABI StepOnePlus Real-Time PCR system. Equal amounts of completely methylated (100%) human CpG-DNA and demethylated human CpG-DNA (Zymo Research) were included as "input" and negative control.

Methylation of Reporter Plasmid. To investigate the effects of CpG-DNA methylation on *IL10* promoter activity, we methylated the 630-bp *IL10* reporter construct and the empty pGL3 plasmid, using CpG-DNA methylase (Zymo Research) according to the manufacturer's instructions.

Cotransfection of T Cells with Stat3 or Stat5 Expression Plasmids and p300 siRNA. Three million Jurkat T cells were transfected with a total amount of 3 μg of Stat3 or Stat5 expression plasmid 10 nM irrelevant control siRNA and p300-specific siRNA (OriGene) using Lipofectamine 2000 (Invitrogen, Life Technologies). Before these experiments, experimental conditions were optimized using Cy-3–labeled control siRNA (OriGene). Transfection efficiency was >70%. Cells were collected after overnight culture (24 h) and processed for mRNA analysis as indicated in the *qRT-PCR* section. All experiments were repeated six times, and values in the bar diagrams are given as mean and SDs. Later, cells were transfected with p300 expression plasmids and Stat3 or Stat5 siRNAs to reconfirm our findings following the same protocols.

ChIP Assays. Anti-H3K18ac (Abcam), anti-p300 (Santa Cruz), anti-Stat3 (EMD Millipore), and anti-Stat5 (Abcam) antibodies and nonspecific normal rabbit IgG were obtained from Upstate (EMD Millipore). ChIP assay was carried out according to the manufacturer's instructions (Invitrogen, Life Technologies). Briefly, cells were cross-linked with 1% formaldehyde, washed with cold PBS, and lysed in buffer containing protease inhibitors

(Roche). Cell lysates were sonicated to shear DNA and sedimented, and diluted supernatants were immunoprecipitated with the indicated antibodies. A proportion (10%) of the diluted supernatants was kept as input control. After the recovery of ChIP-DNA, real-time qPCR was performed. The amount of immunoprecipitated DNA was subtracted by the amplified DNA that was bound by the nonspecific normal IgG and subsequently calculated as relative to the respective input DNA. Where indicated, cells were pretreated with C646, a selective small-molecule inhibitor of the p300 histone acetyltransferase (Origene) according to the manufacturer's protocols.

Intracellular Stat Phosphorylation Assays. To determine intracellular Stat3 levels and Stat3 phosphorylation, Thermo Scientific Pierce STAT3 In-Cell ELISA Kits were applied following the manufacturer's protocols. Briefly, colorimetric and fluorescent microplate assays were used to measure total and phosphorylated (Tyr705) Stat3 in whole cells. Primary human T cells were cultured in 96-well microplates, and some cells were stimulated with anti-CD3 and anti-CD28 antibodies or phorbol 12-myristate 13-acetate and ionomycine as indicated for 4 h.

Intracellular staining for phosphorylated Stat3 (Tyr705) (BD Biosciences) or phosphorylated Stat5 (Tyr694) (BD Biosciences) was carried out in primary human T cells from controls and SLE patients with phycoerythrin (PE)-labeled antibodies (clones 4/P-Stat3, and 47/Stat5) as indicated. Some cells were stimulated with anti-CD3 and anti-CD28 antibodies for 4 h. After stimulation, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, resuspended in ice-cold methanol, washed in PBS, and stained following standard procedures. Finally, cells were subjected to flow cytometry on an LSR II flow cytometer (BD Biosciences), and data were analyzed using FloJo version 7.2.2 (TreeStar, Inc.).

Proximity Ligation Assay. HEK 293T cells (70×10^3) were cultured on eight-well cell culture slides (BD Falcon) and transfected with expression plasmids (on a pcDNA3 backbone) for Stat3, Stat5, p300, or the combination of Stat3 or Stat5 and p300 (0.3 μg of each plasmid per transfection) using the X-tremeGENE 9 DNA Transfection Reagent (Roche) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were harvested and subjected to the Duolink proximity ligation assay (PLA) following the manufacturer's instructions (Olink). Briefly, anti-Stat3 or-Stat5 and anti-p300 antibodies were labeled with PLUS and MINUS oligonucleotide tails. Cells were fixed with 3.7% formaldehyde, washed with PBS, permeabilized with Triton X (Life technologies), and incubated with PLUS and MINUS oligonucleotide labeled antibodies. According to the manufacturer's instructions, cells were washed several times and incubated with ligase (30 min) and polymerase solution (100 min). Then, cells were mounted with DAPI containing medium (Olink) and read on a fluorescence microscope (Zeiss). Experiments with primary human T cells were performed using 5×10^6 T cells in Eppendorf tubes. After all washes and incubations, required according to the manufacturer's protocol, cells were transferred to microscopy slides using a cytospin centrifuge (Shandon), mounted with DAPI containing medium (Olink), and read on a fluorescence microscope (Zeiss). The number of PLA signals per cell was quantified using ImageJ software (http://rsb.info.nih.gov/ij/disclaimer.html).

Statistical Analysis. The paired two-tailed Student *t* test was used for statistical analysis. The Pearson product moment correlation coefficient (r) was used to determine the correlation between IL-10 mRNA levels and individual SLE disease activity indices (SLEDAIs).

Demographic Information. T-cell samples from 22 female SLE patients and appropriate age-, sex-, and ethnicity-matched controls were collected for DNA methylation analysis. SLEDAI, systemic lupus erythematosus disease activity index (0, inactive; 4, mild activity; 8, moderate activity; 10 and above, high activity); F, female; GC, glucocorticoids; HCQ, hydroxychloroquine; Aza, azathioprine; MTX, methotrexate; MMF, mycofenolate mofetil; RNA, RNA from total T lymphocytes for qRT-PCR; DNA, DNA from total T lymphocytes for methyl-cytidine-phosphate-guanine DNA immunoprecipitation.

No.	SLEDAI	GC, mg/d	HCQ, mg/d	Aza, mg/d	MTX, mg/d	MMF, mg/d
1	0	—	—	—	15	—
2	0	—	200	100	—	—
3	0	—	—	—	—	—
4	0	10	400	100	—	—
5	0	—	400	—	—	—
6	0	—	400	—	—	—
7	4	15	400	—	—	—
8	4	10	400	—	—	2,500
9	4	5	400	150	—	—
10	4	10	400	—	—	—
11	4	3.33	400	—	—	—
12	8	17.5	—	—	—	3,000
13	8	—	400	—	—	2,000
14	8	—	—	—	—	1,000
15	8	15	400	150	—	—
16	8	0	400	—	—	3,000
17	10	40	400	—	—	—
18	10	5	—	—	—	1,500
19	14	50	400	—	—	2,000
20	10	5	400	150	—	—
21	14	—	400	—	—	—
22	10	25	400	—	10	—

Primers.

Primer name	Sequence	Application
IL10_SRI-741_F	TCAGCAAGTGCAGACTACTC	ChIP PCR
IL10_SRI-741_R	TGTGTTCCAGGCTCCTTTAC	ChIP PCR
IL10_SRI-149_F	GGAGGAGCTCTAAGCAGAA	ChIP PCR
IL10_SRI-149_R	AAGCCCCTGATGTGTAGAC	ChIP PCR
IL10_I-SRI_F	TCAGCAAGTGCAGACTACTC	ChIP PCR
IL10_I-SRI_R	TCCTCTCACCGTCTTGCTTTCG	ChIP PCR
hIL10_PP_F-Mlu	gcacgcgtCCAAATTCTCAGTT-GGCACTGGTGTACC	Amplification
hIL10_PP_R-Bgl	gcagatctCCAGTCAGGAGGAC-CAGGCAACA	Amplification
hIL10_Int4_F-Mlu	gcacgcgtGCTTCGAAAGCAAGA	Amplification
hIL10_Int4_R-Bgl	gcagatctGATGGTATGACCTGCTCA	Amplification

Primer pairs for ChIP-PCR and PCR-amplification of promoter or enhancer elements are displayed. Underscored lower case letters within the amplification primers indicate attached restriction sites for MluI or BglII.

1. Hedrich CM, et al. (2012) cAMP response element modulator alpha controls IL2 and IL17A expression during CD4 lineage commitment and subset distribution in lupus. *Proc Natl Acad Sci USA* 109(41):16606–16611.

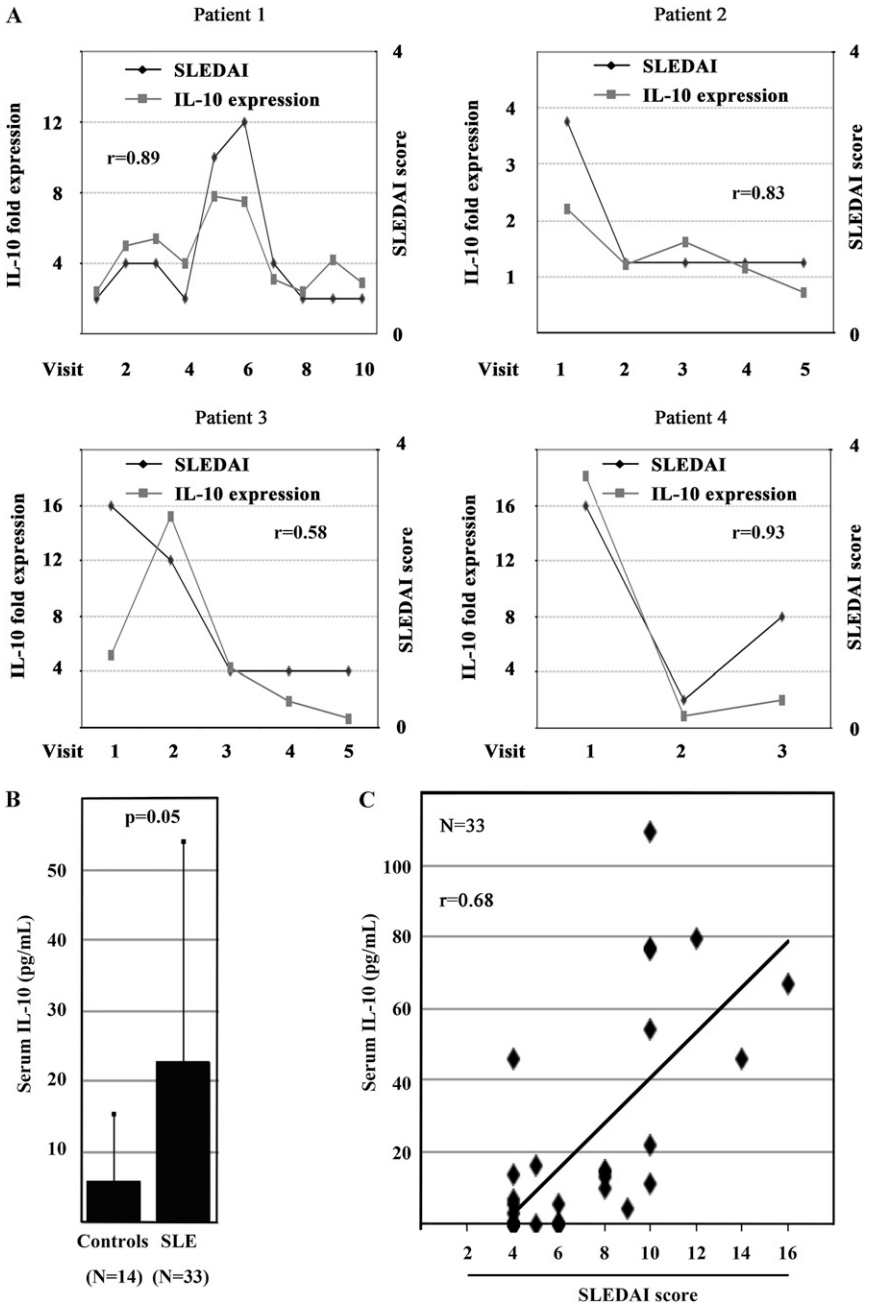

Fig. S1. T cells from SLE patients express increased amounts of IL-10. (*A*) In select individual patients, T cell-derived IL-10 mRNA expression reflected and followed disease activity as assessed by SLEDAI. Results from four individuals are provided. (*B*) IL-10 protein levels are increased in the serum of SLE patients (n = 33) compared with controls (n = 13). (*C*) IL-10 serum expression in SLE patients correlated with disease activity.

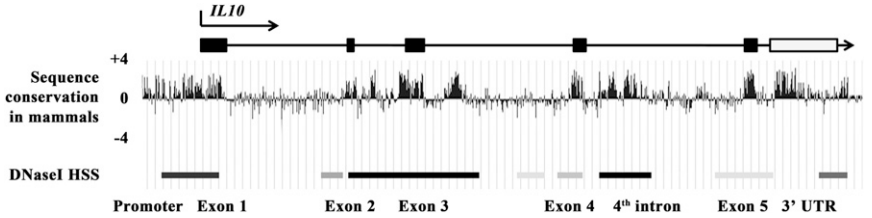

Fig. S2. Bioinformatic analysis of the *IL10* gene. The *IL10* gene is located on chromosome 1q31-32. It consists of five exons and one 3′ untranslated region. The degree of sequence conservation through mammals is relatively high compared with most other genes, with the highest conservation over the 5′ proximal promoter, the five exons, the 3′ UTR, and two intronic regions in the 5′ regions of introns 3 and 4. These highly conserved regions map previously reported DNase hypersensitivity sites (DNase I HSS).

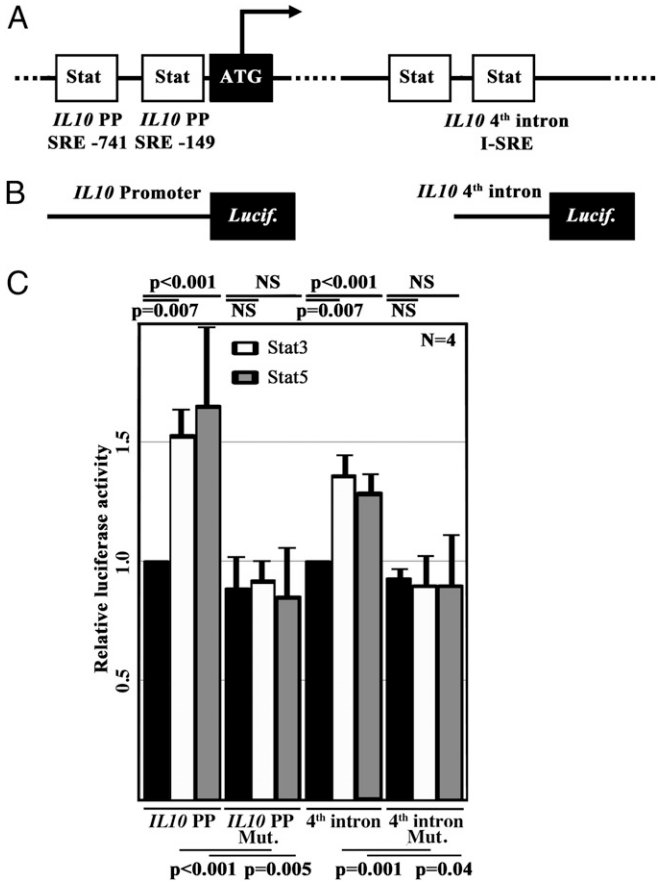

Fig. S3. In silico predicted Stat-responsive elements and reporter construct design. (*A*) The IL10 5′ proximal promoter contains two putative Stat-responsive elements (SREs), −741 and −149 upstream of the start codon (ATG). A previously reported enhancer element within the fourth intron harbors two predicted SREs, one of which has been demonstrated to recruit Stat5 in human T cells and Stat4 in murine natural killer (NK) cells (I-SRE). (*B*) Thus, we generated luciferase reported constructs of the 5′ proximal promoter, covering two SREs, and the intronic enhancer, covering the I-SRE. (*C*) Deletion of SRE-741 within the promoter construct or I-SRE within the enhancer construct significantly reduced luciferase activity to levels that were comparable with the activity of pGL3 empty vectors in Jurkat T cells. Forced Stat3 or Stat5 expression did not have additional effects.

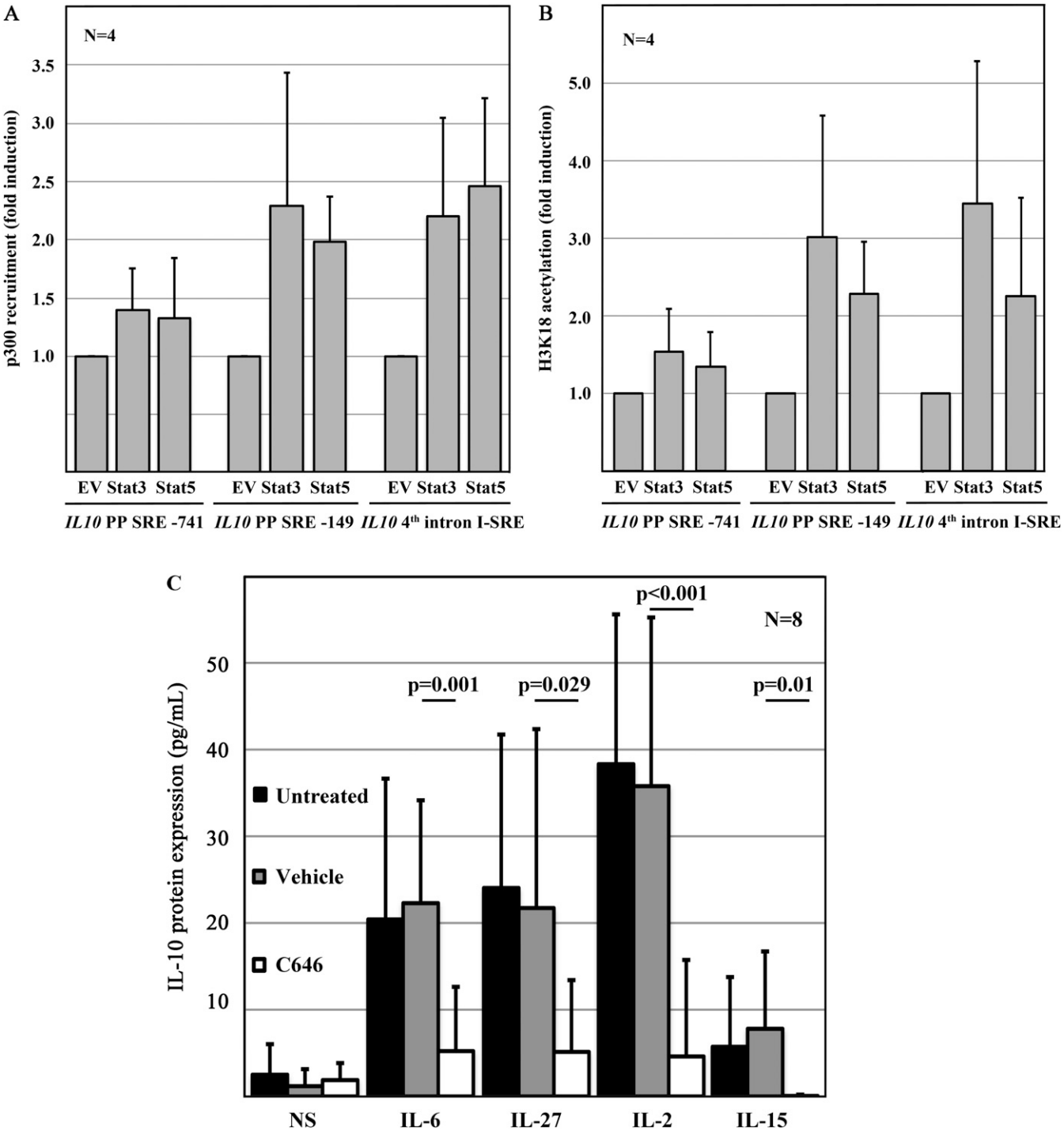

Fig. S4. Stat transcription factors mediate histone acetylation through p300. Forced Stat3 (*A*) or Stat5 (*B*) expression mediate p300 recruitment to SRE-149 and I-SRE in Jurkat T cells that is reflected by chromatin remodeling as assessed by H3K18ac. (*C*) Stimulation of primary human T cells with Stat-inducing cytokines (Stat3, IL-6 and IL-27; Stat5, IL-2 and IL-15) in the presence of the p300 inhibitor C646 is significantly impaired, indicating additional Stat-induced and p300-mediated effects on top of *trans*-activation. EV, empty vector; NS, not stimulated.

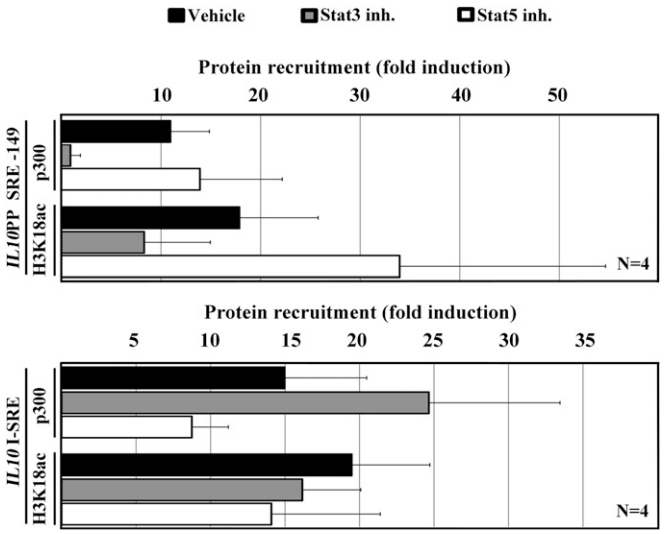

Fig. S5. Stat3/5 deficiency results in impaired histone acetylation. Primary human T cells from healthy individuals were stimulated with anti-CD3 and anti-CD28 antibodies in the absence (Vehicle control DMSO) or presence of Stat3 or Stat5 inhibitors as indicated. P300 recruitment to the *IL10* promoter at SRE-149 (*Upper*) or the intronic enhancer (I-SRE, *Lower*) and resulting histone acetylation at H3K18 was monitored through ChIP. Stat3 inhibition results in failure to recruit p300 to the *IL10* promoter (SRE-149) but not to the I-SRE, which in healthy individuals is occupied with Stat5. Inhibition of Stat5 does not affect p300 recruitment to the *IL10* promoter (SRE-149), which is occupied by Stat3. Weak effects of Stat5 inhibition on p300 recruitment to the I-SRE suggest a partial replacement with Stat3 secondary to an imbalance between Stat5 and Stat3 in analogy to SLE T cells.

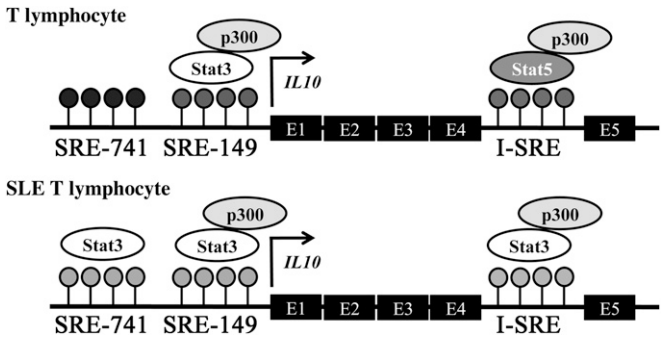

Fig. S6. Working model indicating the relationship between Stat3 and Stat5 recruitment and the epigenetic state of the *IL10* gene. (*A*) In T cells from healthy controls, intermediate DNA methylation of the proximal *IL10* promoter and an intronic enhancer allow for Stat3 recruitment to SRE-149 and I-SRE. Stat5 recruitment to the I-SRE results in epigenetic remodeling of the same regions through p300. (*B*) In T cells from SLE patients, reduced DNA methylation of the proximal *IL10* promoter and the intronic enhancer allows for Stat3 recruitment to SRE-741, SRE-149, and I-SRE. Stat3 replaces Stat5. Stat3 recruitment to SRE-149 and the I-SRE results in epigenetic remodeling through p300.