

cAMP-responsive Element Modulator α (CREM α) Suppresses IL-17F Protein Expression in T Lymphocytes from Patients with Systemic Lupus Erythematosus (SLE)*

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Background: CREM α is increased in SLE T cells, and although it suppresses IL-2, it enhances IL-17A production.

Results: CREM α binds to the *IL17F* promoter and suppresses its expression in SLE T cells.

Conclusion: CREM α disrupts the balance between IL-17A and IL-17F in SLE T cells in favor of IL-17A.

Significance: Understanding the molecular basis of the aberrant cytokine network in SLE will help in devising approaches to correct it.

The proinflammatory cytokines IL-17A and IL-17F are primarily produced by Th17 lymphocytes. Both are involved in host defense mechanisms against bacterial and fungal pathogens and contribute to the development of various autoimmune diseases. T lymphocytes from patients with systemic lupus erythematosus (SLE) display increased expression of transcription factor cAMP-responsive element modulator α (CREM α), which has been documented to account for aberrant T cell function and contributes to the pathogenesis of SLE. Here, we provide evidence that IL-17F expression is reduced in SLE T cells. We demonstrate that CREM α binds to a yet unidentified CRE site within the proximal promoter. This results in reduced IL-17F expression in SLE T lymphocytes and is independent of activating epigenetic patterns (increased histone H3 Lys-18 acetylation, reduced histone H3 Lys-27 trimethylation, and CpG-DNA demethylation). Forced CREM α expression in human T lymphocytes results in reduced IL-17F expression. Our findings demonstrate extended involvement of CREM α in cytokine dysregulation in SLE by contributing to a disrupted balance between IL-17A and IL-17F. An increased IL-17A/IL-17F ratio may aggravate the proinflammatory phenotype of SLE.

The IL-17 family of cytokines consists of six members: IL-17A through IL-17F (1). IL-17 cytokines play a key role in immune responses, and dysregulation of cytokine expression contributes to autoimmune disorders (2). The namesake cyto-

kine IL-17A was the first member to be described in 1993 (3). The remaining cytokines, IL-17B through IL-17F, have been identified subsequently, based on their homology with IL-17A, with IL-17F being the most conserved family member (50% amino acid conservation). The *IL17A* and *IL17F* genes are located on chromosome 6p12. IL-17A and IL-17F are produced by various immune cells, including T lymphocytes, natural killer cells, invariant natural killer cells, $\gamma\delta$ T cells, and neutrophils. Both cytokines are secreted as disulfide-linked homodimers or IL-17A/F heterodimers. They share key biological properties, including involvement in host defense mechanisms against bacteria and fungi, and IL-17F exerts proinflammatory functions, including the induction of chemokines, cytokines, and the recruitment of neutrophils to the site of inflammation (2). However, IL-17A homodimers seem to promote more robust proinflammatory responses when compared with IL-17F homodimers and IL-17A/F heterodimers (2, 4–7). Over the past decade, a specialized subset of IL-17-producing T helper lymphocytes, denoted Th17 cells, has been reported and extensively studied. Th17 lymphocytes are the predominant cell type, producing IL-17A and IL-17F (4, 5, 8, 9). Th17 subsets play a central role in adaptive immune responses and are involved in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE)⁴ (10). SLE is a chronic autoimmune disease that affects multiple organs and is characterized by severe T cell signaling abnormalities (11).

Dysregulation in IL-17A expression contributes to the pathophysiology of autoimmune disorders. Increased expression of IL-17A has been documented in SLE (11–14), rheumatoid arthritis (15), psoriasis (16), and multiple sclerosis (17, 18). Recently, we demonstrated the involvement of the transcriptional regulatory factor cAMP-responsive element modulator α (CREM α) in the induction of IL-17A expression in SLE T lymphocytes (14). The involvement of IL-17F in autoimmune

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⁴ The abbreviations used are: SLE, systemic lupus erythematosus; cAMP-responsive element modulator α ; CRE, cAMP-responsive element; H3K18, histone H3 Lys-18; H3K27, histone H3 Lys-27; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; CNS, conserved non-coding sequence(s).

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disease remains to be clarified. However, its expression has been reported to be elevated in rheumatoid arthritis, inflammatory bowel disease, and psoriasis (2).

In the present report, we link overexpression of the transcription regulatory factor CREM α to reduced IL-17F production in SLE T lymphocytes. CREM α belongs to a superfamily of transcription factors that includes cAMP-responsive element (CRE)-binding protein, the inducible cAMP-response element repressor, and activating transcription factors (14). In response to activation, these transcription factors bind to CREs (consensus sequence: TGACGTCA) or CRE half-sites (5' (TGAC) and/or 3' (GTCA)) in *cis*-regulatory regions. The presence of multiple CREM isoforms is caused by the presence of various promoters, alternative initiation sites, and differential splicing (19).

We previously reported that T lymphocytes from SLE patients display increased CREM α expression that is due to increased CREM promoter activity and reflects disease activity (20–23). Several target genes have been identified that are relevant for immune cell function and undergo *trans*-regulation by CREM α . These include the cytokine genes *IL17A* and *IL2* that are regulated antithetically (14, 24), the transcription factor *c-Fos* (20, 25), TCR/CD3 ζ , (26), and the antigen-presenting cell molecule CD86 (27). We further demonstrated that CREM α is involved in epigenetic remodeling of cytokine genes through histone deacetylase 1 (HDAC1) and DNA methyltransferase 3a (DNMT3a) recruitment to regulatory gene sequences.

Here, we demonstrate reduced IL-17F expression from SLE T lymphocytes. We show that CREM α binds to a yet unidentified CRE site within the proximal *IL17F* promoter (–127/–123 bp upstream of the transcriptional start site). CREM α recruitment to this site is associated with reduced IL-17F expression in T lymphocytes from SLE patients. We provide evidence that CREM α suppresses *IL17F* promoter activity. Reduced IL-17F expression is independent of activating epigenetic patterns in SLE T lymphocytes, including increased histone H3 Lys-18 (H3K18) acetylation, decreased H3K27 trimethylation, and cytosine-phosphate-guanosine (CpG)-DNA demethylation of the human *IL17* locus. Our data support the importance of CREM α in regulating the transcriptional machinery of SLE T lymphocytes and constitute the first report of reduced IL-17F expression in SLE. Because IL-17A/IL-17F heterodimers have reduced proinflammatory activities when compared with IL-17A, imbalances of the IL-17A/IL-17F ratio toward IL-17A may further contribute to the inflammatory phenotype of SLE.

EXPERIMENTAL PROCEDURES

Study Subjects and T Lymphocyte Culture—All SLE patients included in this study 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) after written informed consent under protocol 2006-P-0298. All included patients were female. Average SLE disease activity scores were 10.5, reflecting active disease. Epidemiological information including immunosuppressive medications is given in Table 1. Healthy ethnicity-, age-, and gender-matched individuals were

TABLE 1

Epidemiological data of the included SLE patients

Samples from the same patients (patients 1–6 and 7–10) were also used in Ref. 24. F, female; SLEDAI, systemic lupus erythematosus disease activity index (0, inactive; 1–5, mild activity; 6–10, moderate activity; 11–19, high activity; >20, very high activity). Aza, azathioprine; MMF, mycophenolate mofetil; HCQ, hydroxychloroquine; GC, glucocorticoids. IP, immunoprecipitation.

Patient	Gender	Age	SLEDAI	Treatment	Application
1	F	30	4	MMF, HCQ, GC	C ^m pG-IP
2	F	37	10	HCQ, GC	C ^m pG-IP
3	F	36	10	MMF, GC	C ^m pG-IP
4	F	39	8		C ^m pG-IP
5	F	38	36	GC	C ^m pG-IP
6	F	54	14	HCQ	C ^m pG-IP
Average		39	13.67		
Range		(30–54)	(4–36)		
7	F	36	4	MMF	ChIP
8	F	27	6	GC	ChIP
9	F	39	5	HCQ	ChIP
10	F	32	8	Aza	ChIP
11	F	45	4	HCQ, GC	ChIP
12	F	45	4	HCQ, GC	ChIP
13	F	48	4	MMF	ChIP
Average		33.5	5.00		
Range		(27–39)	(4–8)		

chosen as controls. Peripheral venous blood was collected in heparin-lithium tubes, and total human T lymphocytes were purified as described previously (14). Primary human T cells and human Jurkat T cells were maintained in RPMI medium supplemented with 10% fetal bovine serum. Naive CD4⁺ T cells from healthy controls were purified from total T cell suspension, using the Human Naive CD4⁺ T Cell Isolation kit II (Miltenyi Biotec). For activation assays, T lymphocytes were incubated in the absence or presence of plate-bound anti-CD3 and anti-CD28 antibodies (BioXCell; both at 1 μ g/ml) for 72 h.

mRNA Extraction and Quantitative RT-PCR—Total RNA was isolated, using the RNeasy minikit (Qiagen). Residual genomic DNA contamination was removed by DNase I (Qiagen). RNA was reverse-transcribed into cDNA, using the reverse transcription system (Promega). Sequences for primers used for qRT-PCR are given in Table 2.

IL-17A and IL-17F ELISAs—Primary human T lymphocytes were cultured for 72 h (in the presence or absence of plate-bound anti-CD3 and anti-CD28 antibodies as indicated). Supernatants were collected and measured for secreted IL-17A and IL-17F by ELISAs following the manufacturer's instructions (eBioscience, San Diego, CA).

Plasmids and Generation of Luciferase Reporter Constructs—An expression plasmid for human CREM α (on a pcDNA3.1/V5-His-TOPO backbone, Invitrogen) has been kindly provided by G. N. Europe-Finner (Faculty of Medical Sciences, Newcastle upon Tyne, UK) (28). Reporter constructs spanning the proximal 636 and 166 bp of the human *IL17F* promoter were PCR-amplified and cloned into luciferase vector pGL3-Basic (Promega), using primers with attached restriction sites for MluI and BglII. All plasmid DNA preparations were carried out with DNA purification kits (Qiagen) and sequence-verified (Genewiz, Cambridge, MA). Site-directed mutagenesis at the –127/–123 CRE site within reporter construct *IL17A_p(–166)_luc* was performed using a DNA oligonucleotide harboring a mutated CRE (5'-gaccggagttaccctgcgaatgcagggtttttttttattcc-3'; MWG Operon) and *PfuTurbo*[®]

TABLE 2
qRT-PCR primers

Primer	Forward (5'–3')	Reverse (5'–3')	Annealing temperature °C
IL-17A	ACCAATCCCAAAAGGTCTC	CACTTTGCCTCCCAGATCAC	60
IL-17F	CACGTAACATCGAGAGCCG	AGCCCAAGTTCCTACACTGG	60
18 S rRNA	ACTCAACACGGGAAACCTCA	AACCAGACAAATCGCTCCAC	60
IL17_CNS4	TTGGGTCAGATGGTGAGAAAG	GAAAGCAAGCAGCAAGGATG	55
IL17_CNS5	CCTGTGTATGCCTGTGTTC	GTCAGTAAC'TCCGGGTCAA	55
IL17_CNS6	CAGGAGCTGACCTTTCTA	TAGTTCTCCCAGCAGGTA	55

DNA polymerase (Stratagene) following the manufacturer's instructions.

Forced Expression of CREM α in Primary Human T Lymphocytes and Jurkat T Cells—Primary human T lymphocytes or Jurkat T cells (as indicated) were transfected with a total amount of 3.0 μ g of plasmid DNA, using the Amaxa transfection system (Lonza). Five hours after transfection, cells were collected and lysed, and mRNA was harvested and processed as indicated previously (14). All experiments were repeated at least three times, and values in the bar diagrams are given as mean \pm S.D.

Transfection of Jurkat T Cells with Control and CREM siRNA—Jurkat T cells were transfected with 5 nM irrelevant control siRNA and CREM-specific siRNA (OriGene) using Lipofectamine (Invitrogen). Prior to these experiments, experimental conditions were optimized, applying Cy-3-labeled control siRNA (OriGene) through transfection with Lipofectamine (Invitrogen). Transfection efficiency was >90%. Cells were collected after culture for 5 h in the absence and presence (as indicated) of soluble anti-CD3/CD28 antibodies (3 μ g/ml) and processed for mRNA analysis as indicated previously (14). All experiments were repeated three times, and values in the bar diagrams are given as mean \pm S.D.

Luciferase Assays in Jurkat T Cells—One million 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 \pm S.D.

ChIP Assays—Anti-HDAC1, anti-H3K18ac, and anti-H3K27me3 antibodies, and nonspecific normal rabbit mouse IgG were obtained from Upstate (Millipore). Polyclonal anti-CREM α antibody detecting human CREM α has been described before (23). ChIP grade Protein A/G Plus-agarose has been purchased from Pierce (ThermoScientific). ChIP assay was carried out according to the manufacturer's instructions (Upstate Biotechnology/Millipore). Briefly, cells were cross-linked with 1% formaldehyde, washed with cold phosphate-buffered saline, and lysed in buffer containing protease inhibitors (Roche Applied Science). Cell lysates were sonicated to shear DNA and sedimented, and diluted supernatants were immunoprecipitated with the indicated antibodies. A proportion (20%) of the diluted supernatants was kept as input control. Protein-DNA

complexes were eluted in 1% SDS, 0.1 M NaHCO₃ and reverse-cross-linked at 65 °C. DNA was recovered, using the QIAamp DNA minikit (Qiagen) and subjected to PCR analysis on an ABI OneStepPlus real-time PCR system. Real-time qPCR primer sequences are given in Table 2. The amount of immunoprecipitated DNA was subtracted by the amplified DNA that was bound by the nonspecific normal IgG and subsequently calculated relative to the respective input DNA.

IL-17A Secretion Assay—Human naive CD4⁺ T cells were activated, using anti-CD3/anti-CD28 antibodies for 72 h and PMA (50 ng/ml)/ionomycin (500 ng/ml) for another 5 h. Because IL-17A and IL-17F are largely co-expressed in human CD4⁺ T lymphocytes, the commercially available IL-17A secretion assay was performed according to the manufacturer's instructions (Miltenyi Biotec).

Methyl-CpG-DNA Immunoprecipitation—The methyl-CpG-DNA immunoprecipitation assay was carried out following the manufacturer's instructions (Zymo Research). Briefly, genomic DNA from T lymphocytes obtained from SLE patients and healthy control individuals was purified, applying the AllPrep RNA/DNA/protein minikit (Qiagen), and sheared to fragments of ~200-bp length, using DNA shearase (Zymo Research). Subsequently, 100 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 OneStepPlus real-time PCR system, using primers as listed in Table 2. Equal amounts (100 ng) of completely methylated (100%) human CpG-DNA and demethylated human CpG-DNA (Zymo Research) were included as "input" and negative control.

Statistical Analysis—Paired two-tailed Student's *t* test was used for statistical analysis.

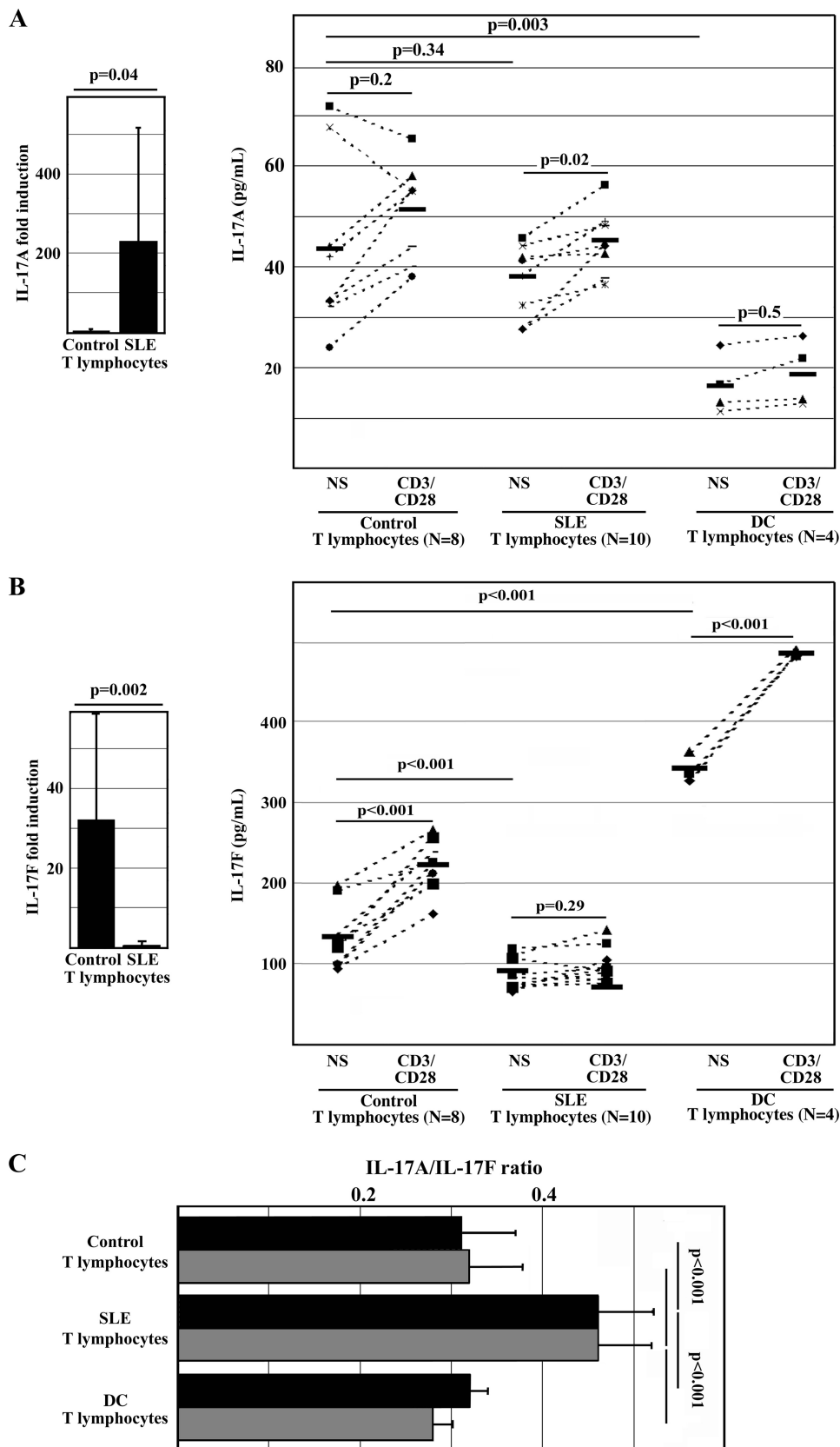
RESULTS

T Lymphocytes from SLE Patients Fail to Express IL-17F—Because IL-17A is overexpressed by T cells from SLE patients (11–13), and IL-17A and IL-17F are usually co-expressed in IL-17 expressing T lymphocytes (9), we asked whether this is also the case in SLE T cells. Thus, we cultured total T lymphocytes from healthy controls, SLE patients, and autoimmune disease control patients (psoriatic arthritis and rheumatoid arthritis) for 72 h in the presence or absence of anti-CD3/CD28 antibodies and determined IL-17A and IL-17F expression at the mRNA and protein level. In response to stimulation, T lymphocytes from SLE patients produced significantly more IL-17A mRNA when compared with healthy controls (Fig. 1A, left, *p* = 0.04). Interestingly, resting T lymphocytes from healthy controls and SLE patients expressed comparable amounts of

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IL-17A protein in the cell culture supernatant. IL-17A expression was inducible in T lymphocytes from SLE patients ($p = 0.02$), whereas the changes in IL-17A expression in control T

cells were variable and did not reach statistical significance (Fig. 1A, right, $p = 0.2$). Interestingly, T lymphocytes from autoimmune disease control patients produced significantly less



IL-17A protein when compared with control and SLE T lymphocytes ($p = 0.003$). In contrast to these observations, T lymphocytes from SLE patients failed to produce IL-17F mRNA when compared with control T cells (Fig. 1*B*, left, $p = 0.002$). Resting T lymphocytes from healthy donors and autoimmune disease control patients expressed significantly more IL-17F protein when compared with SLE T lymphocytes (Fig. 1*B*, left, $p < 0.001$). Stimulation with anti-CD3/CD28 resulted in increased IL-17F expression in T lymphocytes from all control individuals ($p < 0.001$), whereas the IL-17F expression from SLE T lymphocytes did not increase further ($p = 0.29$).

In order to further assess differences in the regulation of IL-17A and IL-17F expression in T lymphocytes from controls, SLE patients, and autoimmune disease control individuals, we calculated IL-17A/IL-17F protein expression ratios of cell culture supernatants in resting total T lymphocytes and in response to T cell activation with anti-CD3/CD28 antibodies (Fig. 1*C*). Interestingly, the IL-17A/IL-17F ratio of both control and autoimmune disease control T lymphocytes was significantly lower when compared with T lymphocytes from SLE patients ($p < 0.001$). This reflects “symmetrically” increased IL-17A and IL-17F protein expression from autoimmune disease control T lymphocytes. However, T lymphocytes from SLE patients produce significantly increased amounts of IL-17A but fail to produce IL-17F.

Chromatin Modifications in Conserved Regions of Human *IL17F* Gene—We previously reported that epigenetic modifications of *IL2* and *IL17* contribute to the dysregulation of these genes in SLE T lymphocytes. Next, we aimed to investigate epigenetic patterns across the *IL17F* gene in naive and activated CD4⁺ T lymphocytes from healthy control individuals and total T lymphocytes from SLE patients and controls. Therefore, we defined regions of interest within and around the *IL17F* gene, based on bioinformatic approaches. We aligned the mouse and human *IL17F* genes (VISTA Genome Browser, available on the World Wide Web) and determined conserved non-coding sequences (CNS; Fig. 2*A*). CNS regions were defined as regions with sequence homology of >75% between the human and mouse genes over at least 200 bp. Based on the degree of conservation, we identified three regions of interest, one of which was ~15 kbp upstream of the *IL17F* gene (CNS6), one mapping to the proximal promoter (CNS5) that contains a yet unidentified CRE site (−127/−123), and one 3′ of the *IL17F* gene (CNS4). We performed ChIP analyses in naive and activated CD4⁺ T cells, using antibodies against activating histone H3 acetylation (H3K18ac) or repressive H3 methylation (H3K27me3). Throughout the analyzed regions, we detected decreased H3K27 trimethylation and enriched H3K18 acetyla-

tion following T cell activation (Fig. 2*B*). Subsequently, we compared these histone modifications in total T cells obtained from SLE patients and matched healthy individuals. T lymphocytes from SLE patients display reduced H3K27 trimethylation and increased H3K18 acetylation when compared with control T lymphocytes (Fig. 2*C*). Thus, our data suggest a similar pattern in SLE T cells as observed in activated naive CD4⁺ T cells.

CpG-DNA Methylation in Activated T Lymphocytes from SLE Patients and Healthy Controls—Histone hypermethylation is frequently accompanied by concordant CpG-DNA methylation (29–31). Because the investigated CNS regions and the proximal promoter of the human *IL17F* gene contain CpG-rich regions (Fig. 2*A*), we investigated CpG-DNA methylation patterns across these regions in resting and activated naive CD4⁺ T lymphocytes from healthy donors and in total T lymphocytes from SLE patients and matched control individuals.

Because IL-17A and IL-17F are usually co-expressed in T lymphocytes from healthy donors (9), we sorted between IL-17A-secreting and -non-secreting cells, using cytokine capture assays. Therefore, naive CD4⁺ T cells from healthy blood donors were stimulated with anti-CD3/anti-CD28 antibodies for 72 h, followed by PMA/ionomycin treatment for another 5 h. CD4⁺ T lymphocytes were enriched for IL-17A-secreting cells, applying antibodies that yielded a purity of ~30% within the “IL-17A secretor” group (as reported previously (14) and measured by flow cytometry (data not shown)). Cells in the non-IL-17A secretor group did not stain for intracellular IL-17A. CpG-DNA methylation within and around the *IL17F* gene was significantly decreased among the IL-17A-secreting T cells, suggesting an “open” chromatin conformation that is accessible for transcription regulatory factors (Fig. 2*D*). Subsequently, we analyzed CpG-DNA methylation of the human *IL17F* gene in total T lymphocytes from six age-, gender-, and ethnicity-matched SLE and control individuals (Fig. 2*E*). Total T cells were isolated and activated by subsequent CD3/CD28 and PMA/ionomycin stimulation as aforementioned. Throughout the analyzed regions, SLE T cells displayed lower degrees of CpG-DNA methylation in response to T cell activation as compared with control T lymphocytes. These findings suggest that IL-17F production in human T lymphocytes can be regulated by CpG-DNA methylation and that T cell activation in SLE T lymphocytes results in remodeling of the *IL17* locus and increases the accessibility for *trans*-regulatory factors. However, this does not explain why IL-17F expression is reduced in SLE T lymphocytes.

FIGURE 1. Activated T lymphocytes from SLE patients fail to produce IL-17F. Primary T lymphocytes from healthy control individuals, SLE patients, and control individuals with other autoimmune diseases (DC; rheumatoid arthritis and psoriatic arthritis) were cultured in the presence or absence (not significant; NS) of anti-CD3 and anti-CD28 antibodies. After 72 h, cells were harvested and subjected to qRT-PCR; supernatants were subjected to IL-17A and IL-17F ELISA. *A*, T lymphocytes from SLE patients express significantly more IL-17A mRNA in response to stimulation with anti-CD3/CD28 antibodies when compared with T lymphocytes from healthy controls (left). T lymphocytes from healthy controls and SLE patients produce comparable amounts of IL-17A protein in response to T cell activation (72 h), whereas resting and activated T lymphocytes from DC patients produce lower amounts of IL-17A (right). *B*, T lymphocytes from SLE patients express significantly lower amounts of IL-17F mRNA in response to stimulation with anti-CD3/CD28 antibodies when compared with T lymphocytes from healthy controls (left). T lymphocytes from healthy controls and DC patients produce significantly more IL-17F in response to T cell activation, whereas resting and activated T lymphocytes from SLE patients fail to produce IL-17F (right). *C*, failure to express IL-17F protein results in an increased IL-17A/IL-17F (protein) ratio of SLE T lymphocytes when compared with healthy control and autoimmune disease control T cells. Black bars, IL-17A/IL-17F ratio in unstimulated cells; gray bars, cells after stimulation with anti-CD3/CD28 antibodies for 72 h. Error bars, S.D.

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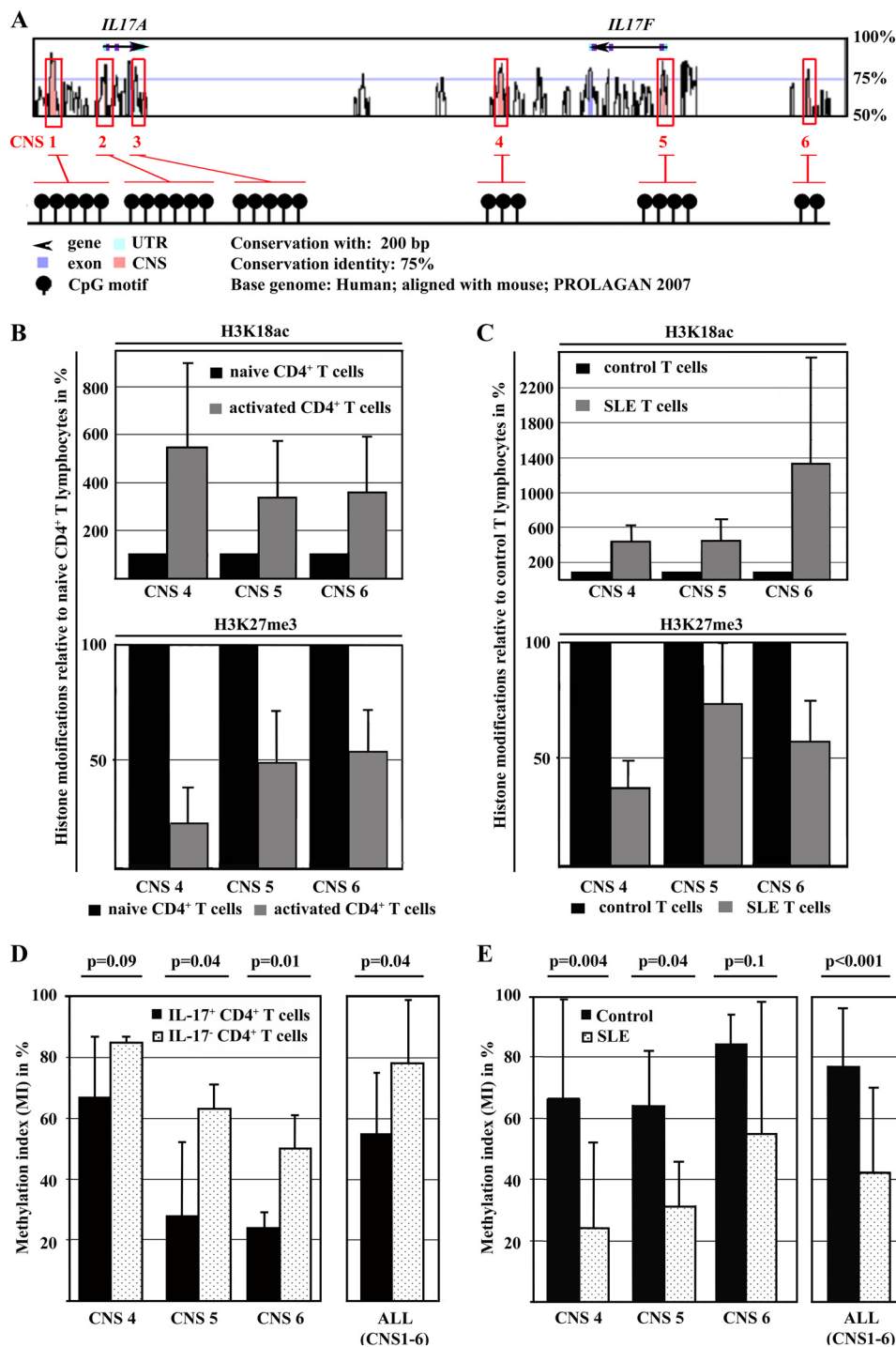


FIGURE 2. Epigenetic remodeling of the human *IL17F* gene in response to T cell activation and in SLE T lymphocytes. *A*, alignment of the human and mouse *IL17* gene locus. Conserved CNS (pink) were defined as regions with sequence homology of >75% between humans and mice over a length of at least 200 bp. Exons of the human *IL17A* and *IL17F* genes are displayed in purple, conserved non-coding sequences are shown in pink, and untranslated regions (UTR) are shown in turquoise. *B*, H3K18 acetylation (top) and H3K27 trimethylation (bottom) were analyzed in resting (black bars) and activated (gray bars) naive CD4⁺ T cells from four healthy individuals by ChIP assays. The ratio of relative histone acetylation/methylation was determined between unstimulated and the activated naive T cells (from the same individual). Black bars represent histone methylation/acetylation in unstimulated cells (set to 100%). Changes in the methylation or acetylation status following T cell activation are given in the bar diagram (mean ± S.D. (error bars)). *C*, H3K18 acetylation (top) and H3K27 trimethylation (bottom) were analyzed in T lymphocytes from four matched healthy controls (black bars) and four SLE patients (gray bars) by ChIP assays. The ratio of relative histone acetylation/methylation was determined between control and SLE T cells. Black bars represent histone methylation/acetylation in healthy control T cells (set to 100%). *D*, naive CD4⁺ T cells from healthy controls that had been sequentially stimulated with anti-CD3/anti-CD28 antibodies for 72 h and with PMA/ionomycin for another 5 h were subjected to an IL-17A secretion assay. ChIP was performed in both IL-17A-enriched T cells (black bars) and non-IL-17A-secreting T cells (dotted bars), using antibodies that specifically bind methyl-CpG-DNA. Methylated DNA was recovered, and CNS regions were amplified by real-time qPCR. Completely methylated (input; 100%) and unmethylated human DNA samples (negative control; 0%) were included. Values are given as mean ± S.D. from six independent experiments. *E*, total T cells from six matched SLEs (gray bars) and healthy control individuals (CON; black bars) were subjected to CpG-DNA immunoprecipitation. Percentages of methylated DNA are given as mean ± S.D.

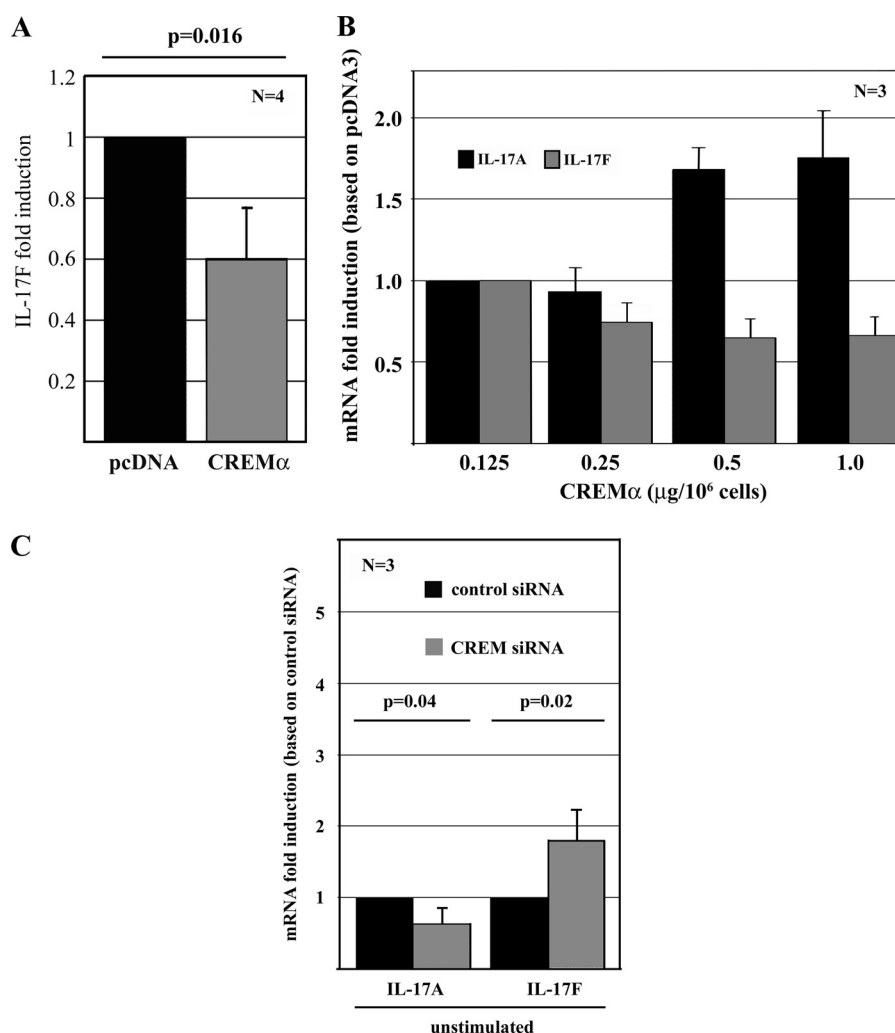


FIGURE 3. CREM α down-regulates IL-17F expression. *A*, pcDNA3 or CREM α expression plasmid were transfected into primary human T cells and 5 h after transfection, RNA was analyzed for IL-17A and 18 S rRNA expression, using qRT-PCR. Experiments were performed in T cells from four different healthy blood donors. The *bar diagram* displays the mean relative IL-17F expression (after CREM α overexpression) \pm S.D. (*error bars*) from four experiments. *B*, different concentrations (as indicated) of pcDNA3 or CREM α expression plasmid were transfected into Jurkat T cells. Five hours after transfection, mRNA was analyzed for IL-17A and 18 S rRNA expression, using qRT-PCR. The *bar diagrams* display the mean relative IL-17A (*black bars*) and IL-17F (*gray bars*) expression as induced by CREM α overexpression \pm S.D. from three experiments. *C*, Jurkat T cells were transfected with 5 nM control and CREM α siRNA. Five hours after transfection, mRNA was analyzed for IL-17A and 18 S rRNA expression, using qRT-PCR. Cytokine expression levels in response to transfection with control siRNA were determined as a relative value of 1. *Bar diagrams* display the mean relative IL-17A and IL-17F expression as induced by CREM α knockdown \pm S.D. from three unrelated experiments.

Forced CREM α Expression Suppresses IL-17F Expression in Primary Human T Lymphocytes—SLE T cells display reduced IL-17F production and elevated cellular CREM α protein levels (Fig. 1A) (21). Increased CREM α expression has been demonstrated to contribute to decreased IL-2 and increased IL-17A production in T lymphocytes from SLE patients. Thus, we considered that CREM α may also be involved in *IL17F* gene regulation. Primary human T cells from healthy individuals display low levels of CREM α . In order to mimic the conditions in SLE T lymphocytes, we transiently transfected primary human T cells (from healthy blood donors) with a His₆-tagged CREM α expression plasmid (as previously reported in Ref. 14). mRNA obtained from these cells was tested for relative IL-17F expression and proved to express significantly reduced IL-17F transcript numbers after forced CREM α expression as assessed by real-time quantitative RT-PCR (Fig. 3B; relative change of 0.6; $p = 0.016$).

In order to assess dose-dependent effects of CREM α on IL-17A and IL-17F expression, we transfected Jurkat T cells with increasing amounts of CREM α expression plasmids. We detected a dose-dependent increase in IL-17A mRNA expression, whereas IL-17F mRNA expression was subject to transcriptional repression (Fig. 3C). This reflects the characteristics of T lymphocytes from SLE patients that show elevated cellular levels of CREM α , resulting in overexpression of IL-17A and failure to express IL-17F.

CREM α Knockdown with CREM siRNA Suppresses IL-17A and Enhances IL-17F mRNA Expression—To further establish the involvement of CREM α in the regulation of IL-17A and IL-17F, we transfected Jurkat T cells with unrelated control siRNA and siRNA directed against CREM α . mRNA obtained from transfected cells was tested for relative IL-17A and IL-17F expression and proved to express significantly reduced IL-17A ($p = 0.04$) and increased IL-17F ($p =$

SLE T Lymphocytes Fail to Produce IL-17F

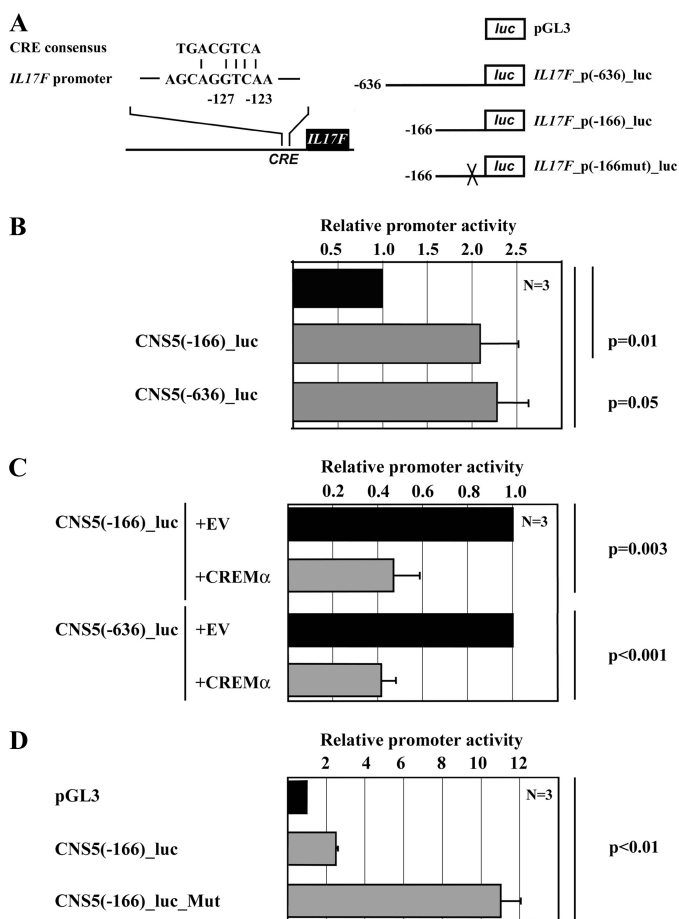


FIGURE 4. CREM α binding reduces *IL17F* promoter activity. *A*, alignment of the CRE consensus sequence with the CRE site (–127/–123) of the proximal human *IL17F* promoter. The schematic on the right displays *IL17F* reporter plasmids used for luciferase assays (636 and 166 bp). *IL17F*_p(–166mut)_luc indicates a reporter plasmid containing a site-directed mutation at the CRE site (–127/–123). *B*, human Jurkat T cells were transfected with the empty pGL3-Basic reporter (black bar) or the *IL17F* promoter-driven reporter plasmids (gray bars). Cells were collected 5 h after transfection, and firefly luciferase activity was measured and normalized by *Renilla* luciferase activity. $n = 3$ independent experiments. *C*, human Jurkat T cells were co-transfected with the indicated reporter constructs and either pcDNA3 empty vector (EV; black bars) or CREM α expression plasmid (gray bars). For each reporter, pcDNA3 empty vector (EV) co-transfection was set to 1.0, and the relative effect mediated by CREM α was calculated. Each experiment was performed three times, and values are given as mean \pm S.D. *D*, mutation of the CRE site (–127/–123) results in a significantly increased spontaneous promoter activity. $n = 3$ independent experiments.

0.02) transcript numbers (Fig. 3C). This provides further proof of the involvement of CREM α in the regulation of IL-17A and IL-17F.

CREM α Negatively Regulates *IL17F* Promoter Activity—A bioinformatic analysis of the human *IL17F* promoter revealed a putative CRE site located between 127 and 123 bp upstream of the transcription initiation site, thus denoted the –127/–123 CRE site (Fig. 4A). This site defines a 4-bp sequence that matches the perfect CRE 3'-half-site (GTCA), which has been shown to serve as a minimum binding motif for CREM proteins in other *cis*-regulatory target sequences (14, 21, 27). To investigate whether CREM α regulates IL-17F expression at the transcriptional level, we performed reporter studies, using promoter constructs spanning the proximal 636 or 166 bp of the human *IL17F* promoter (Fig. 4B). Indeed, CREM α overexpres-

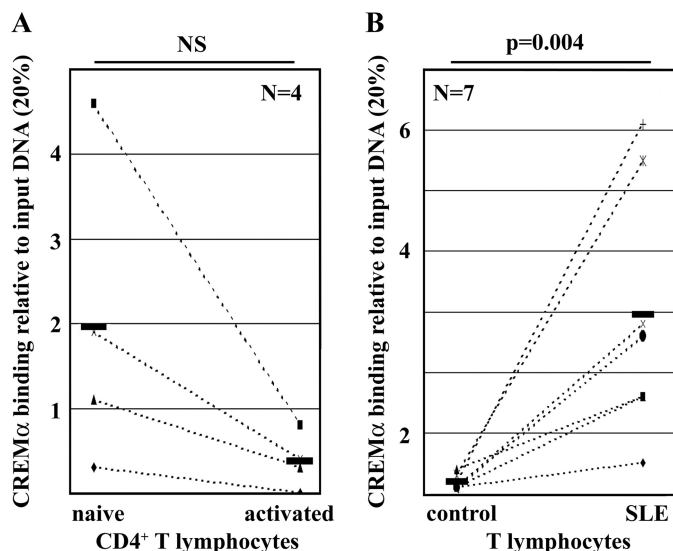


FIGURE 5. CREM α binds to the –127/–123 CRE site within the proximal *IL17F* promoter. *A*, naive human CD4⁺ T lymphocytes were isolated from four healthy individuals and cultured in the absence or presence of anti-CD3/anti-CD28 antibodies for 72 h. ChIP assays were performed in order to detect CREM α recruitment to the –127/–123 CRE site. Immunoprecipitated DNA was analyzed by real-time qPCR, amplifying a region that covers the CRE site within the proximal *IL17F* promoter. Ratios between anti-CREM α -immunoprecipitated and input DNA are shown. Dotted lines associate data between unstimulated and corresponding activated cells obtained from the same individual. Black bars represent the mean of the four experiments. *B*, CREM α ChIP was performed with total T lymphocytes from seven individually matched pairs of SLE patients and healthy controls. Immunoprecipitated DNA was analyzed by real-time qPCR. Ratios between anti-CREM α -immunoprecipitated and input DNA are shown. Dotted lines associate data between matched control/SLE pairs. Black bars, mean values. NS, not significant.

sion resulted in significantly down-regulation of *IL17F* promoter activities in both constructs (–fold change of 0.47 in *IL17F*_p(–166)_luc, $p = 0.003$, and 0.41 in *IL17F*_p(–636)_luc, $p < 0.001$) (Fig. 4C). Next, we mutated the –127/–122 CRE site within the 166 bp-spanning *IL17F* reporter construct and noted a significantly increased activity of the *IL17F* promoter construct (–fold change of 10.99, $p < 0.001$) (Fig. 4D). These results suggest that this site is crucial for CREM α -mediated down-regulation of *IL17F* transcription in human T lymphocytes.

CREM α Binding to the –127/–123 CRE Site Is Increased in Activated Naive CD4⁺ T Cells and T Lymphocytes from SLE Patients—We addressed the *in vivo* relevance of CREM α binding to the –127/–123 CRE site in the human *IL17F* promoter by ChIP assays, using a CREM α -specific polyclonal antibody. We used naive CD4⁺ T cells that were freshly isolated from total human T lymphocytes and compared them with naive CD4⁺ T cells that were stimulated with anti-CD3 and anti-CD28 antibodies for 72 h (Fig. 5, A and B). We observed a trend toward reduced CREM α binding to this site in activated naive T lymphocytes after individual comparison with CREM α binding in unstimulated naive CD4⁺ T cells (–fold increase of 0.05, not significant (NS); Fig. 5A). Because T lymphocytes from SLE patients display signaling abnormalities resembling an activated phenotype, we aimed to determine CREM α recruitment to the *IL17F* promoter in total T lymphocytes from seven SLE patients and seven healthy control individuals that were individually matched by age, gender, and ethnicity (Fig. 5B). In agreement with the reduced expression of IL-17F in SLE T lym-

phocytes and reduced IL-17F expression in response to forced CREM α overexpression, we determined significantly increased CREM α binding to this site in SLE T lymphocytes (-fold increase of 9.28, $p = 0.004$). Our data suggest that increased CREM α binding to the *IL17F* promoter in SLE T cells results in potent transcriptional repression of *IL17F* gene expression.

DISCUSSION

Here we present evidence that the transcription regulatory factor CREM α mediates reduced IL-17F expression in T lymphocytes from SLE patients, regardless of an “open” chromatin conformation of the *IL17* locus. CREM α levels were documented to be increased in T lymphocytes from SLE patients (21, 32). This is mediated by increased mRNA expression of the *CREM* gene in a disease activity-dependent manner (20, 21, 32). We previously documented that overexpression of CREM α in human T lymphocytes results in increased IL-17A expression, which is mediated by direct transcriptional effects (14).

IL-17 cytokines have been documented to be key players in adaptive immune responses (2). The namesake cytokine IL-17A was first discovered as “cytotoxic T lymphocyte-associated antigen 8” but has been documented to be expressed by multiple cells, including immune cells, fibroblasts, and synovio-cytes (2, 3, 15). Over the past decade, the IL-17 cytokine family has been expanded by five new members, IL-17B through IL-17F, with IL-17A and IL-17F being the most closely related and well studied (2). Both cytokines, IL-17A and IL-17F, are co-expressed by specialized T helper subsets, which are referred to as Th17 cells, and exhibit similar expression patterns (9). To this point, it remained unclear whether IL-17A and IL-17F underlie similar, overlapping, or divergent expression profiles in autoimmune disorders. IL-17A and IL-17F have been reported to induce proinflammatory responses through chemokine and cytokine induction as well as the recruitment of proinflammatory immune cells to the site of inflammation (2, 33). Both cytokines are involved in the pathogenesis of several autoimmune diseases, including rheumatoid arthritis (15), psoriasis (16), and multiple sclerosis (17, 18). In rheumatoid arthritis, IL-17A and IL-17F exert proinflammatory functions through Stat3, NF- κ B, MAPK pathways, and CCAAT/enhancer-binding protein transcription factors. Thereby, IL-17A and IL-17F enhance innate immune responses that mediate inflammation.

Imbalances between pro- and anti-inflammatory cytokines that result from a disrupted transcriptional network are hallmarks of the pathogenesis of various autoimmune diseases, including SLE (34). We previously documented that SLE patients display increased IL-17A levels and that IL-17A-producing T lymphocytes infiltrate target organs, including the kidneys (12, 13). Recent reports from our group suggest anti-thetic effects of the transcription factor CREM α on *IL2* and *IL17A* gene expression at both the transcriptional and epigenetic level (14, 20). Interestingly, CREM α recruitment is increased to a comparable extent at CRE sites of the *IL2*, *IL17A*, and *IL17F* promoter (~4–6-fold). However, the functional downstream effects of increased CREM α binding on gene expression of these cytokines are diametric. CREM α contrib-

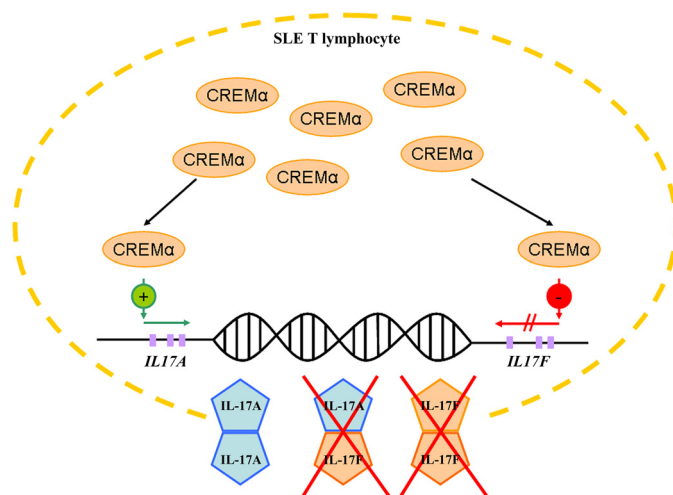


FIGURE 6. CREM α effects on IL-17A/IL-17F expression in SLE T lymphocytes. Overexpression of CREM α in T lymphocytes from SLE patients results in increased CREM α recruitment to both *IL17A* and *IL17F* promoter. As we reported previously (14), CREM α binding results in increased expression of IL-17A in SLE T cells. CREM α recruitment to the *IL17F* promoter mediates reduced IL-17F expression. These diametric mechanisms result in an “SLE-characteristic” imbalance between IL-17A and IL-17F levels and probably impaired assembly of IL-17A/IL-17F heterodimers, which have reduced pro-inflammatory activity as compared with IL-17A homodimers. This mechanism may have severe implications for the inflammatory cascades in SLE disease expression.

utes to increased IL-17A expression through 1) direct *trans*-activating effects on the *IL17A* promoter and 2) associated activating epigenetic modifications, including increased H3K18 acetylation, reduced H3K27 trimethylation, and reduced CpG-DNA methylation of the *IL17A* gene in SLE T cells (14). *IL2* undergoes 1) direct transcriptional repression at the –180 CRE element of the *IL2* promoter, 2) HDAC1 recruitment to the same site, and 3) CpG-DNA methylation because CREM α recruits DNMT3a to the *IL2* –180 CRE element (24, 35–37). Furthermore, reduced expression of the transcription factor AP-1 (c-Fos) results in impaired IL-2 expression (25).

Activated CD4⁺ T lymphocytes from healthy donors exhibit chromatin modifications that reflect an “open” chromatin conformation, because it is accessible for transcription regulatory factors, and express IL-17F. In T lymphocytes from SLE patients that fail to produce IL-17F, CREM α binds to the *IL17F* promoter and induces direct transcriptional repression at the –127/–123 CRE element. This results in reduced IL-17F expression that is independent of the aforementioned “open” chromatin conformation of the entire *IL17* locus. Whether our observations have the potential to be used as biomarkers for disease activity or disease outcome remains to be elucidated in a larger cohort. All patients included in our studies had moderate to high disease activity, and the pharmacological treatment of the single patients was rather heterogeneous. Because environmental factors, including medication, have been reported to impact epigenetic patterns, this could be a limitation of this study. However, epigenetic patterns and CREM α recruitment to the *IL17F* promoter was comparable in all investigated individuals. Further studies in larger cohorts are warranted in order to investigate the influence of 1) disease activity, 2) disease progression, and 3) pharmacological treatment on epigenetic

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marks and cytokine expression in T lymphocytes from SLE patients.

Our findings may well be significant in the pathophysiology of SLE because reduced IL-17F expression in SLE T lymphocytes may result in reduced assembly of IL-17A/F heterodimers. It has been documented that IL-17F homodimers and IL-17A/F heterodimers exert reduced proinflammatory capacities when compared with IL-17A homodimers (2, 4–7). In concert with increased IL-17A expression in SLE T lymphocytes, reduced IL-17F expression contributes to a disrupted IL-17A/IL-17F ratio toward IL-17A (Fig. 6). It appears likely that this imbalance orchestrates the inflammatory phenotype of SLE and is responsible for at least some of the differences in organ-specific symptoms between SLE and other (less severe) autoimmune disorders. Our observation that forced expression of CREM α mediates diametric effects on the expression of IL-17A and IL-17F, mimicking the phenotype of SLE T lymphocytes, and that this can be reversed by the application of CREM-specific siRNA stresses the involvement of CREM α in the pathophysiology of SLE and its role as a putative therapeutic target.

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