THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 4, pp. 2361–2370, January 24, 2014

cAMP Responsive Element Modulator (CREM) α Mediates Chromatin Remodeling of *CD8* during the Generation of $CD3^+CD4^-CD8^-$ T Cells*

Received for publication, October 3, 2013, and in revised form, November 20, 2013 Published, JBC Papers in Press, December 2, 2013, DOI 10.1074/jbc.M113.523605

Christian M. Hedrich[‡§1], José C. Crispín[‡], Thomas Rauen[‡¶], Christina Ioannidis[‡], Tomohiro Koga[‡], Noe Rodriguez Rodriguez[‡], Sokratis A. Apostolidis[‡], Vasileios C. Kyttaris[‡], and George C. Tsokos[‡2]

From the [‡]Department of Medicine, Division of Rheumatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115, the [§]Department of Pediatric Rheumatology and Immunology, University Children's Hospital Dresden, 01307 Dresden, Germany, and the [¶]Department of Nephrology and Clinical Immunology, RWTH University of Aachen, 52074 Aachen, Germany

Background: Expanded double negative T cells in systemic lupus erythematosus (SLE) originate from $CD8^+$ T cells.
Results: cAMP responsive element modulator (CREM) α induces epigenetic remodeling of the *CD8* cluster through DNMT3a and G9a.
Conclusion: CREMα centrally contributes to double negative T cell expansion in SLE pathogenesis.
Significance: CREMα governs T cell distribution in SLE.

TCR-$\alpha\beta^+CD3^+CD4^-CD8^-$ "double negative" T cells are expanded in the peripheral blood of patients with systemic lupus erythematosus (SLE) and lupus-prone mice. Double negative T cells have been claimed to derive from $CD8^+$ cells that down-regulate CD8 co-receptors and acquire a distinct effector phenotype that includes the expression of proinflammatory cytokines. This, along with the fact that double negative T cells have been documented in inflamed organs, suggests that they may contribute to disease expression and tissue damage. We recently linked the transcription factor cAMP responsive element modulator (CREM) α, which is expressed at increased levels in T cells from SLE patients and lupus prone MRL/*lpr* mice, with trans-repression of a region syntenic to the murine *CD8b* promoter. However, the exact molecular mechanisms that result in a stable silencing of both *CD8A* and *CD8B* genes remain elusive. Here, we demonstrate that CREMα orchestrates epigenetic remodeling of the *CD8* cluster through the recruitment of DNA methyltransferase (DNMT) 3a and histone methyltransferase G9a. Thus, we propose that CREMα is essential for the expansion of double negative T cells in SLE. CREMα blockade may have therapeutic value in autoimmune disorders with DN T cell expansion.

Systemic lupus erythematosus (SLE)[3] is an autoimmune disorder that can affect any organ or system and cause severe complications. Regardless of recent advances in the search for disease mechanisms, the molecular pathophysiology of SLE remains largely unknown. TCR-$\alpha\beta^+CD3^+CD4^-CD8^-$ "double negative" (DN) T cells are expanded in the peripheral blood of SLE patients and lupus-prone MRL/*lpr* mice. We recently demonstrated that DN T cells in humans and MRL/*lpr* mice derive from $CD8^+$ T cells by down-regulating CD8 surface-receptor expression (1).

The regulation of CD8 has been studied in humans and mice. In both species, mature $CD4^+$ and $CD8^+$ T cells derive from $CD4^-CD8^-$ double negative thymocytes that convert into $CD4^+CD8^+$ double positive progenitor cells, which later on during their differentiation into mature T cells down-regulate either CD4 or CD8 (2, 3). Thymus-derived $CD8^+$ T cells express heterodimers of CD8α and CD8β on their surface, whereas gut-derived $CD8^+$ T cells or $CD8^+$ dendritic cells express CD8α homodimers (2, 3). We have reported that during the TCR activation-induced transformation of $CD8^+$ T cells into peripheral DN T cells, the transcription factor cAMP responsive element modulator (CREM) α *trans*-represses a region syntenic to the murine *CD8b* promoter in human $CD8^+$ T cells. *Trans*-repression of this regulatory element results in transcriptional silencing and subsequently down-regulation of CD8 surface expression (1). This is of special interest, because CREMα is expressed at increased levels in T cells from SLE patients where it affects several T cell functions, including cytokine expression (1, 4). However, our findings did not completely explain the down-regulation of both CD8α and CD8β in response to TCR stimulation.

Phenotypes of specialized T cell subsets correlate with the exclusive expression of either CD4 or CD8, suggesting that the molecular mechanisms regulating CD4 or CD8 expression may also be involved in the definition of the phenotype of $CD4^+$ helper or $CD8^+$ cytotoxic T cells (5). Four clusters with increased DNase sensitivity have been identified within the murine *CD8* locus, which are syntenic with six in the human cluster (2, 3). Transgenic reporter systems allowed the identifi-

* This work was supported, in whole or in part, by National Institutes of Health Grants R01 AI42269, R01 AI49954, and R01 AI85567 (to G. C. T.).
[1] To whom correspondence may be addressed. E-mail: chedrich@bidmc.harvard.edu.
[2] To whom correspondence may be addressed. E-mail: gtsokos@bidmc.harvard.edu.
[3] The abbreviations used are: SLE, systemic lupus erythematosus; DN, double negative; CREM, cAMP responsive element modulator; MeDIP, methylated CpG-DNA immunoprecipitation; PLA, proximity ligation assay; DNMT, DNA methyltransferase; CREB, cAMP-response element-binding protein; RUNX, Runt-related transcription factor; co-IP, co-immunoprecipitation.

cation of several enhancer elements within the *CD8* cluster ($E8_I$–$E8_{IV}$) (2, 3, 5–15). This enhancer network is required for lineage-specific regulation of CD8α and CD8β during T cell development and its elements undergo epigenetic remodeling during T cell development either allowing or prohibiting the expression of CD8A and/or CD8B (3). Epigenetic mechanisms regulate gene expression by influencing the accessibility of chromatin to transcription factors and RNA polymerases (16). The addition of methyl groups to the 5′-carbon end of cytidine residues in cytidine-phosphate-guanosine sequences of the genomic DNA, and post-translational modifications to the amino terminus of histone proteins represent the two main mechanisms during chromatin remodeling (16). It has been demonstrated that the *CD8* cluster in mice undergoes epigenetic remodeling during T cell development in the thymus (4). Low degrees of DNA methylation in $CD4^+CD8^+$ double positive and $CD8^+$ T cells allow the expression of murine *CD8a* and *CD8b*, whereas increased levels of DNA methylation around the *CD8a* and *CD8b* genes in $CD4^+$ and DN T cells prohibit gene expression (4).

In this study we asked whether the *CD8* cluster undergoes epigenetic remodeling in $CD8^+$ T cells in response to TCR stimulation. We investigated whether the transcription factor CREMα, which is induced in response to TCR stimulation and expressed at increased levels in T cells from SLE patients induces chromatin remodeling of the CD8 cluster in response to TCR activation. We demonstrate that CREMα is recruited to several conserved non-coding regions within the human *CD8* cluster, mediating epigenetic silencing of *CD8A* and *CD8B*. We conclude that the transcriptional regulator CREMα plays a central role in mature $CD8^+$ T cell function and contributes to the expansion of DN T cells in patients with SLE.

MATERIALS AND METHODS

Cell Culture—Peripheral blood mononuclear cells were enriched for T lymphocytes by precipitation of non-T cells (Rosettesep, Stemcell Technologies) followed by density gradient centrifugation (Lymphoprep, Nycomed). From these T lymphocyte-enriched peripheral blood mononuclear cells, $CD4^-CD8^+$ T lymphocytes were isolated by negative selection (Dynabeads, Invitrogen). $CD8^+$ T lymphocytes were cultured at a concentration of 1×10^6 cells/ml in RPMI1640 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 120 h and harvested for quantitative RT-PCR, flow cytometry, methylated DNA immunoprecipitation (MeDIP), or chromatin immunoprecipitation (ChIP) as indicated.

Human Subjects—All 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, gender, 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).

Mice—MRL/*lpr* mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions. Experimental procedures were approved by the BIDMC Animal Care and Use Committee.

Flow Cytometry and Cell Sorting—Anti-CD4-PB, anti-CD8-PE, and anti-CD3-APC/Cy7 were purchased from BioLegend. Samples were acquired on a LSR II flow cytometer (BD Biosciences) and data were analyzed FlowJo version 7.2.2 (Tree Star). For the analysis of T lymphocyte populations, a first gate that included live cells was used. $CD3^+$ T lymphocytes were then plotted in a $CD4^+$ *versus* $CD8^+$ graphic that allowed the identification of discrete $CD4^+$, $CD8^+$, and double negative T lymphocyte populations. For some experiments, stained cells were sorted in a FACSAria flow cytometer (BD Biosciences), post-sorting purity was >98%.

Semi-quantitative Real-time Polymerase Chain Reaction—Total RNA from control and SLE T lymphocytes was isolated, using the Qiagen RNeasy Mini Kit (Qiagen). cDNA was generated using a first strand cDNA synthesis kit (Invitrogen). For gene expression analyses, real-time PCR was performed using SYBR Green site-specific primers on an ABI OneStepPlus Real-time PCR System. Results were normalized to 18S. Primer sequences for quantitative RT-PCR, plasmid generation, MeDIP, and ChIP PCR will be provided upon request.

Gene Expression Plasmids—Expression plasmids for human CREMα, DNMT3a, and G9a have been described previously (17, 18, 19). Three million primary human $CD8^+$ T lymphocytes were transfected with a total amount of 3 μg of the indicated expression plasmids using the Amaxa transfection system (Lonza) or Lipofectamine (Invitrogen) as indicated. After 24 (RNA, ChIP and MeDIP analyses) or 120 h (flow cytometry), cells were harvested and assayed.

Methylated CpG-DNA Immunoprecipitation (MeDIP)—The MeDIP assay was carried out according to the manufacturer's instructions (Zymo Research). Briefly, genomic DNA from T lymphocytes obtained from healthy individuals or SLE patients was purified using the AllPrep RNA/DNA/protein Mini kit (Qiagen), sheared to fragments of ~200 bp using DNA shearase (Zymo Research). Subsequently, 100 ng of sheared genomic DNA were used for methylated CpG-DNA immunoprecipitation. The same amounts (100 ng) of (100%) methylated human DNA, and unmethylated human DNA (Zymo Research) were included as "input" and negative control. Methylated DNA was recovered and subjected to PCR analysis on an ABI OneStepPlus real-time PCR system.

Chromatin Immunoprecipitation (ChIP) Assays—Anti-H3K9m3e, anti-H3K27me3, and anti-G9a antibodies, nonspecific normal rabbit, and normal mouse IgG were obtained from Upstate (Millipore) and anti-DNMT3a antibody was from Abcam. Polyclonal anti-CREMα antibody detecting human CREMα has been described (20, 5).

Most ChIP experiments were carried out with the Upstate Biotechnology/Millipore Chip kit according to the manufacturer's instructions (Upstate Biotechnology/Millipore). For this assay, ChIP grade Protein A/G Plus-agarose was purchased from Pierce (ThermoScientific). For some experiments Forced CREMα expression combined with DNMT3a or G9a knockdown were carried out using Magnify ChIP assays (Invitrogen)

according to the manufacturer's protocol. Briefly, 1–2 million 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, sedimented, and diluted supernatants were immunoprecipitated with the indicated antibodies. A proportion (20% for Millipore, 10% for Invitrogen) of the diluted supernatants were kept as input (input represents PCR amplification of the total sample). 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.

Co-immunoprecipitation of DNMT3a or G9a with CREMα—One million HEK293T cells were transfected with expression plasmids (on a pcDNA3 backbone) for CREMα, DNMT3a, G9a, or a combination as indicated (2 μg of each plasmid per transfection) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. 24 h after transfection, cells were harvested and lysed in 400 μl of RIPA buffer including protease inhibitors (Roche Applied Science). Cell lysates were subjected to centrifugation (14,000 × *g*, 10 min, 4 °C) and 500 μg of total protein were incubated with anti-CREMα antibodies at 4 °C overnight and subjected to co-immunoprecipitation with the Pierce Co-IP kit, following the manufacturer's instructions (Pierce). Co-IP solutions were subjected to SDS-PAGE as described before (17, 18). Proteins were transferred to PVDF membranes and detected by anti-DNMT3a or G9a antibodies as indicated, applying suitable secondary peroxidase-linked anti-rabbit antibody (Santa Cruz) and ECL (Amersham Biosciences) as chemiluminescent. Input controls to confirm overexpression of the respective proteins was performed by immunblotting the non-immunoprecipitated cell lysates. Densitometry of protein-specific bands was performed on a Bio-Rad gel dock and quantified with Quantity One software (Bio-Rad).

Proximity Ligation Assay (PLA)—70×10^3 HEK 293T cells were cultured on 8-well cell culture slides (BD Falcon), and transfected with expression plasmids (on a pcDNA3 backbone) for CREMα, G9a, or a combination of CREMα and G9a (0.3 μg of each plasmid per transfection) using X-tremeGENE 9 DNA Transfection Reagent (Roche) according to the manufacturer's instructions. 24 h after transfection, cells were harvested and subjected to the Duolink proximity ligation assay following the manufacturer's instructions (Olink). Shortly thereafter, anti-CREM and anti-G9a antibodies were labeled with PLUS and MINUS oligonucleotide tails. Cells were fixed with 3.7% formaldehyde, washed with PBS, permeabilized with Triton X-100 (Invitrogen), 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 $CD8^+$ T cells were performed using 5×10^6 T cells in Eppendorf tubes. After all the washing and incubations, 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 has been quantified using ImageJ software.

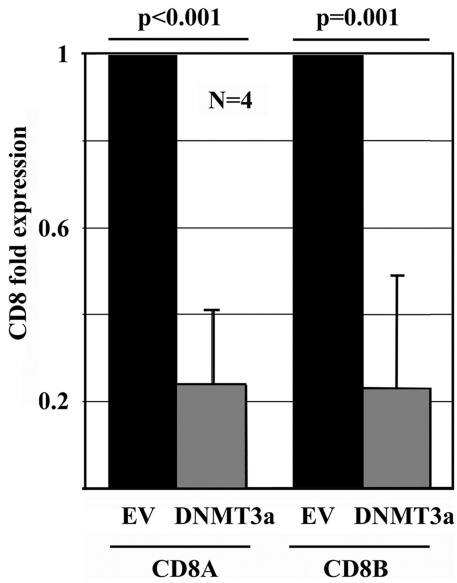

FIGURE 1. **DNA methylation through DNMT3a regulates CD8 expression.** CD8A and CD8B mRNA expression was assessed by quantitative RT-PCR after forced CpG-DNA methylation through DNMT3a (24 h).

Forced Expression of CREMα and DNMT3a or G9a Knock-down—Three million primary human $CD8^+$ T cells were transfected with a total amount of 3 μg of expression plasmid and 20 nM scrambled control siRNA or DNMT3a- or G9a-specific siRNA (OriGene) using Lipofectamine (Invitrogen) as indicated. Prior to these experiments, experimental conditions were optimized using Cy3-labeled control siRNA (OriGene) after transfection with Lipofectamine (Invitrogen). Transfection efficiency was >70%. Cells were collected after an overnight culture and processed for mRNA, MeDIP, or ChIP analysis as indicated. All experiments were repeated four to six times as indicated. Values in the bar diagrams are given as mean ± S.D.

Statistical Analysis—Paired two-tailed Student's *t* test was used for statistical analysis of all flow cytometry and transfection experiments as indicated. A *p* value of 0.05 was considered statistically significant. Results are indicated as the mean ± S.D., unless noted otherwise.

RESULTS

DNA Methylation of the CD8 Gene Cluster in $CD8^+$ and $CD8^-$ T Cells—DNA methylation plays a well established role in the regulation of gene expression (16). To investigate whether DNA methyltransferase (DNMT)3a-mediated *de novo* DNA methylation affects CD8 expression, we over-expressed DNMT3a in primary human $CD8^+$ T cells from healthy individuals (17, 18) and detected significantly reduced *CD8A* ($p < 0.001$) and *CD8B* ($p = 0.001$) transcription (Fig. 1).

Thus, we aimed to determine DNA methylation of the human and murine *CD8* genes in $CD8^+$ T cells as compared with $CD4^+$ and DN T cells. For the study of the human *CD8* cluster, $CD4^+$ and $CD8^+$ T cells were sorted. Secondary to their low abundance in the peripheral blood, human DN T cells were *in vitro* induced by stimulating $CD8^+$ T cells with anti-CD3 and anti-CD28 antibodies as reported previously (1). Murine $CD4^+$,

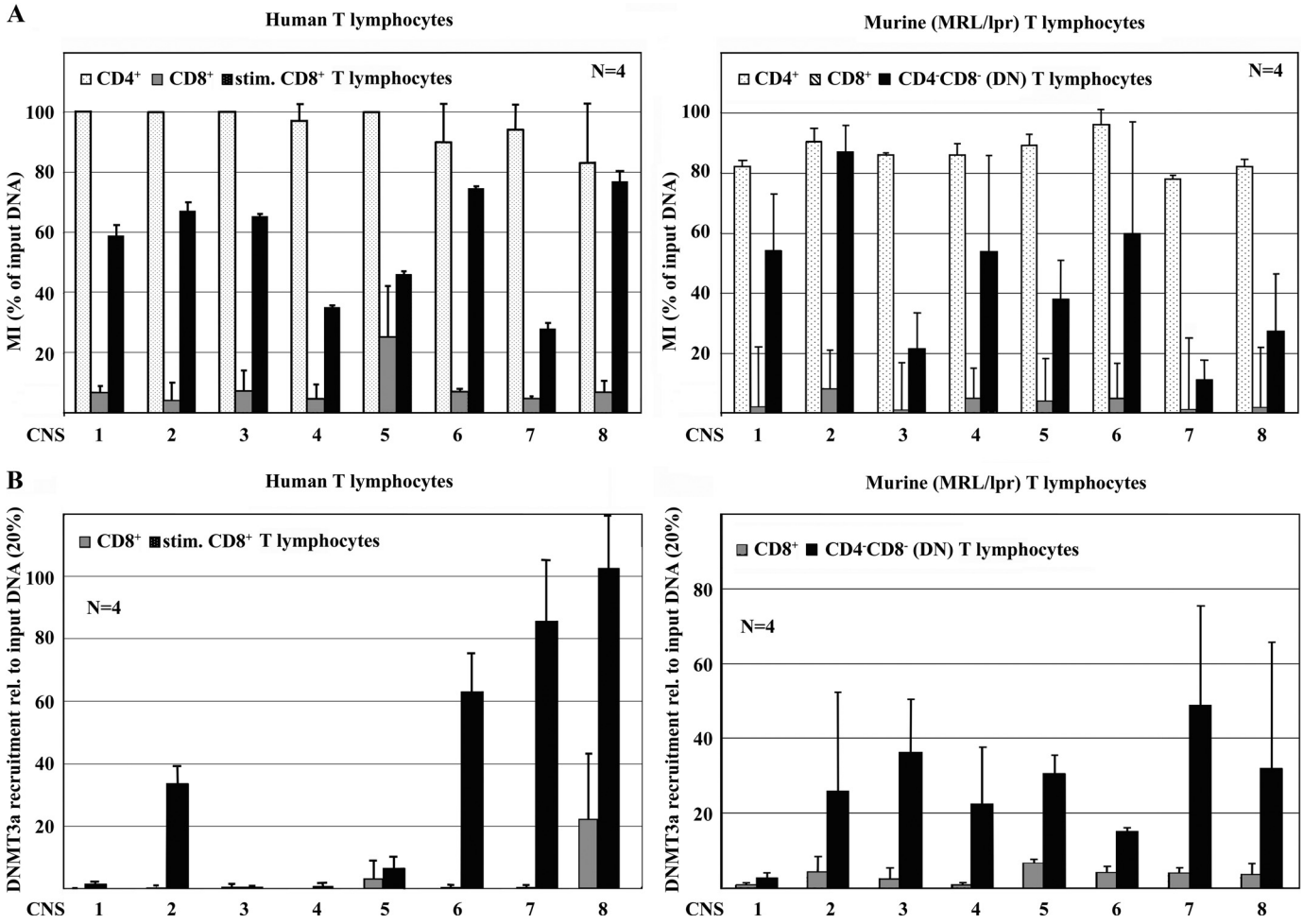

FIGURE 2. **CD8 expression is determined by DNA methylation.** *A*, $CD4^+$ and $CD8^+$ T cells from healthy humans and MRL/*lpr* mice were sorted for the assessment of DNA methylation. Murine DN T cells were sorted; human DN T cells were induced by TCR stimulation. In human (*left*) and murine (*right*) $CD4^+$ T cells, the *CD8* cluster was methylated. In $CD8^+$ T cells from both species, the *CD8* cluster was largely unmethylated. Stimulated human $CD8^+$ T cells and murine DN T cells exhibited increased DNA methylation of the *CD8* cluster. *B,* recruitment of the DNMT3a to the *CD8* cluster was assessed using ChIP. In human and murine DN T cells, DNMT3a is recruited to the *CD8* cluster. DNA methylation of the human *CD8* cluster is more homogenous when compared with mice. In both species DNMT3a recruitment is particularly strong at CNS2, -6, -7, and -8.

$CD8^+$, and DN T cells were sorted from spleens of MRL/*lpr* mice on a FACSAria flow cytometer. Reflecting our gene expression data, human and murine $CD4^+$ T cells displayed high levels of DNA methylation of the entire *CD8* cluster, whereas *CD8A* and *CD8B* in $CD8^+$ T cells were largely unmethylated (Fig. 2*A*). Murine and "induced" human DN T cells exhibited methylation levels that were between those in $CD4^+$ and $CD8^+$ T cells, suggesting that the *CD8* genes are undergoing DNA methylation during the CD8 down-regulation process in DN T cell generation. Of note, DNA methylation of the human *CD8* cluster was more homogenous when compared with murine *CD8*.

DNA Methylation of the CD8 Cluster Is Mediated by DNMT3a—We previously demonstrated that CREMα interacts with the *de novo* methyltransferase DNMT3a, mediating epigenetic remodeling (17, 18). Similar to the silencing of *IL2* in effector T cells, the *CD8* cluster undergoes *de novo* DNA methylation during generation of DN T cells. Thus, we wondered whether this could also be mediated by the interaction of CREMα with DNMT3a. We performed DNMT3a ChIP assays in human $CD8^+$ T cells from healthy individuals and induced DN T cells as well in murine $CD4^+$, $CD8^+$, and DN T cells. As indicated in Fig. 2*B*, DNMT3a is recruited to the human and murine *CD8* cluster in non-CD8 expressing T cells, particularly to CNS2, -7, and -8.

To establish the physiological relevance of the interaction between CREMα and DNMT3a, we over-expressed CREMα in primary human $CD8^+$ T cells from healthy individuals, whereas knocking down DNMT3a (Fig. 3). Indeed, knock-down of DNMT3a resulted in increased *CD8A* ($p = 0.027$) and *CD8B* ($p = 0.06$) transcription, whereas forced CREMα expression mediated reduced CD8A ($p = 0.003$) and CD8B ($p < 0.001$) expression (Fig. 3, *A* and *B*). As suggested by previous studies and the finding that DNA methylation plays a role in the regulation of CD8, CREMα-mediated effects on CD8A and CD8B expression were reversible by DNMT3a knock-down (Fig. 3*A*). This was reflected by DNA methylation of CNS2, -7, and -8, which was increased in response to CREMα over-expression and reversible by DNMT3a knock-down (Fig. 3*B*).

Histone 3 Methylation (H3K9me3 and H3K27me3) Controls CD8 Expression—DNA methylation and histone methylation frequently follow the same patterns and can affect one another

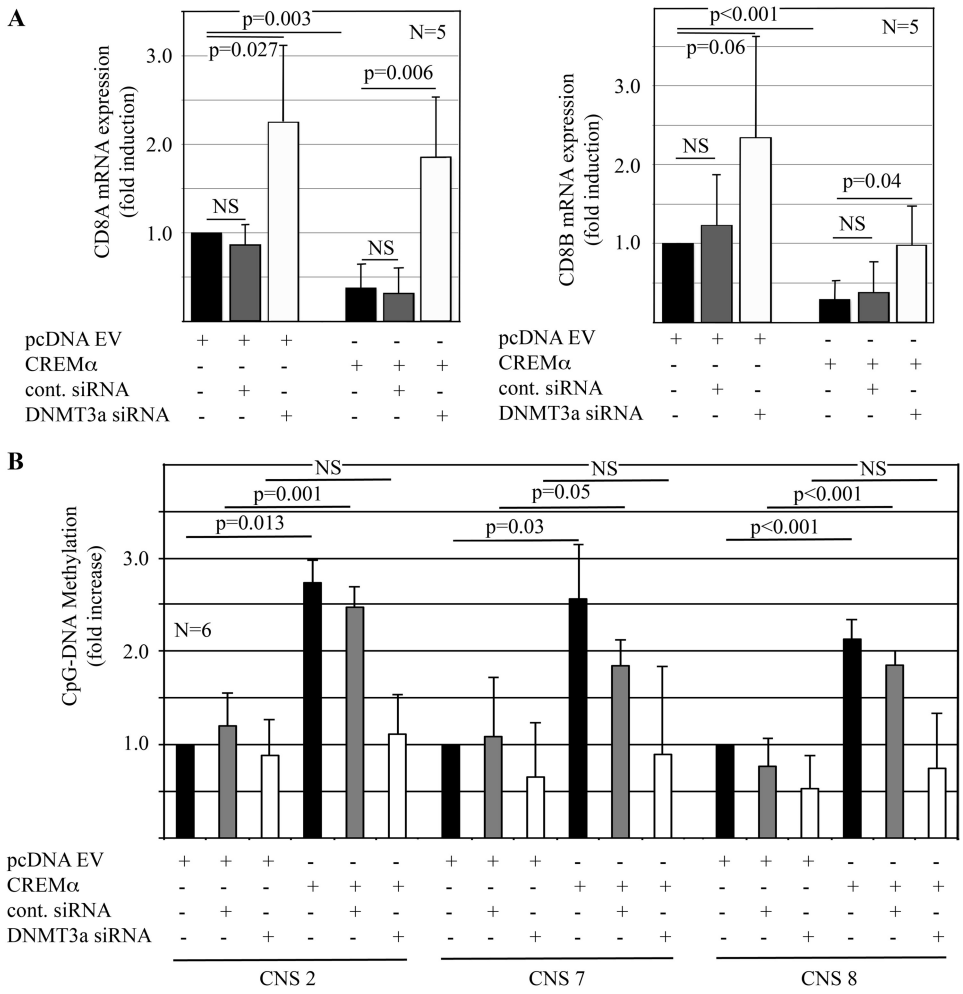

FIGURE 3. **CREMα recruits DNMT3a to the *CD8* cluster.** *A*, DNMT3a knock-down results in increased CD8A and CD8B mRNA expression in human $CD8^+$ T cells (24 h) when compared with controls (*left*: CD8A, *right*: CD8B, *first to third lanes*). CREMα reduces CD8A and CD8B mRNA expression (24h) (*fourth* and *fifth lanes*). DNMT3a knock-down reverses CREMα-mediated suppression of CD8A and CD8B mRNA (24 h) (*sixth lane*). *B,* CD8A and CD8B mRNA expression in response to CREMα with or without DNMT3a knock-down are reflected by DNA methylation.

(21, 16). Thus, we asked whether the epigenetic silencing of *CD8A* and *CD8B* is facilitated also by histone modifications. Reflecting DNA methylation, human and murine $CD4^+$ T cells displayed high levels of histone H3 tri-methylation at lysine residues 9 (H3K9me3) and 27 (H3K27me3) over the entire *CD8* cluster, whereas *CD8A* and *CD8B* in $CD8^+$ T cells were largely unmethylated. Furthermore, murine and induced human DN T cells exhibited increased H3K9me3 and H3K27me3 when compared with $CD8^+$ T cells (Fig. 4). Interestingly, histone H3K9 and H3K27 methylation patterns were more consistent over the murine *CD8* cluster, whereas histone H3 methylation peaks around CNS2, -6, -7, and -8 in human DN T cells. These differences may be the result of less "pure" DN T cell populations applied in the human experiments. As mentioned, human DN T cells were induced by TCR stimulation of $CD8^+$ T cells over 5 days, reaching a DN T lymphocyte purity of usually around 50% (1).

CREMα Recruits Histone Methyltransferase G9a to the CD8 Cluster—During the differentiation of cells and tissues, epigenetic remodeling is orchestrated by transcription factors, such as CREMα that interacts with DNMT3a (16, 21, 22). Because DNA and histone methylation patterns of the *CD8* cluster follow the same patterns during peripheral DN T cell generation, we wondered which histone methyltransferase(s) were responsible for the methylation of histone H3K9 and H3K27 and whether they are mediated by CREMα, connecting DNA methylation and histone code. We performed ChIP assays, analyzing histone methyltransferase G9a recruitment to the *CD8* cluster in $CD8^+$, $CD4^+$, and DN T cells from healthy humans and MRL/*lpr* mice. We chose G9a as a histone methyltransferase that has been reported relatively specific for histone methylation at H3K9 and H3K27 (23). As displayed in Fig. 5, G9a recruitment largely reflects H3K9 and H3K27 methylation of the *CD8* cluster, suggesting these modifications are being mediated by G9a. To test this hypothesis, we forced G9a expression in primary human $CD8^+$ T cells and monitored gene expression and histone H3K9 and H3K27 methylation (Fig. 5, *C* and *D*). Indeed, forced G9a expression resulted in a significant reduction of CD8A ($p = 0.01$) and CD8B ($p = 0.008$) mRNA expression that was mirrored by an increase in H3K9me and H3K27me3 around CNS2, -7, and -8, which exhibited the strongest recruitment of G9a.

Because CREMα recruits DNMT3a to target genes in health and disease (1, 4, 5, 24, 25) and histone and DNA methylation

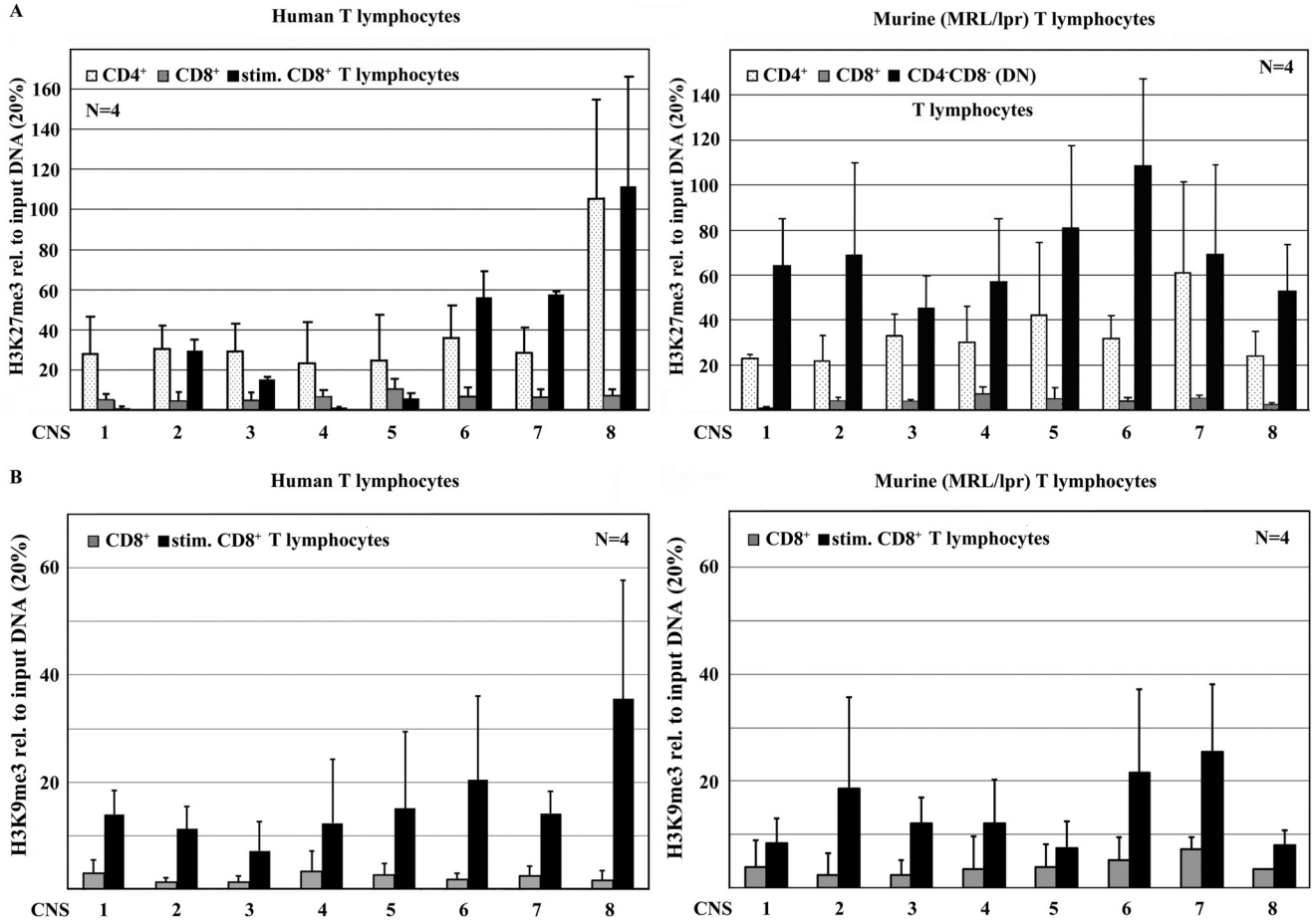

FIGURE 4. **CD8 expression is reflected by H3K27 and H3K9 methylation.** $CD4^+$ and $CD8^+$ T cells from healthy humans and MRL/LPR mice were sorted for the assessment of histone methylation of the *CD8* cluster, using ChIP. Murine DN T cells were sorted; human DN T cells were induced by TCR stimulation. In human (*left*) and murine (*right*) $CD4^+$ T cells, histone H3 was tri-methylated at residues lysine 27 (H3K27me3) (*A*) and at lysine 9 (H3K9me3) (*B*) over the entire *CD8* cluster. In $CD8^+$ T cells from both species, almost no H3K9me3 orH3K27me3 was detected, whereas induced human and murine DN T cells exhibited an increase in H3K27me3 and H3K9me3. Histone H3K9 and H3K27 methylation is more consistent over the murine *CD8* cluster, whereas histone H3 methylation peaks around CNS2, -6, -7, and -8 in human DN T cells.

usually follow the same patterns, we hypothesized that CREMα may also recruit G9a to the *CD8* cluster, thus orchestrating epigenetic remodeling. To test our hypothesis, we investigated whether CREMα is differentially recruited to CNS2, -7, and -8 in $CD8^+$ T cells from humans and MRL/*lpr* mice (Fig. 6, *A* and *B*). Indeed, CREMα is recruited to the *CD8* cluster in both $CD8^+$ T cells from healthy controls and SLE patients. However, T cells from SLE patients exhibit enhanced CREMα recruitment to CNS2, -7, and -8 when compared with controls, suggesting CREMα contributing to the enhanced generation of DN T cells in SLE (Fig. 6*A*). As SLE patients, MRL/*lpr* mice exhibit increased CREMα expression in T cells (Fig. 6*B*, *upper panel*) and exhibit enhanced recruitment of CREMα to CNS2, -7, and -8 was determined in DN T cells when compared with $CD8^+$ T cells (Fig. 6*B*, *lower panel*).

To investigate whether CREMα and G9a physically interact, we performed co-immunoprecipitation assays with anti-CREMα antibodies. In HEK293T cells that were transfected with an empty pcDNA3.1 plasmid we co-immunoprecipitated only small amounts of G9a with CREMα (Fig. 6*C*, *first lane*). Forced expression of G9a allowed increased amounts of G9a with CREMα to co-immunoprecipitate (Fig. 6*C*, *second lane*). Overexpression of both CREMα and G9a allowed strong co-immunoprecipitation of EHMT2/G9a with CREMα (Fig. 6*C*, *third lane*). This suggests a direct interaction between CREMα and G9a. To exclude the possibility of G9a interacting directly with DNMT3a, thus allowing co-immunoprecipitation with CREMα antibodies, we transfected cells with DNMT3a and G9a expression plasmids and tried to co-immunoprecipitate G9a with anti-DNMT3a antibodies (Fig. 6*C*, *fourth lane*). Failure to co-immunoprecipitate G9a with DNMT3a suggests that G9a interacts with CREMα rather than interacting with CREMα indirectly through DNMT3a. To reconfirm these findings with another technique, we forced the expression of CREMα and G9a in the same (HEK293T) cells, followed by PLA (Olink). Forced expression of either CREMα or G9a resulted in an increased signal when compared with controls. Forced expression of both CREMα and G9a resulted in the strongest signal, indicating *in situ* interaction (not shown). To translate our preliminary findings into the biological context of our study, we performed PLA in freshly isolated $CD8^+$ T cells and after stimulation with anti-CD3 and anti-CD28 antibodies for 120 h. Indeed, CREMα and G9a also co-localized in primary human $CD8^+$ T cells and

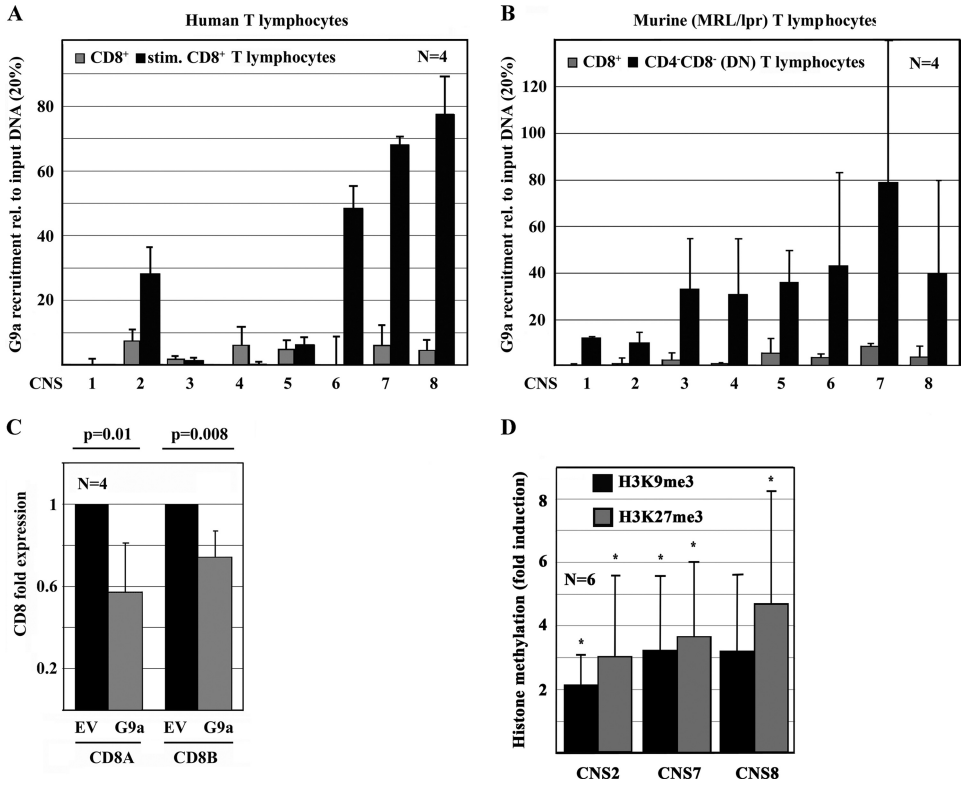

FIGURE 5. **CREMα interacts with G9a mediating chromatin remodeling of the *CD8* cluster.** *A* and *B*, based on histone methylation patterns, the recruitment of G9a to the *CD8* cluster has been tested, using ChIP. In both healthy humans (*A*) and MRL/*lpr* mice (*B*), the recruitment of G9a to the *CD8* cluster in DN T cells is largely increased when compared with $CD8^+$ T cells. *C*, forced expression of the histone methyltransferase G9a mediates a significant reduction of CD8A and CD8B mRNA expression through a significant increase of H3K9me3 and H3K27me3 (*D*) (*, indicates $p < 0.05$).

the interaction was enhanced in response to stimulation for 120 h (Fig. 6*D*).

To determine the physiological relevance of the interaction between CREMα and G9a, we over-expressed CREMα in primary human $CD8^+$ T cells while knocking down G9a (Fig. 6, *E–Gs*). G9a knock-down resulted in increased CD8A and CD8B mRNA expression, whereas reduced CD8A and CD8B expression in response to CREMα was reversible targeting G9a with siRNAs (Fig. 6*E*). This was reflected by increased H3K9me3 and H3K27me3 in response to CREMα, which was reversed by G9a knock-down (Fig. 6, *F* and *G*). Thus, our findings indicate that the interaction between CREMα and G9a plays a role in the regulation of *CD8A* and *CD8B* gene expression.

DISCUSSION

In the peripheral blood of SLE patients, DN T cells are expanded and contribute to disease expression and tissue damage (4, 5, 24, 25). DN T cells in SLE derive from $CD8^+$ T cells by down-regulating CD8 surface expression and the acquisition of distinct effector phenotypes (4, 5, 24, 25). However, the molecular mechanisms instructing the transformation of $CD8^+$ T cells into DN T cells remain largely unclear.

We previously demonstrated that the transcription factor CREMα *trans*-regulates a region syntenic to the murine *CD8b* promoter, thus contributing to the generation of DN T cells (1). Studies demonstrated that transcriptional silencing can be achieved by epigenetic remodeling of the murine *CD8* cluster with a "closed" chromatin conformation prohibiting the recruitment of *trans*-activating signals (4, 5, 24, 25). However, the inducing stimuli and molecular mechanisms remained unknown. It has been shown that the presence of DNA methyltransferase (DNMT) 1 is essential for the silencing of *CD8* in $CD8^-$ tissues. As a result, DNMT1-deficient mice exhibit enhanced CD8 expression secondary to a loss of inhibition (5, 26). Interestingly, this selectively occurred in TCR-γδ T cells and not in $CD4^+$ TCR-αβ T cells that usually express the α-chain of the CD8αβ heterodimer, suggesting the chromatin conformation and resulting expression patterns of CD8 to be more variable when compared with CD4, which was constitutively repressed in non-CD4 expressing tissues (5, 26). This indicates that epigenetic remodeling of the *CD8* cluster may be a central step in the transformation of $CD8^+$ into DN T cells. We demonstrate that the *CD8* cluster undergoes epigenetic remodeling in response to stimulation of $CD8^+$ T cells, contributing to the generation of DN T cells. In both induced human and sorted MRL/*lpr* DN T cells DNA methylation along the entire *CD8* cluster is mirrored by H3K9 and H3K27 tri-methylation, indicating that chromatin remodeling could be involved in the down-regulation of CD8 in mature DN T cells. Although the entire *CD8* cluster in human DN T cells exhibits a consistently high methylation index, DNA methylation in murine DN T cells is region-specific with a low methylation index of CNS7 and -8. This is agreement with early findings of increased DNase hypersensitivity in DN T cells from MRL/*lpr* and CBA/CaH WEHI mice in the same regions (27, 28). Interestingly, histone H3K9 and H3K27 methylation is more consistent over the murine *CD8* cluster, whereas histone H3 methylation peaks

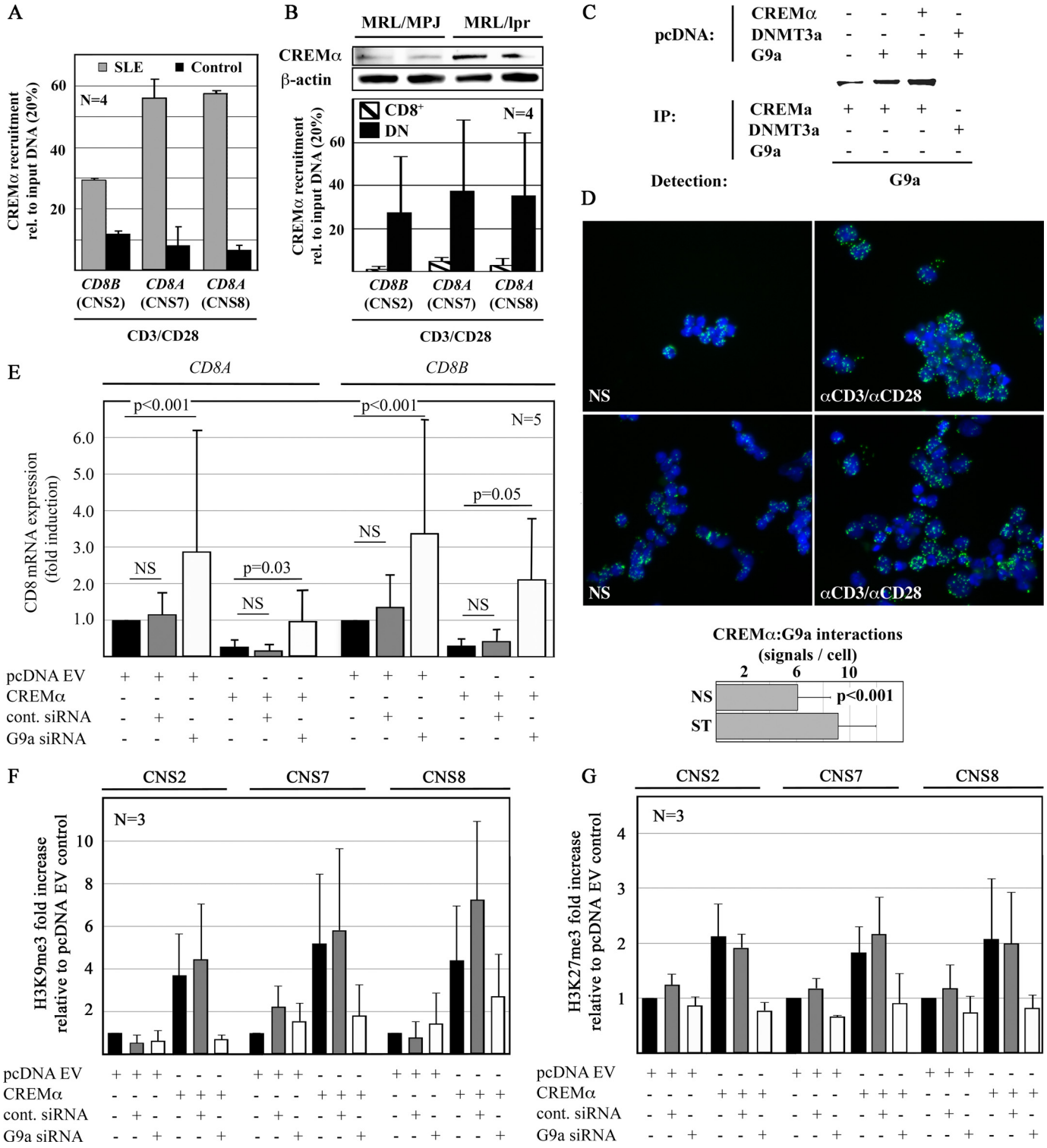

FIGURE 6. **CREMα recruits G9a to the *CD8* cluster.** *A*, CREMα recruitment to CNS2, -7, and -8 is enhanced in CD8$^+$ T cells from SLE patients. *B*, in analogy to SLE T cells, CREMα expression is increased in T cells from MRL/*lpr* mice (*upper panel*). In DN but not in CD8$^+$ T cells, CREMα is recruited to CNS2, -7, and -8 suggesting CREMα is involved in the down-regulation of CD8 (*lower panel*). *C*, HEK293T cells were transfected with empty pcDNA3.1 plasmids. Proteins were co-immunoprecipitated with anti-CREMα or DNMT3a antibodies as indicated. Lysates were subjected to Western blotting with G9a antibodies. Representative results from one of three independent experiments are displayed. *D*, an interaction between CREMα and G9a has been established applying PLA. *Ex vivo* isolated CD8$^+$ T cells exhibit interactions between CREMα and G9a that are enhanced after TCR stimulation (120 h). The number of PLA signals per unstimulated (*NS*) or stimulated (*ST*) CD8$^+$ T cell was quantified using ImageJ software. Displayed is the number of signals per CD8$^+$ T cell in 50 visual fields from 5 independent experiments (*lower panel*). *E*, G9a knock-down results in an increase of CD8A and CD8B mRNA expression in primary human CD8$^+$ T cells (24 h) when compared with controls siRNA (*left*, CD8A; *right*, CD8B, *first* to *third lanes*). CREMα reduces CD8A and CD8B mRNA expression (24h) (*fourth* and *fifth lanes*). G9a knock-down reverses the CREMα effects on *CD8A* and *CD8B* (*sixth lane*). *F* and *G*, CD8A and CD8B expression patterns in response to CREMα with or without G9a knock-down are reflected by histone H3K9 (*F*) and H3K27 (*G*) methylation of CNS2, -7, and -8.

around CNS2, -6, -7, and -8 in human DN T cells. Taken together, CNS2, -6, -7, and -8 undergo the most striking epigenetic changes during the generation of DN T cells in both species and map to previously reported elements that are the target of chromatin remodeling during $CD8^+$ lineage determination in the thymus of mice (4, 25). Species-specific characteristics in the distribution of DNA and histone H3 methylation remain to be understood. However, the discrete differences add up to a "common" epigenetic pattern that results in silencing of *CD8* in DN T cells.

Transcription factors influence chromatin conformation through the recruitment of DNA and/or histone methyltransferases (16–18, 21). We and others demonstrated that CREMα and its counteractor CREB diametrically influence the transcriptional activity of cytokine genes through both *trans*-regulation and the recruitment of epigenetic modulators (22). The ATF transcription factor CREB has been demonstrated to interact with the p300 co-activator that has histone acetyltransferase activity (29). We recently reported that CREMα mediates epigenetic remodeling of cytokine genes during the priming of $CD4^+$ T cells, including diametric effects on DNA methylation in effector T cells (18, 22). Because both CREB and CREMα orchestrate epigenetic remodeling and exhibit the aforementioned antithetic effects on CNS2, CREMα was a promising candidate in the search for regulators governing chromatin remodeling of the *CD8* cluster during DN T cell generation. Indeed, we demonstrate that CREMα recruits both DNMT3a and the histone methyltransferase G9a to regulatory regions within the human and murine *CD8* cluster (CNS2, 7, and 8), instructing chromatin remodeling and transcriptional silencing. Our finding that CREMα regulates CD8 expression through both *trans*-repression and the induction of epigenetic remodeling is in agreement with recent studies, targeting transcriptional regulation of murine *CD4*, *CD8a*, and *CD8b* during T cell development in the thymus. The observation that the transcription factor Runt-related transcription factor (RUNX) 1 is required for *trans*-repression of *CD4* and that the closely related transcriptional regulator RUNX3 instructs chromatin remodeling of *CD4* in DN thymocytes and $CD8^+$ T cells provides further evidence that transcription factors orchestrate epigenetic priming of immune cells (4, 5, 22, 24, 30). Interestingly, RUNX3 and the Runx/core binding factor-β are necessarily required for CD8 co-receptor expression in activated $CD8^+$ T cells through the recruitment to the $E8_I$ enhancer (31). The absence of $E8_I$ resulted in chromatin remodeling of the entire *CD8* cluster with enhanced H3K27me3 and reduced histone H3 acetylation, both reflecting a "closure" of the murine *CD8a* gene (31). This is of special interest, because $E8_I$ maps to our CNS6 and -7, and is in close proximity to CNS8, all of which undergo CREMα-instructed epigenetic remodeling in response to TCR activation. Thus, reduced recruitment of RUNX3 to this region could also play a role in the transformation of $CD8^+$ T cells into peripheral induced DN T cells and will be the focus of future studies.

Taken together, our data solidify the role of CREMα in the regulation of CD8. CREMα in addition to the previously reported *trans*-repression of CNS2 contributes to the down-regulation of CD8 through and the recruitment of DNMT3a and G9a. Because CREMα is increased in T cells from SLE patients and MRL/*lpr* mice, these mechanisms appear central for the generation of DN T cells in SLE and potentially other autoimmune diseases with increased numbers of DN T cells. This underlines the potential of CREMα as disease biomarker and putative therapeutic target in SLE. It remains to be determined whether CREMα instructs chromatin remodeling during the priming and differentiation of T cells in the thymus or if CREMα exclusively regulates CD8 in peripheral $CD8^+$ T cells in response to activation.

REFERENCES

1. Hedrich, C. M., Rauen, T., Crispin, J. C., Koga, T., Ioannidis, C., Zajdel, M., Kyttaris, V. C., and Tsokos, G. C. (2013) cAMP responsive element modulator (CREM)α trans-represses the transmembrane glycoprotein CD8 and contributes to the generation of $CD3^+CD4^-CD8^-$ T cells in health and disease. *J. Biol. Chem.* **288,** 31880–31887
2. Hostert, A., Tolaini, M., Festenstein, R., McNeill, L., Malissen, B., Williams, O., Zamoyska, R., and Kioussis, D. (1997) A CD8 genomic fragment that directs subset-specific expression of CD8 in transgenic mice. *J. Immunol.* **158,** 4270–4281
3. Kieffer, L. J., Yan, L., Hanke, J. H., and Kavathas, P. B. (1997) Appropriate developmental expression of human CD8β in transgenic mice. *J. Immunol.* **159,** 4907–4912
4. Bilic, I., Koesters, C., Unger, B., Sekimata, M., Hertweck, A., Maschek, R., Wilson, C. B., and Ellmeier, W. (2006) Negative regulation of CD8 expression via Cd8 enhancer-mediated recruitment of the zinc finger protein MAZR. *Nat. Immunol.* **7,** 392–400
5. Kioussis, D., and Ellmeier, W. (2002) Chromatin and *CD4*, *CD8A* and *CD8B* gene expression during thymic differentiation. *Nat. Rev. Immunol.* **2,** 909–919
6. Ellmeier, W., Sunshine, M. J., Losos, K., Hatam, F., and Littman, D. R. (1997) An enhancer that directs lineage-specific expression of CD8 in positively selected thymocytes and mature T cells. *Immunity* **7,** 537–547
7. Ellmeier, W., Sunshine, M. J., Losos, K., and Littman, D. R. (1998) Multiple developmental stage-specific enhancers regulate CD8 expression in developing thymocytes and in thymus-independent T cells. *Immunity* **9,** 485–496
8. Ellmeier, W., Sunshine, M. J., Maschek, R., and Littman, D. R. (2002) Combined deletion of CD8 locus cis-regulatory elements affects initiation but not maintenance of CD8 expression. *Immunity* **16,** 623–634
9. Garefalaki, A., Coles, M., Hirschberg, S., Mavria, G., Norton, T., Hostert, A., and Kioussis, D. (2002) Variegated expression of CD8α resulting from in situ deletion of regulatory sequences. *Immunity* **16,** 635–647
10. Hostert, A., Tolaini, M., Roderick, K., Harker, N., Norton, T., and Kioussis, D. (1997) A region in the *CD8* gene locus that directs expression to the mature CD8 T cell subset in transgenic mice. *Immunity* **7,** 525–536
11. Hostert, A., Garefalaki, A., Mavria, G., Tolaini, M., Roderick, K., Norton, T., Mee, P. J., Tybulewicz, V. L., Coles, M., and Kioussis, D. (1998) Hierarchical interactions of control elements determine CD8α gene expression in subsets of thymocytes and peripheral T cells. *Immunity* **9,** 497–508
12. Kieffer, L. J., Bennett, J. A., Cunningham, A. C., Gladue, R. P., McNeish, J., Kavathas, P. B., and Hanke, J. H. (1996) Human CD8α expression in NK cells but not cytotoxic T cells of transgenic mice. *Int. Immunol.* **8,** 1617–1626
13. Zhang, X. L., Seong, R., Piracha, R., Larijani, M., Heeney, M., Parnes, J. R., and Chamberlain, J. W. (1998) Distinct stage-specific cis-active transcriptional mechanisms control expression of T cell coreceptor CD8α at double- and single-positive stages of thymic development. *J. Immunol.* **161,** 2254–2266
14. Zhang, X. L., Zhao, S., Borenstein, S. H., Liu, Y., Jayabalasingham, B., and Chamberlain, J. W. (2001) CD8 expression up to the double-positive CD3(low/intermediate) stage of thymic differentiation is sufficient for development of peripheral functional cytotoxic T lymphocytes. *J. Exp. Med.* **194,** 685–693
15. Feik, N., Bilic, I., Tinhofer, J., Unger, B., Littman, D. R., and Ellmeier, W.

(2005) Functional and molecular analysis of the double-positive stage-specific CD8 enhancer E8III during thymocyte development. *J. Immunol.* **174,** 1513–1524
16. Hedrich, C. M., and Tsokos, G. C. (2011) Epigenetic mechanisms in systemic lupus erythematosus and other autoimmune diseases. *Trends Mol. Med.* **17,** 714–724
17. Hedrich, C. M., Rauen, T., and Tsokos, G. C. (2011) cAMP-responsive element modulator (CREM)α protein signaling mediates epigenetic remodeling of the human interleukin-2 gene. Implications in systemic lupus erythematosus. *J. Biol. Chem.* **286,** 43429–43436
18. Hedrich, C. M., Crispin, J. C., Rauen, T., Ioannidis, C., Apostolidis, S. A., Lo, M. S., Kyttaris, V. C., and Tsokos, G. C. (2012) cAMP response element modulator α controls IL2 and IL17A expression during CD4 lineage commitment and subset distribution in lupus. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 16606–16611
19. Rauen, T., Hedrich, C. M., Juang, Y. T., Tenbrock, K., and Tsokos, G. C. (2011) cAMP-responsive element modulator (CREM)α protein induces interleukin 17A expression and mediates epigenetic alterations at the interleukin-17A gene locus in patients with systemic lupus erythematosus. *J. Biol. Chem.* **286,** 43437–43446
20. Juang, Y. T., Wang, Y., Solomou, E. E., Li, Y., Mawrin, C., Tenbrock, K., Kyttaris, V. C., and Tsokos, G. C. (2005) Systemic lupus erythematosus serum IgG increases CREM binding to the IL-2 promoter and suppresses IL-2 production through CaMKIV. *J. Clin. Invest.* **115,** 996–1005
21. Brenner, C., and Fuks, F. (2007) A methylation rendezvous. Reader meets writers. *Dev. Cell.* **12,** 843–844
22. Rauen, T., Hedrich, C. M., Tenbrock, K., and Tsokos, G. C. (2013) cAMP responsive element modulator. A critical regulator of cytokine production. *Trends Mol. Med.* **19,** 262–269
23. Tachibana, M., Sugimoto, K., Fukushima, T., and Shinkai, Y. (2001) Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. *J. Biol. Chem.* **276,** 25309–25317
24. Sato, T., Ohno, S., Hayashi, T., Sato, C., Kohu, K., Satake, M., and Habu, S. (2005) Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. *Immunity* **22,** 317–328
25. Harker, N., Garefalaki, A., Menzel, U., Ktistaki, E., Naito, T., Georgopoulos, K., and Kioussis, D. (2011) Pre-TCR signaling and *CD8* gene bivalent chromatin resolution during thymocyte development. *J. Immunol.* **186,** 6368–6377
26. Lee, P. P., Fitzpatrick, D. R., Beard, C., Jessup, H. K., Lehar, S., Makar, K. W., Pérez-Melgosa, M., Sweetser, M. T., Schlissel, M. S., Nguyen, S., Cherry, S. R., Tsai, J. H., Tucker, S. M., Weaver, W. M., Kelso, A., Jaenisch, R., and Wilson, C. B. (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* **15,** 763–774
27. Landolfi, M. M., Van Houten, N., Russell, J. Q., Scollay, R., Parnes, J. R., and Budd, R. C. (1993) $CD2^-CD4^-CD8^-$ lymph node T lymphocytes in MRL lpr/lpr mice are derived from a $CD2^+CD4^+CD8^+$ thymic precursor. *J. Immunol.* **151,** 1086–1096
28. Wu, L., Pearse, M., Egerton, M., Petrie, H., and Scollay, R. (1990) $CD4^-CD8^-$ thymocytes that express the T cell receptor may have previously expressed CD8. *Int. Immunol.* **2,** 51–56
29. Lee, J. S., Zhang, X., and Shi, Y. (1996) Differential interactions of the CREB/ATF family of transcription factors with p300 and adenovirus E1A. *J. Biol. Chem.* **271,** 17666–17674
30. Taniuchi, I., Osato, M., Egawa, T., Sunshine, M. J., Bae, S. C., Komori, T., Ito, Y., and Littman, D. R. (2002) Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* **111,** 621–633
31. Hassan, H., Sakaguchi, S., Tenno, M., Kopf, A., Boucheron, N., Carpenter, A. C., Egawa, T., Taniuchi, I., and Ellmeier, W. (2011) Cd8 enhancer E8I and Runx factors regulate CD8α expression in activated $CD8^+$ T cells. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 18330–18335