Pyruvate dehydrogenase phosphatase catalytic subunit 2 limits Th17 differentiation

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Th17 cells favor glycolytic metabolism, and pyruvate dehydrogenase (PDH) is the key bifurcation enzyme, which in its active dephosphorylated form advances the oxidative phosphorylation from glycolytic pathway. The transcriptional factor, inducible cAMP early repressor/cAMP response element modulator (ICER/CREM), has been shown to be induced in Th17 cells and to be overexpressed in CD4$^+$ T cells from the patients with systemic lupus erythematosus (SLE). We found that glycolysis and lactate production in in vitro Th17-polarized T cells was reduced and that the expression of pyruvate dehydrogenase phosphatase catalytic subunit 2 (PDP2), an enzyme that converts the inactive PDH to its active form, and PDH enzyme activity were increased in Th17 cells from ICER/CREM-deficient animals. ICER was found to bind to the *Pdp2* promoter and suppress its expression. Furthermore, forced expression of PDP2 in CD4$^+$ cells reduced the in vitro Th17 differentiation, whereas shRNA-based suppression of PDP2 expression increased in vitro Th17 differentiation and augmented experimental autoimmune encephalomyelitis. At the translational level, PDP2 expression was decreased in memory Th17 cells from patients with SLE and forced expression of PDP2 in CD4$^+$ T cells from lupus-prone MRL/*lpr* mice and patients with SLE suppressed Th17 differentiation. These data demonstrate the direct control of energy production during Th17 differentiation in health and disease by the transcription factor ICER/CREM at the PDH metabolism bifurcation level.

ICER | PDP2 | Th17 | SLE | glycolysis

Systemic lupus erythematosus (SLE) is a multiorgan disorder of largely unknown etiology characterized by an autoreactive cellular and humoral immune response. While most types of immune cells have been reported to be involved in the development and progression of SLE, abnormalities of T cells including Th17 cells appear to play a major role in the pathogenesis (1, 2). Patients with SLE have a higher frequency of Th17 cells, which contribute to the establishment of proinflammatory conditions by infiltrating multiple organs (3, 4).

The cAMP response element modulator (CREM) controls the transcription of cAMP-responsible genes (5). Interestingly, expression of CREM splice variants that repress cAMP transcription, such as CREMα and inducible cAMP early repressor (ICER), are increased in CD4$^+$ T cells from patients with SLE (6). Several lines of evidence suggest a strong association between ICER/CREM and Th17 cell differentiation (6), including the facts that forced expression of CREMα in human T cells enhances IL-17A expression (7), ICER is most exclusively induced in Th17 cells and promotes Th17 cell differentiation, and ICER/CREM-deficient mice display less Th17-related autoimmune pathology. This has also been underscored by the genome-wide analysis of the Th17 transcription regulatory network that has revealed that *Crem* was induced among other genes during Th17 differentiation and that silencing of *Crem* led to reduced Th17 differentiation (8).

Activated T cells including Th17 cells use glucose metabolism to fulfill the metabolic requirements for rapid proliferation and biosynthesis supporting cellular growth and differentiation (9, 10). A previous metabolic profiling study has revealed that pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA, is a key bifurcation enzyme between T cell glycolytic and oxidative metabolism. Because Th17 cells favor pyruvate to lactate conversion for rapid nonmitochondrial ATP generation, Th17 cells were shown to be uniquely regulated by this enzyme (11). Accordingly, the enzymatic activity of PDH is repressed in Th17 cells to promote conversion of pyruvate to lactate by enhancing the activity of PDH kinase (PDHK) that phosphorylates PDH (active form) to phospho-PDH (inactive form) (11). However, whether PDH phosphatase (PDP), the balancing counterpart of PDHK that dephosphorylates phospho-PDH (inactive form) to PDH (active form), is also affected during Th17 differentiation is not known.

Since genome-wide analysis of cAMP-response element (CRE) binding protein occupancy has indicated the possibility that CRE-binding proteins can regulate genes involved in cell metabolism (12), we considered that ICER/CREM may control the activity of metabolic enzymes. We observed reduced glycolysis and lactate production in in vitro Th17-polarized ICER/CREM-deficient T cells. We found that *Pdp2*, one of the subunits of PDP, is increased in Th17 cells from ICER/CREM-deficient mice, and its expression is controlled directly by ICER/CREM at the transcriptional level. Forced PDP2 expression reduced the in vitro Th17 differentiation, whereas shRNA-based suppression of PDP2 expression increased in vitro Th17 differentiation and augmented experimental autoimmune encephalomyelitis (EAE) in mice. Importantly, PDP2 expression was decreased in memory Th17 cells from patients with SLE and forced expression of PDP2 to naïve CD4$^+$ T cells from the patients with SLE reduced IL-17 production during Th17 differentiation. Our data demonstrate that ICER accomplishes Th17 differentiation through direct control of a metabolic enzyme, which diverts energy production to the glycolytic pathway.

Significance

Th17 cells favor glycolytic metabolism. Pyruvate dehydrogenase, which facilitates entry into the oxidative phosphorylation circle, is inhibited by pyruvate dehydrogenase phosphatase catalytic subunit 2 (PDP2). Our studies demonstrate that the transcription factor ICER/CREM, which is known to promote Th17 differentiation and related pathology, suppresses the expression of PDP2 and diverts energy production into the glycolytic pathway. In lupus-prone mice and people with systemic lupus erythematosus, PDP2 levels are decreased and its replenishment suppresses Th17 differentiation.

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Results

ICER Promotes Glycolysis and Lactate Production in in Vitro Th17 Cells. Previously, we had shown that ICER/CREM promotes Th17 cell differentiation (6). Th17 cells depend on glycolysis more than other T cell subsets (11). Glycolysis involves the conversion of glucose to pyruvate, which is subsequently converted to lactate by lactate dehydrogenase (LDH), and lactate itself has been shown to induce the generation of Th17 cells (13). We hypothesized that ICER/CREM promotes Th17 differentiation through the control of enzymes that enable lactate production. To examine the function of ICER in glycolysis and lactate production, naïve $CD4^+$ T cells from ICER/CREM-sufficient or ICER/CREM-deficient mice were cultured under Th17 conditions in vitro and extracellular acidification rate (ECAR) was analyzed by an extracellular flux analyzer (Fig. 1*A*). Glycolysis can be measured as a change in ECAR that reflects lactate production (11). Th17 cells from ICER/CREM-sufficient mice had more glycolysis as reflected by the increased lactate production and more glycolytic capacity than cells from ICER/CREM-deficient mice (Fig. 1*B*). To confirm these observations, we overexpressed ICERγ in naïve $CD4^+$ T cells from ICER/CREM-deficient mice and polarized them to Th17 cells. Indeed, ICERγ overexpression restored the glycolysis and glycolytic capacity (Fig. 1 *C* and *D*), indicating that ICER promotes glycolysis and lactate production in in vitro-differentiated Th17 cells.

ICER Decreases PDP2 in in Vitro Th17 Cells. PDH is a key enzyme controlling the levels of T cell glycolytic and oxidative metabolism. PDH activity is inhibited by PDHK and promoted by PDP (Fig. 2*A*). PDHK3 is expressed in Th1, Th17, and Tregs, while PDHK1 is expressed more in Th17 cells than other T cell subsets, and inhibition of PDHK1 suppressed Th17 cell differentiation (11). PDP is a dimeric enzyme consisting of catalytic and regulatory subunits. There are two types of the catalytic subunit of PDP. PDP catalytic subunit 1 (PDP1) is predominantly expressed in mitochondria from skeletal muscle, whereas PDP catalytic subunit 2 (PDP2) is expressed in the liver (14) and many other cells, including white blood cells.

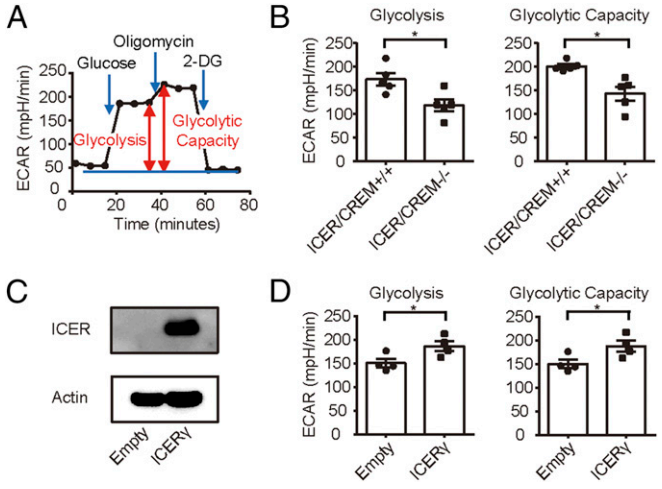

Fig. 1. ICER increases glycolysis and lactate production in Th17 cells. (*A*) Schematic representation of the experiments performed to measure glycolysis and glycolytic capacity. ECAR, a measurement of lactate, was measured by extracellular flux analyzer. (*B*) ICER/CREM-deficient or ICER/CREM-sufficient naïve $CD4^+$ T cells were cultured under Th17-polarizing conditions. Glycolysis and glycolytic capacity were assessed on day 3. Cumulative data of ECAR were shown (mean ± SEM); $n = 5$. (*C* and *D*) ICER/CREM-deficient naïve $CD4^+$ T cells were cultured under Th17-polarizing conditions. Empty vector (Empty) or ICERγ-expressing (ICERγ) plasmids were transfected to cultured T cells on day 1. (*C*) ICER and actin protein expression on day 3 was assessed by Western blotting. Representative blots are shown. Data are representative of three experiments. (*D*) ECAR was measured by extracellular flux analyzer on day 3. Cumulative data of ECAR were shown (mean ± SEM); $n = 4$. $^*P < 0.05$.

To investigate how ICER promotes glycolysis and lactate production in Th17 cells, we assessed the expression levels of the *Pdhk1*, *Pdhk3*, *Pdp2*, and pyruvate dehydrogenase phosphatase regulatory subunits (*Pdpr*) in in vitro polarized Th17 cells using qRT-PCR. As shown in Fig. 2*B*, *Pdhk1* expression was decreased in ICER/CREM-deficient Th17 cells compared with ICER/CREM-sufficient Th17 cells, whereas *Pdp2* expression was increased in ICER/CREM-deficient Th17 cells. There were no significant differences in *Pdhk3* or *Pdpr* between the two groups (Fig. 2*B* and *SI Appendix*, Fig. S1*A*). However, when empty or ICERγ vector was overexpressed in Th17-polarized ICER/CREM-deficient cells, *Pdp2* expression was significantly decreased in ICERγ-overexpressed Th17 cells, while the expression of *Pdhk1* was not affected (Fig. 2*D*). In agreement with the changes in mRNA levels, PDP2 protein expression was increased in ICER/CREM-deficient Th17 cells (Fig. 2*C*) and was decreased in ICERγ-overexpressing Th17 cells (Fig. 2*E*). Next, LDH and PDH enzyme activity are determined. LDH activity is reduced in ICER/CREM-deficient Th17-polarized cells compared with ICER/CREM-sufficient Th17-polarized cells (Fig. 2*F*), while PDH activity is increased in ICER/CREM-deficient Th17-polarized cells (Fig. 2*G*). Phospho-PDH, PDH, and actin levels were examined by Western blotting using lysates from ICER/CREM-sufficient or ICER/CREM-deficient Th17-polarized cells. Phospho-PDH (inactive PDH) is decreased in ICER/CREM-deficient Th17-polarized cells compared with their sufficient counterparts (Fig. 2*H*), and ICERγ overexpression restored phospho-PDH (Fig. 2*I*).

In a previous report, we had shown that ICER induces Gls1 expression and promotes glutaminolysis. To show that the limited glutaminolysis in ICER/CREM-deficient mice does not affect *Pdp2* gene expression, we examined its levels in DMSO-treated and Gls1 inhibitor [Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES)]-treated Th17 cells. Indeed, BPTES treatment did not change the *Pdp2* gene expression (*SI Appendix*, Fig. S1*B*). These results suggested that the increase of pdp2 expression in ICER/CREM-deficient Th17-polarized cells is independent of glutaminolysis. We also assessed the effect of ICER for glucose uptake: 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBGD) uptake was examined by flow cytometry using ICER/CREM-sufficient and ICER/CREM-deficient Th17-polarized cells. 2-NBGD uptake in ICER/CREM-deficient Th17-polarized cells was almost same as ICER/CREM-sufficient cells (*SI Appendix*, Fig. S1*C*). These experiments indicate that ICER inhibits PDP2 expression and increases PDH enzyme activity, which in turn increases lactate production (Fig. 2*A*) and Th17 cell differentiation (13).

ICER Is a Transcriptional Suppressor for PDP2. We asked whether the transcriptional factor ICER can inhibit PDP2 expression directly by binding to the *Pdp2* promoter that defines a CRE. We constructed luciferase reporter vectors driven by the full-length *Pdp2* promoter or the *Pdp2* promoter in which the CRE (Δ-74) had been mutated (Fig. 3*A*). The *Pdp2* promoter reporter activity in ICER/CREM-sufficient mice was increased in the Th17-polarized cells, which had been transfected with the mutated vector compared with those transfected with the reporter vector with the full *Pdp2* promoter (Fig. 3*B*). Moreover, *Pdp2* promoter activity was increased in ICER/CREM-deficient cells compared with full-length *Pdp2* promoter in ICER/CREM-sufficient cells transfected with full-length *Pdp2* promoter reporter, while the activity of the mutated *Pdp2* promoter was not increased in ICER/CREM-deficient cells compared with ICER/CREM-deficient cells transfected with full-length (Fig. 3*B*). To confirm that ICERγ accessed the *Pdp2* promoter at the CRE site, we transfected a Flag-tagged ICERγ overexpression vector into Th17-polarized ICER/CREM-deficient T cells and measured the recruitment of ICERγ to the *Pdp2* promoter using chromatin immunoprecipitation (ChIP) assays. As shown in Fig. 3*C*, ICERγ accumulated at the promoter region of

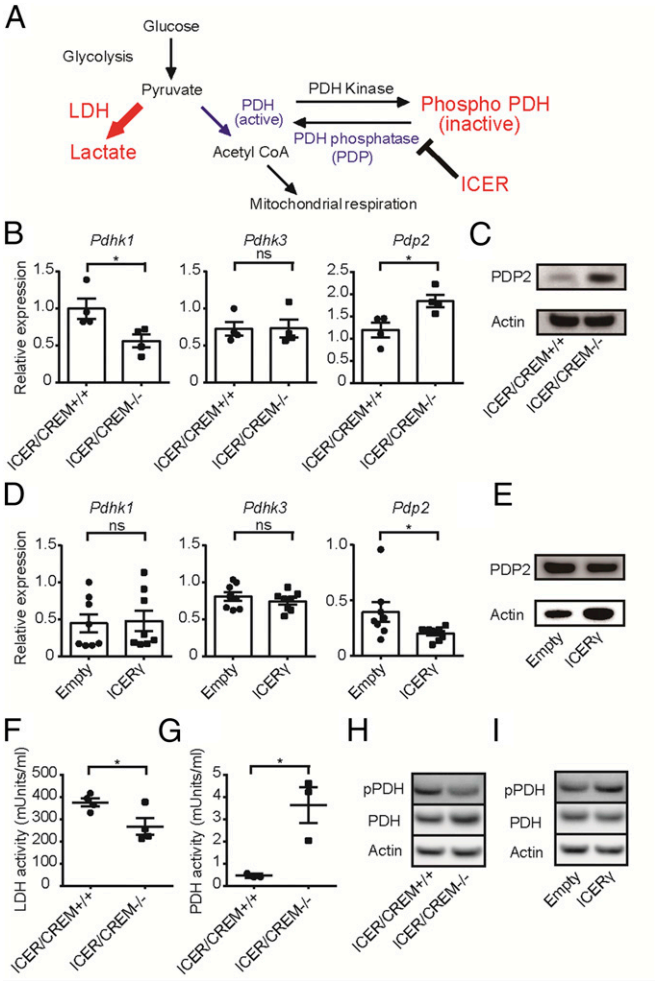

Fig. 2. ICER limits PDP2 expression in Th17 cells. (*A*) Regulation of PDH, PDH kinase, and PDH phosphatase in the glycolysis pathway and possible role of ICER/CREM. PDH catalyzes pyruvate to acetyl-CoA. PDH kinase phosphorylates PDH (active form) to phospho-PDH (inactive form), whereas PDH phosphatase dephosphorylates phospho-PDH to PDH. Th17 cells are known to favor pyruvate-to-lactate conversion by LDH for rapid nonmitochondrial ATP generation. ICER/CREM suppresses PDH phosphatase and leads PDH stays in inactive form. (*B*, *C*, and *F*–*H*) ICER/CREM-deficient or ICER/CREM-sufficient naïve CD4$^+$ T cells were cultured under Th17-polarizing conditions. (*B*) The relative gene expression of the indicated molecules on day 3 was measured by qRT-PCR. Cumulative data are shown (mean ± SEM); $n = 4$. (*C*) PDP2 and actin protein expression on day 3 were assessed by Western blotting. Representative blots are shown. Data are representative of three experiments. (*D*, *E*, and *I*) ICER/CREM-deficient naïve CD4$^+$ T cells were cultured under Th17-polarizing conditions. Empty vector (Empty) or ICERγ expressing (ICERγ) plasmids were transfected to cultured T cells on day 1. (*D*) The relative gene expression of the indicated molecules on day 3 was measured by qRT-PCR. Cumulative data are shown (mean ± SEM); $n = 4$. (*E*) PDP2 and actin protein expression on day 3 was assessed by Western blotting. Representative blots are shown. Data are representative of three experiments. (*F* and *G*) LDH (*F*) and PDH (*G*) enzyme activity on day 3 were examined. Cumulative data are shown (mean ± SEM); $n = 3$–4. (*H* and *I*) Phospho-PDH, PDH, and actin protein expression on day 3 was assessed by Western blotting. Representative blots are shown. Data are representative of three experiments. *$P < 0.05$. ns, not significant.

Pdp2, which contains the CRE but not at the exon 2 region of *Pdp2*, which also contains a putative CRE, suggesting that ICERγ selectively accumulates at the promoter region of *Pdp2* in Th17-polarized T cells. These data show that the transcription factor ICER inhibits PDP2 expression by binding directly to the *Pdp2* promoter in Th17 cells (Fig. 2*A*).

PDP2 Reduces Glycolysis, Lactate Production, and Th17 Cell Differentiation in Vitro. To investigate whether PDP2 affects Th17 cell differentiation, we generated a PDP2 overexpression vector. Naïve CD4$^+$ T cells were cultured under Th17-polarized conditions, and empty or PDP2 expression vectors were forced expressed in these cells. After we confirmed that PDP2 expression was increased in PDP2 vector-transfected Th17-polarized cells (*SI Appendix*, Fig. S2*A*), glycolysis and glycolytic capacity were measured. Glycolysis as represented by lactate production and glycolytic capacity in Th17 cells were significantly reduced after PDP2 overexpression compared with cells transfected with the empty vector (Fig. 4*A*), while ATP-coupled OCR without glutamine was increased following PDP2 overexpression (*SI Appendix*, Fig. S2*B*). Th17 cell differentiation of CD4$^+$ cells was suppressed after PDP2 overexpression (Fig. 4*B*). To confirm these observations, we transfected Th17-polarized cells with a *Pdp2*-shRNA, which reduced *Pdp2* expression or with control shRNA (*SI Appendix*, Fig. S2*C*). The glycolysis and glycolytic capacity of the Th17 cells that were transfected with *Pdp2*-shRNA were increased compared with cells transfected with control shRNA (Fig. 4*C* and *SI Appendix*). Th17 cell differentiation was increased in Th17-polarized cells transfected with *Pdp2*-shRNA (Fig. 4*D* and *SI Appendix*, Fig. S2*D*). These data confirmed that PDP2 negatively regulates glycolysis, lactate production, and Th17 cell differentiation in vitro.

***Pdp2*-shRNA Transfected Th17 Cells Exacerbate Disease Activity in Adoptive Transfer EAE Mouse Model.** To investigate further the role of PDP2 in in vivo IL-17–dependent pathology, we performed an adoptive transfer EAE experiment. Naïve CD4$^+$ T cells from 2D2 mice expressing a myelin oligodendrocyte glycoprotein T cell receptor (TCR) were cultured under Th17-polarized conditions and were transfected with *Pdp2*-shRNA or control shRNA and transferred into *Rag1*-deficient mice. The

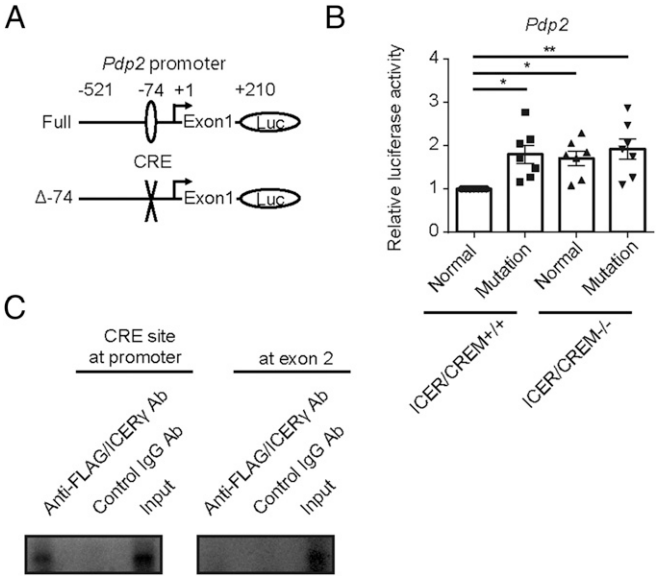

Fig. 3. ICER negatively controls PDP2 expression. (*A*) Schematic representation of the reporter constructs is shown. Numbers represent the position from the exon1 of the murine *Pdp2* gene. (*B*) ICER/CREM-sufficient and ICER/CREM-deficient naïve CD4$^+$ T cells were cultured under Th17-polarizing condition. The full-length *Pdp2* promoter region (Full) or a version containing a mutated CRE binding site (Δ-74) were transfected on day 1. Cells were harvested and lysed on day 2. Cumulative results of seven independent experiments are shown (mean ± SEM). (*C*) ICER/CREM- deficient naïve CD4$^+$ T cells were cultured under Th17-polarizing conditions. FLAG-tagged ICERγ overexpression vectors were transfected on day 1. Cells were harvested and lysed on day 3, and binding of FLAG/ICERγ to the CRE was assessed by ChIP assay. CRE at the exon 2 of the *Pdp2* gene was used as a negative control for ChIP enrichment. Representative blots from four experiments are shown. *$P < 0.05$; **$P < 0.01$.

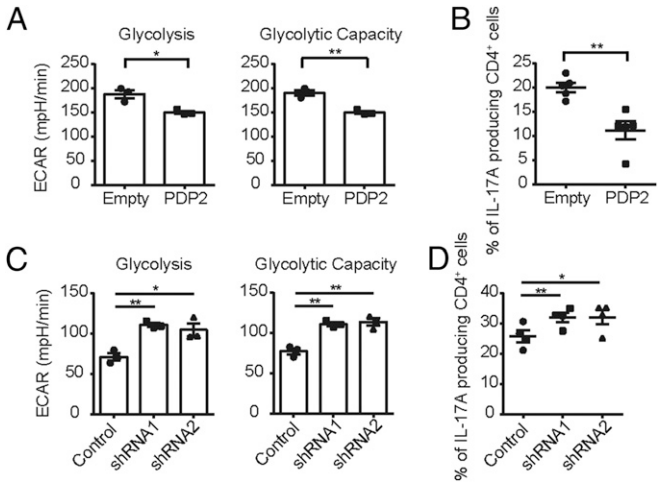

Fig. 4. PDP2 decreases Th17 differentiation by limiting the glycolysis and lactate production in in vitro Th17-polarized T cells. (*A* and *B*) Naïve CD4$^+$ T cells were cultured under Th17-polarizing conditions. Empty vector (Empty) or PDP2 plasmids were transfected into cultured T cells on day 1. (*A*) Glycolysis and glycolytic capacity were assessed by measuring ECAR by extracellular flux analyzer on day 3. Cumulative data of ECAR were shown (mean ± SEM); $n = 3$. (*B*) Percentage of IL-17A–positive cells was measured by flow cytometry. Cumulative data were shown (mean ± SEM); $n = 5$. (*C* and *D*) Naïve CD4$^+$ T cells were cultured under Th17-polarizing conditions. Two types of *Pdp2*-shRNA (shRNA1 and shRNA2) or control shRNA (Control) containing lentiviral particles were infected on day 1. (*C*) ECAR was measured by extracellular flux analyzer on day 3. Cumulative data of ECAR are shown (mean ± SEM); $n = 3$. (*D*) Percentage of IL-17A–positive cells was estimated by flow cytometry. Cumulative data are shown (mean ± SEM); $n = 4$. Typical representative data are shown in *SI Appendix*, Fig. S2*D*. *$P < 0.05$; **$P < 0.01$.

mice that received *Pdp2*-shRNA–transfected 2D2 T cells developed increased EAE disease activity compared with those which received cells transfected with control shRNA (Fig. 5*A*). To confirm these data, the absolute numbers of spinal cord-infiltrating cells were examined by flow cytometry. *Rag*1-deficient mice, which received *Pdp2*-shRNA–transfected 2D2 T cells, had increased numbers of IL-17A–producing CD4$^+$ T cells in the spinal cord compared with those which received cells transfected with control shRNA (Fig. 5*B*).

PDP2 Overexpression Reduced Th17 Differentiation in Vitro in Lupus-Prone Mice and the Patients with SLE. We previously reported that ICER/CREM is induced in Th17 cells from patients with SLE. To clarify the role of PDP2 in SLE pathogenesis, we assessed the effect of PDP2 overexpression in MRL/*lpr* lupus-prone mice. Th17 cell differentiation was reduced following PDP2 overexpression in MRL/*lpr* mice (Fig. 6*A*). Next, we sorted memory Th17 cells (CD4$^+$CD45RA$^-$CCR6$^+$CCR4$^+$) from patients with SLE and healthy donors and compared the expression of PDH-regulating *PDP2* and *PDHK1* expression. Although there was no significant difference in *PDHK1* expression between the two groups, *PDP2* expression was significantly reduced in the patients with SLE compared with health donors (Fig. 6*B*). We previously reported that memory Th17 cells from patients with SLE express higher levels of ICERγ than cells from healthy donors. This observation is in agreement with the results shown in Fig. 2*D* where overexpression of ICERγ in T cells did not affect the expression of *PDHK1* in Th17 cells. Finally, naïve CD4$^+$ T cells from the patients with SLE were polarized to Th17 cells and then transfected with empty or human PDP2 overexpression vectors. Cells transfected with PDP2 decreased Th17 cell differentiation in vitro (Fig. 6*C*).

Discussion

In this study, we demonstrate that PDP2 suppresses Th17 differentiation and Th17 cell-specific PDP2 inhibition accelerates EAE. At the molecular level, we show that ICER represses the expression of PDP2. Memory Th17 cells from patients with SLE have reduced *PDP2* expression, and Th17 differentiation is inhibited by PDP2 overexpression.

Proliferating cells favor the glycolysis pathway because glycolysis produces energy quickly compared with any other metabolic pathways (10). In addition, glycolysis enables the generation of precursor-cell building blocks including DNA, RNA, proteins, and lipids. Conversion of pyruvate to lactate regenerates nicotinamide adenine dinucleotide, which is necessary to continue glycolysis (10).

Th17 cells are important not only in the defense against opportunistic pathogens but also in the pathogenesis of autoimmune diseases including SLE and multiple sclerosis (15, 16). It has been well established that cell metabolism regulates T cell differentiation and function (9, 17, 18) and that Th17 cells depend more on glycolysis and glutaminolysis than other T cell subsets (11). Furthermore, Th17 cells efficiently convert pyruvate to lactate. It was previously reported that PDHK1 is increased in Th17 compared with Th1 and Treg cells. PDHK converts active PDH to inactive form, which suppresses the conversion of pyruvate to acetyl-CoA, and shifts the process toward lactate production (11), which in Th17 cells favors expression of *Rorc* gene expression and IL-17 production (13). PDP is the balancing enzyme that converts inactive PDH to its active form. In this report, we present evidence that introduces PDP2 as an important gate controller between lactate production and oxidative phosphorylation and that its activity is controlled by the transcription factor ICER.

Because cell metabolism regulates the fate in T cells, modulation of metabolic pathways had been considered as a possible therapeutic tool (18). Previous reports have shown that blocking glycolysis with 2-Deoxy-D-glucose (2DG, a glycolysis inhibitor) can inhibit Th17 cell differentiation (11) and treatment of lupus-prone mice with a combination of metformin, a mitochondrial metabolism inhibitor, and 2DG normalized T cell metabolism and reduced disease activity (19). A PDHK inhibitor has also been claimed as a possible drug because it reduced Th17 differentiation and ameliorated colitis and EAE in mice (11). However, our data failed to show a difference in *PDHK1* expression in memory Th17 cells between healthy and SLE subjects. In contrast, *PDP2* expression levels were found decreased in Th17 cells from patients with SLE. Furthermore, since PDP2

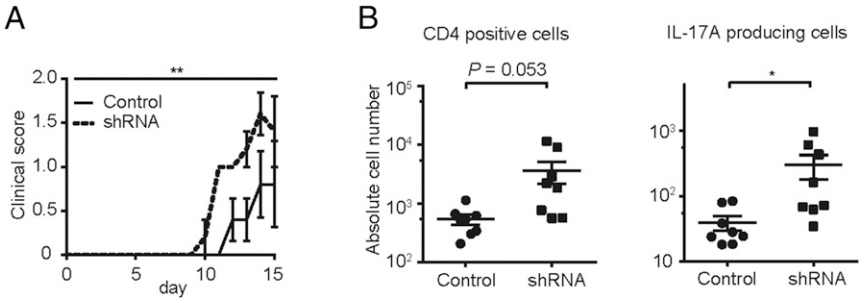

Fig. 5. *Pdp2*-shRNA–transfected Th17 cells exacerbate disease activity in an adoptive cell transfer EAE model. (*A* and *B*) Naïve CD4$^+$ T cells from 2D2 mice were cultured under Th17-polarizing conditions. *Pdp2*-shRNA or control shRNA containing lentiviral particles were infected on day 1. On day 3 of culture, harvested those cells were transferred to recipient *Rag1*-deficient mice i.v. (*A*) The clinical scores of recipient mice are shown. Cumulative results of 10 mice per group are shown (mean ± SEM). (*B*) Absolute cell numbers of spinal cord-infiltrated CD4$^+$ T cells and IL-17A–producing CD4$^+$ T cells from recipient mice were evaluated by flow cytometry on day 14. Cumulative data are shown (mean ± SEM); $n = 8$. *$P < 0.05$; **$P < 0.01$.

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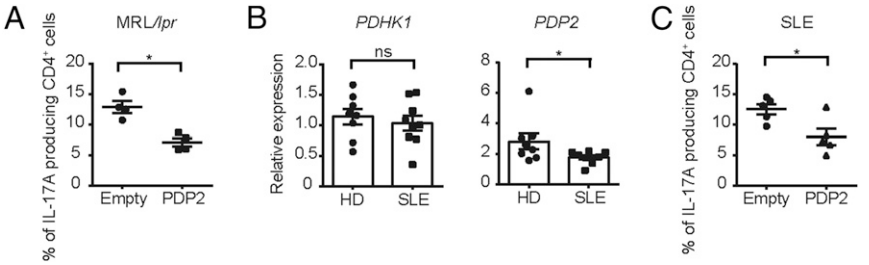

Fig. 6. The amount of PDP2 is decreased in T cells from patients with SLE. (*A*) Naïve CD4$^+$ T cells from 12- to 16-wk-old MRL/*lpr* mice were cultured under Th17-polarizing conditions. Empty vector (Empty) or PDP2 plasmids were transfected to cultured T cells on day 1. Percentage of IL-17A–positive cells were measured by flow cytometry. Cumulative data were shown (mean ± SEM); $n = 4$. (*B*) The relative *PDHK1* and *PDP2* expressions of fleshly isolated memory Th17 cells from healthy donors (HD) and the patients with SLE were measured by qRT-PCR. Cumulative data are shown (mean ± SEM); $n = 8$ (HD) and $n = 9$ (SLE). (*C*) Naïve CD4$^+$ T cells from patients with SLE were cultured under Th17-polarizing conditions. Empty or PDP2 plasmids were transfected to those cultured T cells on day 3. Percentage of IL-17A–positive cells were measured by flow cytometry on day 7. Cumulative data were shown (mean ± SEM); $n = 5$. $*P < 0.05$. ns, not significant.

overexpression in lupus T cells reduced Th17 cell differentiation, we propose that PDP2 manipulation may help patients with SLE.

CREM belongs to CREM/ATF family and has many alternatively spliced transcript variants that are regulated at the epigenetic and posttranscriptional levels (5). One of the splice variants of CREM, ICER, is unique because it has an alternative transcription initiation site and is induced by a private alternative promoter (20). ICER/CREM binds to CRE sites and modulates gene expression positively or negatively (5). We previously documented that ICER promotes Th17 cell differentiation, ICER/CREM-deficient mice have less autoimmune disease, and CD4$^+$ T cells from the patients with SLE express more ICER/CREM than those from health subjects (6). We have also shown that ICER promotes Gls1 expression and glutaminolysis, and glutaminolysis is requisite for Th17 cell differentiation (21). In this work, we present another molecular link whereby the transcription factor ICER controls the expression of a specific metabolic protein PDP2 in the induction of Th17 cells. The effect on PDP2 appears to be independent of the previously reported effect of ICER on Gls1 and glutaminolysis (21).

In summary, we have shown that PDP2 reduces glycolysis, lactate production, and Th17 differentiation and that inhibition of PDP2 accelerates EAE. The expression of PDP2 is controlled directly by the transcription factor ICER, which binds to the CRE site within the *Pdp2* promoter and suppresses its expression. At the translational level we show that Th17 cells from patients with SLE display less PDP2 and that forced PDP2 expression reduces Th17 differentiation in CD4$^+$ cells from lupus-prone MRL/*lpr* mice and patients with SLE.

Materials and Methods

Human Samples. Patients who fulfilled the criteria for the diagnosis of SLE as set forth by the American College of Rheumatology (22) and healthy individuals were enrolled. The Beth Israel Deaconess Medical Center (BIDMC) institutional review board approved the study protocol (2006-P-0298). Informed consent was obtained from all study subjects. The disease activity for each patient was calculated using the clinical and laboratory index SLE Disease Activity Index (23). Age-, sex-, and ethnicity-matched healthy individuals were chosen as controls (*SI Appendix*, Table S1).

Mice. SV129/Bl6.ICER/CREM$^{-/-}$mice were originally cloned by Günther Schuetz, German Cancer Research Center Heidelberg (24). Animals were crossed to C57BL/6J mice for over nine generations to transfer the ICER/CREM$^{-/-}$ locus to the B6 background. C57BL/6-Il17atm1Bcgen/J (IL-17GFP), C57BL/6-Tg(Tcra2D2, Tcrb2D2)1Kuch/J (2D2), MRL/MpJ-Faslpr/J (MRL/*lpr*), and B6.129S7-Rag1tm1Mom/J (*Rag1* KO) mice were purchased from The Jackson Laboratory. B6.ICER/CREM$^{-/-}$.IL-17GFP mice were made by crossing B6.ICER/CREM$^{-/-}$ mice with IL-17GFP mice. Animals were killed at the end of their 8–12 wk of life for in vitro culture experiments and indicated week for in vivo experiments. All mice were maintained in a specific pathogen-free animal facility (BIDMC). Experiments were approved by the Institutional Animal Care and Use Committee of BIDMC.

Single Cell Isolation. For mouse, spleen and lymph node lymphocytes as well as infiltrating lymphocytes in spinal cords were isolated as described (21). For human, peripheral blood was collected and total human T cells were purified as described (25). In short, T cells were isolated by negative selection (RosetteSep; Stem Cell Technologies) before density gradient purification (Lymphoprep; Nycomed).

In Vitro T Cell Differentiation. Murine Th17-polarized culture was performed as described (21). For the human Th17-polarized culture, isolated naive CD4 T cells were stimulated with plate-bound anti-CD3 Ab (1 μg/mL, OKT-3; BioXCell), anti-CD28 Ab (1 μg/mL, CD28.2; BioLegend), IL-6 (50 ng/mL; NM_000600; BioLegend), TGF-β1 (10 ng/mL, NM_003236; BioLegend), IL-1β (10 ng/mL, NM_000576; BioLegend), IL-23 (50 ng/mL, NP_057668 and NP_002178.2; BioLegend), anti-IL-4 Ab (10 μg/mL, MP4-25D2; BioXCell), and anti-IFN-γ Ab (5 μg/mL; B27; BioXCell).

Metabolism Assays. ECAR and OCR were measured using a XFp extracellular flux analyzers. Assay buffer for glycolysis stress test was made of XF base medium with 2 mM glutamine and without glucose or sodium pyruvate. Assay buffer for ATP-coupled OCR was made of XF base medium with 10 mM glucose and 1 mM sodium pyruvate and without glutamine. All other procedures were performed according to the manufacturer's instructions as described (21).

Western Blotting. Cell lysates ware separated on NuPAGE 4–12% Bis-Tris Gel (Life Technologies), and proteins were transferred to a nitrocellulose membrane. Antibodies used were as follows: anti-CREM1 Ab (Santa Cruz), anti-actin Ab (Sigma-Aldrich), anti-PDP2 Ab (Novus Biologicals), PDH Monoclonal Antibody (Thermo Fisher Scientific) Ab, PhosphoDetect Anti-PDH-E1α (pSer^{293}) Rabbit p PhosphoDetect Anti-PDH-E1α (pSer^{293}) Rabbit pAb (Millipore sigma), and goat anti-rabbit IgG coupled with HRP (Jackson Immunoresearch). The ECL system (Amersham) was used for detection.

Flow Cytometry. The following antibodies were used for flow cytometry analysis: For mouse CD4 (GK1.5), CD8a 53-6.7, CD19 605, CD45 30-F11, CD90.2 53-2.1, and IL-17A JC11-18H10.1 were purchased from BioLegend. CD3α 17A2 was purchased from eBioscience. For human, CD45RA HI100, CD25 BC96, CD127 A019D5, CCR4 L291H4, CCR6 G034E3, and CXCR3 G025H7 were purchased from BioLegend. A CD4 SK3 was purchased from eBioscience. A 7AAD (surface) or a Zombie Aqua Fixable Viability Kit (intracellular) staining was performed for eliminating dead cells. Surface staining was performed on ice for 20–30 min. Absolute cell numbers were calculated on the basis of the percentage of each cell population. For intracellular staining, harvested cells were stimulated for 4 h in culture medium with PMA (Sigma-Aldrich), ionomycin (Sigma-Aldrich), and monensin (BD Biosciences). Cytofix/Cytoperm and Perm/Wash buffer (IL-17A/IFN-γ; BD Biosciences) was used for fixation and permeabilization. All flow cytometry data were acquired on a BD LSRII (BD Biosciences) or Cytoflex LX (Beckman Coulter) and analyzed with FlowJo (FlowJo, LLC). All procedures were performed according to the manufacturer's instructions.

Glucose Uptake Assay. 2-NBDG Glucose Uptake Assay Kit (Biovision) was performed according to the manufacturer's instructions.

Enzyme Activity. LDH and PDH enzyme activity on day 3 were examined by LDH activity assay kit and PDH activity assay kit (Sigma-Aldrich). Both were performed according to the manufacturer's instructions.

Human Memory Th17 Cell Sorting. CD4$^+$ T cells were enriched using CD4$^+$ T cells isolation kit II (Miltenyi Biotec) from peripheral blood mononuclear cells. After staining, CD4$^+$CD45RA$^-$CCR6$^+$CCR4$^+$ memory Th17 cells were sorted by BD FACS Aria II (five lasers: 355, 405, 488, 561, 640 nm; BD Biosciences).

Transfection of Overexpression Vectors. For ICERγ overexpression, N′-FLAG-tagged ICERγ overexpressing vectors generated previously were used in this study (6). Mouse and human PDP2 overexpression vectors were made by Genescript. All overexpression vectors were constructed by using pIRES2-DsRed-Express vector. All constructs were verified by DNA sequencing. For ICER and PDP2 overexpression experiments in murine primary T cells, cells were harvested 1 d after starting culture and empty vector, ICERγ, or PDP2-overexpression plasmid were transfected using the Amaxa Mouse T Cell Nucleofector Kit with the X-001 program (Amaxa) as described (21). For the PDP2 overexpression experiment in human primary T cells, cells were harvested 3 d after starting culture, and empty vector or PDP2-overexpression vector was transfected using the Amaxa Human T Cell Nucleofector Kit with the T-020 program (Amaxa) according the manufacturer's protocol. Cells were again cultured in those supernatants for 4 d. The efficacy of the transfection in living cells was tested by flowcytometry and always exceeded 10%.

RNA Isolation and Quantitative PCR. TRIzol Reagent was used for RNA preparation. The following TaqMan probes (Thermo Fisher Scientific) were used to detect target genes [Murine: *Pdp2* Mm01252669_s1, *Pdk1* Mm00554300_m1, *Pdk3* Mm00455220_m1, *Pdpr* Mm01243524_m1, *TATA box-binding protein* (*Tbp*) Mm00446973_m1, and *Gusb* (β-glucuronidase) Mm01197698_m1. Human: *PDP2* Hs01934174_s1, *PDHK1* Hs01561847_m1, *GUSB* Hs00939627_m1, and *TBP* Hs00427620_m1]. Gene expression was assessed by the comparative CT method and normalized to the reference gene *Tbp* and *Gusb* (21).

Luciferase Assays. Mouse *Pdp2* promoter luciferase promoter construct (pGL3_mpdp2_vector; −526/+210 from start of exon1) was purchased from Genescript. Site-directed mutagenesis at the −79/−74 CRE site (TGACG) within pGL3_mgls1_vector was performed by Q5 site-directed mutagenesis kit (New England BioLabs) using the following primers; 5′- AGGGCCCGC-CTCCAAGGA -3′ and 5′- GACATTGAGATTACCCAATGGATGAGGAGGG -3′. All sequences were verified (Genewiz). Luciferase reporter plasmid was transfected using the Amaxa Mouse T Cell Nucleofector Kit with the X-001 program (Amaxa) on day 1 of culture. Each reporter experiment included 200 ng of renilla luciferase construct as an internal control. Luciferase activity was quantified using the Promega Dual Luciferase Assay System (Promega) on day 2 of culture according to the manufacturer's instructions.

ChIP. Freshly isolated naïve $CD4^+$ T cells from ICER/CREM$^{-/-}$ mice were cultured in Th17-polarizing condition for 3 d. N′-FLAG–tagged ICERγ overexpressing vectors were transfected as described above on day 1. Harvested cells were lysed and ChIP assay was performed as described (21). Anti-FLAG antibody produced in rabbit (Sigma-Aldrich) was used for immunoprecipitation. The primer pairs used were as follows: 5′- CACAAAAGCCACGGG-TAAC -3′ and 5′- ATGAGGAGGGCAAAAGGAAG -3′ for promoter region contains CRE site and 5′- CATATGGAAATGGGGCTGAG -3′ and 5′- CTTCCAGAGGAGC-CTGGATT -3′ for exon 2 contains CRE site. All procedures were performed according to the manufacturer's instructions.

Generation of Lentiviral Particles Containing shRNAs. MISSION pLKO.1-puro empty vector control plasmid DNA (Sigma Aldrich) was used for this cloning. We designed two *Pdp2*-shRNAs as listed below and cloned them into the empty vector following the manufacturer's protocols. The following oligonucleotide sequences were used for this cloning: 5′- CCGGCGGAGTAC-CAAATTCAGTGTTCTCGAGAACACTGAATTTGGTACTCCGTTTTTG -3′ and 5′-AATTCAAAAACGGAGTACCAAATTCAGTGTTCTCGAGAACACTGAATTTGGTA-CTCCG -3′ for *Pdp2*-shRNA1, and 5′- CCGGCGTCCAAACGAATGGGATGATC-TCGAGATCATCCCATTCGTTTGGACGTTTTTG -3′and 5′- AATTCAAAAAATCTCGA-CGGGTTGCTATAATCTCGAGATTATAGCAACCCGTCGAGAT -3′ for *Pdp2*-shRNA2. Sequences of cloned vectors were verified (Genewiz).

MISSION pLKO.1-puro nonmammalian shRNA control plasmid DNA control shRNA (Sigma Aldrich) was used for control shRNA. Those vectors were transfected to 40% confluent HEK-293T cells by polyethyleneimine "Max" (Polycycles, Inc.) according the manufacturer's protocol. Culture media with shRNA-contained lentiviral particles was collected on day 3 and 4.

EAE. Naïve $CD4^+$ T cells from 2D2 mice were cultured under Th17 cell conditions. On day 1 of culture, *Pdp2*-shRNA or control shRNA-containing lentiviral particles were added to media with polybrene infection/transfection reagent (Sigma-Aldrich). One day after infection, puromycin was added to media. Cultured cells were harvested and purified on day 4 of culture. Five million cells were suspended in 150 μL of PBS (pH 7.4) and were injected i.v. into each *Rag1*-deficient mouse. Pertussis toxin (300 ng per mouse; List Biological Laboratories) was i.p. injected later on the day of transfer and 2 d later. Mice were monitored and weighted as described (21)

Histological Staining and Analysis. Sections from 10% formalin fixed spinal cords were stained with H&E. Spinal cord sections were scored as described (21).

Statistics. Statistical analyses were performed in GraphPad Prism version 6.0 software. Statistical significance was determined by *t* tests (two-tailed) for two groups or one-way ANOVA with Bonfferoni's multiple comparisons tests for three or more groups. For the EAE experiments, clinical scores and body weight changes of each treatment group were compared using two-way ANOVA. P values of <0.05 were considered statistically significant (**P < 0.01, *P < 0.05).

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1. Tsokos GC (2011) Systemic lupus erythematosus. *N Engl J Med* 365:2110–2121.
2. Moulton VR, Tsokos GC (2015) T cell signaling abnormalities contribute to aberrant immune cell function and autoimmunity. *J Clin Invest* 125:2220–2227.
3. Crispín JC, et al. (2008) Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol* 181: 8761–8766.
4. Pisitkun P, et al. (2012) Interleukin-17 cytokines are critical in development of fatal lupus glomerulonephritis. *Immunity* 37:1104–1115.
5. Foulkes NS, Sassone-Corsi P (1992) More is better: Activators and repressors from the same gene. *Cell* 68:411–414.
6. Yoshida N, et al. (2016) ICER is requisite for Th17 differentiation. *Nat Commun* 7: 12993.
7. Rauen T, Hedrich CM, Juang YT, Tenbrock K, Tsokos GC (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.
8. Ciofani M, et al. (2012) A validated regulatory network for Th17 cell specification. *Cell* 151:289–303.
9. MacIver NJ, Michalek RD, Rathmell JC (2013) Metabolic regulation of T lymphocytes. *Annu Rev Immunol* 31:259–283.
10. Lunt SY, Vander Heiden MG (2011) Aerobic glycolysis: Meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol* 27:441–464.
11. Gerriets VA, et al. (2015) Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *J Clin Invest* 125:194–207.
12. Zhang X, et al. (2005) Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc Natl Acad Sci USA* 102:4459–4464.
13. Haas R, et al. (2015) Lactate regulates metabolic and pro-inflammatory circuits in control of T cell migration and effector functions. *PLoS Biol* 13:e1002202.
14. Huang B, et al. (1998) Isoenzymes of pyruvate dehydrogenase phosphatase. DNA-derived amino acid sequences, expression, and regulation. *J Biol Chem* 273: 17680–17688.
15. Park H, et al. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133–1141.
16. Korn T, Bettelli E, Oukka M, Kuchroo VK (2009) IL-17 and Th17 cells. *Annu Rev Immunol* 27:485–517.
17. Buck MD, O'Sullivan D, Pearce EL (2015) T cell metabolism drives immunity. *J Exp Med* 212:1345–1360.
18. Morel L (2017) Immunometabolism in systemic lupus erythematosus. *Nat Rev Rheumatol* 13:280–290.
19. Yin Y, et al. (2015) Normalization of CD4+ T cell metabolism reverses lupus. *Sci Transl Med* 7:274ra18.
20. Molina CA, Foulkes NS, Lalli E, Sassone-Corsi P (1993) Inducibility and negative autoregulation of CREM: An alternative promoter directs the expression of ICER, an early response repressor. *Cell* 75:875–886.
21. Kono M, Yoshida N, Maeda K, Tsokos GC (2018) Transcriptional factor ICER promotes glutaminolysis and the generation of Th17 cells. *Proc Natl Acad Sci USA* 115: 2478–2483.
22. Hochberg MC (1997) Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 40:1725.
23. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH; The Committee on Prognosis Studies in SLE (1992) Derivation of the SLEDAI. A disease activity index for lupus patients. *Arthritis Rheum* 35:630–640.
24. Blendy JA, Kaestner KH, Weinbauer GF, Nieschlag E, Schütz G (1996) Severe impairment of spermatogenesis in mice lacking the CREM gene. *Nature* 380:162–165.
25. Sunahori K, Juang YT, Tsokos GC (2009) Methylation status of CpG islands flanking a cAMP response element motif on the protein phosphatase 2Ac alpha promoter determines CREB binding and activity. *J Immunol* 182:1500–1508.