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Research Paper

Cyclic AMP-Responsive Element-Binding Protein (CREB) is Critical in Autoimmunity by Promoting Th17 but Inhibiting Treg Cell Differentiation

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ABSTRACT

The molecular mechanisms that govern differential T cell development into pro-inflammatory Th17 *vs.* regulatory T (Treg) cells remain unclear. Here, we show that selective deletion of CREB in T cells or Th17 cells impaired Th17 cell differentiation *in vitro* and *in vivo*, and led to resistance to autoimmune diseases. Mechanistically, CREB, activated by CD3-PKC-Θ signaling, plays a key role in regulating Th17 cell differentiation, at least in part through directly binding to the *Il17-Il17f* gene locus. Unexpectedly, although dispensable for FOXP3 expression and for the homeostasis and suppressive function of thymus-derived Treg cells, CREB negatively regulates the survival of TGF-β-induced Treg cells, and deletion of CREB resulted in increased FOXP3 + Treg cells in the intestine and protection in a colitis model. Thus, CREB is critical in autoimmune diseases by promoting Th17 cell and inhibiting *de novo* Treg cell generation.

1. Introduction

Th17 cells are important in immunity against bacterial and fungal infections but also critical in inflammatory and autoimmune diseases. Foxp3$^+$ regulatory T (Treg) cells function to suppress various immune and autoimmune diseases. Therefore, the balance of Th17 and Treg cell function has to be tightly controlled. However, these two types of cells are regulated reciprocally in their development: Th17 cell differentiation is initiated by IL-6 and TGF-β, whereas TGF-β alone directs *de novo* differentiation of naive T cells into FOXP3$^+$ Treg (inducible Treg cells or iTreg) cells (Dong, 2008). Though with highly significant implications in autoimmune diseases, the molecular mechanisms for controlling Th17 and Treg cell divergence remain not well understood.

cAMP response element binding protein (CREB) is one of the best characterized phosphorylation-activated transcription factors in the basic leucine zipper (bZIP) superfamily, comprising of CREB, ATF1 and CREM. CREB can be phosphorylated by diverse protein kinases, including PKA, CamKII, CaMKIV, RSK2, MAPK, MSK1/2 and PKC, and is negatively regulated by the Ser/Thr phosphatases protein phosphatase 1 (PP1) and PP2A. Phosphorylation of serine 133 in the CREB's kinase inducible domain (KID) promotes the recruitment of the co-activator protein CBP (CREB binding protein)/p300, and induces activation of CREB (Kandel, 2012; Wen et al., 2010).

CREB has many important physiological functions and regulates various developmental and cellular processes, and is particularly important in regulating neuronal and brain functions (Ortega-Martinez, 2015). In the immune system, CREB has been shown to positively regulate the development, survival, activation, or proliferation of macrophages, dendritic cells, B cells and T cells (Wen et al., 2010), and deletion of CREB caused severe developmental defect of αβ T cell lineages (Rudolph et al., 1998), whereas disruption of CREB by expressing the dominant negative form of CREB – ACREB significantly impaired the survival and proliferation of activated B cells and T cells, likely due to reduced expression of Bcl-2 or immediate early genes (Barton et al., 1996; Rudolph et al., 1998; Zhang et al., 2002, 2000). In addition, CREB can also regulate cytokine production from both innate and adaptive immune cells in response to various immune stimulations, including IL-10, IL-23, TNF-α, IFN-γand IL-2 (Barton et al., 1996; Kocieda et al., 2012; Wen et al., 2010). These studies highlight the importance of CREB in immune system.

Emerging evidences indicate that the CREB/ATF1 family transcription factors may regulate the differentiation and function of Th17 and Treg cells. For examples, CREB has been shown to bind the promoter and conserved non-coding sequence (CNS) 2 region in the *Foxp3* gene locus and activate its promoter activity (Kim and Leonard, 2007),

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whereas CREMα was found to increase the human *Il17* gene transcription through binding to the promoter region (Rauen et al., 2011), though it inhibits *Il17f* gene transcription (Hedrich et al., 2012). In addition, disruption of CRTC2 in T cells, a coactivator for CREB/ATF1 family transcription factors, reduced Th17 cell differentiation *in vitro* and *in vivo* (Hernandez et al., 2015). Prostaglandin E2 (PGE2), a paracrine hormone that can activate CREB/ATF1 family transcription factors through the cAMP/PKA signaling pathway, was able to enhance the transcription of the *Il17* gene and stabilize IL-17 expression in mature Th17 cells upon secondary stimulation (Boniface et al., 2009; Hernandez et al., 2015; Yao et al., 2009). Additionally, overexpression of ACREB (Hernandez et al., 2015) or inhibition of CBP/p300 (Hammitzsch et al., 2015), a dominant negative inhibitor or coactivator for the CREB/ATF1 family transcription factors, significantly reduced IL-17 expression in Th17 cells. However, due to lack of genetic experimental evidence and alternative pathways involved in the above studies, whether and how CREB regulates Th17 cell differentiation, as well as Treg cell differentiation, is unclear.

In this study, we utilized various CREB conditional knockout mice to systematically examine the role of CREB in $CD4^+$ T cells *in vitro* and *in vivo*. Our data demonstrate that CREB plays a critical role in regulating Th17 cell differentiation *in vitro* and *in vivo*, as well as in Th17 cell-mediated autoimmune diseases. Although not essential for FOXP3 gene expression in nTreg or iTreg cells, or for the suppressive activity and homeostasis of nTreg cells, CREB appears to be a negative regulator for the survival or proliferation of iTreg cells. Our studies thus identify CREB as a critical regulator of the autoimmune diseases by controlling Th17/Treg cell ratios.

2. Materials and Methods

2.1. Primers and Inhibitors and Antibodies

The primers used for ChIP-PCR, mutagenesis, constructing P27p-PGL3 luciferase reporters and CREB genotyping primers were listed in Supplementary Table 1. The CREB gene was cloned into the *Bgl*II sites of pRVKM plasmid using primers listed in Supplementary Table 1. The real-time RT-PCR primers used to quantify Il17, Il17f, Il21, RORα, RORγt, IkBζ, Batf, AHR and β-actin, and the Il17p-PGL3, CNS2-Il17p-PGL3 luciferase reporter constructs were described previously (Chang et al., 2011; Yang et al., 2008). Most of the protein kinase inhibitors were purchased from Selleck Chemicals LLC, including H89 (Cat# S1582) for PKA and MSK1/2, PD325901 (Cat#S1036) for ERK, SB203580 (Cat#S1076) for p38, sotrastaurin (Cat#S2791) for PKC-Θ and KN-93 (Cat#S7423) for CamKIV. The PI3K inhibitor LY294002 (Cat#L9908) was purchased from Sigma. The CREB and p27 antibodies were purchased from Cell Signaling: CREB (Cat#9197, Clone#48H2) & phospho-CREB (Cat#9198, Clone#87G3) for ChIP assays, phospho-CREB (Cat#9187, Clone#87G3) for flowcytometry assays and p27 antibody (Cat#3688, Clone#D37H1) for western blotting.

2.2. Mice

The $CREB^{fl/fl}$ mice were initially generated by Prof. Günther Schütz (German Cancer Research Center, Heidelberg, Germany) (Mantamadiotis et al., 2002), and provided to us by Prof. Gerard Karsenty (Department of Genetics and Development, Columbia University College of Physicians and Surgeons, New York, New York, USA). The $CREB^{fl/fl}$ mice has been backcrossed to C57BL/6 background (Yoshizawa et al., 2009), and then crossed with CD4-Cre (Lee et al., 2001), FOXP3-Cre (Rubtsov et al., 2008) and IL-17F-Cre mice (Ichiyama et al., 2016) to generated various conditional knockout mice. All the mice were housed in the SPF animal facility at the M. D. Anderson Cancer Center or Tsinghua University. All the animal experiments were performed at the age of 8–12 weeks with the use of protocols approved by the Institutional Animal Care and Use Committee.

2.3. Animal Disease Models

Active EAE were induced and analyzed as previously described (Wang et al., 2012). For adoptive T cell transfer EAE, ~150,000 naïve 2d2 + CD4 + T cells/mouse were first injected into RAG1 KO mice intravenously, and EAE was induced in the recipient mice the next day same as in the active EAE model. For adoptive T cell transfer colitis, ~1.5–4 million naïve CD4 + T cells were injected into RAG1 KO mice with or without Treg cells at a 10:1 ratio, and the weight loss was monitored in recipient mice weekly for up to 3–4 months, and then sacrificed for analysis.

2.4. RNAseq Analysis

The total cellular RNA was extracted by using TRIZOL reagent (Invitrogen). The mRNA was enriched by using the Dynabeads® mRNA DIRECT™ Micro Kit (Thermos Fisher Scientific, catalog NO. 61021). The RNAseq library was prepared and amplified using NEXTflex™ RNA-Seq Kit (Bioo Scientific, catalog NO. 5129-02), and sequenced with PE125 bp reads on an illumine Hiseq 2500. Sequence reads were mapped to the *Mus musculus* genome (version mm10) used SOAP2, and >23 M unique mapping reads were obtained (GEO accession numbers: GSE80375). The gene profilings were calculated and analyzed by the RPKM method, with a threshold of 1.4 fold changes.

2.5. Luciferase Reporter, ChIP, Realtime PCR, and Retroviral Assays

All these assays were performed according to previous publications (Wang et al., 2012).

2.6. Calculations and Statistic Analysis

All our *in vitro* and *in vivo* data were repeated at least 2–3 times with consistent results. The data in this study were shown as mean + SD where applicable, and the statistical significance was determined by Student's *t*-test, or Anova analysis when indicated. (* represents $p < 0.05$; ** represents $p < 0.03$; *** represents $p < 0.01$).

3. Results

3.1. CREB is Activated by TcR-PKC-Θ Signaling in CD4 + T Cells

As a first step to determine the role of CREB in $CD4^+$ T cells, we sorted mouse $CD4^+CD44^-CD25^{lo}CD62L^{hi}$ naive T cells and tested CREB phosphorylation following various stimulations. Similar to previous findings (Zhang et al., 2000), antibodies to CD3 and CD28 induced persistent phosphorylation of CREB in naïve T cells, and the peak level of pCREB was achieved around 4 h after TcR stimulation (Suppl. Fig. 1a). Moreover, the early stage of CREB phosphorylation in activated $CD4^+$ T cells was largely dependent on TcR/CD3 signaling, but independent of IL-6, IL-2, TGF-β or CD28 (Suppl. Fig. 1b).

To assess the kinase or signaling pathway responsible for CREB activation, we tested a number of pharmacological inhibitors for the serine and threonine kinases downstream of TcR stimulation, including H89 for PKA and MSK1/2, PD325901 (PD32) for ERK, SB203580 (SB20) for p38, sotrastaurin (PKCi) for PKC-Θ, KN-93 for CamKIV and LY294002 (PI3Ki) for PI3K. Only PKC-Θ inhibitor significantly inhibited CREB phosphorylation in anti-CD3-activated T cells at 4 h (Suppl. Fig. 1c/1d). LY294002, though alone had a modest effect on CREB phosphorylation, further inhibited CREB phosphorylation when used together with sotrastaurin (Suppl. Fig. 1d), suggesting a synergistic effect between PKC-Θ and PI3K in mediating CREB phosphorylation. In addition, the effect of PKC-Θ and PI3K inhibitors on CREB phosphorylation was more apparent at 24 h after TcR stimulation (Suppl. Fig. 1d).

3.2. CREB Deficiency in Th17 Cells Led to Resistance to EAE

Recent studies suggest the presence of CRE (CREB response element) sites in the *Il17-Il17f* gene locus, and CREB/ATF1 family transcription factors may regulate IL-17 and IL-17F expression in T cells or non-T cells, positively or negatively. (Hedrich et al., 2012; Kotla et al., 2013; Rauen et al., 2011). To genetically analyze the exact role and the relative importance of CREB in regulating Th17 cell differentiation and Th17 cell-driven autoimmunity, T cell-specific CREB conditional knockout mice were generated by crossing Cd4-Cre mice with $CREB^{fl/fl}$ mice (Mantamadiotis et al., 2002), in which the exon 10 of *Creb* (containing the first part of bZIP domain) was specifically deleted in T cells (CREB-CD4KO). These CREB-CD4KO mice developed normally and did not have any obvious defect in the frequencies and numbers of $CD4^+$ T cells or $FOXP3^+$ Treg cells, although the $CD8^+$ T cells were slightly increased in the spleen (data not shown).

The CREB-CD4KO and control $CREB^{fl/fl}$ (WT) mice were then subject to experimental autoimmune encephalomyelitis (EAE) – a mouse model of Th17 cell-mediated autoimmune disease. WT mice developed severe disease symptoms (all scored 2.5 or above), whereas the CREB-CD4KO mice only developed very mild disease and showed recovery from EAE at later stage (Fig. 1a). In the central nervous system (CNS), the numbers of infiltrating $CD4^+$ T cells were reduced by >80% in CREB-CD4KO mice compared to the WT group (data not shown). In addition, these $CD4^+$ T cells barely produced IL-17 and IFN-γ (Fig. 1b). Taken together, the numbers of CNS-infiltrating Th17 cells were reduced by >95% in CREB-CD4KO mice (Fig. 1c).

Surprisingly, the percentages of $FOXP3^+$ Treg cells were increased in the CNS and spleens of CREB-deficient mice in EAE, especially after developing EAE symptoms (Fig. 1d/e). To determine whether CREB expression in Treg cells was responsible for the observed EAE phenotype, $CREB^{fl/fl}$xFOXP3Cre (FOXP3KO) mice were generated and used for EAE experiments. Treg-specific ablation of CREB did not have any effect on IL-17 expression and EAE disease (Fig. 2a), suggesting that lack of CREB in Treg cells did not contribute to EAE resistance in the CREB-CD4KO mice. For further confirmation, we bred 2d2 transgenic mice (with MOG-specific TcR) with $CREB^{fl/fl}$ mice, and naïve $CD4^+$ T cells obtained from both $CREB^{fl/fl}$x2d2 and CREB-CD4KOx2d2 mice were adoptively transferred into Rag1 KO mice, followed by EAE induction. KO 2d2 T cells, though expressing normal levels of IFN-γ, failed to develop into Th17 cells and to induce EAE in Rag1 KO mice, whereas WT 2d2 T cells induced very severe EAE diseases (Fig. 2b).

Finally, the $CREB^{fl/fl}$ mice were crossed with the Il17f–Cre knock-in mice we recently constructed to generate CREB-IL-17FKO mice (Ichiyama et al., 2016) in which CREB was selectively ablated in IL-$17F^+$ Th17 cells. Consistent to the CREB-CD4KO mice, CREB deficiency in Th17 cells significantly ameliorated EAE disease, as well as IL-17 production in CNS-infiltrating T cells (Fig. 2c). These data collectively demonstrate a critical, Th17 cell-intrinsic role by CREB in directing Th17 cell differentiation and Th17 cell-mediated autoimmunity *in vivo*.

3.3. Th17 Cell-Specific Ablation of CREB Dampened Th17 Cell Differentiation In Vitro

To understand how CREB regulates Th17 cell response, naïve $CD4^+$ T cells were isolated from both WT control and CREB-CD4KO mice, and cultured under different Th-skewing conditions. Surprisingly, in contrast to the findings in *in vivo* disease model, CREB deficiency did not

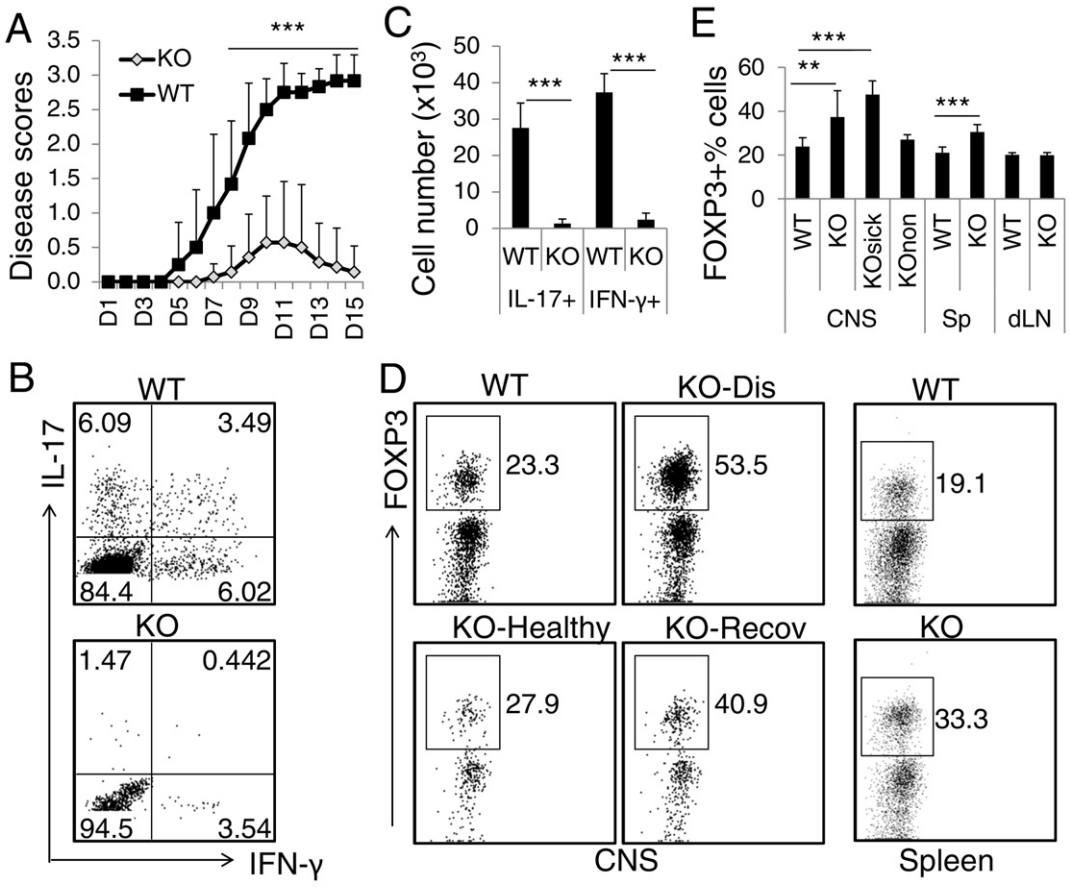

Fig. 1. CREB-deficient mice are resistant to EAE. $CREB^{fl/fl}$ (WT, n = 6) and CREB-CD4KO (KO, n = 7) mice were immunized twice with MOG35-55 for EAE induction. A, Mean clinical scores are shown *versus* days after second MOG immunization. B, Intracellular staining of IL-17 and IFN-γ in CNS of EAE mice (Gated on $CD4^+$ cells). C, Cellularity data in CNS. D, Intracellular staining of FOXP3 in CNS, spleen, and dLN (healthy = never developed EAE diseases; Dis = developed EAE diseases; Recov = recovered from EAE diseases). E, Statistics of FOXP3 staining results. The experiment was repeated twice with consistent results.

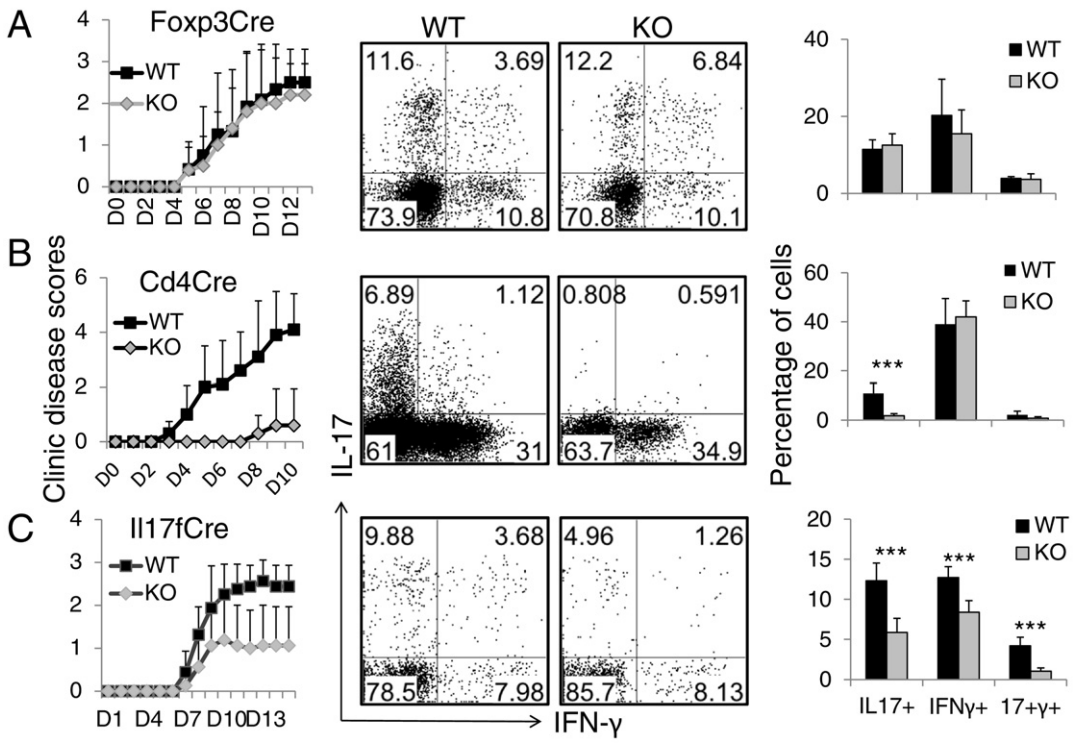

Fig. 2. CREB expression in Th17 but not Treg cells is required for EAE development. A, EAE was induced in $CREB^{fl/fl}$ (WT, n = 6) and CREB-FOXP3KO (KO, n = 5). B, EAE was induced in RAG1 KO mice transferred with WT 2d2 naïve T cells (n = 5) or CREB-deficient 2d2 naïve T cells (n = 5). C, EAE was induced in $CREB^{fl/fl}$ (WT, n = 8) and $CREB^{fl/fl}$xIL-17F-Cre mice (n = 8). Left column: EAE disease scores; Middle column: IL-17 and IFN-γ staining in CNS (Gated on $CD4^+$); Right column: statistic data of IL-17 and IFN-γ staining. All the experiments were repeated twice with consistent results.

affect Th17 cell differentiation, as well as Th1 and Th2 differentiation (Suppl. Fig. 2). Since deletion of CREB often resulted in compensatory increase of CREM or ATF1 (Hummler et al., 1994), and CREB is known to be a critical regulator in T cell development and activation (Barton et al., 1996; Rudolph et al., 1998). To avoid the possibility of developmental compensation, naïve $CD4^+$ T cells from WT control and CREB-IL17FKO mice were isolated and cultured under Th17 cell-polarizing conditions, in which the CREB gene will be deleted only after IL-17F expression is induced. Interestingly, Th17 cell-specific ablation of CREB reduced both IL-17 and IL-17F expression, with a more prominent effect on the IL-17/17F double positive population (Fig. 3a).

To further understand the role of CREB in regulating Th17 cell differentiation, RNAseq was performed with the WT and CREB-IL17FKO Th17 cells. In total, we identified 1704 genes regulated by CREB by >1.4 fold changes (Fig. 3b). As expected, the *Il17* mRNA level was significantly reduced whereas the expression of key Th17 cell-related transcription factors remained unchanged, including *Rorc* (Fig. 3c). Among the genes positively regulated by CREB (Fig. 3c), *Nr4a2* is known to be a downstream target of CREB and has been shown to play an important role in regulating Th17 cell differentiation (Doi et al., 2008), and *Cd24a* and *Klrc1* are reported to be susceptible genes for various autoimmune diseases in human, and regulate autoimmunity in mouse disease models (Fang et al., 2010; Ren et al., 2007). Retroviral overexpression of Cd24a or Nr4a2 could not fully restore IL-17 expression in CREB-CD4KO T cells to WT cell level, though enhanced Th17 cell differentiation more or less in *in vitro* cultures (Suppl. Fig. 3b), indicating CREB could regulate IL-17 expression *via* both a direct and indirect manner. Within the genes negatively regulated by CREB (Fig. 3c), *Foxp1* has been shown to inhibit IL-21 expression in follicular T cells (Wang et al., 2014), and may potentially inhibit Th17 cell differentiation. It was also noted that the Th1 and Th2 cell signature genes, including *Ifng*, *Il4* and *Il13, as well as Il2,* showed increased expression in the CREB-deficient cells, though mildly (Fig. 3c). Through comparing published Th17 cell microarray data (Lee et al., 2012), we identified a total of 176 CREB-regulated genes that showed more than two fold changes in Th17 *vs* Th0 cells (Lee et al., 2012), and clustering analysis suggests that CREB regulates these genes in a way highly similar to their up- or down- expression pattern in Th17 cells (Fig. 3b).

3.4. CREB Directly Binds to the Il17-17f Gene Locus and Functions in Synergy With RORγt

The CREB/ATF1 family transcription factors binds to a highly conserved consensus 8 bp DNA sequence TGACGTCA. Computational analysis revealed multiple CRE sites within the CNS2 and promoter regions of the *Il17* gene, either clustered together with the RORE sites, or near the TATA box region (Suppl. Fig. 3a), suggesting that CREB may cooperate with RORγt in controlling *Il17* transcription. To assess this possibility, we constructed the CNS2-17p mutant reporter constructs in which the CRE sites in the CNS2 region and the *Il17* promoter regions were mutated. Mutation of the CRE sites at both CNS2 and the *Il17* promoter regions abolished RORα- and RORγt-dependent reporter activity in both EL4 and Th17 cells (Fig. 4a/b), suggesting CREB was necessary for the function of ROR factors in directing IL-17 expression. In addition, ChIP assays demonstrated that pCREB bound to multiple sites in the *Il17-Il17f* gene locus in Th17 cells, including CNS2 and the *Il17* and *Il17f* gene promoters, but not irrelevant sequences (Fig. 4c). Lastly, CREB retroviral overexpression enhanced IL-17 and IL-17F expression in T cells cultured under Th17 cell polarizing condition, or RORγt-dependent IL-17/17F expression in neutral culture condition (Suppl. Fig. 4).

Taken together, these data demonstrated that CREB directed Th17 cell differentiation through direct binding to the *Il17-Il17f* gene locus and synergizing with RORs.

3.5. CREB Selectively Regulates the Survival of iTreg Differentiation

In addition to the *Il17-Il17f* locus, the CRE sites have also been identified in the promoter and the CNS2 enhancer regions of the *Foxp3* gene,

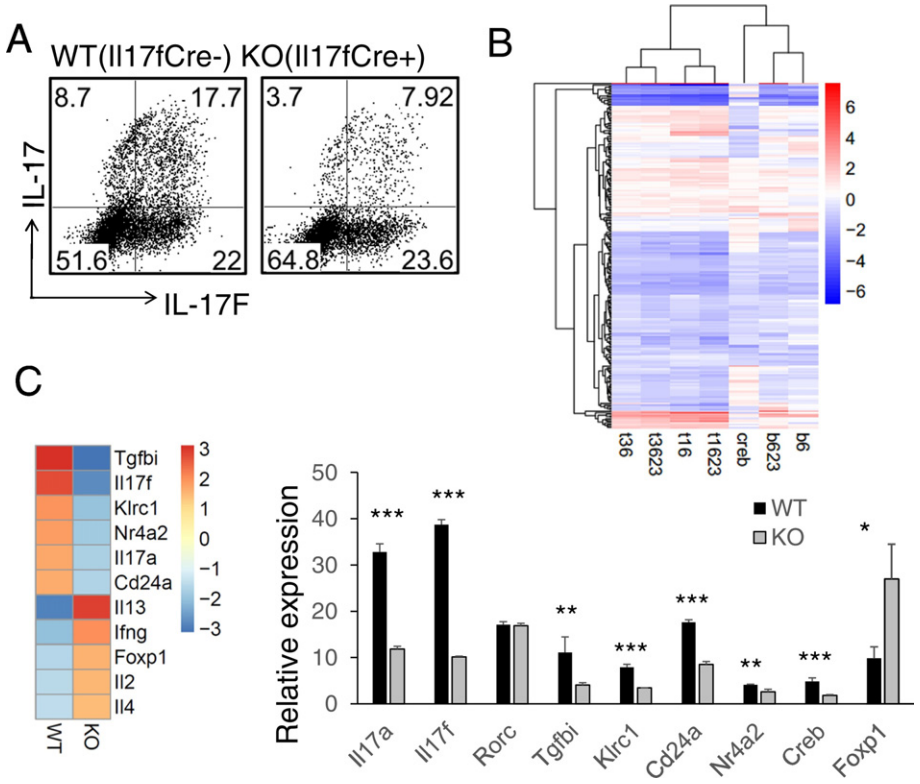

Fig. 3. CREB controls Th17 cell differentiation *in vitro*. A, Th17 cell differentiation was performed under optimal conditions (TGF-β + IL-6 + IL-β + IL-23) for 6 days, using WT and CREB-IL-17FKO naïve CD4 + T cells, and then restimulated and analyzed for IL-17 and IL-17F staining. The differentiation was performed for >5 times with consistent results. B, Clustering analysis of CREB regulated genes *vs* published microarray data of Th17 cells generated under different *in vitro* culture conditions (b6 = IL-1β + IL-6; b623 = IL-1β + IL-6 + IL-23; T16 = TGF-β + IL-6; T1623 = TGF-β + IL-6 + IL-23; T36 = TGF-β3 + IL-6; T3623 = TGF-β3 + IL-6 + IL-23). C, Left: heat map of selected CREB regulated genes; Right: validated realtime PCR results.

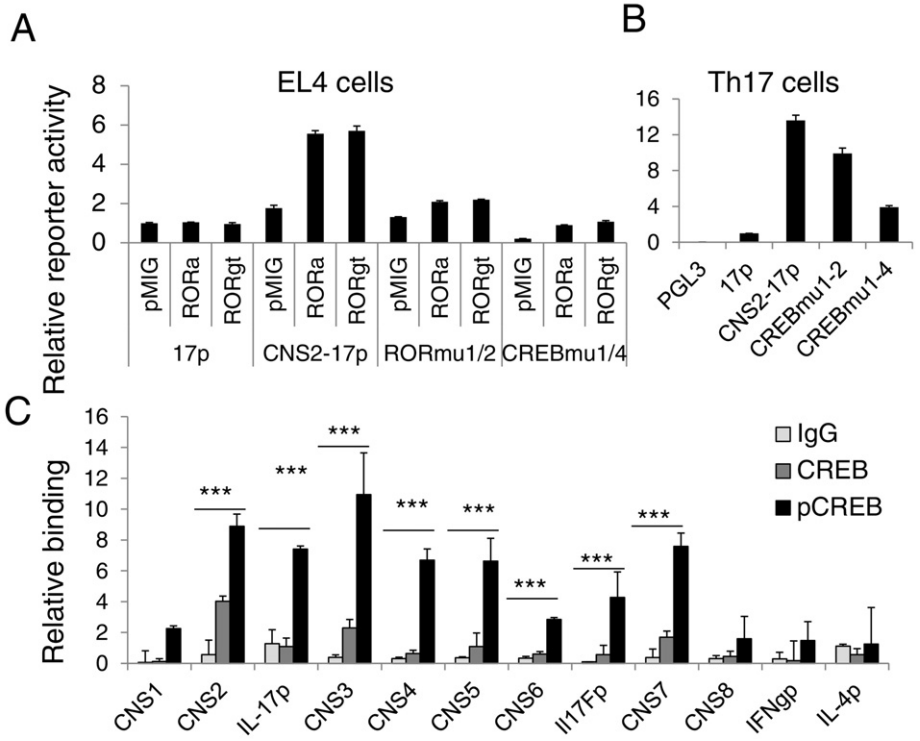

Fig. 4. CREB regulates ROR-dependent IL-17 expression. A, The luciferase activities of the PGL3, IL-17 promoter (17p)-PGL3, CNS2-17p-PGL3, CNS2$^{RORmu1/2}$-17p-PGL3 (RORmu1/2) (in which the two RORE sites in CNS2 were mutated), CNS2-17p-CREB$^{mu1/4}$ (CREBmu1/4) (in which the two CRE sites in both CNS2 and Il17p were mutated) reporter constructs were performed in EL4 cells in the absence or presence of RORα and RORγt. B, The luciferase activities of the PGL3, Il17p, CNS2-Il17p, CNS2-17p-CREB$^{mu1/2}$ (CREBmu1/2) (in which the two CRE sites in CNS2 were mutated), and CREBmu1/4 reporter constructs were performed in Th17 cells. C, ChIP assay was performed in Th17 cells with antibodies against CREB and pCREB, and realtime PCR was used to quantify the relative bindings to the *Il17-17f* gene locus. The assays were repeated twice with consistent results.

and it was proposed that the binding of CREB to the *Foxp3* CNS2 enhancer was essential for Foxp3 expression in Treg cells based on *in vitro* assays (Kim and Leonard, 2007; Ruan et al., 2009). Unexpectedly, CREB deficiency in $CD4^+$ T cells did not have any effect on FOXP3 expression and Treg cell homeostasis in both thymus and spleen, or in Peyer's patches in young mice (~6–8 weeks old) (data not shown). Furthermore, CREB deficiency did not affect the suppressive activity of nTreg or iTreg cells *in vitro* or in the adoptive T cell transfer colitis (Suppl. Fig. 5). These data suggest that CREB was not essential for *Foxp3* gene expression in both nTreg and iTreg cells, as well as for their suppressive functions.

In contrast to nTreg cells, CREB deficiency greatly enhanced the survival of iTreg cells induced by TGF-β as determined by FSC/SSC-scattering, and 7-AAD/annexin V staining, but did not significantly alter FOXP3 expression (Fig. 5). Addition of exogenous IL-2 was able to improve the survival of WT cells to a level comparable to CREB-deficient iTreg cells (Fig. 5), indicating IL-2 can overcome the pro-apoptotic effect of CREB on iTreg cells.

To analyze the underlying mechanism, we examined the expression of a number of cell cycle regulators in iTreg cells, including Cyclin A1, Cyclin D1, p15, p16, p19, p21 and p27, because of the presence of putative CRE sites near or within the promoter regions of these genes. Among all the genes studied, deficiency of CREB only affected p27 gene expression in iTreg cells (Suppl. Fig. 6a). To confirm this finding, western blot was performed using WT and CREB-deficient iTreg cells (Fig. 6a). Resting naïve $CD4^+$ T cells expressed high levels of p27, and the persistent expression of p27 in activated T cells was dependent on TGF-β (Suppl. Fig. 6b). TcR stimulation lowered the expression of p27 in both WT and CREB-deficient iTreg cells in the first 12 h. However, p27 expression was increased at 24–48 h only in WT but not CREB-deficient iTreg cells (Fig. 6a). This finding was also supported by the time course analysis of p27 mRNA levels in iTreg cells (Fig. 6b and Suppl. Fig. 6a). p27 is known as a potent inhibitor for G1 cell cycle progression through targeting cyclin-dependent kinases. Consistently, the majority of WT T cells was inhibited at G1/G0 phase, whereas CREB-deficient T cells showed increased number of cells entering the G2/M and S phases when cultured with TGF-β alone. In line with the previous results (Fig. 5), exogenous IL-2 overcomes the cell cycling inhibitory effect of TGF-β (Suppl. Fig. 6c).

Consistently, ChIP assays revealed that pCREB strongly bound to the p27 gene promoter in iTreg cells (Fig. 6d). In the reporter gene assay, transfecting CREB only mildly enhanced the p27 promoter activity in 293 T cells, possibly due to the presence of endogenous CREB. In contrast, mutation of the predicated CREB binding site at the −351/−347 bp significantly reduced the p27 promoter activity (Suppl. Fig. 6d). Considering the negative role of p27 in regulating cell cycle progression, these data together suggest that selective modulation of p27 contributed to the increased survival of CREB-deficient iTreg cells generated in the absence of exogenous IL-2.

3.6. CREB Deficiency Alters Intestinal Treg/Th17 Cell Ratio and Ameliorates T Cell-induced Colitis

The above studies clearly demonstrated crucial, opposing roles of CREB in regulating Th17 and iTreg cells, which prompted us to examine the effect of CREB deficiency on T cells in the intestine where abundant Th17 and iTreg cells are present. CREB did not affect Treg cell homeostasis in young (6–8 weeks old) mice (data not shown). However, in old mice (6–9 month old), CREB deficiency led to significantly increased $FOXP3^+$ Treg cell population selectively in the intestine (Peyer's patch and lamina propria) but not in other lymphoid organs or tissues, despite the ratio of Helios+ *vs* Helio- FOXP3 Treg cells remained unaltered (Suppl. Fig. 7). Consistently, CREB-deficiency significantly reduced the expression of IL-17 in lamina propria derived T cells (Suppl. Fig. 7).

To determine whether CREB plays a role in regulating intestinal immune response, an adoptive T cell transfer colitis model was employed by using naïve $CD4^+$ T cells isolated from the WT and CREB-CD4 KO mice. RAG1 KO mice receiving WT, but not CREB-deficient T cells, showed a significant weight loss (Fig. 7a). CREB-deficient T cells exhibited greatly reduced IL-17 but not IFN-γ expression in both spleen and mesenteric lymph nodes (mLNs) (Fig. 7b/c). In addition, CREB-deficiency led to significantly increased FOXP3+ cells in the spleen but not mLNs (Fig. 7b/c). Considering that CREB-deficiency Treg cells were equally efficient in suppressing T-cell mediated wasting diseases (Suppl. Fig. 5b), increased Treg cells in the absence of CREB thus may also contribute to alleviation of colitis diseases, in addition to its effect on Th17 cell differentiation.

4. Discussion

The reciprocal relationship of Th17 and iTreg cell development has been shown but the underlying molecular mechanisms are far beyond understanding. In this study, we demonstrated a positive role of CREB in regulating Th17 cell differentiation partially *via* direct binding to the *Il17-17f* gene locus, as well as a negative role in controlling the survival of iTreg cells *via* controlling TGF-β-dependent expression of cyclin-dependent kinase inhibitor p27. It has been proposed that binding of CREB to the demethylated *Foxp3* enhancer CNS2 is important for FOXP3 expression in Treg cells (Kim and Leonard, 2007; Ruan et al., 2009). However, our results showed that CREB was dispensable for

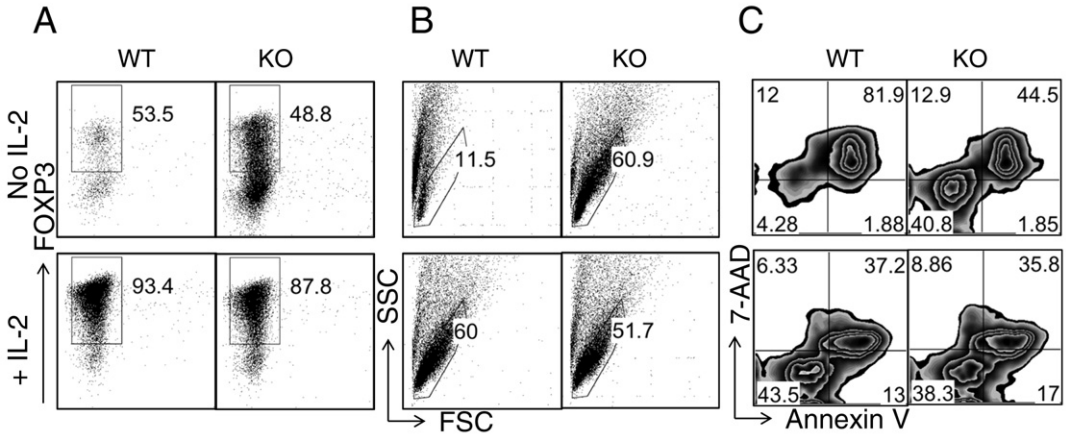

Fig. 5. CREB regulates the survival of iTreg cells. WT and CREB-deficient (KO) naïve $CD4^+$ T cells were induced to iTreg cells in the presence of TGF-β with or without exogenous IL-2. A, The expression of FOXP3 was determined by flow cytometry. B, The viable cells were determined by SSC-/FSC-scattering. C, Cell apoptosis was determined by 7-AAD and Annexin V staining. The experiment was repeated three times with consistent results.

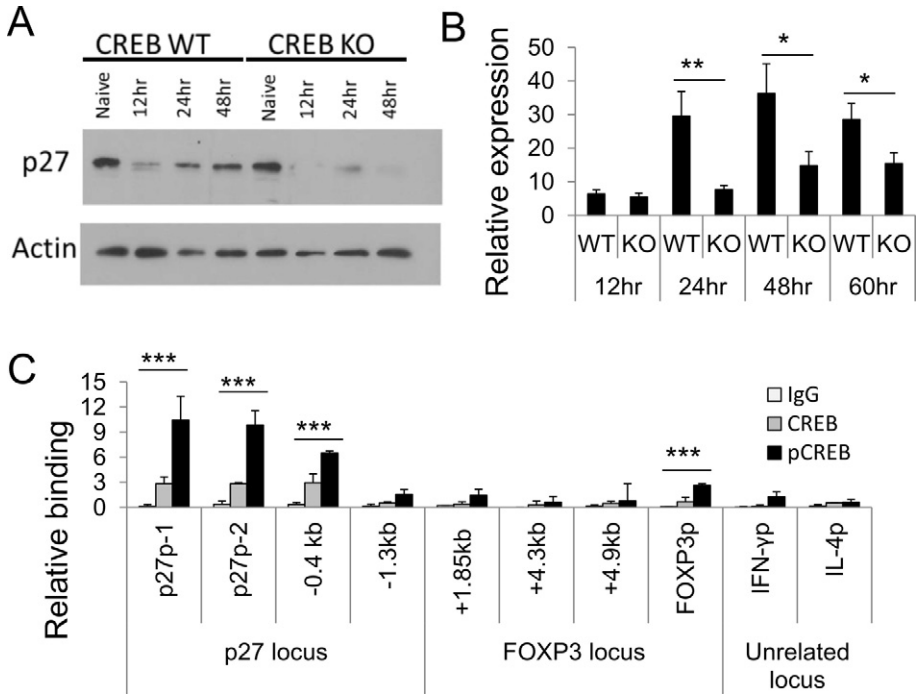

Fig. 6. CREB positively regulates p27 expression in iTreg cells. WT and CREB-CD4KO T naïve CD4$^+$ T cells were induced to iTreg cells using TGF-β in the absent of exogenous IL-2, and the p27 expression was determined by western blotting (A) and real-time RT-PCR (B) at various time points. ChIP assay was performed in iTreg cells with antibodies against CREB and pCREB, and real-time RT-PCR was used to quantify the relative bindings to the FOXP3 and p27 gene loci. All the assays were repeated twice with consistent results.

FOXP3 expression in both iTreg cells and nTreg cells, as well as for the suppressive activity of nTreg cells. Accordingly, CREB deficiency resulted in significantly reduced IL-17 expression and increased FOXP3$^+$ T cells *in vivo* at both steady phase (in the intestine of old mice) and inflammatory settings, and caused resistance to EAE and T-cell dependent colitis. These data together highlight a crucial role of CREB in controlling Th17/Treg cell ratios and Th17 cell-mediated autoimmune diseases.

The concept that CREB may regulate Th17 cell differentiation arose from our previous studies on CNS2 – a *cis*-regulatory enhancer element essential for controlling IL-17 expression in Th17 cells (Wang et al.,

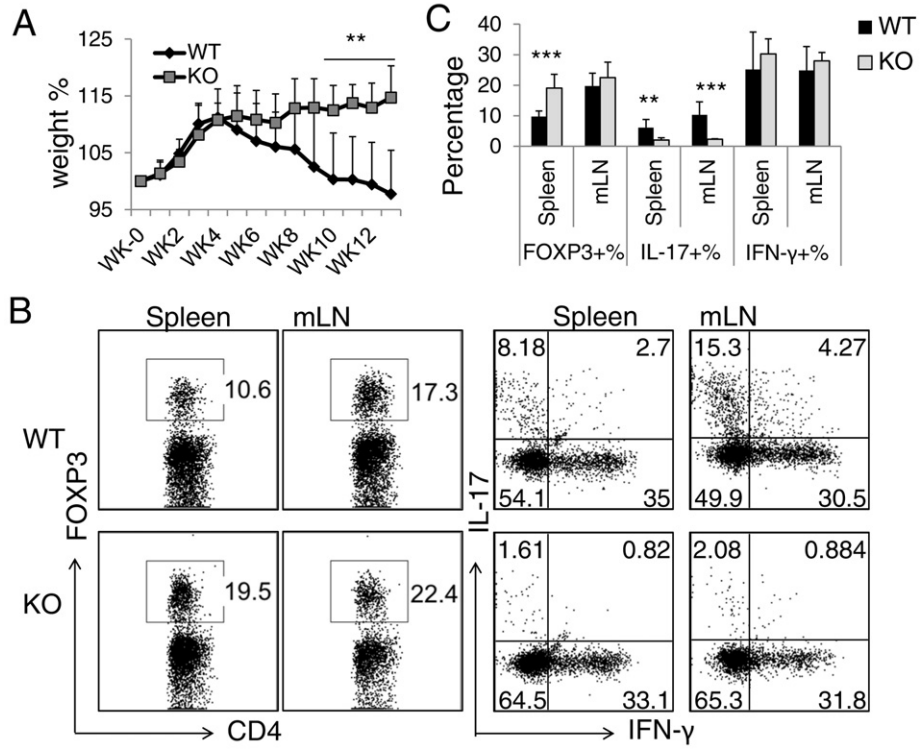

Fig. 7. CREB is required for T cell-dependent colitis. WT and CREB-CD4KO naïve CD4+ T cells were first transferred into RAG1 KO mice, and then monitored for developing colitis in recipient mice. A, Percentage of weight loss. B, Staining data of FOXP3, IL-17 and IFN-γ expression in the spleen and mesenteric lymph nodes. C. Statistic data of (B). The results were repeated twice with similar results.

2012). A careful scrutiny of the CNS2 sequence identified two CRE sites clustered together and entangled with the two RORE sites, and computational analysis identified multiple CRE sites within the *Il17-17f* gene locus, including two CRE sites nearby the TATA box of the *Il17* gene (Suppl. Fig. 3). Interestingly, early studies showed that HTLV1 infected T lymphocytes or Tax transduced Jurkat cells highly expressed *Il17* mRNA in which CREB was persistently activated (Dodon et al., 2004), and a high prevalence of autoimmune diseases, including rheumatoid arthritis, was often observed in HTLV1 infected patients (Goncalves et al., 2010; Shoeibi et al., 2013). These early findings promoted us to speculate a possible role of CREB in regulating IL-17 and Th17 cell differentiation.

To ascertain this, a reporter gene assay was performed with the CNS2-17p luciferase reporter construct. Mutating the four CRE sites within the CNS2 and the *Il17* promoter region completely abolished RORα/RORγt-dependent reporter activity (Fig. 4a), suggesting an indispensable role of these CRE sites in regulating ROR-dependent *Il17* transcription. Our ChIP and retroviral overexpression assays demonstrated that CREB bound to multiple regions within the *Il17-17f* locus, including CNS2 and the *Il17* promoter, and synergized with RORγt to enhance Th17 cell differentiation (Fig. 4c and Suppl. Fig. 4). Despite not affecting *in vitro* Th17 cell differentiation, CD4 + T cell-specific ablation of CREB drastically reduced *in vivo* Th17 cell differentiation and led to resistance to EAE. It is possible that the *in vitro* and *in vivo* Th17 cell differentiations are regulated through distinct mechanisms, or deletion of CREB in CD4 + T cells resulted in compensatory increase of functionally redundant genes in *in vitro* cultures. As a comparison, deletion of CREB by the *Il17f promoter*-mediated Cre expression dampened IL-17 expression *in vitro* and *in vivo*, and caused resistance to EAE (Figs. 2c and 3), suggesting that CREB plays a Th17 cell-instrinsic role in controlling Th17 cell differentiation and related autoimmune diseases. Considering that deletion of *Creb* begins only when IL-17F is expressed in the *Il17f-Cre* system, which is at least 24 h after Th17 cell differentiation (data not shown), and phosphorylation of CREB can last up to 5 days after TCR stimulation (Zhang et al., 2000), our results suggested a persistent role of CREB in regulating the differentiation and function of Th17 cells. Through genome-wide RNAseq analysis, a total of 1704 genes were found to be regulated by CREB by >1.4 fold, directly or indirectly, among which several genes have been suggested to play a role in regulating Th17 cell differentiation or related autoimmune diseases, including *Cd24a*, *Nr4a2*, *Klrc1* (Doi et al., 2008; Fang et al., 2010; Ren et al., 2007), as well as *Il17* and *Il17f*. Interestingly, among the CREB-regulated genes, 176 genes were already reported to be up- or down-regulated in Th17 cells when compared with activated T cells (Lee et al., 2012) (Fig. 3b), suggesting that CREB may regulate Th17 cell differentiation and shaping its pathogenic features in a broad manner, besides regulating *Il17* expression.

Early studies suggest a negative role of CREB in regulating IFN-γ expression and Th1 differentiation *via* direct binding to the *Ifnγ* promoter (Zhang et al., 1998). However, expression of ACREB inhibited Th1 and Th2 differentiation, largely due to defect in T cell activation/survival (Zhang et al., 2000). A recent study pointed a positive role of CREB in Th1 differentiation, yet by inducing the receptors for IL-12 and IFN-γ (Yao et al., 2013). Therefore, whether or not CREB is able to directly regulate IFN-γ production in Th1 cells remains unclear. In our studies, CREB-deficiency in $CD4^+$ T cells did not affect Th1 differentiation *in vitro* (Suppl. Fig. 2), and in the adoptive T cell transfer EAE and colitis models (Fig. 2b and Fig. 7b/c). These results highlight the importance of CREB in regulating Th17 cell differentiation, but not Th1 cell differentiation.

CREB was suggested to positively regulate FOXP3 expression in Treg cells through interacting with the *Foxp3* enhancer sequence CNS2 (Kim and Leonard, 2007; Ruan et al., 2009). However, our results showed that CREB-deficiency did not result in notable down-regulation of FOXP3 in Treg cells generated *in vitro* and *in vivo* (Fig. 1d/e, Fig. 5, Fig. 7b/c), as well as any apparent inflammatory symptoms in mice up to 6–8 months old (data not shown). To the opposite, deletion of CREB significantly improved the survival of iTreg cells generated *in vitro* in the absence of exogenous IL-2 (Fig. 5), and led to increased $FOXP3^+$ Treg cells selectively in the intestine of old mice (~6–8 months old) (Suppl. Fig. 7), where abundant iTreg cells are present. In the EAE model, increase of FOXP3 + % Treg cells in the CNS or spleen of CREB-deficient mice was also observed (Fig. 1d/e), though their origin remained unclear at this moment. Our findings thus argue against a direct role of CREB in regulating FOXP3 expression through targeting CNS2.

iTreg cells play an important role in controlling mucous immune homeostasis in the gastrointestinal tract and lung (Josefowicz et al., 2012). In the adoptive T cell transfer colitis model, we found that CREB-deficient naïve CD4 + T cells failed to express IL-17 after transfer into RAG1 mice, but showed increased FOXP3 expression (Fig. 7b/c). It is known that IL-17 deficiency in T cells had no effect or even caused more severe colitis in RAG KO mice (Leppkes et al., 2009; O'Connor et al., 2009), and iTreg cells were equally efficient in suppressing T-cell mediated colitis as nTreg cells (Haribhai et al., 2009). These data together suggest an important role of CREB in regulating the function of iTreg cells.

ChIP assay showed that CREB did not bind the *Foxp3* CNS2 enhancer in iTreg cells as previously reported, but weakly interacted with the promoter region. A possible explanation is that the binding of CREB to the *Foxp3* gene locus including promoter and enhancer regions is highly dynamic and transient (Ruan et al., 2009), and differential experimental settings could result in different results. As a contrast, CREB strongly interacted with the promoter of p27 (Fig. 6c) – a cyclin-dependent kinase inhibitor that negatively regulates cell cycle progression (Wells and Morawski, 2014). Mutation of the putative CRE sites in the p27 promoter region significantly decreased the p27 gene promoter activity (Suppl. Fig. 6d), and CREB-deficiency reduced p27 expression in iTreg cells at both protein and mRNA level (Fig. 6a/b and Suppl. Fig. 6a/b). In contrast, IL-2 was shown to inhibit p27 expression in T cells (Nourse et al., 1994), and thus was able to overcome the growth inhibitory effect of CREB on iTreg cells (Fig. 5). The regulation of p27 may be responsible for the observed increased survival of iTreg cells generated *in vitro* (Fig. 5), or increased FOXP3 +% Treg cells under different experimental settings (Fig. 1d and Fig. 7b/Suppl. Fig. 7), when CREB was ablated.

CREB can be rapidly activated in thymocytes and T cells by TcR stimulation, and its phosphorylation was shown to depend on MSK1/2, but not PKA (Rudolph et al., 1998; Yoshizawa et al., 2009). In addition, cytokines like IL-2 and TGF-β were also able to induce CREB phosphorylation (Feuerstein et al., 1995). However, our inhibitory studies suggest that the early activation of CREB in naïve $CD4^+$ T cells was largely dependent on CD3-PKC-Θ signaling pathway, and independent of IL-2, IL-6, TGF-β and CD28, as well as the serine and threonine kinases MSK1/2, ERK, p38MAPK, CamKIV and PI3K (Suppl. Fig. 1). The major difference underlying these studies is that the previous studies often used total T cells, T cell lines or activated T cells, whereas in our studies we used purified naïve CD4 + T cells and stimulated them with plate-bound anti-CD3 and anti-CD28. Interestingly, a recent study shows that PKC-Θ-deficiency selectively impaired Th17 cell differentiation, but not Th1 and Th2 cells (Kwon et al., 2012), and PKC-Θ has recently emerged as a promising drug target in treatment of Th17 cell-related diseases, including psoriasis and graft-*versus*-host-disease (GVHD)(Altman and Kong, 2012). Our results show that the role of PKC-Θ in regulating Th17 cells is at least partly mediated through CREB. Considering the importance of CREB in regulating Th17 cells and related autoimmunity, further investigation of how CREB is activated by PKC-Θ in T cells may reveal drug targets for treatment of Th17 cell-related immune diseases.

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Conflicts of Interest

The authors declare no competing financial or other interests.

Author Contributions

X.W. and C.D. designed the project and analyzed the data. X.W., L.N., D.C., Y.J., B.S.K., A.W., X.L., B.Z. and X. Y. performed the experiments. H. L. analyzed the RNAseq data. X.W. and C.D. prepared the manuscript.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2017.10.010.

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