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Regulatory T cells facilitate the nuclear accumulation of inducible cAMP early repressor (ICER) and suppress nuclear factor of activated T cell c1 (NFATc1)

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Regulatory T Cells Induce the Nuclear Accumulation of ICER/CREM and Suppress the Induction of NFATc1 in Conventional CD4$^+$ T Cells

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Running Title: ICER/CREM in nTreg function

Abstract

ICER (*i*nducible *c*AMP *e*arly *r*epressor) is a transcriptional repressor, which, due to alternate promoter usage, is generated from the 3' region of the *Crem* (*cAMP response modulator*) gene. Its expression and nuclear occurrence is elevated by high cAMP levels in naturally occurring regulatory T (nTreg) cells. By using two mouse models we demonstrate that nTreg cells control the cellular localization of ICER/CREM and, thereby, inhibit the IL-2 synthesis in conventional $CD4^+$ T cells. Depletion of nTreg cells in DEREG ("*DE*pletion of *REG*ulatory T cell") mice resulted in cytosolic localization of ICER/CREM and increased IL-2 synthesis. However, direct contacts between nTregs and conventional $CD4^+$ T cells led to nuclear accumulation of ICER/CREM and suppression of IL-2 synthesis upon administration of CD28 superagonistic (CD28SA) antibody. In a similar way, nTreg cells communicated with B cells and induced the cAMP-driven nuclear localization of ICER/CREM. High levels of ICER suppressed the induction of *N*uclear *f*actor of *a*ctivated *T* *c*ell *1* (*Nfatc1)* gene in T cells whose inducible *Nfatc1* P1 promoter bears two highly conserved cAMP-responsive elements (CREs) to which ICER/CREM can bind. These findings suggest that nTreg cells suppress T cell responses by the cAMP-dependent nuclear accumulation of ICER/CREM and inhibition of NFATc1 and IL-2 induction.

Introduction

\body nTreg cells are of crucial importance for preventing autoimmunity (1). It is widely accepted that nTregs exert their regulatory capacity via cell contact-dependent and cytokine-independent mechanisms. The inhibition of *Il2* gene expression in effector $CD4^+$ T cells is a characteristic feature of nTreg-mediated suppression (2). nTreg cells harbor high levels of cAMP (3), and the contact-dependent transfer of cAMP from nTregs to effector $CD4^+$ T cells was shown to contribute to nTreg-mediated suppression of effector $CD4^+$ T cells (4, 5). Inhibition of cAMP degradation by the phosphodiesterase (PDE)-4 inhibitor rolipram enhanced the nTreg-mediated suppression of effector $CD4^+$ T cells both *in vitro* and *in vivo* (6). Unlike conventional $CD4^+$ T cells, which express relatively low ICER/CREM levels, nTregs show a marked increase in ICER/CREM mRNA and protein levels due to the enhancing effects of Foxp3 (7). ICER binds specifically to multiple NFAT/AP-1 sites within the *Il2* promoter (8) which correlates with a strong decrease in the number of IL-2 expressing effector $CD4^+$ T cells (7). It was proposed that such composite NFAT/AP-1 binding motifs generate NFAT/Foxp3 inhibitor complexes, which suppress the *Il2* gene expression in nTreg cells (9).

In vitro, NFAT and ICER form inhibitory ternary complexes on several composite NFAT/AP-1 DNA binding sites which, in addition to suppression of IL-2 transcription, are essential for the inhibition of other cytokines, such as TNF-α, IL-4, IL-13, and GM-CSF (10). Therefore, NFAT/ICER complexes seem to be instrumental for the transcriptional attenuation of numerous NFAT-driven cytokine promoters in conventional $CD4^+$ T cells. A critical role of NFAT factors for inhibitory complex formation is further strengthened by observations indicating that combined NFATc2/c3 deficiency rendered conventional $CD4^+$ T cells unresponsive to suppression, although normal nTreg development was detected in those mice (11). Moreover, targeting ICER/CREM in RNAi and antisense RNA approaches antagonized the nTreg cell-mediated suppression and/or inhibition of IL-2 production in conventional $CD4^+$ T cells rendering these effector T cells refractory to suppression (7, 12).

Activation of effector $CD4^+$ T cells results in a strong transcriptional induction and nuclear translocation of NFATc1 (13). By contrast, nTreg cells are

unable to induce NFATc1 at the transcriptional level (14). They express relatively low levels of cytoplasmic NFATc1 and do not translocate efficiently NFATc1 to the nucleus upon CD3/CD28 stimulation (15). This property of nTreg cells is associated with a reduced calcium flux, diminished calcineurin activation, and increased activity of the glycogen synthase kinase-3β, a negatively acting NFAT protein kinase. These observations suggest that the signals leading to the generation of suppressive transcription complexes in nTreg cells differ markedly from those critical for the generation of NFAT/AP-1 and other NFAT complexes which activate cytokine promoters in conventional $CD4^+$ T cells.

By using monoclonal antibodies (mAbs) raised against CD28, including a CD28 superagonistic (CD28SA) antibody (16), we investigated the relationship between the activation of nTregs and ICER-mediated suppression in conventional $CD4^+$ T cells. Depletion of nTreg cells from the T cell compartment of DEREG ("*DE*pletion of *REG*ulatory T Cell") mice expressing the diphtheria toxin receptor in $Foxp3^+$ T cells prior to CD28SA stimulation led to cytosolic localization of ICER/CREM in CD28SA-stimulated conventional $CD4^+$ T cells. This correlated with an increase in IL-2 expression. Interaction of nTreg cells with conventional $CD4^+$ T cells *in vivo* resulted in the nuclear localization of ICER/CREM and cessation of IL-2 synthesis. Moreover, contacts of nTreg cells with B cells led to an increase in nuclear localization of ICER/CREM, in a similar fashion as detected for conventional $CD4^+$ T cells. One mechanism of ICER/CREM-mediated suppression of conventional $CD4^+$ T cells is the binding of ICER/CREM to the inducible *Nfatc1* P1 promoter and its suppression in response to increased cAMP levels. This leads to low NFATc1 concentrations, a block in cellular proliferation and IL-2 synthesis and, thereby, to the suppression of $CD4^+$ T cells.

Results

Stimulation of conventional CD4⁺ T cells with CD3/CD28 mAbs leads to cytosolic localization of ICER/CREM

Immunohistochemical stainings of freshly isolated conventional $CD4^+$ T cells and nTreg cells with Abs specific for ICER/CREM or NFATc1 revealed a predominant nuclear occurrence of ICER/CREM and a cytosolic localization of NFATc1 in both cell types (Fig. 1A and B, see “fresh”). A similar cellular distribution was observed in paraffin-embedded sections of murine spleens: almost all follicular lymphocytes showed ICER/CREM in nucleus and NFATc1 in cytoplasm (Fig. S1A). However, upon priming and/or re-stimulation with CD3/CD28 mAbs we detected ICER/CREM in the cytoplasm of conventional $CD4^+$ T cells whereas in nTreg cells ICER/CREM remained in the nucleus (Figs. 1A and B, see “primed”; quantified in Fig. S1B-G). This cytosolic localization of ICER/CREM in conventional $CD4^+$ T cells was partially impaired in T cells from CD28-deficient mice (Fig. S2) suggesting a role for co-stimulatory CD28 signals in the cytosolic localization of ICER/CREM. In the absence of such signals, upon stimulation with phorbol ester plus ionomycin, ectopically expressed ICER could not be detected in cytosol of human embryonal kidney (HEK) 293 T cells (Fig. S3A). Similar to the function of Foxp3, the nuclear localization of ICER led to inhibition of *Il2* promoter linked to a luciferase reporter gene (Fig.S3B). When conventional $CD4^+$ T cells re-stimulated by CD3/CD28 mAbs were also treated with forskolin (which elevates intracellular levels of cAMP through activation of adenylyl cyclase; Fig. 1C) ICER/CREM was detected in the nucleus. A similar effect was observed upon blocking of cAMP degradation by IBMX, an inhibitor of phosphodiesterases (PDEs) (Fig. 1A). In addition, direct contacts between nTreg and conventional $CD4^+$ T cells in standard Treg assays led to increased presence of ICER/CREM in the nucleus of conventional $CD4^+$ T cells (Fig. S4).

The nuclear localization of ICER/CREM in conventional $CD4^+$ T cells corresponded with a marked suppression of endogenous IL-2 mRNA synthesis upon forskolin (or IBMX) treatment of conventional $CD4^+$ T cells re-stimulated with CD3/CD28 mAbs (Fig. 1D). Under both conditions, NFATc1 was found in nuclei of conventional $CD4^+$ T cells (Fig. 1A) whereas in nTreg cells, NFATc1 was predominantly in cytosol and remained unaffected by any of the treatments mentioned above (Fig. 1B).

Direct contacts with nTreg cells induce cAMP-dependent nuclear localization of ICER/CREM in conventional CD4$^+$T cells in vivo

nTreg cells exhibit inherently high levels of intracellular cAMP (Fig. 1C) and are capable to confer cAMP into conventional CD4$^+$ T cells in a contact-dependent fashion (3). Therefore, we asked whether direct contacts between nTreg cells and conventional CD4$^+$ T cells would induce the nuclear localization of ICER/CREM upon activation *in vivo*. To test this, we used the vital dye calcein that spreads from donor to recipient cells via gap junctions (17). RAG-2-deficient, CD90.1-positive OT-II mice that bear CD4$^+$ T cells expressing a transgenic (tg) $OVA_{323-339}$ -specific T cell receptor (TCR) but lacking nTreg cells, were immunized with $OVA_{323-339}$ peptide in complete Freund's adjuvant by injection into the left hind footpad. Six hours later, calcein-loaded congenic $OVA_{323-339}$-specific nTreg cells expressing CD90.2 were adoptively transferred. After 24 h, CD4$^+$ T cells from draining and non-draining lymph nodes were isolated and sorted by FACS (Fig. 2). A substantial part of the CD4$^+$, CD90.1-positive T cells from draining lymph nodes of immunized mice exhibited high calcein staining (Fig. 2B). In contrast, CD4$^+$, CD90.1-positive T cells from the non-draining lymph nodes prepared from the same mouse did not exhibit any or low calcein staining (Fig. 2A). After sorting, intracellular cAMP concentration was assessed in calcein-low and calcein-high cells using a cAMP-specific ELISA (Fig. 2C). Total RNA was extracted for determination of IL-2 mRNA levels (Fig. 2D), and the cells were stained for the intracellular localization of ICER/CREM and NFATc1 (Fig. 2E).

By confocal microscopy a predominant nuclear staining of ICER/CREM was observed in calcein-high conventional CD4$^+$ T cells upon interaction with calcein-loaded nTreg cells, whereas ICER/CREM was in cytosol of T cells that did not contact nTreg cells (calcein-low cells, Fig. 2E and Fig. S5). Moreover, NFATc1 was prevalently nuclear in draining lymph nodes and co-localized with ICER/CREM in calcein-high cells (Fig. 2E). The calcein-high conventional CD4$^+$ T cells showed a significant increase in intracellular cAMP concentration (Fig.2C), which correlated with the transcriptional attenuation of IL-2 expression (Fig. 2D). These findings indicate a close correlation between nTreg cell-mediated increase in intracellular cAMP levels, the nuclear localization of ICER/CREM, and the suppression of IL-2 synthesis in conventional CD4$^+$ T cells *in vivo*.

Ablation of nTreg cells drives ICER/CREM into the cytosol of conventional CD4⁺ T cells upon CD28SA administration

To substantiate these findings, we investigated the cellular distribution of ICER/CREM in lymph nodes from DEREG mice. Those mice express the human diphteria toxin (DT) receptor (as well as green fluorescent protein [GFP]) under the control of the *Foxp3* locus and, therefore, allow the specific ablation of nTreg cells by DT (18). DT-treated DEREG mice retained only 0.3 percent of nTregs compared to control mice, which bear approximately eight percent of nTreg cells in their $CD4^+$ T cell compartment (Fig. 3A; see also Fig. S6F). CD28SA treatment led to a moderate expansion and activation of nTreg cells expressing GFP ($Foxp3^+CD25^+$ or $CTLA4^+$) (15), and, in particular, to up-regulation of CD69 in conventional $CD4^+$ T cells (Fig. 3A). Conventional $CD4^+$ T cells isolated from lymph nodes of CD28SA-treated DEREG mice revealed the cytosolic localization of ICER/CREM in the absence of nTreg cells (-nTreg) but nuclear localization of ICER/CREM in their presence (+nTreg) (Fig. 3B and D). ICER/CREM was mostly cytosolic in CD28SA-treated DEREG mice pre-treated with DT (quantified in Fig. S6A-C). Serum samples obtained from those nTreg-depleted and CD28SA-treated mice after 1.5, 3, and 4 h revealed a significant increase of circulating IL-2 and TNFα levels (Fig. 3C). These data indicate a strong correlation between the cytosolic localization of ICER/CREM in $CD4^+$ conventional T cells and cytokine release upon activation and depletion of nTreg cells in DT+CD28SA-treated DEREG mice, implicating ICER/CREM in nTreg cell-mediated suppression.

nTreg – B cell interactions lead to the nuclear presence of ICER/CREM in B cells

In the spleen of untreated mice, $Foxp3^+$ nTreg cells are in close proximity of B cells (Fig. S7A). When activated B cells were co-incubated with calcein-loaded nTreg cells *in vitro*, they exhibited increased intracellular levels of calcein (Fig. S7B-I). This indicates that nTreg cells could confer cAMP into B cells, in a similar fashion as into conventional $CD4^+$ T cells. B cells also induce ICER mRNA in a cAMP-dependent fashion upon forskolin treatment (Fig. S7C). When we examined the subcellular localization of ICER/CREM upon B cell receptor (BCR)

triggering by anti-IgM mAb (Fig. S7D), ICER/CREM was predominantly cytosolic. As in conventional $CD4^+$ T cells, forskolin or IBMX co-treatment of anti-IgM-activated B cells directed ICER/CREM to the nucleus (Fig. S7E-G) suggesting that contact-dependent transfer of cAMP from nTreg cells to anti-IgM and/or LPS-activated B cells lead to nuclear localization of ICER/CREM (Fig. S7H and I) which could modulate B cell activity. To assess this notion further, in standard Treg assay we substituted T cell-depleted splenocytes with purified anti-IgM-activated B cells whose B7 expression was monitored by FACS (Fig. S8). Under these conditions, the proliferation of purified B cells was impaired, and the expression of CD80 (B7.1), but not CD86 (B7.2), was reduced (Fig. S8). These data suggest that, in analogy to conventional $CD4^+$ T cells, nTreg cells communicate with B cells, which leads to nuclear localization of ICER/CREM.

ICER/CREM binds to the Nfatc1 P1 promoter and represses TCR-mediated NFATc1/αA induction

The induction of the *Nfatc1* gene in effector lymphocytes is mediated by promoter P1 whose activity is strongly enhanced by immunoreceptor signals. P1 harbors two highly conserved cAMP-responsive elements, which are denoted as CRE-145 and CRE-640 (or proximal and distal CRE) (13) (Fig. 4A). In conventional $CD4^+$ T cells, stimulated by CD3/CD28 mAbs, forskolin treatment or co-cultivation with nTreg cells attenuated the induction of the P1 promoter, which directs the generation of NFATc1/α isoforms, in particular the short isoform NFATc1/αA (Fig. 4B). Under the same conditions, no effect was detected on the constitutively active P2 promoter. When we examined ICER binding to the CRE-145 and CRE-640 motifs in electrophoretic mobility shift assays (EMSAs) the transcriptional attenuation of NFATc1/αA correlated with the binding of recombinant ICER protein to the CRE-145 and, to a lower degree, to the CRE-640 (Fig. 4C). As previously reported for the CD28 responsive element (CD28RE) of the *Il2* promoter (8), the CRE-145 motif of P1 is associated with an NFAT binding site. Both the CRE-145 and CD28RE motif can form inhibitory NFAT/ICER complexes *in vitro* (Fig. 4C and S9A). Moreover, NFATc1/αA and ICER interacted via protein-protein interactions (Fig. S9B). After forskolin and CD3/CD28 re-stimulation of conventional $CD4^+$ T cells (prepared in the same way as cells used

for confocal imaging in Fig. 1A) we detected a marked increase in ICER/CREM binding to the P1 promoter in chromatin immunoprecipitation (ChIP) assays (see Fig. 4D, lane 4). Only a weak ICER/CREM binding was detected after CD3/CD28 re-stimulation alone (Fig. 4D, lane 3). Co-transfections of a luciferase reporter gene directed by P1 with increasing concentrations of a vector expressing ICER led to the suppression of P1 induction whereas a leucine-zipper mutant of ICER (ICER-LZ) failed to suppress P1 (Fig. 4E). This shows that dimerization and binding of ICER to DNA play a critical role for the transcriptional attenuation of the P1 promoter. Taken together, these data indicate that nTreg cells direct the nuclear accumulation of ICER/CREM via elevating cAMP levels and, thereby, attenuate the TCR-mediated induction of NFATc1 in $CD4^+$ effector T cells.

Discussion

In this study we showed that in conventional $CD4^+$ T cells an increase in intracellular cAMP levels leads to nuclear localization of ICER/CREM and the repression of NFATc1 and IL-2 induction. This is due to direct cell-to-cell contacts between nTreg cells and $CD4^+$ T cells *in vivo*. nTreg cells harbor constitutively high levels of cAMP and are able to confer cAMP through gap junctions into conventional $CD4^+$ T cells (4). The increase in cellular cAMP levels enhances the expression and nuclear localization of ICER (22). This results in the binding of ICER to the inducible P1 promoter of the *Nfatc1* gene and the repression of NFATc1/αA induction. ICER binds also to the *Il2* and other NFAT-driven cytokine promoters and interferes with their activity upon T cell activation.

In antigen-driven immune responses, effector $CD4^+$ T cells utilize IL-2 as an autocrine growth factor and provide IL-2 to nTreg cells in a paracrine fashion, thereby increasing the number and suppressive activity of nTregs (19). One explanation for the absence of systemic cytokine release in CD28SA-treated mice is the suppression of IL-2 production in $CD4^+$ effector T cells by ICER/CREM. We tested this view by depleting nTregs from DEREG mice. The data from these experiments confirmed a direct role for cAMP in nTreg-mediated maintenance of ICER/CREM in the nuclei of $CD4^+$ T cells. They correspond with the findings of adoptive transfer experiments using calcein-loaded nTreg cells. In draining lymph nodes of immunized mice that received calcein-loaded nTregs a substantial part

of $CD4^+$ T cells showed a high calcein staining and increased cAMP levels upon nTreg transfer. Confocal microscopy revealed a nuclear staining of ICER/CREM in calcein-high cells (Fig. 2). This is in striking contrast to cytosolic localization of ICER/CREM in calcein-low cells indicating a critical role for enhanced cAMP levels in the expression, localization and function of ICER during nTreg-mediated suppression.

How nTreg cells dampen $CD4^+$ effector T cell responses is still a matter of dispute (1). In nTreg cells, the activity of the *Nfatc1* P1 promoter and, therefore, the induction of inducible short NFATc1/αA isoform is significantly reduced (14). We showed that nTreg cells could suppress $CD4^+$ effector T cells by conferring cAMP via intercellular gap junctions (3), which appears to be instrumental for the induction and nuclear accumulation of ICER. By the binding of ICER alone or together with NFATs to the inducible P1 promoter, ICER interferes with the strong induction of NFATc1/αA, a molecular marker of activation of $CD4^+$ effector T cells and splenic B cells (13). Increased nuclear levels of ICER have also a major impact on the transcriptional silencing of the *Il2* promoter and other NFAT-driven promoters leading to impaired $CD4^+$ T cell proliferation and function (20, 21). The experimental findings presented here provide a novel view on molecular mechanism(s) how nTreg cells control the activity of $CD4^+$ effector T cells (and splenic B cells) in a cAMP-dependent fashion.

Materials and Methods

Mice. BALB/c, C57BL/6 ($CD28^{-/-}$, OT-II mice (DO11.10 CD90.1) and $Rag2^{-/-}$ mice were obtained from Charles River. DEREG mice were published previously (18).

Preparation of T and B cell subsets. $CD4^+$ T cells were isolated using the Dynal mouse T cell negative isolation protocol. $CD25^+$ or $CD25^-CD4^+$ and $CD19^+$ B cells were enriched using the MACS system.

FACS staining. Staining of surface molecules was performed on ice using direct flourochrome-conjugated mAbs or indirectly by biotin-labeled mAbs and APC-conjugated steptavidin. Samples were acquired on the FACS Calibur or sorted on the FACS Aria flow cytometer and analyzed using FlowJo software.

nTreg cell depletion in DEREG mice and treatment with CD28SA. DEREG mice were treated daily with intraperitoneal injections of 1 μg diphteria toxin (DT) for five consecutive days before administration of 100 μg of CD28SA mAb D665.

Cytokine measurements using CBA technology. For detecting cytokines in the serum of DEREG mice, the Cytometric Bead Array (CBA) technology from BD Pharmingen (San Diego, CA) was used.

Cell culture and stimulations. HEK 293T cells were cultured and stimulated as described before (22). Priming and re-stimulation of $CD25^-CD4^+$ T cells as well as $CD25^+$ nTreg cells was performed using plate-bound CD3/CD28 mAbs. After 72 h the cells were harvested, cultured for additional 96 h (priming) and re-stimulated.

Transfection and luciferase assays. HEK 293T cells were transfected with DNA vectors encoding ICER, NFATc1, or FoxP3 as indicated. Luciferase activity was measured as described earlier (14). For immunofluorescence, HEK 293T cells were transfected with constructs encoding ICER, NFATc1, and FoxP3 using Superfect according to Qiagen`s protocol.

Quantitative RT-PCR. Total RNA was extracted using Ambion`s RNAqueous Kit, and first strand cDNA was synthesized with Superscript II reverse transcriptase and oligo (dT)12-18 primers. RNA levels were quantified in real-time PCR assays using the ABI/PRISM 7700 system with primers described in SI Materials and Methods.

cAMP ELISA. To assess cellular cAMP levels, 1.5 x 10^6 T cells were snap-frozen in liquid nitrogen before lysis in 0.1 N HCl. The cAMP-specific EIA Direct Cyclic

AMP Enzyme Immunoassay was subsequently performed according to manufacturer's instructions.

Immunoprecipitations and Western blot analysis. Immunoprecipitations of transfected HEK 293T cells were performed as described previously (14). For immunodetection primary Abs were used with peroxidase-coupled secondary antibodies developed with enhanced chemiluminiscence system.

Chromatin Immunoprecipitation (ChIP). ChIP analysis of expanded conventional $CD4^+$ T cells was carried out according to manufacturer's instructions (Cell Signaling, Cambridge, MA).

Electromobility Shift Assay (EMSA). Binding reactions were performed in a 15μl reaction as described previously (8) using recombinant proteins. Samples were incubated with ^{32}P-labeled oligonucleotides (see SI Materials and Methods) followed by electrophoresis on a native polyacrylamide gel.

Immunofluorescence of single cells or tissue sections. Stainings of ICER/CREM, NFAT, and Foxp3 in primary T cells collected on slides or in HEK 293T cells transfected transiently were performed as described earlier (14) and in SI Materials and methods. Formalin-fixed, paraffin-embedded tissue samples from spleens of DEREG mice were prepared as described in Ref. (23).

Calcein^{AM} staining and *in vivo* calcein transfer. nTreg cells were incubated with 1 mM Calcein^{AM} and stimulated in co-culture with $CD19^+$ B cells or $CD4^+$ T cells for 4 or 20 h, respectively. Calcein transfer was analyzed by FACS. For *in vivo* calcein transfer DO11.10, Rag2$^{-/-}$, CD90.2-positive (or CD90.1-positive, respectively) mice were immunized by subcutaneous injection of 50 μg $OVA_{323-339}$. After 6 h, 1 x 10^7 pre-activated calcein-loaded nTregs from DO11.10, CD90.1-positive (or CD90.2 positive, respectively) mice were injected i.v. into the same mice. 24 h after immunization, lymph node cells were stained for the expression of CD4 and CD90.1. Calcein-high and calcein-low $CD4^+$ T cells were isolated by using a FACSAria cell sorter.

Proliferation assays. Along with APCs (or purified B cells as indicated) T cells ($2.5x10^4$ per well in U bottomed 96-well plates) were cultured for 72 h in the presence of CD3 mAb and evaluated as described in SI Materials and Methods.

Statistical analysis. More than 100 cells from at least three independent experiments were counted and MFI was calculated using the Leica TCS SP5 confocal laser scanning microscopy software. Groups were compared with Prism software (GraphPad, La Jolla, CA) using unpaired or paired Student's t test.

Supporting Information

For more detailed information see SI Materials and Methods.

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Author Contributions

M.V., T.G. and T. B. performed experiments, interpreted results and contributed to the writing of the manuscript. M.K. and N.G. performed real time PCR assays and cAMP ELISAs, S.G. provided expertise for paraffin sections, F.B.-S. for DNA transfections and the analysis of protein-protein interactions, and A.A. for retroviral infections. T.S. provided the DEREG mice, E. Sch. expertise for cAMP studies, and T.H. for CD28SA assays. E.S. and J.B. directed the study and wrote the manuscript.

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Figure Legends

Figure 1. CD3/CD28 mAb stimulation directs ICER/CREM to cytoplasm in conventional CD4$^+$ T cells but not in nTreg cells whereas elevated cAMP levels direct ICER/CREM to the nucleus. (A and B) Conventional CD4$^+$ T cells (A; "fresh") or nTreg cells (B; "fresh") primed and expanded by CD3/CD28 mAbs (primed) were re-stimulated either alone (CD3/CD28) or in the presence of forskolin (CD3/CD28+Forsk) or IBMX (CD3/CD28+IBMX) (see SI Materials and Methods) leading to elevated intracellular cAMP levels (C). Treated cells were analyzed for ICER/CREM and NFATc1 localization by confocal microscopy. Neither ICER nor NFATc1 shuttle in nTregs, but in conventional CD4$^+$ T cells. (C) Intracellular cAMP levels were assessed in conventional CD4$^+$ T cells and nTregs using ELISA. (D) Quantification of endogenous IL-2 mRNA levels in conventional CD4$^+$ T cells treated as indicated by quantitative real-time PCR. Data are shown as mean +/- SEM (n=3-4, $p<0.05$).

Figure 2. Direct contacts between nTreg cells and conventional CD4$^+$ T cells induce the nuclear localization of ICER/CREM and inhibit IL-2 expression in vivo. (A and B) Detection of interactions between nTreg and conventional CD4$^+$ T cells. CD90.1$^+$, RAG-2$^{-/-}$ OTII mice that contain CD4$^+$ T cells, which lack nTreg cells, were immunized subcutaneously with $OVA_{323-339}$ peptide in the left hint footpad. 6 h later, calcein-loaded congenic $OVA_{323-339}$ - specific nTregs expressing CD90.2 (B) were injected intravenously. 24 h after immunization, the intracellular levels of calcein and CD90.1 expression of CD4$^+$ cells from non-draining (A) and draining lymph nodes (B) were analyzed. Cells were sorted by gating on propidium iodide-negative and CD4$^+$ lymph node cells, and their intracellular cAMP concentration was assessed using ELISA in calcein-low and calcein-high cells (C). In (D) total RNA was extracted from sorted calcein-low and calcein-high cells, respectively, and IL-2 mRNA levels were quantified by real-time PCR. Data are shown as mean +/- SEM (n=3, $p<0.05$). (E) Immunofluorescence of calcein-low and calcein-high cells was performed using Abs specific for ICER/CREM and NFATc1 and analyzed by confocal microscopy.

Figure 3. nTreg cells direct ICER/CREM to the nucleus upon administration of CD28SA in vivo. (A) DEREG mice were treated with diphteria toxin (DT) for 5 days before administration of CD28SA. Lymph node cells were isolated, analyzed for the expression of CD25, GFP/Foxp3, CD69, and CTLA-4, and $CD4^+CD25^-$ cells were sorted 3 days later by FACS and harvested by cytospin. (B) Immunofluorescence was performed with Abs specific for ICER/CREM and NFATc1 and analyzed by confocal microscopy. Conventional $CD4^+$ T cells (Tcon) from Treg-depleted and CD28SA-treated DEREG mice (DT+CD28SA), revealed cytoplasmic localization of ICER/CREM (-nTreg). In contrast, ICER/CREM was nuclear in $CD4^+$ T cells isolated from lymph nodes of DEREG mice after CD28SA administration (CD28SA) in the presence of nTregs (+nTreg). (C) nTreg depletion results in systemic cytokine release after CD28SA stimulation. Mice were stimulated with CD28SA as indicated. Cytokine measurements of blood serum using CBA technology are shown for IL-2 and TNFα. Data are shown as mean +/- SEM (n=3-4, $p<0.05$). (D) Representative single cell analyses showing cytosolic localization of ICER/CREM (blue line) in $CD4^+$ T cells after CD28SA and DT treatment (-nTreg). In contrast, ICER/CREM is nuclear upon CD28SA administration without prior DT treatment (+nTreg). Nucleus is defined by DAPI staining (cyan blue line) and cytosolic distribution of NFATc1 is indicated in red.

Figure 4. ICER/CREM binds to the Nfatc1 P1 promoter and represses TCR-mediated NFATc1/αA induction. (A) Scheme of the *Nfatc1* gene and its P1 promoter. Underlined are the CRE motifs at the positions -145 and -640, respectively. (B) Evaluation of NFATc1 mRNA expression initiated at the P1 and P2 promoters in unstimulated and CD3 or CD3/CD28 Ab-stimulated conventional $CD4^+$ T cells treated without or with forskolin (upper panel). Conventional $CD4^+$ T cells stimulated with CD3/CD28 Abs were cultivated alone or in the presence of nTregs (lower panel). RT-PCR assays are shown detecting transcripts directed by the P1 (NFATc1/α) and P2 (NFATc1/β) promoters. (C) Recombinant ICERII protein binds specifically to the CRE-145 and CRE-640 motifs of the NFATc1 P1 promoter in EMSAs, as shown in supershift experiments (sICER) (left panel). In the right panel, the generation of inhibitory NFAT/ICER (NF/IC) complexes with

recombinant proteins at the CREs of P1 promoter is shown (for IL-2 promoter see Fig. S9). (D) ChIP assays demonstrate increased binding of ICER/CREM to the NFATc1 P1 promoter upon forskolin and CD3/28 mAb re-stimulation of conventional $CD4^+$ T cells treated in the same fashion as cells used for confocal analysis in Fig. 1A. Crosslinked chromatin was immunoprecipitated with an ICER/CREM-specific Ab (CS4). As a control, IgG of normal rabbit serum (NRS) was used in parallel. (E) ICER represses induction of P1 promoter of NFATc1. A luciferase construct driven by the P1 promoter (NFATc1 P1 Luc) was co-transfected with increasing concentrations of a vector expressing ICER into EL-4 T cells. Cells were stimulated by forskolin and ionomycin (Forsk+Iono) or PMA and ionomycin (PMA+Iono). In parallel, a vector encoding a leucin zipper-deficient ICER mutant (ICER-LZ) was transfected. Error bars show S.D. values of at least 3 experiments.