Deletion Timing of Cic Alleles during Hematopoiesis Determines the Degree of Peripheral CD4+ T Cell Activation and Proliferation

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ABSTRACT

Capicua (CIC) is a transcriptional repressor that regulates several developmental processes. CIC deficiency results in lymphoproliferative autoimmunity accompanied by expansion of CD44hiCD62Llo effector/memory and follicular Th cell populations. Deletion of Cic alleles in hematopoietic stem cells (Vav1- Cre-mediated knockout of Cic) causes more severe autoimmunity than that caused by the knockout of Cic in CD4+CD8+ double positive thymocytes (Cd4-Cre-mediated knockout of Cic). In this study, we compared splenic CD4+ T cell activation and proliferation between whole immune cell-specific Cic-null (Cicf/f;Vav1-Cre) and T cell-specific Cic-null (Cicf/f;Cd4-Cre) mice. Hyperactivation and hyperproliferation of CD4+ T cells were more apparent in Cicf/f;Vav1-Cre mice than in Cicf/f;Cd4-Cre mice. Cicf/f;Vav1-Cre CD4+ T cells more rapidly proliferated and secreted larger amounts of IL-2 upon TCR stimulation than did Cicf/f;Cd4-Cre CD4+ T cells, while the TCR stimulation-induced activation of the TCR signaling cascade and calcium flux were comparable between them. Mixed wild-type and Cicf/f;Vav1-Cre bone marrow chimeras also exhibited more apparent hyperactivation and hyperproliferation of Cic-deficient CD4+ T cells than did mixed wild-type and Cicf/f;Cd4-Cre bone marrow chimeras. Taken together, our data demonstrate that CIC deficiency at the beginning of T cell development endows peripheral CD4+ T cells with enhanced T cell activation and proliferative capability.

Keywords: Capicua; T-lymphocytes; Lymphocyte activation; Cell proliferation

INTRODUCTION

CD4+ Th cells play a pivotal role in the adaptive immune system. CD44hiCD62Llo naïve CD4+ T cells are activated by interactions with Ag-presenting cells (APCs) and eventually differentiate into CD44hiCD62Llo effector/memory T cells. Initially, TCR and co-stimulatory receptors on naïve CD4+ T cells interact with Ag-presented MHC II molecules and co-stimulatory ligands on APCs, respectively, to provide activation signals to naïve CD4+ T cells (1). Activated CD4+
T cells then rapidly proliferate and differentiate into various Th subsets such as Th1, Th2, Th17, and follicular helper T (Tfh) cells according to the cytokines within their surrounding environment (2).

Expansion of the CD44hiCD62Llo effector/memory T cell population is one of the hallmarks of autoimmune diseases (3-6). Hyperactivation and hyperproliferation of CD4+ T cells potentially generates a larger population of autoreactive and pathogenic effector Th cells that contribute to the onset of autoimmune diseases. Therefore, alterations in the TCR signaling cascade and downstream target gene expression are associated with the pathogenesis of autoimmune diseases (7,10). Among the Th subsets, Tfh cells mediate the germinal center (GC) response to generate high-affinity Ab-producing plasma cells and memory B cells (11,12). Given the role of Tfh cells in this process, excessive formation and/or hyperactivation of Tfh cells are closely associated with the pathogenesis of Ab-mediated systemic autoimmune diseases such as systemic lupus erythematosus (SLE) (13-15). In support of this, it has been observed that blocking the development or function of Tfh cells alleviates the severity of disease symptoms in lupus mouse models (16,17).

Capicua (CIC) is a high-mobility group box transcription factor that is required for several developmental and physiological processes, including lung and brain development, abdominal wall closure during embryogenesis, and bile acid homeostasis (18-22). CIC also functions as a tumor suppressor in various types of cancer, including prostate, lung, liver, breast, and colorectal cancers, oligodendroglioma, and T cell lymphoma (18,22-30). CIC functions within immune cells have also been studied (6,30,31). Whole immune cell-specific Cic-null (Cicfl/fl;Vav1-Cre) mice develop lymphoproliferative systemic autoimmunity accompanied by increased frequency of CD44hiCD62Llo effector/memory T cell subsets in the spleen (6). Genetic ablation of Cic in T cells by Cd4-Cre, which deletes floxed alleles of a specific gene in CD4+CD8+ double positive (DP) thymocytes by Cicfl/fl;Cd4-Cre mice, or 2) Vav1-Cre-mediated Cic deletion in hematopoietic stem cells results in systemic autoimmunity (6). However, autoimmunity-like phenotypes such as hyperglobulinemia and increased serum autoantibody levels are more severe in Cicfl/fl;Vav1-Cre mice than in T cell-specific Cic-null (Cicfl/fl;Cd4-Cre) mice (6). This phenomenon could be attributed to 2 possibilities: 1) Cic-deficient immune cells other than T cells may contribute to the development of autoimmune-like symptoms in Cicfl/fl;Vav1-Cre mice; or 2) Vav1-Cre-mediated Cic deletion in hematopoietic stem cells may produce peripheral T cells with stronger autoimmunity-inducing potential than that caused by the knockout (KO) of Cic in DP thymocytes by Cd4-Cre.

In this study, we examined the activation and proliferative properties of peripheral CD4+ T cells in Cicfl/fl;Vav1-Cre and Cicfl/fl;Cd4-Cre mice of the same age. We also compared the effects of Vav1-Cre- versus Cd4-Cre-mediated Cic deletion in hematopoietic stem cells on T cell activation and proliferation both in vitro and in vivo.

MATERIALS AND METHODS

Mice

Cicfl/fl;Cd4-Cre and Cicfl/fl;Vav1-Cre mice have been described previously (6). For the experiments, littermates or appropriate age/sex-matched mice were used. FLAG-tagged Cic knock-in (CicFLAG/FLAG) mice were created using the CRISPR/Cas9 system, where a single guide RNA (sgRNA) targets the CRISPR/Cas-derived RNA-guided endonuclease (RGEN) site of Cic exon 21 to insert 3XFLAG sequences at the 3’ end of the Cic open reading frame prior to the stop codon.
All mice were maintained in a specific pathogen-free animal facility under a standard 12-h light/12-h dark cycle. Mice were fed standard rodent chow and provided with water ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee of Pohang University of Science and Technology.

**Flow cytometry analysis**

For cell surface staining, cells were stained with fluorochrome-conjugated Abs against cell surface proteins in FACS buffer (PBS with 2% FBS). The following Abs were used: anti-CD4 (RM4-5; BioLegend, San Diego, CA, USA), anti-CD25 (PC61; BioLegend), anti-CD62L (MEL-14; BioLegend), anti-CD69 (H1.2F3; BioLegend), anti-PD-1 (RMP1-30; BioLegend), anti-FLAG (L5; BioLegend), anti-Rat IgG2aκ (RTK2758; Biolegend), anti-CD44 (IM7; BD Bioscience, San Jose, CA, USA), anti-CD62L (MEL-14; BD Bioscience), anti-ICOS (7E.17G9; BD Bioscience), anti-OX-40 (OX-86; eBioscience, San Diego, CA, USA), anti-GITR (DTA-1; eBioscience), anti-Thy1.1 (H1S51; eBioscience), anti-Thy1.2 (53-2.1; eBioscience), anti-CD4 (RM4-5; Tonbo Biosciences, San Diego, CA, USA), and anti-CD44 (IM7; Tonbo Biosciences). For intracellular staining of Ki-67, cells were fixed and permeabilized using Foxp3 Fixation/Permeabilization solution (eBioscience) according to the manufacturer’s instructions, and they were then stained with anti-Ki-67 (SO1A15; eBioscience). Dead cells were identified using Ghost Dye (Tonbo Biosciences). The expression of surface and intracellular markers was analyzed using either a CantoII flow cytometer (BD Biosciences) or an LSRII flow cytometer (BD Biosciences).

**In vitro T cell proliferation assay**

Naïve CD4+ T cells were obtained from pooled spleens and lymph nodes of Cic-/-, Cic-/-;Cd4-Cre, and Cic-/-;Vav1-Cre mice through negative selection (Stemcell Technologies, Vancouver, Canada), and to ensure perfect purity, naïve CD4+CD25−CD44lo T cells were sorted. The cells were labeled with 5 μM Cell Trace Violet (CTV; Invitrogen, Carlsbad, CA, USA) for 15 min at 37°C. To remove any free dye remaining in the reaction tubes, 5 times the original staining volume of culture medium (containing 10% FBS) was added, and this was followed by incubation for 5 min. The CTV-labeled cells were activated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) in complete RPMI-1640 medium, incubated at 37°C in a 5% CO2 incubator, and then collected at 48 h and 72 h after stimulation. The samples were analyzed using a CantoII flow cytometer (BD Biosciences).

**ELISA**

Sorted naïve CD4+ T cells were activated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml), and the cell supernatants were collected from cultures at 48 h and 72 h after stimulation. Then, 96-well plates (Corning, New York, NY, USA) were coated with 2 μg/ml anti-IL-2 (14-7022; eBioscience) at 4°C overnight. The plates were washed with PBS with 0.05% Tween 20 (PBST) and blocked with blocking buffer (PBST containing 2% BSA) at room temperature for 2 h. Standards and diluted samples were added and incubated at room temperature for 2 h. After washing, the plates were incubated with biotin-conjugated anti-IL-2 (13-7021; eBioscience) for 1.5 h followed by incubation with avidin-HRP (18-4100; eBioscience) for 30 min. The plates were developed with 3,3,5,5-tetramethylbenzidine (TMB) substrate (Surmodics, Eden Prairie, MN, USA) for 30 min and then stopped by adding 1 M H2SO4. Absorbance was measured at 450 nm.

**Calcium flux measurement**

Splenocytes were incubated with 4 μM Indo-1 (Invitrogen) in RPMI-1640 medium at 37°C for 30 min, and this was followed by washing with RPMI-1640 medium. Indo-1-loaded
Splenocytes were incubated with soluble anti-CD3 (0.5 or 1.0 μg/mL) and fluorochrome-conjugated Abs for surface markers (CD4, CD44, and CD62L) in RPMI-1640 medium containing 10% FBS on ice for 20 min and then warmed before cross-linking. A concentration of 25 μg/ml anti-hamster IgG (Jackson Immunoresearch, West Grove, PA, USA) was added to cross-link anti-CD3 Abs, and the signals were measured by flow cytometry. Ionomycin was added to ensure that T cells were effectively loaded with Indo-1. The emission wavelength ratios of Ca²⁺-bound to unbound Indo-1 were analyzed using an LSRII flow cytometer (BD Biosciences).

**Western blot analysis**

Sorted naïve CD4⁺ T cells were incubated with soluble anti-CD3 (5 μg/mL) and cross-linked with 25 μg/ml anti-hamster IgG. The cells were incubated at 37°C and collected 2, 10, and 30 min after cross-linking for western blot analysis. Samples were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1x Roche Complete Protease Inhibitor Cocktail, and 1x Roche Phosphatase Inhibitor Cocktail). Protein concentrations were measured using a BCA kit (Pierce). Equal amounts of protein were prepared and boiled with sample buffer (250 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 5% β-mercaptoethanol, and 0.1% bromophenol blue) for 5 min. The protein samples were separated using 8% SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), blocked with 5% dry non-fat milk in Tris buffered saline pH 7.4, 0.1% Tween-20 (TBST), washed with TBST, and probed with the following primary Abs in Can get signal solution (Toyobo, Osaka, Japan): anti-CIC (homemade; 1:1,000), anti-p-ZAP70 (SC-12946-R; 1:500), anti-ZAP70 (SC-32760; 1:1,000), anti-p-PLCγ (CST 2821S; 1:500), anti-PLCγ (SC-407; 1:500), anti-p-AKT (CST 9271S; 1:500), anti-AKT (CST 9272; 1:1,000), anti-p-ERK (CST 4370S; 1:500), anti-ERK (CST 9102S; 1:1,000), anti-p-IkBα (CST 2859S; 1:500), anti-IkBα (CST9242S; 1:1,000), and anti-β-actin (SC-32233; 1:2,000). Membranes were incubated with secondary anti-rabbit or anti-mouse IgG Abs conjugated with HRP at room temperature for 1 h, and this was followed by detection using ECL (Bio-Rad) or West DURA (Thermo Scientific, Waltham, MA, USA). Blot images were obtained using ImageQuant LAS 500 (GE Healthcare, Chicago, IL, USA).

**Mixed bone marrow (BM) chimera**

Bone marrow cells were collected from the tibias and femurs of Cic⁻/⁻, Cic⁻/⁻;Cd4-Cre, and Cic⁻/⁻;Vav1-Cre mice in RPMI-1640 medium containing 10% FBS and depleted red blood cells with RBC lysis buffer. Equal numbers of BM cells (1×10⁶) from Cic⁻/⁻ (Thy1.1/Thy1.2) and Cic⁻/⁻;Cd4-Cre (Thy1.1/Thy1.1) mice or Cic⁻/⁻ (Thy1.1/Thy1.2) and Cic⁻/⁻;Vav1-Cre (Thy1.1/Thy1.2) mice were mixed and transferred to C57BL/6 mice (Thy1.2/Thy1.2) that were lethally irradiated at 10 Gy. Eight weeks later, the mixed BM chimeric mice were sacrificed, and their spleens were analyzed by flow cytometry.

**Statistical analysis**

Statistical analyses were performed using Prism 8.0 (GraphPad Software, San Diego, CA, USA). One-way ANOVA with Tukey’s *post hoc* test was used for multigroup comparisons. Paired 2-tailed Student’s *t*-tests were used to compare two groups of mixed BM chimeras. Error bars indicate SEM. A value of p<0.05 was considered significant.
RESULTS AND DISCUSSION

Enhanced peripheral CD4⁺ T cell activation in Cic<sup>fl</sup>/Vav1-Cre mice

To directly compare the severity of autoimmunity-related phenotypes caused by hematopoietic lineage cell-specific versus T cell-specific deletion of Cic alleles, we simultaneously analyzed Cic<sup>fl</sup> (WT), Cic<sup>fl</sup>/Vav1-Cre, and Cic<sup>fl</sup>/Cd4-Cre mice of the same age. Splenomegaly was clearly observed in Cic<sup>fl</sup>/Vav1-Cre mice and not in Cic<sup>fl</sup>/Cd4-Cre mice irrespective of sex at 12 weeks of age (Fig. 1A). We also analyzed splenic CD4⁺ T cells in these mice. We confirmed our previous findings that the frequency and number of CD44<sup>+CD62L<sup>−</sup> effector/memory cells were both significantly increased in Cic<sup>fl</sup>/Vav1-Cre mice at the expense of CD44<sup>−CD62L<sup>+</sup> naïve CD4⁺ T cells; however, this phenotype was much milder in Cic<sup>fl</sup>/Cd4-Cre mice (Fig. 1B). Consistent with these results, the levels of surface molecules representing T cell activation status (CD25<sup>−</sup>CD44<sup>−</sup>CD62L<sup>+</sup>CD69<sup>+</sup>CD44<sup>−</sup>CD62L<sup>−</sup>) were more dramatically and significantly altered in CD4⁺ T cells from Cic<sup>fl</sup>/Vav1-Cre mice (Vav1-Cre Cic KO CD4⁺ T cells) compared to those from Cic<sup>fl</sup>/Cd4-Cre mice (Cd4-Cre Cic KO CD4⁺ T cells) (Fig. 1C). The levels of co-stimulatory molecules, including ICOS, PD-1, OX-40, and GITR, were also higher in Vav1-Cre Cic KO CD4⁺ T cells than they were in Cd4-Cre Cic KO CD4⁺ T cells (Fig. 1D). Taken together, Cic<sup>fl</sup>/Vav1-Cre mice clearly exhibit increased peripheral CD4⁺ T cell hyperactivation compared to that observed in these cells derived from Cic<sup>fl</sup>/Cd4-Cre mice.

Increased cell proliferative activity in Vav1-Cre Cic KO CD4⁺ T cells

We next examined the cell proliferative activity of Vav1-Cre and Cd4-Cre Cic KO naïve CD4⁺ T cells by performing an in vitro T cell proliferation assay using cell trace violet (CTV) staining. Vav1-Cre Cic KO naïve CD4⁺ T cells proliferated faster and secreted higher amounts of IL-2 than did wild-type (WT) cells upon TCR stimulation with an anti-CD3 Ab, and these effects were overridden by co-stimulation with an anti-CD28 Ab (Fig. 2A and B). These findings verified

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**Figure 1.** CD4⁺ T cell hyperactivation in Cic<sup>fl</sup>/Vav1-Cre mice.

(A) Image of spleens from 12 week-old male (M) and female (F) Cic<sup>fl</sup> (WT), Cic<sup>fl</sup>/Cd4-Cre, and Cic<sup>fl</sup>/Vav1-Cre mice. (B) FACs analysis showing the proportion of CD44<sup>−CD62L<sup>+</sup> naïve CD4⁺ T cells and CD44<sup>−CD62L<sup>−</sup> effector/memory CD4⁺ T cells in the spleens of 12 week-old WT, Cic<sup>fl</sup>/Cd4-Cre, and Cic<sup>fl</sup>/Vav1-Cre mice. Bar graphs show not only the frequency (left) but also the number (right) of naïve and effector/memory CD4⁺ T cells. (C and D) Surface expression levels of activation markers (CD25, CD44, CD62L, and CD69) (C) and co-stimulatory molecules (ICOS, PD-1, OX-40, and GITR) (D) on CD4⁺ T cells in the spleen of WT, Cic<sup>fl</sup>/Cd4-Cre, and Cic<sup>fl</sup>/Vav1-Cre mice. Bar graphs show data as mean plus or minus the SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Effect of Cic Loss in HSCs on Peripheral CD4+ T Cell Activation

Figure 2. Increased CD4+ T cell proliferative capability in Cicf/f;Vav1-Cre mice.
(A) In vitro T cell proliferation assay. Naïve CD4+ T cells purified from pooled spleens and lymph nodes of WT, Cicf/f;Cd4-Cre, and Cicf/f;Vav1-Cre mice were labelled with CTV dye and stimulated with plate-bound anti-CD3 (5 μg/ml) in the presence (right) or absence (left) of plate-bound anti-CD28 (2 μg/ml). The cells were analyzed at 48 h and 72 h after stimulation. Data are representative of three independent experiments. Shaded area: unstimulated control, black line: Cicf/f;Cd4-Cre, and red line: Cicf/f;Vav1-Cre. (B) ELISA of IL-2. Naïve CD4+ T cells purified from pooled spleens and lymph nodes of WT, Cicf/f;Cd4-Cre, and Cicf/f;Vav1-Cre mice were stimulated with plate-bound anti-CD3 (5 μg/ml) in the presence (right) or absence (left) of plate-bound anti-CD28 (2 μg/ml). The supernatants were taken at 48 h and 72 h after stimulation and subjected to ELISA to determine IL-2 concentration. N = 4 per each sample. (C) FACS analysis of Ki-67+ CD4+ T cells in the spleens of 12 week-old WT, Cicf/f;Cd4-Cre, and Cicf/f;Vav1-Cre mice. n=9 per each genotype of mice. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

our previous results (6). However, the proliferation rate and IL-2 production were comparable between WT and Cd4-Cre Cic KO naïve CD4+ T cells (Fig. 2A and B), suggesting that the sensitivity to TCR stimulation is different between Vav1-Cre and Cd4-Cre Cic KO naïve CD4+ T cells. We also determined the frequency of in vivo proliferating CD4+ T cells in the spleens of Cicf/f;Cd4-Cre and Cicf/f;Vav1-Cre mice. The proportion of Ki-67+ CD4+ T cells was significantly increased in both Cicf/f;Cd4-Cre and Cicf/f;Vav1-Cre mice (Fig. 2C). Notably, Cicf/f;Vav1-Cre mice exhibited a higher frequency of Ki-67+ CD4+ T cells than did Cicf/f;Cd4-Cre mice (Fig. 2C).

These results indicate that Vav1-Cre Cic KO CD4+ T cells possessed enhanced cell proliferative capability compared to that of Cd4-Cre Cic KO CD4+ T cells.

Comparative calcium flux and activation of TCR signaling cascades in CD4+ T cells from WT, Cicf/f;Cd4-Cre, and Cicf/f;Vav1-Cre mice

As TCR stimulation-induced cell proliferation and IL-2 secretion were significantly increased in Vav1-Cre Cic KO naïve CD4+ T cells compared to these processes in WT or Cd4-Cre Cic KO CD4+ naïve T cells (Fig. 2A and B), we examined the activation of the TCR signaling pathway in the same set of cells when treated with an anti-CD3 Ab. Unexpectedly, neither calcium flux nor the levels of phosphorylated TCR signaling components, including ZAP70, PLCγ, AKT, ERK, and IκBα, were increased in Vav1-Cre Cic KO CD4+ T cells (Fig. 3A and B). The levels of phosphor-ERK were slightly decreased in both Cd4-Cre and Vav1-Cre Cic KO CD4+ T cells (Fig. 3B). This result may be due to derepression of CIC target genes such as Spry4, Sprd1, and Dusp4 that are involved in the dephosphorylation of ERK, as levels of these genes have been reported to be upregulated in Cic-deficient naïve CD4+ T cells (6). These findings suggest that CIC deficiency-induced CD4+ T cell activation and proliferation may occur independently of activation of TCR signaling cascade components and calcium flux.

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T cell-intrinsic effect of CIC loss on the hyperactivation and hyperproliferation of peripheral CD4+ T cells in Cicf/f;Vav1-Cre mice

We showed that hyperactivation and hyperproliferation of CD4+ T cells were more apparent in Cicf/f;Vav1-Cre than in Cicf/f;Cd4-Cre mice (Figs. 1B and 2C). Because Vav1-Cre deletes floxed alleles of a specific gene not only in T cells but also in other types of immune cells (33), it is unclear if the enhanced hyperactivation and hyperproliferation of splenic CD4+ T cells in Cicf/f;Vav1-Cre mice were T cell-intrinsic or extrinsic. To clarify this issue, we generated mixed BM chimeric mice by transferring the same number of BM cells from Thy1.1/Thy1.2 heterozygous WT and Thy1.1/Thy1.1 homozygous Cicf/f;Cd4-Cre mice (WT: Cicf/f;Cd4-Cre BM chimera) or Thy1.1/Thy1.1 homozygous WT and Thy1.1/Thy1.2 heterozygous Cicf/f;Vav1-Cre mice (WT: Cicf/f;Vav1-Cre BM chimera) into irradiated Thy1.2/Thy1.2 homozygous WT recipient mice (Fig. 4A), and analyzed splenic CD4+ T cells in these chimeric mice. Consistent with the results from the analyses of CD4+ T cells in WT, Cicf/f;Cd4-Cre, and Cicf/f;Vav1-Cre mice (Fig. 1B), not only Cicf/f;Vav1-Cre but also Cicf/f;Cd4-Cre BM cells preferentially gave rise to CD44hiCD62Llo effector/memory CD4+ T cells at the expense of CD44loCD62Lhi naïve CD4+ T cells when compared to those from WT mice (Fig. 4B and C). Importantly, the fold changes in the frequency of CD44loCD62Lhi naïve and CD44hiCD62Llo effector/memory CD4+ T cells between WT and Cic-deficient CD4+ T cell compartments were more dramatic in the WT: Cicf/f;Vav1-Cre BM chimeras than they were in the WT: Cicf/f;Cd4-Cre BM chimeras (Fig. 4C). Consistent with this result, the surface expression levels of CD44 and CD62L were more dramatically increased and decreased, respectively, on the Vav1-Cre Cic KO CD4+ T cells compared to those on the Cd4-Cre Cic KO CD4+ T cells (Fig. 4D and E). These data demonstrate that the enhanced CD4+ T cell hyperactivation in Cicf/f;Vav1-Cre mice in comparison with that in Cicf/f;Cd4-Cre mice is at
Figure 4. Vav1-Cre-mediated T cell-intrinsic loss of CIC leads to hyperactivation and hyperproliferation of CD4+ T cells. (A) Schematic illustration for the generation of chimeric mice with WT, Cioff;Cd4-Cre, and Cioff;Vav1-Cre BM cells. (B) FACS analysis for the proportion of splenic CD44+CD62L+ naive and CD44+CD62L- effector/memory cells in the CD4+ T cell compartments of either WT (Thy1.1/Thy1.2) or WT (Thy1.1/Thy1.1) or WT (Thy1.1/Thy1.1) or Cioff;Cd4-Cre (Thy1.1/Thy1.1) or Cioff;Vav1-Cre (Thy1.1/Thy1.1) mixed BM chimeric mice. Graphs show a pairwise comparison between WT and Cic-deficient (cKO) CD4+ T cells in the same mixed BM chimeric mouse. (C) Relative fold changes in the frequency of CD44+CD62L+ naive and CD44+CD62L- effector/memory cells between WT and Cic-deficient (cKO) CD4+ T cell compartments of the mixed BM chimeric mice. The relative fold changes were calculated based on the results in (B). (D) Relative fold changes in the frequency of Ki-67+ cells between WT and Cic-deficient (cKO) CD4+ T cell compartments of the mixed BM chimeric mice. The relative fold changes were calculated based on the results in (B). (E) Surface expression levels of CD44 and CD62L on splenic CD4+ T cells from the mixed BM chimeric mice. Graphs show a pairwise comparison between WT and Cic-deficient (cKO) CD4+ T cells in the same mixed BM chimeric mouse. (F) Relative fold changes in the levels of CD44 and CD62L on splenic CD4+ T cells between WT and Cic-deficient (cKO) CD4+ T cell compartments of the mixed BM chimeric mice. The relative fold changes were calculated based on the results in (B). (F) FACS analysis for the frequency of splenic Ki-67+ cells in the CD4+ T cell compartments of either WT (Thy1.1/Thy1.2) or Cicoff;Cd4-Cre (Thy1.1/Thy1.1) or WT (Thy1.1/Thy1.1) or Cioff;Vav1-Cre (Thy1.1/Thy1.1) mixed BM chimeric mice. Graphs show a pairwise comparison between WT and Cic-deficient (cKO) CD4+ T cells in the same mixed BM chimeric mouse. The relative fold changes were calculated based on the results in (F). WT (Thy1.1/Thy1.1) or Cioff;Cd4-Cre (Thy1.1/Thy1.1) mixed BM chimeric mice (n=9) and WT (Thy1.1/Thy1.1) or Cicoff;Vav1-Cre (Thy1.1/Thy1.1) mixed BM chimeric mice (n=5–6).

*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
chimeras (Supplementary Fig. 1), suggesting that the increased expression levels of co-stimulatory molecules on CD4+ T cells in Cicf/f;Vav1-Cre mice (Fig. 1D) might be attributed to both T cell-intrinsic and -extrinsic loss of CIC. We also examined CD4+ T cell proliferation in these mice by conducting Ki-67 staining. Consistent with the previous data (Fig. 2C), the frequency of Ki-67+ cells was significantly increased in the compartments of Vav1-Cre and Cd4-Cre Cic KO CD4+ T cells compared to that in WT CD4+ T cell compartments (Fig. 4F and G). Moreover, the fold increase in the frequency of Ki-67+ cells between WT and Cic-deficient CD4+ T cell compartments was greater in the WT:Cicf/f;Vav1-Cre BM chimeras than in the WT:Cicf/f;Cd4-Cre BM chimeras (Fig. 4G). Taken together, these results demonstrate that both hyperactivation and hyperproliferation of peripheral CD4+ T cells in Cicf/f;Vav1-Cre mice were due to T cell-intrinsic loss of CIC rather than CIC deficiency in other types of immune cells.

Our study revealed that Vav1-Cre-mediated removal of Cic alleles endows peripheral CD4+ T cells with stronger activation potential and proliferative capability than that mediated by Cd4-Cre, and that the hyperactivation and hyperproliferation of Vav1-Cre Cic KO CD4+ T cells are T cell-intrinsic phenotypes. In line with this idea, we observed that the frequency of CD44+CD62L- naive and CD44-CD62L+ effector/memory CD4+ T cells was normal in the spleens of B cell-specific (Cicf/f;Cd19-Cre) and dendritic cell-specific (Cicf/f;Cd11c-Cre) Cic null mice (unpublished data). These findings raise the interesting possibility that CIC deficiency prior to the DP stage of thymic T cell development could affect mature CD4+ T cell activation and proliferation properties, as Vav1 is expressed in hematopoietic stem cells and Cd4 is expressed in DP thymocytes (32,33). Tan et al. (30) have shown that CIC deficiency increases the frequency of CD4-CD8- double negative 1 (DN1) thymocytes, suggesting a potential role of CIC in the regulation of thymic T cell development. Consistent with this notion, we found that CIC levels were higher in DN thymocytes than they were in DP and single positive (SP) thymic T cells (Supplementary Fig. 2). A more detailed study examining the role of CIC in thymic T cell development will improve our understanding of how CIC suppresses peripheral T cell activation and proliferation to ultimately regulate autoimmunity.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1
Surface expression levels of ICOS, PD-1, and OX-40 on splenic CD4+ T cells from mixed BM chimeric mice.

Click here to view
Supplementary Figure 2
CIC protein levels in CD4−CD8− DN, CD4+CD8+ DP, CD4+ SP (4SP), and CD8+ SP (8SP) thymocytes.

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