Published in final edited form as:

Clin Immunol. 2011 March ; 138(3): 321–330. doi:10.1016/j.clim.2010.12.020.

Divergent Effects of Calcineurin A β on Regulatory and Conventional T-cell Homeostasis

Thomas Doetschman^{a,b,d}, Allyson Sholl^e, Hwu dau rw Chen^{a,b,1}, Connie Gard^a, David A. Hildeman^e, and Ramireddy Bommireddy^{a,c,d,*}

^aBIO5 Institute, University of Arizona, Tucson, AZ 85724-5217

^bDept. of Cell Biology, University of Arizona, Tucson, AZ 85724-5217

^cDept. of Immunobiology, & Anatomy, University of Arizona, Tucson, AZ 85724-5217

^dArizona Cancer Center, University of Arizona, Tucson, AZ 85724-5217

^eDivision of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229

Abstract

Calcineurin (CN) is a phosphatase that activates nuclear factor of activated T cells (NFAT). While the CN inhibitors cyclosporine A (CsA) and tacrolimus (FK506) can prevent graft rejection, they also cause inflammatory diseases. We investigated the role of calcineurin using mice deficient in the CN catalytic subunit A β (CNA β). *Cnab*^{-/-} mice exhibit defective thymocyte maturation, splenomegaly and hepatomegaly. Further, as *Cnab*^{-/-} mice age, they exhibit spontaneous T-cell activation and enhanced production of proinflammatory cytokines (IL-4, IL-6, and IFN γ). FOXP3⁺ T_{reg} cells were significantly decreased in *Cnab*^{-/-} mice likely contributing to increased T-cell activation. Interestingly, we found that CNA β is critical for promotion of BCL-2 expression in FOXP3⁺ T_{reg} and for permitting TGF β signaling, as TGF β induces FOXP3 in control but not in *Cnab*^{-/-} T-cells. Together, these data suggest that CNA β is important for the production and maintenance of T_{reg} cells and to ensure mature T-cell quiescence.

Keywords

Calcineurin; Cyclosporin A; Inflammation; knockout; FOXP3; T_{reg} cells

1. Introduction

CN, a serine/threonine protein phosphatase, is activated by Ca²⁺-calmodulin upon elevation of intracellular Ca²⁺ levels by T-cell receptor (TCR) signaling, and it activates NFAT molecules by dephosphorylation. CN is a well characterized drug target for prevention of graft rejection in transplant patients [1–3]. The immunosuppressive drugs FK506 and CsA inhibit NFATs which are involved in lymphocyte responses [4]. It has been shown that

© 2010 Elsevier Inc. All rights reserved.

*Corresponding author: Ramireddy Bommireddy, PhD, BIO5 Institute, MRB 323, 1656 E Mabel St., P.O. Box 245217, Tucson, AZ 85724-5217, USA. Fax: 520-626-7600, ramiredb@email.arizona.edu.

¹Current address.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

FK506 and CsA inhibit positive selection but not negative selection of thymocytes suggesting that CN activation is critical for T-cell development in the thymus [5]. Although CsA and FK506 are effective in treating transplant patients for graft acceptance, long-term side effects include frequent infections, kidney failure, inflammatory bowel disease (IBD) and lymphomas [6–8].

The administration of CsA to new-born BALB/c mice induces organ-specific autoimmunity, inhibits *Foxp3* expression, and blocks T_{reg}-cell generation, both *in vitro* and *in vivo* [9–11]. *FOXP3* is induced by NFAT and AP-1 in human T cells, and CsA inhibits *FOXP3* expression through inhibition of NFAT activation [10]. Under optimal stimulatory conditions which activate AP-1, NFAT induces not only *Il2*, but also *Il4* and *Ifng* resulting in a productive immune response in mice [12–14]. Together these observations suggest that low levels of TCR signaling leads to T-cell differentiation into T_{reg} cells whereas higher levels of TCR signaling leads to effector T-cell generation. Consistently, CNA β -deficient mice do not reject allogenic tumors, whereas CNA α -deficient mice succumb to inflammation and kidney failure [15;16]. A defect in *FasL* expression and impaired apoptosis of T cells leads to splenomegaly and lymphadenopathy in mice doubly deficient for *Nfatc2* and *c3* [17]. These studies suggest that CN signaling is required for T-cell activation, activation-induced cell death (AICD) and T-cell tolerance [12;18].

Under tolerogenic conditions, NFAT forms a complex with FOXP3 and together induces T_{reg}-cell generation by induction of *Il2ra* (CD25) and *Ctla4* (CD152) [19]. Recently, Tone Y *et. al.* have found that the *Foxp3* enhancer contains SMAD3 and NFAT binding sequences, suggesting cooperative positive regulation of *Foxp3* by TGF β and TCR signals [20]. CN signaling also seems to play an important role in T-cell regulation as the proportion of CD4⁺CD25⁺ cells are increased in *Nfatc2* and *Nfatc3* double knockout (DKO) mice. Although the suppressor activity of the DKO T_{reg} cells (CD4⁺CD25⁺⁺GITR⁺⁺) is comparable to that of their wildtype (WT) CD4⁺CD25⁺⁺GITR⁺⁺ T-cell counterparts, their CD4⁺CD25⁻ T cells exhibit activated phenotype suggesting that they are not inhibited by T_{reg} cells *in vivo*. These data suggest that an intrinsic defect makes the DKO T cells resistant to tolerance induction [17]. Since CsA inhibits not only T-cell activation but also T_{reg}-cell generation in mice [9] and humans [10], whether the inhibitory effect of CsA is through inhibition of CN signaling or through its effect on other CN-independent signaling pathways is still unclear.

CNA α -deficient mice have normal thymocyte maturation and T-cell responses to polyclonal stimulation, but they exhibit defective T-cell responses to antigenic stimulation [21;22]. CNA α KO mice die within 2 months after birth due to kidney failure [15]. Although CNA α compensates to a certain extent for the loss of CNA β in mature T cells, CNA β plays a non-redundant role in T-cell homeostasis and T-cell regulation [16]. The present study is a follow up to our recent study in which we have demonstrated that T cells in *Cnab*^{-/-} mice are activated and produce increased levels of IL-6 and IFN γ [23]. Since young CNA β -deficient mice exhibit defective T-cell maturation [16] but they develop splenomegaly, and hepatomegaly we hypothesize that defective T-cell regulation increases inflammatory response resulting in lymphoid hyperplasia. In this study we present evidence that CNA β deficiency affects T_{reg}-cell-generation and leads to expansion of mature T cells with activated phenotype, suggesting that while CNA β signaling is required for T-cell maturation and anti-tumor responses [16], it is also important for T-cell regulation and homeostasis.

2. Materials and Methods

2.1 Mice

Cnab^{-/-} mice (C57BL/6) are obtained from Dr. Jeffery Molkentin (Cincinnati Children's Hospital Medical Center) [16] were mated to *DO11.10* TCR Tg *Rag1*^{-/-} mice on a BALB/c background [24]. Mice were backcrossed 4 times to BALB/c mice. All experiments were reviewed and approved by the University of Arizona IACUC committee.

2.2 PCR Genotyping

The genotype of newborn pups from double heterozygous matings was determined by PCR amplification of tail DNA and size fractionation on agarose gels [25].

2.3 Flow cytometry analysis of splenocytes

Phenotype analysis of splenocytes was determined by four-color flow cytometry as described [24]. FITC-, PE-, PerCP, PerCP-Cy5.5, Pacific Blue, or APC-conjugated antibodies to cell surface molecules were purchased from either BD Biosciences (San Diego, CA) or eBioscience (San Diego, CA). FOXP3 staining kit (clone FKJ-16s) was purchased from eBioscience (San Diego, CA). Thymocytes and splenocytes were surface stained with fluorochrome-conjugated antibodies after blocking with Fc blocking antibodies in FACS staining buffer at 4°C in the dark. Cells were washed once with FACS buffer, fixed in 2% paraformaldehyde and acquired using a BD-LSR or BD-LSR-II flow cytometer. Intracellular FOXP3 staining was performed according to manufacturer's protocol as described in our earlier studies [26]. Flow cytometry data were analyzed by Cell-Quest or FACS DIVA software.

2.4 Splenocyte culture and Cytokine measurements

For FOXP3 induction, splenocytes were isolated from *Cnab*^{-/-} and control mice and 1×10⁶ cells were cultured in RPMI-1640 medium supplemented with 10% FBS in the presence of anti-CD3/CD28 beads (Dyna Beads, Invitrogen), IL-2 (100 IU/ml) and TGFβ1 (2–5 ng/ml) in 48 well plate for 3 days. Cells were harvested and stained for surface expression of CD4, CD25 and intracellular FOXP3 as described above. For cytokine measurements, culture supernatants were harvested and assayed for cytokines by ELISA kits from BD Biosciences as described in our recent study [23].

2.5 Intracellular cytokine staining

Splenocytes were isolated from *Cnab*^{-/-} and control mice and 1×10⁶ cells were cultured in s-MEM supplemented with 10% FBS in 96 well plates coated with anti-CD3/CD28 antibodies and Brefeldin A (10 µg/ml). Cells were harvested and stained for surface expression of CD4, washed, fixed in BD Cytofix/Cytoperm buffer (BD Biosciences, San Diego, CA) on ice, washed in Perm/wash buffer and resuspended in BD Cytoperm Plus buffer, washed with BD Perm/Wash buffer and then stained intracellularly with antibodies against FOXP3, IFNγ, and IL-17. Flow cytometry data were analyzed by FACS DIVA software.

2.6 Statistical analysis

The significance of the differences between wild-type and mutant mouse responses was calculated using Student's *t*-test.

3. Results

3.1 CNA β deficiency results in increased mature T cells with age

CNA β deficiency causes a severe reduction in mature T-cell populations both in the thymus and periphery, and it also causes a defect in T-cell responsiveness [16]. *Cnab*^{-/-} mice also fail to reject allogenic tumors suggesting an impaired immune response [16]. In order to understand the cause of the splenomegaly in older *Cnab*^{-/-} mice we have analyzed T-cell populations at different ages. Consistent with earlier observations, flow cytometry analysis of thymocytes indicates decreased mature CD4⁺ and CD8⁺ single positive (SP) thymocytes in 2 wk-old *Cnab*^{-/-} mice [16]. Although the proportion of mature SP thymocytes is much smaller (30–50% of control) in *Cnab*^{-/-} pre-weanlings (<2 wk) compared to their control mice (Figure 1A) and mature splenic T cells (Figure 1B, upper panels), the numbers steadily increase and reach comparable levels as the mice become older (Figure 1B, middle and lower panels). Thus, despite defects in thymocyte development and production, the total numbers of splenocytes (Figure 1C), CD4⁺ T cells (Figure 1D & E) and CD8⁺ T cells (data not shown) increase with age albeit to a lesser extent (compare upper left quadrants in Figure 1B) suggesting that with age the KO mice are overcoming the lymphopenia that is observed in younger KO mice.

3.2 CNA β is critical to maintain T-cell quiescence

Calcineurin signaling has been shown to be important for T-cell maturation and activation as evidenced by gene ablation studies and use of pharmacological agents that target calcineurin [16;27;28]. As we found increased numbers of T cells in older *Cnab*^{-/-} mice, we analyzed T-cell activation by flow cytometry. Already by 2 months of age, we found that the frequency and total numbers of activated CD4⁺ T cells were significantly increased in *Cnab*^{-/-} mice as assessed by expression of CD44 and CD62L (Figure 2A–C). As we have previously shown that Bcl-2 levels decrease in T cells activated by strong stimuli *in vivo* [29], and a recent report suggested that CNA β was required for expression of BCL-2 [30] we next measured Bcl-2 levels within T cells from *Cnab*^{-/-} versus control mice by intracellular flow cytometry using a BCL-2-specific antibody (see supplemental figure 1). We found that both naïve and activated T cells in *Cnab*^{-/-} mice have significantly increased levels of BCL-2 compared to WT controls (Figure 2 D,E), consistent with their increased accumulation. Further, most of these cells also expressed IL-7R α at relatively high levels (data not shown), and a small percentage of them express CD69 at this time point [23]. However, the increase in BCL-2 levels did not correlate with T-cell accrual as BCL-2 levels were increased to a greater extent in naïve T cells that did not accumulate compared to activated T cells which did accumulate (Figure 2 D, E). Thus, our data suggest that CNA β is critical to maintain peripheral T-cell quiescence, but not to maintain normal expression of BCL-2 in conventional CD4⁺ T cells.

3.3 CNA β is required for Foxp3 expression and T_{reg}-cell generation

CN activates NFAT and NFAT induces *Il2* and *Foxp3* expression and cooperates with TGF β signaling for T_{reg}-cell generation and T-cell tolerance [20]. To test whether T-cell activation in *Cnab*^{-/-} mice is due to a defect in T_{reg}-cell generation, we have analyzed mRNA for FOXP3 via RT-PCR and protein expression of FOXP3, CD25 and other T-cell markers via flow cytometry. Our RT-PCR data show that *Foxp3* expression is down-regulated in the thymus of 2-week-old *Cnab*^{-/-} mice (Figure 3A). We further analyzed T_{reg}-cell generation in two-three-month-old adult mice. The proportion of FOXP3⁺CD4⁺CD25⁺T_{reg} cells is decreased in KO mice, and the absolute numbers of FOXP3⁺T_{reg} cells are significantly reduced in *Cnab*^{-/-} mice at 2–3 months of age (Figure 3B & C). Interestingly, we found that intracellular levels of BCL-2 within FOXP3⁺T_{reg} was decreased in *Cnab*^{-/-} compared to WT controls (Figure 4A & B), consistent with the decreased accumulation of T_{reg} in

Cnab^{-/-} mice. Since BCL-2 levels are decreased in activated T cells compared to naïve T cells, we have determined the activation status of FOXP3⁺ T_{reg} cells. We have observed that the majority of FOXP3⁺ T cells are of the effector/memory phenotype, and that there is a significant increase of that population in *Cnab*^{-/-} mice (Figure 4C). Together, these data suggest that decreased numbers of T_{reg} cells is due to both production and survival. Further, these data suggest that the reduced numbers of T_{reg} cells may contribute to the enhanced immune activation in *Cnab*^{-/-} mice.

3.4 CNAβ deficiency affects inducible T_{reg}-cell generation

Recent studies have established that TGFβ1 induces FOXP3 expression and that the *Foxp3* enhancer contains SMAD3 and NFAT binding sequences, suggesting cooperative regulation of *Foxp3* expression by TGFβ and TCR signals [20]. Mice with a T cell-specific deficiency in the CN regulatory subunit CNB have a block in thymic T-cell development at the double positive stage [27], and mice deficient in CN catalytic subunit alpha (CNAα) die early due to inflammation and kidney failure [15]. Since mice deficient in CNAβ exhibit T-cell activation and splenomegaly later in the life, we have decided to determine whether CNAβ signaling is required for TGFβ1-induced FOXP3 induction in T cells. Our data revealed that CNAβ deficiency significantly affects the generation of FOXP3⁺ iT_{reg}-cells (Figure 5A & B). The defect in FOXP3 expression by TGFβ1 in *Cnab*^{-/-} cultures could not be due to a defect in IL-2 production since addition of IL-2 (100 IU/ml) to the cultures did not restore FOXP3 expression in *Cnab*^{-/-} cultures. Decreases in FOXP3⁺ T_{reg} cells in the *Cnab*^{-/-} cultures are correlated with an increase in IFNγ (Th1), IL-4 (Th2) and IL-10 (Tfh, Tr1 and Th9) cytokine secretions (Figure 5C (**7-wk-old**); **data not shown**). These data suggest that CNAβ signaling is critical for prevention of spontaneous activation of T cells and maintenance of tolerance through inhibition of inflammatory response.

3.5 CNAβ signaling is required to prevent inflammatory response of T cells

As there was an increased frequency of activated T cells and a decreased frequency of T_{reg} cells in *Cnab*^{-/-} mice we next assessed the inflammatory profile of their T cells. To do this, we performed intracellular cytokine staining for IFN-γ and IL-17 in CD4⁺ and CD4⁺ FOXP3⁺ T cells in *Cnab*^{-/-} and control mice. After brief stimulation with anti-CD3/CD28, we found that the frequencies of CD4⁺ T cells that were IFNγ⁺ or IL-17⁺ were significantly increased in *Cnab*^{-/-} mice (Figure 6A–C). Interestingly, we also found that a substantial fraction of CD4⁺FOXP3⁺ T cells were secreting IL-17, but not IFN-γ (Figure 6D–F), suggesting that in the absence of CNAβ signaling, a subpopulation of T_{reg} cells may be pro-inflammatory rather than suppressive.

3.6 *Cnab*^{-/-} mice do not develop splenomegaly and lymphomas on a DO11.10 Rag1^{-/-} background

Since we have observed activation of T cells and defective T_{reg}-cell generation in *Cnab*^{-/-} mice, we hypothesized that the splenomegaly in these mice is due to expansion of activated T-cell populations. To test this, we mated *Cnab*^{-/-} mice to *DO11.10 Rag1*^{-/-} TCR transgenic mice which have no other lymphocytes except transgenic TCR-bearing CD4⁺ T cells that will recognize ovalbumin peptides in the context of I-A^d. We have observed that *DO11.10 Rag1*^{-/-} *Cnab*^{-/-} mice did not develop splenomegaly and lymphomas. However, T cells in these mice responded well to stimulation compared to *Cnab*^{+/+} *DO11.10 Rag1*^{-/-} control T cells as assessed by CD25 expression, but exhibited a decrease in FOXP3 expression (26% in control vs. 13% in KO) upon addition of TGFβ1 (Figure 7). Presence of *Cnab*^{-/-} B cells (*Cnab*^{-/-} *DO11.10 Rag1*^{+/+} mice) resulted in a severe reduction in FOXP3 expression in T cells (Figure 7, **lower panels**). These data suggest that FOXP3 induction by TGFβ1 is partly dependent on CNAβ signaling.

Our results presented here suggest a regulatory role for CNA β in T cells in addition to its known function for T-cell maturation and development. Our data has shown that CNA β deficiency results in divergent effects on T_{reg} versus conventional T cells (T_{conv}). In T_{conv}, CNA β is critical to promote T-cell quiescence and inhibit proinflammatory cytokine production. In T_{reg} cells, CNA β is critical for expression of BCL-2 and permitting responsiveness to TGF β 1. Combined, these defects translate to altered overall control of T-cell homeostasis and quiescence.

4. Discussion

Our data suggest that CNA β has multiple anti-inflammatory effects in the immune system. We found that CNA β (i) limits spontaneous pro-inflammatory T_H1 and T_H17-cell generation; (ii) controls T_{reg}-cell generation from the thymus; (iii) is critical for generation of inducible T_{reg} cells; and reciprocally affects expression of BCL-2 in T_{regs} vs. conventional T cells. Additionally, *Cnab*^{-/-} T cells produce increased amounts of IL-6 which is known to suppress lymphokine-activated killer (LAK)-cell response [23;31].

T-cell homeostasis is achieved by production and survival of distinct T-cell populations (naïve, effector and memory). Mice deficient in either BIM, BCL-2, FAS or FASL all develop defects in T-cell homeostasis [32–36]. Since CN mediates TCR activation signals and since T-cell activation down-modulates BCL-2 expression, we reasoned that CN also may regulate T-cell homeostasis by modulating expression of BCL-2. Indeed, a recent report showed that BCL-2 protein expression is downregulated in *Cnab*^{-/-} mouse lymphocytes [30]. Surprisingly, our data revealed that there is no such down-regulation of BCL-2 expression in naïve T cells in *Cnab*^{-/-} mice compared to control mice. Since BCL-2 deficiency causes lymphopenia [33] and early lethality [37], and CNA β -deficient mice develop splenomegaly and lymphomas, the previously observed loss of BCL-2 levels may have reflected a loss of a population of CD4⁺ T cells that express lower levels of BCL-2. Indeed, as we observed a significant decrease in BCL-2 levels in effector/effector-memory CD4⁺ T cells compared to naïve T cells (Figure 2D–E) in both control and CNA β KO mice, it is possible that Western-blot analysis of total T cells will yield lesser signal intensity (BCL-2) in the CNA β KO T cells since there is a significant increase in activated T cells in CNA β KO mice. Nonetheless, our data clearly show that BCL-2 is expressed in conventional *Cnab*^{-/-} T cells. Dysregulation of these normal survival/apoptotic processes could foster expansion of activated T cells, inflammation and inflammation-induced cancers such as lymphoma and IBD-associated colon cancer.

Even though T_{reg} cells are reduced in CNA β -deficient mice, they are not absent. As we observed that T cells undergo activation in the presence of T_{reg} cells, our data suggest that either natural T_{reg} cells are not functioning normally, or that the observed defect in inducible T_{reg} (iT_{reg})-cell generation may contribute to the immune dysregulation. A cardinal feature of the immune dysregulation in CNA β -deficient mice we have observed is the increased production of IL-6 and IFN γ [23]. Intracellular cytokine data presented here confirm that conventional CD4⁺ T cells are differentiated towards T_H1 and T_H17 in the absence of CNA β (Figure 6), identifying a novel anti-inflammatory role for CNA β . Our data would suggest that the loss of T_{reg} cells in *Cnab*^{-/-} mice leads to spontaneous activation of T cells and B cells that promotes an inflammatory environment that favors lymphoma development. In support of this, we find that (i) T cells from *Cnab*^{-/-} mice have significantly increased production of IL-6 when stimulated *ex vivo*; (ii) that the majority of lymphomas that develop are B-cell lymphomas; and (iii) that mice overexpressing IL-6 generate B-cell lymphomas [38]. Although more work is required to determine the role of IL-6 on lymphoma development, our data here are supportive of such a model and describe for the first time, an anti-inflammatory role for CNA β in both conventional and T_{reg} cells. It is possible that

CNA β signaling is required for normal selection of T cells in the thymus during development and that a defect in negative selection causes peripheral dysregulation.

Our observation that the splenomegaly was prevented in *Rag1*^{-/-} *Cnab*^{-/-} *DO11.10* mice suggests that endogenous antigens are driving the defects in T-cell homeostasis in *Cnab*^{-/-} mice. However, it is still unclear whether lymphomas develop in the absence of either of the lymphocyte subsets in *Cnab*^{-/-} mice. The data also suggest that *Cnab*^{-/-} mice that have no B cells do not produce abnormally high levels of proinflammatory cytokines, with the exception of IL-17. This could be an indirect effect of defective FOXP3 expression which is known to inhibit IL-17 induction through its interaction with ROR γ t [39]. These data suggest that CNA β signaling is involved in limiting the inflammatory response of T cells through induction of FOXP3.

Since CsA inhibits the expression of *Il2* and *Foxp3* [40], and since NFAT binds to promoter/enhancer elements of *Tgfb1*, *Foxp3* and *Il2* and induces their expression (IL-2 is required for *Foxp3* induction by TGF β 1 in CD4⁺CD25⁻ T cells), it is possible that the FOXP3⁺ T_{reg} cells in *Cnab*^{-/-} mice could also be defective in TGF β 1 production resulting in a functional deficiency of these cells. Understanding the specific roles of CN in T_{reg}-cell generation and function and its role in T-cell homeostasis is important for finding the relationship between T-cell activation and B-cell lymphoma in CNA β -deficient mice. Since T_{reg}-cell deficiency is known to lead to T-cell activation and increased anti-tumor responses, it is generally believed that inhibition of T_{reg} cells is a potential therapy for most cancers. However, *Cnab*^{-/-} mice do not reject allogenic tumors [16]. IL-6 and Th2 cells have been found to induce activation and transformation of B cells since chronic activation of B cells is observed in lymphomas and all the tumors observed in *Cnab*^{-/-} mice are of B-cell origin [41–43]. These observations shed light on the CN role in T-cell regulation which is important for controlling inflammatory responses. Further studies are required for understanding the signaling mechanisms involved in lymphomagenesis under inflammatory conditions.

Since inflammation plays a significant role in tumorigenesis and inflammatory cytokines are elevated in transplant patients who are treated with CsA [31;42], developing therapeutic approaches to block the inflammation in these patients would definitely reduce their chance of developing lymphomas. Thus, the CNA β -deficient mouse is a unique model to study the immune dysregulation and lymphomagenesis observed in transplant patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AICD	activation-induced cell death
CNA β	calcineurin A β
CsA	cyclosporine A
KO	knockout

Acknowledgments

This study was supported by NIH AI067903 and U01CA084291 to TD, and ACS-IRG7400128, NCI-CCSG (CA 023074) and NCI-Lymphoma SPORE (P50CA3080501A1) Career Development Award from AZCC to RB. The authors thank Dr. Lisa M. Rimsza for reading and her thoughtful comments and suggestions.

References

1. Ferguson R. FTY720 immunomodulation: optimism for improved transplant regimens. *Transplant. Proc* 2004;36:549S–553S. [PubMed: 15041404]
2. Hopkins PM, McNeil K. Evidence for immunosuppression in lung transplantation. *Curr. Opin. Organ Transplantation* 2008;13:477–483.
3. Serfling E, Klein-Hessling S, Palmetshofer A, Bopp T, Stassen M, Schmitt E. NFAT transcription factors in control of peripheral T cell tolerance. *Eur. J Immunol* 2006;36:2837–2843. [PubMed: 17039563]
4. Matsuda S, Shibasaki F, Takehana K, Mori H, Nishida E, Koyasu S. Two distinct action mechanisms of immunophilin-ligand complexes for the blockade of T-cell activation. *EMBO Rep* 2000;1:428–434. [PubMed: 11258483]
5. Wang CR, Hashimoto K, Kubo S, Yokochi T, Kubo M, Suzuki M, Suzuki K, Tada T, Nakayama T. T cell receptor-mediated signaling events in CD4+CD8+ thymocytes undergoing thymic selection: requirement of calcineurin activation for thymic positive selection but not negative selection. *J. Exp. Med* 1995;181:927–941. [PubMed: 7532685]
6. Sommerer C, Konstandin M, Dengler T, Schmidt J, Meuer S, Zeier M, Giese T. Pharmacodynamic monitoring of cyclosporine a in renal allograft recipients shows a quantitative relationship between immunosuppression and the occurrence of recurrent infections and malignancies. *Transplantation* 2006;82:1280–1285. [PubMed: 17130775]
7. Verdonk RC, Haagsma EB, Jonker MR, Bok LI, Zandvoort JH, Kleibeuker JH, Faber KN, Dijkstra G. Effects of different immunosuppressive regimens on regulatory T-cells in noninflamed colon of liver transplant recipients. *Inflamm. Bowel. Dis* 2007;13:703–709. [PubMed: 17230494]
8. Suthanthiran M, Hojo M, Maluccio M, Boffa DJ, Luan FL. Post-transplantation malignancy: a cell autonomous mechanism with implications for therapy. *Trans. Am. Clin. Climatol. Assoc* 2009;120:369–388. [PubMed: 19768190]
9. Sakaguchi S, Sakaguchi N. Organ-specific autoimmune disease induced in mice by elimination of T cell subsets. V. Neonatal administration of cyclosporin A causes autoimmune disease. *J. Immunol* 1989;142:471–480. [PubMed: 2783437]
10. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, Schmidt-Weber CB. Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol* 2006;176:3593–3602. [PubMed: 16517728]
11. Zeiser RS, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, Contag CH, Negrin RS. Inhibition of CD4+CD25+ regulatory T cell function by calcineurin dependent interleukin-2 production. *Blood* 2006;108:390–399. [PubMed: 16522809]
12. Feske S, Giltman J, Dolmetsch R, Staudt LM, Rao A. Gene regulation mediated by calcium signals in T lymphocytes. *Nat. Immunol* 2001;2:316–324. [PubMed: 11276202]
13. Im SH, Rao A. Activation and deactivation of gene expression by Ca²⁺/calcineurin-NFAT-mediated signaling. *Mol. Cells* 2004;18:1–9. [PubMed: 15359117]
14. Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* 2003;17:2205–2232. [PubMed: 12975316]
15. Gooch JL, Toro JJ, Guler RL, Barnes JL. Calcineurin A-alpha but not A-beta is required for normal kidney development and function. *Am. J Pathol* 2004;165:1755–1765. [PubMed: 15509543]
16. Bueno OF, Brandt EB, Rothenberg ME, Molkentin JD. Defective T cell development and function in calcineurin A beta -deficient mice. *Proc. Natl. Acad. Sci. U. S. A* 2002;99:9398–9403. [PubMed: 12091710]
17. Bopp T, Palmetshofer A, Serfling E, Heib V, Schmitt S, Richter C, Klein M, Schild H, Schmitt E, Stassen M. NFATc2 and NFATc3 transcription factors play a crucial role in suppression of CD4+ T lymphocytes by CD4+ CD25+ regulatory T cells. *J Exp. Med* 2005;201:181–187. [PubMed: 15657288]
18. Macian F, Garcia-Cozar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* 2002;109:719–731. [PubMed: 12086671]

19. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, Mathis D, Benoist C, Chen L, Rao A. FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT. *Cell* 2006;126:375–387. [PubMed: 16873067]
20. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat. Immunol* 2008;9:194–202. [PubMed: 18157133]
21. Zhang BW, Zimmer G, Chen J, Ladd D, Li E, Alt FW, Wiederrecht G, Cryan J, O'Neill EA, Seidman CE, Abbas AK, Seidman JG. T cell responses in calcineurin A alpha-deficient mice. *J Exp. Med* 1996;183:413–420. [PubMed: 8627154]
22. Chan VS, Wong C, Ohashi PS. Calcineurin Aalpha plays an exclusive role in TCR signaling in mature but not in immature T cells. *Eur. J. Immunol* 2002;32:1223–1229. [PubMed: 11981809]
23. Bommireddy R, Bueno OF, Martin J, Ormsby I, Chen H, Gard C, Molkentin JD, Boivin GP, Babcock GF, Doetschman T. Calcineurin deficiency decreases inflammatory lesions in transforming growth factor beta1-deficient mice. *Clin. Exp. Immunol* 2009;158:317–324. [PubMed: 19747209]
24. Bommireddy R, Pathak LJ, Martin J, Ormsby I, Engle SJ, Boivin GP, Babcock GF, Eriksson AU, Singh RR, Doetschman T. Self-antigen recognition by TGFbeta1-deficient T cells causes their activation and systemic inflammation. *Lab Invest* 2006;86:1008–1019. [PubMed: 16865088]
25. Engle SJ, Hoying JB, Boivin GP, Ormsby I, Gartside PS, Doetschman T. Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res* 1999;59:3379–3386. [PubMed: 10416598]
26. Bommireddy R, Babcock GF, Singh RR, Doetschman T. TGFbeta1 deficiency does not affect the generation and maintenance of CD4(+)CD25(+)FOXP3(+) putative T(reg) cells, but causes their numerical inadequacy and loss of regulatory function. *Clin. Immunol* 2008;127:206–213. [PubMed: 18308639]
27. Neilson JR, Winslow MM, Hur EM, Crabtree GR. Calcineurin B1 is essential for positive but not negative selection during thymocyte development. *Immunity* 2004;20:255–266. [PubMed: 15030770]
28. Gallo EM, Ho L, Winslow MM, Staton TL, Crabtree GR. Selective role of calcineurin in haematopoiesis and lymphopoiesis. *EMBO Rep* 2008;9:1141–1148. [PubMed: 18818667]
29. Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, Kappler J, Marrack P. Activated T cell death in vivo mediated by proapoptotic Bcl-2 family member Bim. *Immunity* 2002;16:759–767. [PubMed: 12121658]
30. Manicassamy S, Gupta S, Huang Z, Molkentin JD, Shang W, Sun Z. Requirement of calcineurin a for the survival of naive T cells. *J Immunol* 2008;180:106–112. [PubMed: 18097009]
31. Tanner JE, Alfieri C. Interactions involving cyclosporine A, interleukin-6, and 19 Epstein-Barr virus lead to the promotion of B-cell lymphoproliferative disease. *Leuk. Lymphoma* 1996;21:379–390. [PubMed: 9172802]
32. Kamada S, Shimono A, Shinto Y, Tsujimura T, Takahashi T, Noda T, Kitamura Y, Kondoh H, Tsujimoto Y. Bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. *Cancer Research* 1995;55:354–359. [PubMed: 7812968]
33. Wojciechowski S, Tripathi P, Bourdeau T, Acero L, Grimes HL, Katz JD, Finkelman FD, Hildeman DA. Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. *J Exp. Med* 2007;204:1665–1675. [PubMed: 17591857]
34. Siegel RM, Chan FK, Chun HJ, Lenardo MJ. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat. Immunol* 2000;1:469–474. [PubMed: 11101867]
35. Hao Z, Duncan GS, Seagal J, Su YW, Hong C, Haight J, Chen NJ, Elia A, Wakeham A, Li WY, Liepa J, Wood GA, Casola S, Rajewsky K, Mak TW. Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity* 2008;29:615–627. [PubMed: 18835195]
36. Gupta VA, Hermiston ML, Cassafer G, Daikh DI, Weiss A. B cells drive lymphocyte activation and expansion in mice with the CD45 wedge mutation and Fas deficiency. *J. Exp. Med* 2008;205:2755–2761. [PubMed: 19001138]

37. Bouillet P, Cory S, Zhang LC, Strasser A, Adams JM. Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. *Dev. Cell* 2001;1:645–653. [PubMed: 11709185]
38. Rutsch S, Neppalli VT, Shin DM, DuBois W, Morse HC III, Goldschmidt H, Janz S. IL-6 and MYC collaborate in plasma cell tumor formation in mice. *Blood* 2010;115:1746–1754. [PubMed: 20018915]
39. Ichiyama K, Yoshida H, Wakabayashi Y, Chinen T, Saeki K, Nakaya M, Takaesu G, Hori S, Yoshimura A, Kobayashi T. Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. *J. Biol. Chem* 2008;283:17003–17008. [PubMed: 18434325]
40. Nakano N, Hosokawa H, Kohyama M, Hozumi N. NF-AT-mediated expression of TGF-beta1 in tolerant T cells. *J Immunol* 2007;178:3067–3075. [PubMed: 17312153]
41. Zangani MM, Froyland M, Qiu GY, Meza-Zepeda LA, Kutok JL, Thompson KM, Munthe LA, Bogen B. Lymphomas can develop from B cells chronically helped by idiotype-specific T cells. *J. Exp. Med* 2007;204:1181–1191. [PubMed: 17485509]
42. Hilbert DM, Kopf M, Mock BA, Kohler G, Rudikoff S. Interleukin 6 is essential for in vivo development of B lineage neoplasms. *J. Exp. Med* 1995;182:243–248. [PubMed: 7790819]
43. Davis RE, Ngo VN, Lenz G, Tolar P, Young RM, Romesser PB, Kohlhammer H, Lamy L, Zhao H, Yang Y, Xu W, Shaffer AL, Wright G, Xiao W, Powell J, Jiang JK, Thomas CJ, Rosenwald A, Ott G, Muller-Hermelink HK, Gascoyne RD, Connors JM, Johnson NA, Rimsza LM, Campo E, Jaffe ES, Wilson WH, Delabie J, Smeland EB, Fisher RI, Braziel RM, Tubbs RR, Cook JR, Weisenburger DD, Chan WC, Pierce SK, Staudt LM. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. *Nature* 2010;463:88–92. [PubMed: 20054396]

Figure 1.

Mature T cells increase in older *Cnab*^{-/-} mice. Flow cytometry analysis of thymocytes (A) and splenocytes (B) from young (upper panels) and adult *Cnab*^{-/-} mice (middle & lower panels). Total splenocytes from 2–4 mo-old mice (C), total CD4⁺ T cells in the spleens of <3 mo-old (D) and >3 mo-old mice (E). Total cell numbers in the box inside the dot plots; and percentage of mature T cells (upper left and lower right quadrants) are shown.

Figure 2.

CNA β is critical to maintain T-cell quiescence. Splenocytes from 2 mo old *Cnab*^{-/-} and control mice were stained with fluorescently labeled antibodies against CD4, CD8, CD44, CD62L and BCL-2 and analyzed by flow cytometry. Results show the (A) frequency and (B) total numbers of naïve (CD44^{lo}CD62L^{hi}) versus effector/effector memory (EM) (CD44^{hi}CD62L^{lo}) T cells $\pm$ SEM. (C) representative dot plots show CD44 by CD62L expression on gated CD4⁺ T cells from either WT versus *Cnab*^{-/-} mice. (D) Representative histograms show BCL-2 levels in naïve (left panels) versus Eff/EM (right panels) CD4⁺ T cells from WT (top panels) or *Cnab*^{-/-} (bottom panels) mice. (E) Results show that BCL-2 mean fluorescence intensity of the BCL-2 signal within naïve versus Eff/EM CD4⁺ T cells between WT (open bars) and *Cnab*^{-/-} (filled bars) mice $\pm$ SEM.

Figure 3.

Decreased T_{reg}-cell generation in *Cnab*^{-/-} mice. **(A)** RNA was prepared from total thymus tissue isolated from 2-wk old *Cnab*^{+/+} and *Cnab*^{-/-} mice. Expression of *Foxp3*, *Cnaa*, *Cd4* and *Hprt* genes were analyzed by RT-PCR with 3-fold dilutions of template cDNA amplified for 30 cycles. **(B)** Flow cytometry analysis of FOXP3 expression in splenocytes. Total FOXP3⁺ CD4⁺ T cells from 2–3-month-old mice are shown in **(B)**. **(C)** Representative dot plots from 7-wk-old control and *Cnab*^{-/-} mice are shown.

Figure 4.

CNA β is critical to maintain BCL-2 expression in CD4⁺ FOXP3⁺ T_{reg} cells. Groups of 8 week old WT or *Cnab*^{-/-} mice were sacrificed and BCL-2 expression assessed in spleen cells by flow cytometry. Results show intracellular BCL-2 levels in (A) representative histograms of BCL-2 stain in gated CD4⁺ FOXP3⁺ T cells from WT or *Cnab*^{-/-} mice and (B) the overall BCL-2 MFI in CD4⁺ FoxP3⁺ T cells from individual WT versus *Cnab*^{-/-} mice $\pm$ SEM. (C) Results show the staining for CD44 and CD62L expression on CD4⁺FOXP3⁺ T cells in the spleen.

Figure 5.

CNAβ deficiency affects T_{reg}-cell generation by TGFβ1. Splenocytes were cultured in the presence of anti-CD3/CD28 microbeads (Dynal beads, Invitrogen), IL-2 (100 IU/ml) and TGFβ1 (5 ng/ml) for 3 days. Cells were harvested and stained for surface expression of CD4 and CD25, washed and incubated in Perm/Fix buffer followed by intracellular FOXP3 staining. **A.** Numbers in the upper right quadrant indicate % of CD4⁺ cells that express FOXP3 and CD25. **B.** Data obtained from 4 KO mice and 3 control mice (1 heterozygous and 2 WT mice) are shown as mean±SD. **C.** IFNγ levels in culture supernatants from one of the two experiments.

Figure 6.

CNA β signaling is required to prevent inflammatory response of T cells. Splenocytes from 6 mo-old *Cnab*^{-/-} and WT mice were stimulated with anti-CD3 and anti-CD28 Abs in the presence of Brefeldin A for 5 hrs and were surface stained for CD4 and then intracellularly for cytokines (IFN γ and IL-17) and FOXP3. Representative dot plots of CD4⁺ (A) and FOXP3⁺ (D) T cells of 4 mice from each group, mean $\pm$ SD of IFN γ ⁺ cells (B & E) and IL-17⁺ cells (C & F) is shown.

Figure 7.

CNAβ signaling is required for FOXP3 induction by TGFβ1 in T cells. Splenocytes from *DO11.10 Rag1^{-/-}*, *Cnab^{-/-} DO11.10 Rag1^{+/-}* and *Cnab^{-/-} DO11.10 Rag1^{-/-}* mice were cultured in the presence of CD3/CD28 microbeads, IL-2 (100 IU/ml) and TGFβ1 (2 ng/ml) for 3 days and FOXP3 expression was analyzed by intracellular staining and flow cytometry. Numbers in the upper right quadrants indicate % of CD4⁺ T cells expressing FOXP3 and CD25.