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*J Immunol* 2009; 183:4192-4196; doi: 10.4049/jimmunol.0902041
http://www.jimmunol.org/content/183/7/4192

Supplementary Material: [http://www.jimmunol.org/content/suppl/2009/09/14/jimmunol.0902041.DC1](http://www.jimmunol.org/content/suppl/2009/09/14/jimmunol.0902041.DC1)

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Cutting Edge: Dab2 Is a FOXP3 Target Gene Required for Regulatory T Cell Function

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FOXP3-expressing regulatory T (Treg) cells are vital for maintaining peripheral T cell tolerance and homeostasis. The mechanisms by which FOXP3 target genes orchestrate context-dependent Treg cell function are largely unknown. In this study we show that in mouse peripheral lymphocytes the Drosophila Disabled-2 (Dab2) homolog, a gene that is involved in enhancing TGFβ responses, is exclusively expressed in FOXP3+ regulatory T cells. Dab2 is a direct target of FOXP3, and regulatory T cells lacking DAB2 are functionally impaired in vitro and in vivo. However, not all aspects of Treg cell function are perturbed, and DAB2 appears to be dispensable for Treg cell function in maintaining naive T cell homeostasis. The Journal of Immunology, 2009, 183: 4192–4196.

For the forkhead box P3 (FOXP3)4-expressing regulatory T (Treg) cells are a subset of suppressor CD4+ T cells that are necessary for controlling peripheral T cell tolerance (1). Null mutations in the Foxp3 gene in humans and mice leads to fatal, early onset autoimmunity (2). Treg cell-mediated suppression in vivo is postulated to be highly context dependent and likely involves fail-safe, multiple effector components. The absolute requirement for FOXP3 in Treg cell function has stimulated intensive investigations to identify FOXP3 target genes and proteins. Phosphodiesterase 3b (Pde3b), which controls cAMP availability (3), Clda4, which likely regulates APC function (4–6), Ebi3, which encodes for the IL-27 β-chain of the regulatory cytokine IL-35 (7), and microRNA mir155, which maintains IL-2 responsiveness of Treg cells (8), are direct gene targets of FOXP3 that have been shown to be variably required for Treg cell function and homeostasis.

In the current study, we show that Dab2 (Drosophila disabled homolog-2) is a target gene of FOXP3 that is critical for Treg cell function in vitro and in vivo. Unlike previously identified targets of FOXP3, Dab2 expression is restricted to CD4+ Treg cells in peripheral lymphocyte subsets. In nonlymphoid cells, DAB2 has several critical functions in cell development and transformation by enhancing SMAD activation during TGFβ signaling (9, 10), in gap junction functions (11), and in clathrin-coated pit-mediated endocytosis of cell surface receptors (12). Dab2 expression is also regulated by the vitamin A metabolite all-trans retinoic acid (ATRA) (13). Considering the role of TGFβ and ATRA in regulating FOXP3 expression in Treg cells as well as their function (14, 15), we hypothesized that DAB2 is required for Treg cell function.

Analyses of mice with a T cell-restricted Dab2 deficiency showed that they generate normal numbers of Treg cells, but these Treg cells are not functional in vitro. Although Dab2−/− deficient mice appear healthy and do not show significant perturbations in the maintenance of peripheral naive T cell homeostasis, Dab2−/− deficient Treg cells are impaired in controlling colitogenic T cells in an adoptive transfer model.

Materials and Methods

Mice

Dab2fl/fl (B6 X 129) mice (16) were backcrossed four times onto a B6 background. T cell-specific deletion of Dab2 was obtained by crossing Dab2+Flx mice with either Cd2CreTg+ or Cd4CreTg+ mice (where Tg is transgenic; Ref. 17). Foxp3CreGFP reporter mice (where eGFP is enhanced GFP; Ref. 18) were a gift from V. Kuchroo (Harvard Medical School, Boston, MA). Rag1−/− and C57BL/6 mice were obtained from The Jackson Laboratory. All experiments were approved by the University of Massachusetts Institutional Animal Care and Use Committee (Worcester, MA).

Abs, flow cytometry, and cell sorting

Cells were stained for surface markers (Abs from BD Biosciences and eBioscience) followed by FOXP3 staining according to instructions from eBioscience. The CAMP Ab was from Abcam. Samples were acquired on a BD LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (TreeStar). Thymic and peripheral T cell subsets were sorted to >95% purity using a MoFlo cell sorter (DakoCytomation).

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Received for publication June 30, 2009. Accepted for publication August 10, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This project was supported National Institutes of Health Grants AI054670 and AI59880 and Diabetes Endocrinology Research Centre Grant DK32520 (to C.A.C. and J.K.) and Grant GM66257 (to J.A.C.).

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4 Abbreviations used in this paper: FOXP3, Forkhead box P3; AM, acetoxymethylester; Pde3b, phosphodiesterase 3b; Tg, transgenic; Treg, regulatory T (cell); wt, wild type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902041

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Retroviral infection

NFC (αβ; CD4+CD8+) thymoma cells were infected with a control mouse stem cell virus (MSCV) retroviral vector containing GFP or with a MSCV retroviral vector carrying the full-length Foxp3 cDNA cloned upstream of an internal ribosomal entry site and GFP. Stable cell lines that express vector alone and MSCV-Foxp3 were generated by cell sorting and maintained in complete DMEM (10% FBS, 50 μM 2-ME, 2 mM L-glutamine, 20 mM HEPES, and 0.1 mM nonessential amino acids).

RT-PCR and real-time PCR

RNA was isolated from cells with TRIzol Reagent (Invitrogen) and cDNA was prepared using an Omniscript RT-PCR kit (Qiagen). For semiquantitative RT-PCR (sqRT-PCR), 3-fold serial dilutions of cDNA were used. PCR primers are listed in supplemental data 1.5 Real-time PCR amplification was performed using iQ SYBR Green supermix (Bio-Rad). All data were normalized to Actb mRNA expression and are represented as arbitrary units.

Chromatin immunoprecipitation (ChIP) assays

FOXp3-overexpressing NFC cells and vector control cells were stimulated with PMA (50 ng/ml) and ionomycin (100 ng/ml) at 37°C for 24 h. ChIP precipitation was performed using anti-FOXp3 Ab (Santa Cruz Biotechnology). The recovered DNA was dissolved in 20 μl of H2O and analyzed by PCR. PCR primers used are listed in supplemental data 1.

In vitro FOXp3 induction

Sorted CD4+CD25+ T cells from Foxp3EGFP reporter mice were activated with plate-bound anti-CD3 (0.5 μg/ml; clone 500A2) and anti-CD28 (1.0 μg/ml; clone 37N) in the presence of rTGFβ (2 ng/ml; R&D Systems) and ATRA (100 nM; Sigma-Aldrich).

In vitro suppression assay

Sorted CD4+CD25+ T cells were activated in vitro as described above in the presence of 100 U/ml rIL-2 in complete DMEM for 48 h. Poststimulation, the indicated numbers of Treg cells were cocultured with 5 × 10^4 freshly isolated CD4+ T cells and 5 × 10^4 irradiated splenocytes from B6 mice and activated with plate-bound anti-CD3 for 72 h. [3H]TdR incorporation was measured over the last 6 h.

Colitis induction and cure

Naïve CD4+CD25- cells (4 × 10^5) from wild-type (wt) mice and 2 × 10^5 sorted CD4+CD25+ regulatory T cells were co-injected into lymphocyte-deficient (Rag1−/−) mice. For colitis “rescue” experiments, 4 × 10^5 naïve CD4+CD25- CD45RB<sup>bright</sup> T cells were injected into Rag1−/− mice to induce colitis. Four weeks later, 1 × 10^6 CD4+CD25- regulatory T cells were injected. Colonic pathology was determined 2–3 wk after the transfer of Treg cells by standard H&E staining of formalin-fixed tissues. Colitis was scored as described in (19).

Calcine-aeotroxyxymethylster (AM) intercellular transfer and cAMP ELISA

Sorted CD4+CD25+ Treg cells were loaded with calcine as described (20) and cocultured with Ly5.1+ CD4+ T cells. Cells were activated as described above and calcine transfer was detected by flow cytometry after 16 h. To measure intracellular cAMP levels, sorted CD4+CD25+ and CD4+CD25- cells were washed in ice-cold PBS and lysed in 0.1 N HCl and a cAMP-specific ELISA was performed according to the manufacturer’s protocol (BioMol and Promega).

Results and Discussion

Dab2 expression is restricted in FOXP3+ Treg cells

We infected NFL αβTCR+ CD4+CD8- cells with a retrovirus vector containing Foxp3 cDNA, followed by global gene expression profiling to determine genes whose expression was

5 The online version of this article contains supplemental material.
changed by the ectopic Foxp3 expression (data not shown). One of only a few genes that were altered in expression was Dab2, as illustrated by a confirmatory sqRT-PCR assay (Fig. 1A). In peripheral T cell subsets, Dab2 expression was restricted exclusively to CD4+FOXP3+ Treg cells (Fig. 1B). In the thymus, Dab2 was expressed in early precursor cells (data not shown) and in mature FOXP3+ CD4 single positive (CD4SP; CD4+ CD8−) cells (Fig. 1C). Dab2 was also expressed in TGFβ-induced FOXP3+ Treg cells in vitro with or without ATRA (Fig. 1D), an inducer of Dab2 expression in nonlymphoid cells.

To determine whether FOXP3 can bind to the Dab2 regulatory region, we performed ChIP analysis by selecting relevant Dab2 genomic loci. Among four such conserved consensus binding sites tested, only one, which was located in the 5′-untranslated region ~2.3 kb upstream of the transcriptional site of the Dab2 gene, was found to be associated with FOXP3 in FOXP3+ NFFC cells (Fig. 1E). These results identify Dab2 as a potential direct target gene of FOXP3.

Dab2 deficiency alters Treg cell function

To determine the function of DAB2 in Treg cells, we generated mice lacking Dab2 specifically in T cells. We analyzed both Cd2 promoter-Cre Tg+ × Dab2fl/fl and Cd4 promoter-Cre Tg+ × Dab2fl/fl mice that delete the floxed Dab2 gene during the early (CD3+CD4−CD8− triple negative) and late (CD4+CD8+ double positive) stages of intrathymic T cell development, respectively. Treg cells in these conditional Dab2 knockout (CKO) mouse lines lacked Dab2 expression (Fig. 1B). As Treg cells in both models were indistinguishable in function and phenotype, we present data predominantly from the analysis of Dab2fl/flCd4 mice, herewith referred to as Dab2 CKO mice.

Mice lacking DAB2 in Treg cells were healthy in appearance and did not suffer from overt autoimmunity even at 10–12 mo of age, suggesting that the mutant Treg cells were capable of maintaining T cell tolerance. The frequency of thymic and peripheral CD4+FOXP3+ cells was unchanged in Dab2 CKO mice compared with wt mice (data not shown). The Treg cells were also phenotypically similar to wt Treg cells and expressed normal levels of CTLA-4, GITR (glucocorticoid-induced TNF receptor family-related protein), and IL-7R (supplemental data 2A and data not shown).

We first tested the functionality of Dab2 CKO Treg cells in an in vitro suppression assay. Whereas wt Treg cells suppressed the proliferation of responder T cells in a Treg cell number-dependent manner, Treg cells from Dab2 CKO mice were unable to do so (Fig. 2A). Next, we determined the ability of purified Treg cells from Dab2 CKO mice to control coincected pathogenic naive (CD4+CD25−) T cells when transferred into lymphopenic animals. In contrast to the complete lack of function of Dab2-deficient Treg cells in vitro, they were as effective as wt Treg cells in preventing the induction of colitis by naive T cells transferred into Rag1−/− mice (Fig. 2B). However, in a
more stringent model of Treg cell function, Dab2-deficient Treg cells failed to moderate established colitis (21) (Fig. 2C). Although wt Treg cells effectively cured colitis, Dab2 CKO Treg cells were defective in regulating ongoing, aggressive lymphocyte infiltration and accumulation in the colon that led to a progressive wasting disease (Fig. 2D).

**Impaired gap junction function by Dab2-deficient Treg cells**

We next investigated the mechanistic basis for the observed defects in Dab2-deficient Treg cells by examining potential molecules and/or pathways involved in Treg cell-mediated immune suppression. Treg cells from Dab2-deficient mice produced normal amounts of IL-10, expressed comparable amounts of Il10, Tgfβ1, and Ebi3 at the transcriptional level, and expressed normal amounts of granzyme B (supplemental data 2, B and C, and data not shown). One mechanism by which Treg cells suppress target cell proliferation and activation is by transferring cAMP through gap junctions to effector T cells (20). Because DAB2 is known to interact with connexins that make up the gap junctions (11), we tested whether Dab2-deficient Treg cells were poor suppressors in vitro because they had deficiencies in gap junction-mediated intercellular communication. We labeled Treg cells from wt and Dab2 CKO mice with a gap junction transferable dye, calcine-AM, and cocultured them with congenically marked wt responder cells.

Collectively, we have identified Dab2 as a FOXP3 target gene that is required for a subset of Treg cell function. Dab2 expression is higher in CD44+ Treg cells compared with CD44− Treg cells (data not shown), and whether all FOXP3+ Treg cells express Dab2 is currently under investigation. Although Dab2 is expressed only in Treg cells among peripheral lymphocytes, it is also expressed specifically in TN3 thymocytes and is required for normal TGFβ and/or activin responses of lymphocytes (N. Jain, N. Malhotra, H. Nguyen, R. Friedline, J. Cooper, C. Chambers, and J. Kang, manuscript in preparation).

Given the FOXP3-dependent expression of Dab2 in mature Treg cells and the established function of DAB2 in promoting TGFβ signaling (9), one possibility was that Dab2 CKO mice would phenocopy the loss of Treg cells observed in mice with defective TGFβ signaling (22). However, Dab2 CKO mice maintained normal numbers of Treg cells, suggesting that DAB2 may be required for amplification, rather than maintenance, of TGFβ signaling in an as yet undefined context. Dab2-deficient Treg cells do not function in vitro and cannot cure established colitis in vivo, but they retain sufficient suppressive activity to maintain T cell homeostasis in unperturbed mice. This selective Treg cell defect is another indication that Treg cells use multiple, context-dependent modes of immunosuppression. Although the reduced gap junction activities appear to be the only distinguishing feature of Dab2-deficient Treg cells that has previously been correlated with impaired in vitro suppression, whether this is the case of the impaired function of Dab2-deficient Treg cells remains to be established. The in vivo context in which DAB2 is essential for Treg cell function is an outstanding issue that will require further studies.

**Acknowledgments**

We thank the University of Massachusetts Medical School Flow Cytometry Core Facility for cell sorting, Drs. Leslie Berg and Raymond Welsh for helpful discussions, and Dr. Werner Held and Kavitha Narayan for critical reading of the manuscript.

**Disclosures**

The authors have no financial conflict of interest.

**References**


