Immunity
Article

Foxo Transcription Factors Control Regulatory T Cell Development and Function

Yann M. Kerdiles,[1,3,4] Erica L. Stone,[1,3] Daniel L. Beisner,[1,5] Maureen A. McGargill,[2] Irene L. Ch'en,[1] Christian Stockmann,[1,6] Carol D. Katayama,[1] and Stephen M. Hedrick[1,*]
[1]Molecular Biology Section, Division of Biological Sciences and Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093-0377, USA
[2]St. Jude Children's Research Hospital, Department of Immunology, Memphis, TN 38105, USA
[3]These authors contributed equally to this work
[4]Present address: Centre d'Immunologie de Marseille Luminy, CNRS-INSERM-Université de la Méditerranée, Campus de Luminy, Case 906, 13288, Marseille Cedex 09, France
[5]Present address: Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121, USA
[6]Present address: Institut für Physiologie, Universität Duisburg-Essen, D-45122 Essen, Germany
*Correspondence: shedrick@ucsd.edu
DOI 10.1016/j.immuni.2010.12.002

SUMMARY

Foxo transcription factors integrate extrinsic signals to regulate cell division, differentiation and survival, and specific functions of lymphoid and myeloid cells. Here, we showed the absence of Foxo1 severely curtailed the development of Foxp3$^+$ regulatory T (Treg) cells and those that developed were nonfunctional in vivo. The loss of function included diminished CTLA-4 receptor expression as the *Ctla4* gene was a direct target of Foxo1. T cell-specific loss of Foxo1 resulted in exocrine pancreatitis, hind limb paralysis, multiorgan lymphocyte infiltration, antinuclear antibodies and expanded germinal centers. Foxo-mediated control over Treg cell specification was further revealed by the inability of TGF-β cytokine to suppress T-bet transcription factor in the absence of Foxo1, resulting in IFN-γ secretion. In addition, the absence of Foxo3 exacerbated the effects of the loss of Foxo1. Thus, Foxo transcription factors guide the contingencies of T cell differentiation and the specific functions of effector cell populations.

INTRODUCTION

The rate of autoimmune and hypersensitivity diseases in human beings is on the order of 3%–20% of the adult population, respectively (Cooper and Stroehla, 2003; Torres-Borrego et al., 2008), and this implies that, at least in modern society, a loss of immune regulation is common. With respect to autoimmunity mediated by the adaptive immune system, there are at least three mechanisms that moderate self-reactivity. Central tolerance, peripheral tolerance, and dominant regulatory T (Treg) cells are all required to avoid damage from immune effector mechanisms (von Boehmer and Melchers, 2010; Mueller, 2010; Wing and Sakaguchi, 2010). A conclusion is that the immune system is tenuously balanced between preventing and causing disease, and this is almost certainly the result of evolutionary pressure exerted by myriad and ever present infectious agents (Hedrick, 2004).

Immune regulation depends upon differentiation processes that produce different effector-type T cells. In the late stages of thymocyte development, antigen recognition can result in cell death associated with negative selection or it can result in differentiation to natural Treg (nTreg) cells (Hsieh et al., 2006). In peripheral lymphoid organs, CD4$^+$ T cells that recognize antigen differentiate into one of four distinct, though not necessarily stable, phenotypes characterized by signature cytokine secretion: T helper 1 (Th1 producing interferon-γ [IFN-γ]); Th2 (interleukin-4 [IL-4]), Th17 (IL-17); or induced Treg (iTreg) cells (transforming growth factor-β [TGF-β]) (Wan and Flavell, 2009; Zhu and Paul, 2010). In addition, effector T cells found in germinal centers and characterized as T follicular helper (Tfh) cells may constitute a unique T cell subset or a further differentiation state of the effector cells described above (Linterman and Vinuesa, 2010). In some fashion, the conditions of activation guide the developing cells toward an effector state that is self-reinforcing and often appropriate to a particular infectious agent; when differentiation misses the mark, the immune response is likely to be ineffectual or even pathogenic.

An important aspect of Treg cell function is the expression of the transcription factor Foxp3, given that its absence results in immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX syndrome) (Ziegler, 2006). Although initially considered to be a lineage commitment factor, studies have revealed the presence of a higher level of regulation upstream of Foxp3 (Sugimoto et al., 2006; Gavin et al., 2007; Lin et al., 2007; Hill et al., 2007). One aspect of this control may be based upon TGF-β signaling (Rubtsov and Rudensky, 2007; Liu et al., 2008), and recent work has focused on the manner in which signaling through the T cell receptor (TCR), coreceptors, and TGF-βRI or TGF-βRII receptors combine to promote the differentiation of Treg cells (Tone et al., 2008).

Foxo transcription factors regulate many facets of basic cell physiology including cell cycle progression, cell death, differentiation, and DNA repair. In lymphocyte populations subject to dramatic expansion, contraction, and contingency-dependent differentiation, Foxo proteins would be predicted to play an

important role. In addition, Foxo proteins regulate specialized lymphocyte functions such as gene recombination, homing, and cytokine receptor expression, and they control several critical checkpoints in lymphocyte development (Hedrick, 2009; Dejean et al., 2010; Ouyang and Li, 2010).

Here, we investigated widespread autoimmunity resulting from the T cell-specific deletion of Foxo1 and greatly exacerbated by the additional deletion of Foxo3. The origin of the autoimmunity was found to be a loss in dominant tolerance, and experiments showed that the development of both natural and induced Treg cells required Foxo transcription factors. Consistent with these results, Foxo1-deficient T cells stimulated in the presence of TGF-β were misdirected to a Th1 cell phenotype. Furthermore, evidence is presented for the direct Foxo1-mediated regulation of *Ctla4*, a critical coreceptor for Treg cell function (Friedline et al., 2009). Finally, the loss of Foxo1 resulted in the spontaneous appearance of Tfh cells, the expansion of B cell numbers, and autoantibody production. Thus, Foxo transcription factors play an essential role in specifying the program of T cell differentiation, most importantly in the pathway leading to development and function of Treg cells.

RESULTS

Foxo1 Prevents Systemic T Cell Activation

Foxo1 was shown to be necessary for the survival of naive T cells and homing to secondary lymphoid organs (Figures 1A and 1B, upper panel, and Gubbels Bupp et al., 2009; Kerdiles et al., 2009; Ouyang et al., 2009). In addition, we found that mice with a T cell-specific deletion of *Foxo1* (*Cd4Cre Foxo1*$^{f/f}$) harbor an expanded population of effector memory CD4$^+$CD44^{hi} T cells. Given that CD44^{lo} Foxo1-deficient naive T cells have decreased CD62L, lymph node (LN) CD4$^+$ T cells were further characterized for the expression of CD69. LNs from *Cd4Cre Foxo1*$^{f/f}$ mice possessed a considerably enhanced proportion of effector memory CD4$^+$ T cells when compared with wild-type littermates (Figure 1A), and these cells steadily increased in number with age (Figure 1B, lower panel).

Foxo1 regulates sphingosine-1-phosphate receptors through KLF2 (Fabre et al., 2008) (data not shown), and sphingosine 1-phosphate receptor-1 (S1P1) was shown to complex with CD69 to prevent CD69 cell surface expression (Shiow et al., 2006). Thus, diminished expression of S1P1 could allow ectopic expression of CD69. Nevertheless, Foxo1-deficient mature (TCRβ^{hi}HSA^{lo}) CD4$^+$ single-positive (SP) thymocytes downregulated CD69 expression (Figure 1C). In addition, T cells from OTII *Foxo1*$^{f/f}$ *Cd4Cre Rag1*$^{-/-}$ mice, which are monoclonal, ovalbumin-specific, and unable to recognize endogenous or commensal antigens, displayed a typical CD44^{lo} naive phenotype lacking CD69 (Figure 1D). Thus, in the absence of Foxo1, CD69 expression is normally regulated and we can conclude that the effector memory CD4$^+$ T cells are expanded in peripheral lymphoid organs.

The rescue of naive T cells in mice with a mono-specific T cell repertoire shows that a polyclonal receptor repertoire is required for the spontaneous T cell activation. This, in turn, suggested an ongoing immune response, a notion further supported by several observations. In short term BrdU-pulse experiments we found a significantly higher proportion of cycling CD4$^+$ CD44$^+$ T cells in the peripheral lymphoid organs of *Cd4Cre Foxo1*$^{f/f}$ mice compared to wild-type mice (Figure 1E). Moreover, purified Foxo1-deficient CD4$^+$ T cells, restimulated in vitro with PMA and ionomycin, secreted high quantities of IFN-γ, IL-4, and IL-17, but not IL-2, indicative of increased T cell differentiation biased toward Th1 effector cells (Figure 1F). Histological examination also revealed mild mononuclear cell infiltration in nonlymphoid organs including the heart, salivary glands, kidney, and liver of 1-year-old *Cd4Cre Foxo1*$^{f/f}$ mice (Figure 1G). Notably, by 6 to 8 months of age, *Cd4Cre Foxo1*$^{f/f}$ mice developed signs of spontaneous encephalitis revealed by partial hind-limb paralysis (Yamamoto et al., 2000) (data not shown), and this was found to be associated with an age-progressive peripheral nerve or spinal cord T cell infiltrate (Figures 1H and 1I). Thus, Foxo1 expression is essential to prevent systemic and pathological T cell activation.

T Cell-Specific Foxo1 Deletion Leads to Tfh Cell Development and B Cell Autoimmunity

Further examination of LN populations revealed increased numbers of B cells in *Cd4Cre Foxo1*$^{f/f}$ mice already by 3 weeks of age (Figure 2A). Yet, as we showed previously (Kerdiles et al., 2009), B cells in these mice were not deleted for Foxo1 (Figure S1 available online). The B cell population expansion was associated with an increased proportion of cycling B cells (Figure 2B) and B cell differentiation characterized by enlarged proportions of germinal center (GL-7$^+$FAS$^+$) and isotype-switched (IgM$^-$IgD$^-$) B cells (Figure 2C and data not shown) (Han et al., 1997). Consistent with these results, immunofluorescence analysis of frozen spleen sections revealed the spontaneous development of IgD$^-$ PNA$^+$ germinal centers in the spleens of *Cd4Cre Foxo1*$^{f/f}$ mice (Figure 2D). This apparent B cell activation was associated with substantially increased quantities of circulating IgA, IgG1, IgG2a, and IgG2b isotypes (Figure 2E) and, in addition, a 10-fold increased titer of dsDNA antibodies (Figure 2F). Thus, a T cell-specific deletion of Foxo1 is sufficient to drive the development of B cell autoimmunity.

Germinal center formation, isotype switching, and somatic hypermutation rely on cognate B cell-T cell interactions involving a specialized population designated as follicular helper cells (Tfh cells) (McHeyzer-Williams et al., 2009). These cells are characterized by expression of the chemokine receptor CXCR5, the cytokine IL-21, and the transcription factor Bcl-6. A subpopulation of Tfh cells, expressing high CXCR5 and PD-1, are localized to the germinal centers and important for B cell activation (Linterman and Vinuesa, 2010).

Initial experiments confirmed that CXCR5 expression is restricted to CD44^{hi}CD62L^{lo} CD4$^+$ T cells (data not shown), and analysis revealed that *Cd4Cre Foxo1*$^{f/f}$ mice have an increased proportion and absolute number of CXCR5-expressing cells, including CXCR5^{hi}PD-1^{hi} cells (Figures 2G and 2H and data not shown). Furthermore, analysis of sorted effector memory CD4$^+$ cells revealed a significantly increased expression of *Bcl6* and *Il21* in Foxo1 deficient cells (Figure 2I), although no reduction in *Prdm1* (encoding Blimp1) was noted. Thus, T cell-specific Foxo1 deletion results in the appearance of Tfh cells correlated with the development of B cell autoimmunity.

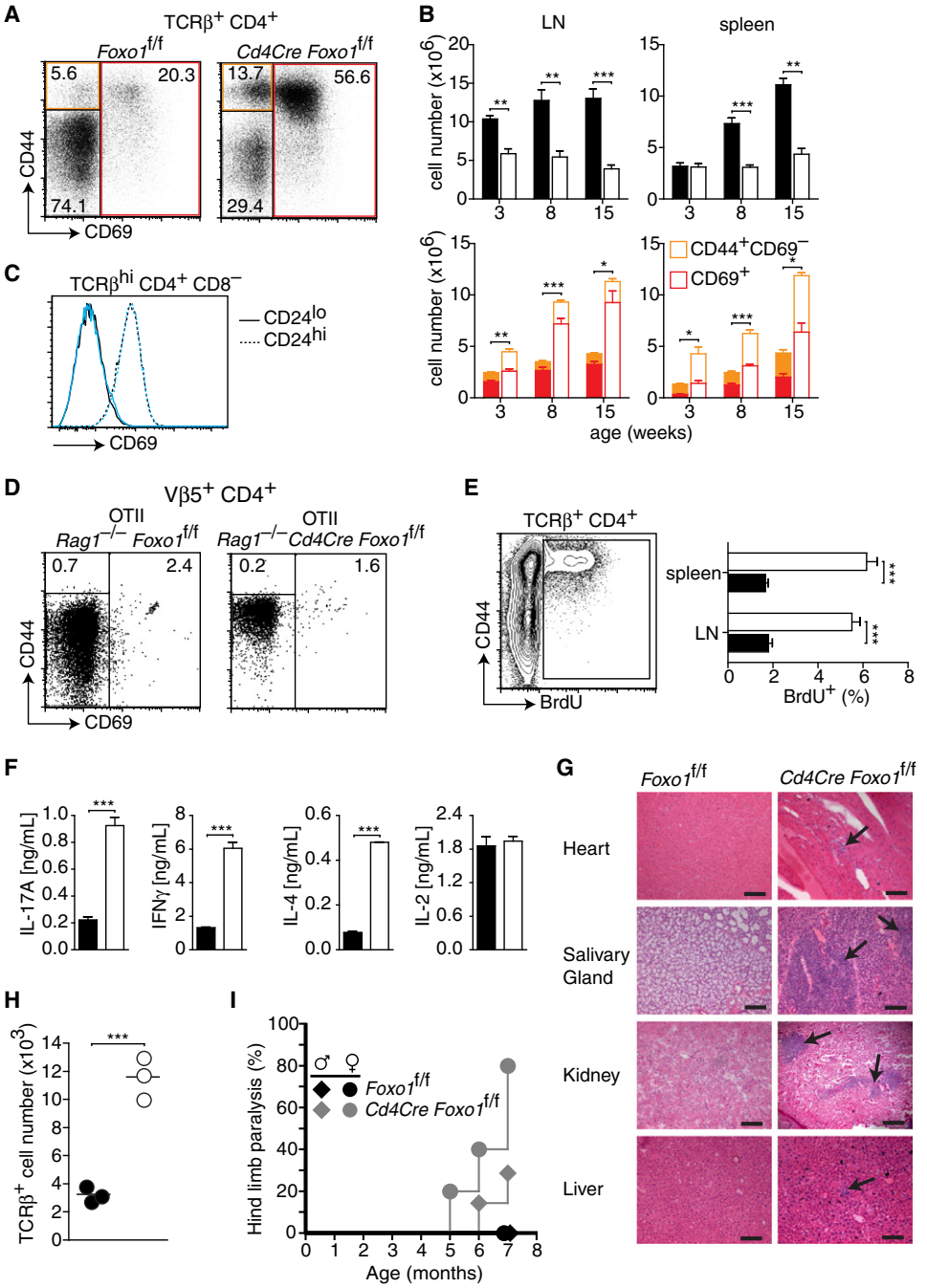

Figure 1. Foxo1 Controls T Cell Tolerance In Vivo

(A–F) *Foxo1*$^{f/f}$ (filled bars or symbols) and *Cd4Cre Foxo1*$^{f/f}$ mice (open bars or symbols) from 8-week- old mice unless otherwise indicated.

(A) Analysis of LN CD4$^+$ T cells (n ≥ 5 mice per genotype analyzed in two independent experiments).

(B) Enumeration of naive (upper panel) and effector memory (lower panel) CD4$^+$ T cells in peripheral lymphoid organs (mean + SEM; n ≥ 3 mice per genotype and time point).

(C) Flow cytometry profile of thymic TCRβ^{hi} CD4$^+$ single-positive cells from 8-week-old *Foxo1*$^{f/f}$ (black lines) and *Cd4Cre Foxo1*$^{f/f}$ mice (blue lines) (n = 3 mice analyzed per genotype).

(D) Splenic OTII cells (n ≥ 4 mice per genotype analyzed in two independent experiments).

(E) Analysis of BrdU incorporation (18 hr pulse) into CD4$^+$ T cells from *Cd4Cre Foxo1*$^{f/f}$ mice. Compiled data (mean + SEM; n = 5–6 mice per genotype analyzed in two independent experiments).

(F) Cytokine secretion by total LN CD4$^+$ T cells stimulated for 24 hr with PMA+ionomycin (mean + SD of duplicate cultures, one representative experiment out of three).

(G) H&E staining of nonlymphoid tissues from 1-year-old mice (n = 3–4 mice per genotype).

(H) Enumeration of total infiltrating T cells in 7- to 8-month-old *Foxo1*$^{f/f}$ (filled circles) and *Cd4Cre Foxo1*$^{f/f}$ mice (open circles).

(I) Hind limb paralysis over time.

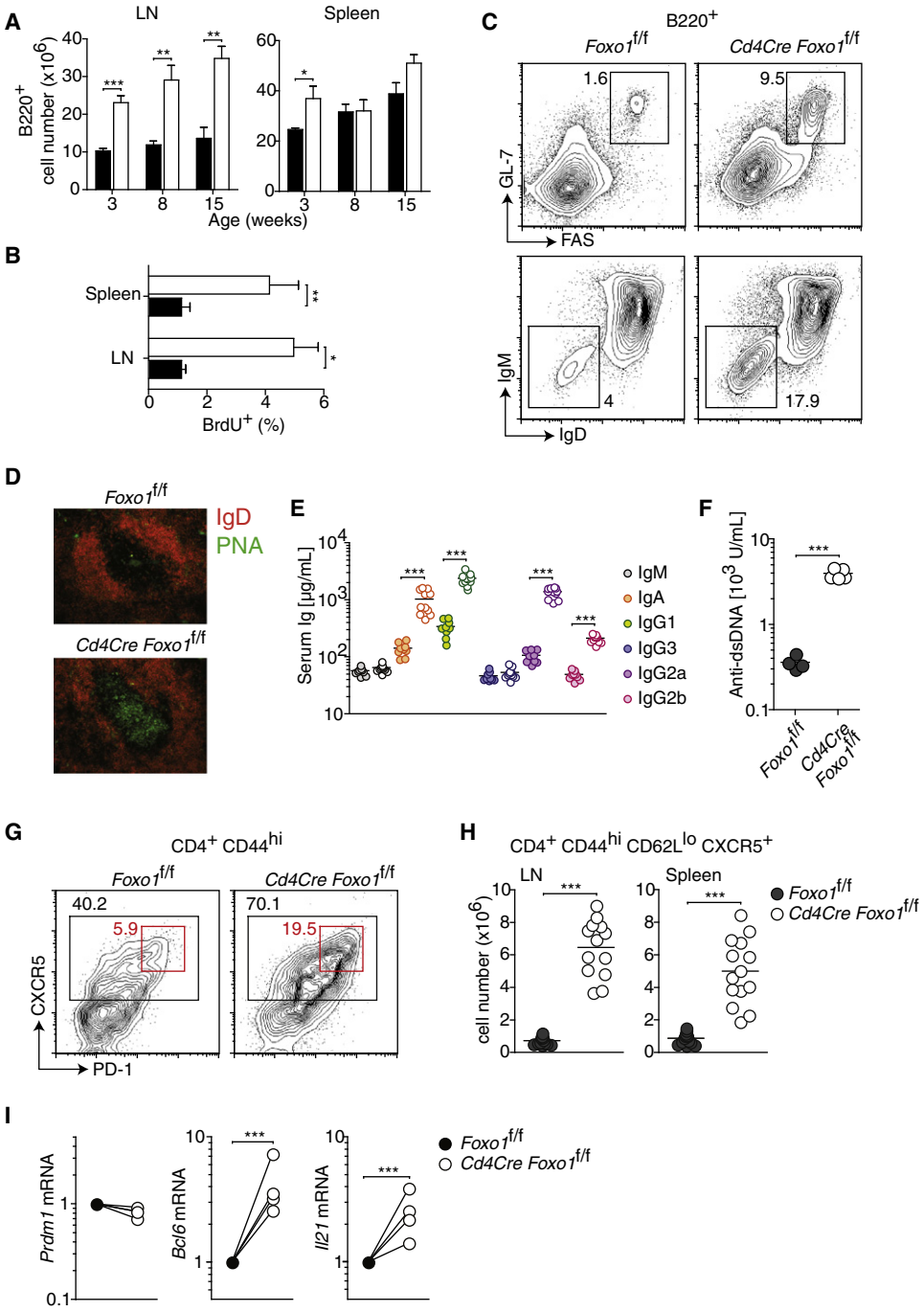

Figure 2. Foxo1 Expression in T Cells Is Required to Prevent Secondary B Cell Activation and Autoimmunity

(A–F) *Foxo1*$^{f/f}$ (filled bars or symbols) and *Cd4Cre Foxo1*$^{f/f}$ mice (open bars or symbols) from 8-week-old mice unless otherwise indicated.

(A) B220^{+} cells in peripheral lymphoid organs at ages indicated (mean + SEM; n ≥ 3 mice per genotype and time point).

(B) BrdU incorporation by B220^{+} cells in peripheral lymphoid organs (mean + SEM; n ≥ 5 mice per genotype analyzed in two independent experiments).

(C) LN B220^{+} cells from 8-week-old mice (n ≥ 10 per genotype analyzed in three independent experiments).

(D) Immunofluorescence of frozen spleen sections (n = 5 to 6 mice per genotype analyzed in two independent experiments).

(E) Serum Ig amounts by isotype.

(F) ELISA quantification of anti-dsDNA antibodies (pooled results from two independent experiments).

(G) Flow cytometry profile of CD4^{+} CD44^{hi} T cells from 8-week-old mice (n ≥ 5 mice per genotype analyzed in two independent experiments).

(H) Enumeration of cells as indicated (pooled results from three independent experiments).

(I) Relative expression (RT-qPCR) of *Prdm1* (Blimp-1), *Bcl-6*, and *Il-21* mRNA, normalized to *B2m* mRNA expression, from sorted effector memory cells (see Figure 1A) (pooled results from four independent experiments).

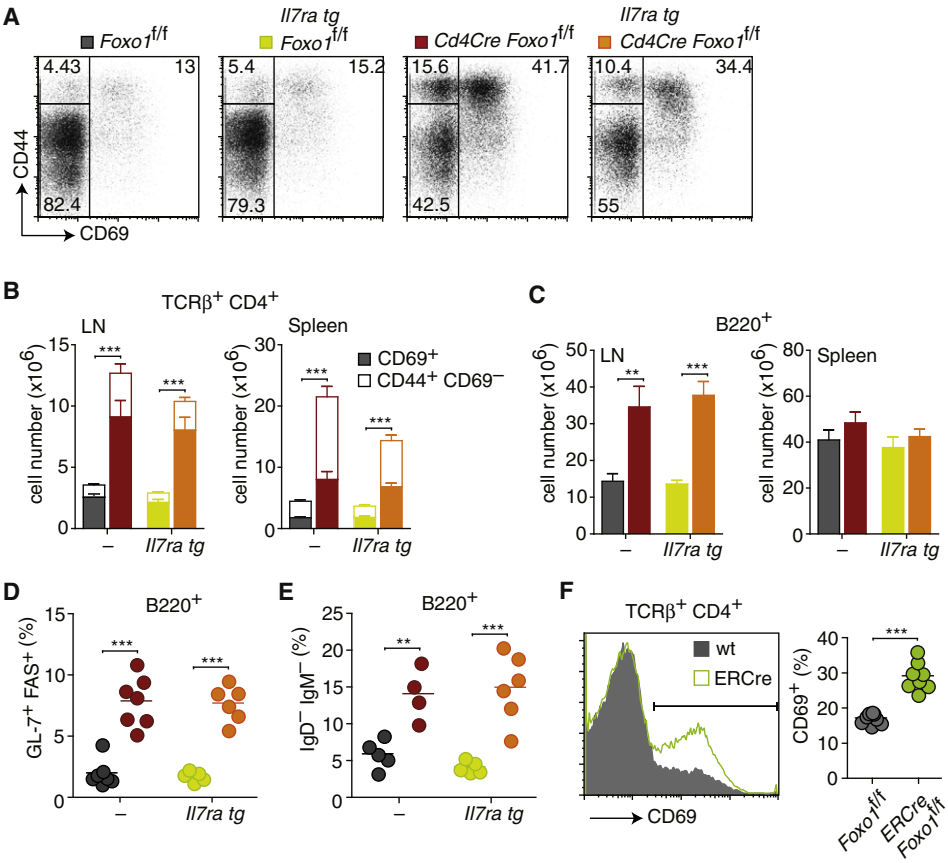

Figure 3. T Cell Activation in Foxo1-Deficient Mice Is Not a Result of an Altered Homeostasis

(A) LN CD4$^+$ T cells from 8- to 12-week-old mice (n $\geq$ 5 mice per genotype analyzed in two independent experiments).

(B and C) Enumeration of effector memory CD4 T cells (B) and B cells (C) in peripheral lymphoid organs (mean ± SEM; n $\geq$ 5 mice per genotype from two independent experiments).

(D and E) Phenotypic analysis of B cells in peripheral lymphoid organs (pooled results from two independent experiments).

(F) Profile of LN CD4 T cells and proportions of CD69$^+$ CD4 T cells in 8-week-old *Foxo1*$^{f/f}$ (gray) and *ERCre Foxo1*$^{f/f}$ (green) mice treated with tamoxifen for 5 days and rested for 5 days (pooled results from two independent experiments).

T Cell Activation Is Not a Consequence of Disrupted Homeostasis

A possibility was that the autoimmunity noted was affected by the requirement for Foxo1 in *Il7ra* transcription (encoding IL-7Rα) and its necessity for naive T cell survival. We thus sought to determine whether a transgene encoding IL-7Rα would rescue the naive T cell population in *Cd4Cre Foxo1*$^{f/f}$ mice (Yu et al., 2004). As expected, the transgene expression fully restored IL-7Rα and Bcl2 expression (Figures S2A–S2C) in vivo, and it reestablished the sensitivity of Foxo1-deficient T cells to IL-7 in culture (Figures S2D). Nonetheless, CD4$^+$ T cells from *Cd2-Il7ra Cd4Cre Foxo1*$^{f/f}$ mice retained an increased proportion and absolute number of activated-memory cells compared to *Cd2-Il7ra Foxo1*$^{f/f}$ control mice (Figures 3A and 3B). In addition, the *Cd2-Il7ra* transgene did not restore a normal number of B cells in the LN, nor did it relieve the increases in germinal center and isotype-switched B cells (Figures 3C–3E).

As previously described, we used the *ERCre* transgene to acutely delete *Foxo1* (Guo et al., 2007; Kerdiles et al., 2009). Tamoxifen treatment regimen resulted in normal numbers of naive T cells in peripheral lymphoid organs, yet this acute deletion of *Foxo1* led to a markedly increased population of CD69$^+$ CD4$^+$ T cells (Figure 3F). Thus, even in nonlymphopenic conditions, deletion of *Foxo1* rapidly led to T cell activation.

Foxo1 Controls Dominant T Cell Tolerance

The control of autoimmune T cells involves central tolerance, cell-intrinsic peripheral tolerance, and dominant tolerance mediated by regulatory T cells. The process of negative selection was thus analyzed in several different forms. The CD5 expression on thymocytes is correlated to the strength of TCR signaling, and it can reveal the process of negative selection (Dutz et al., 1995). A comparison of thymocyte subpopulations from *Foxo1*$^{f/f}$ and *Cd4Cre Foxo1*$^{f/f}$ mice showed that CD4$^+$CD8$^+$ (double positive [DP]), CD4$^+$CD8$^-$ (CD4SP), and CD4$^-$CD8$^+$ (CD8SP) subsets exhibited identical CD5 expression profiles (Figure S3A). In addition, DP thymocytes from OTI *Cd4Cre Foxo1*$^{f/f}$ and control mice were equally sensitive to OVA-peptide-mediated deletion in culture (Figure S3B), and there did not appear to be a disruption in the peripheral TCR repertoire, at least by the criterion of Vβ expression (Figure S3C). Finally, to directly test negative

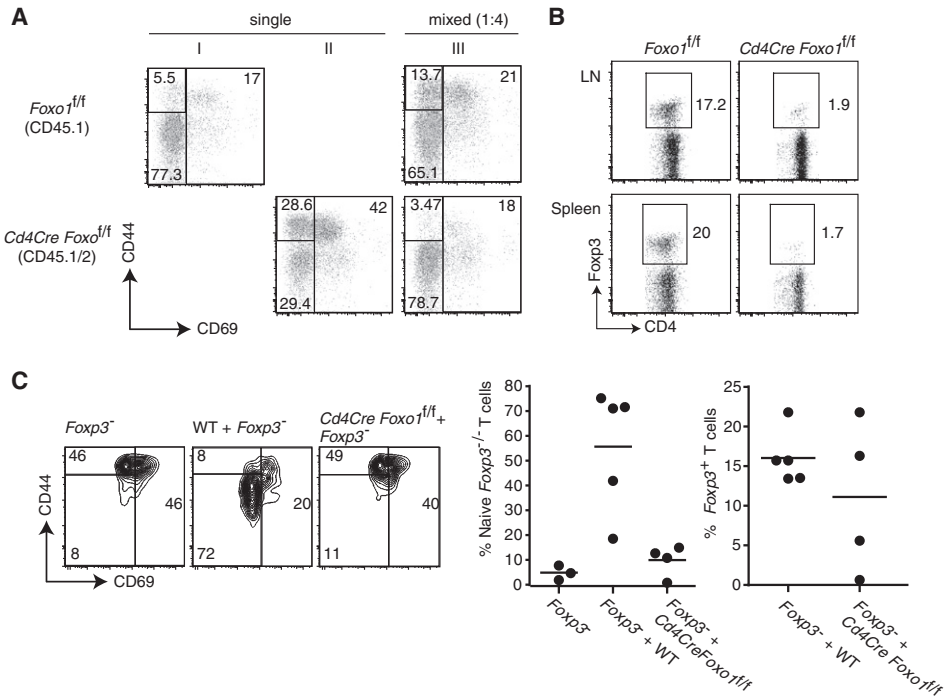

Figure 4. Foxo1 Controls Dominant T Cell Tolerance

(A) LN CD4 T cells 8 weeks after irradiation and (1:4 WT:KO) bone-marrow cell transfer into *Tcra*$^{-/-}$ mice. Different radiation chimeras are as follows: (I) *Foxo1*$^{f/f}$ (CD45.1) BM → *Tcra*$^{-/-}$ (CD45.2) host; (II) *Cd4Cre Foxo1*$^{f/f}$ (CD45.1/2) BM → *Tcra*$^{-/-}$ (CD45.2) host; (III) 1:4 *Foxo1*$^{f/f}$ (CD45.1) + *Cd4CreFoxo1*$^{f/f}$ (CD45.1/2) BM → *Tcra*$^{-/-}$(CD45.2) host. Representative results for 2–4 mice analyzed per condition. Recovery of TCR-β^{+}CD4^{+} T cells in the mixed chimera was 74% wt, 36% KO. Shown are results from one of two experiments.

(B) Foxp3^{+} cell recovery from mixed bone marrow chimeras described in (A) (n = 3 mice analyzed). Shown are results from one of two experiments.

(C) Irradiated *Tcra*$^{-/-}$ mice were reconstituted with bone marrow as indicated. Analysis gated on T cells originating from the *Foxp3*$^{-}$ donor. Results are pooled from two independent experiments.

selection in vivo, mice were bred to express *H2E*^{k} in order to examine the efficiency of *Mtv* superantigen-mediated deletion of T cells bearing Vβ5 and Vβ11 (Woodland et al., 1991). Vβ5- and Vβ11-bearing T cells were selectively deleted in mice expressing *H2E*^{k}, and this was not affected by the loss of Foxo1 (Figure S3D).

Foxo transcription factors also control the expression of Fas ligand (FasL) (Brunet et al., 1999), and T cell-specific expression of FasL has been demonstrated to play a role in autoimmunity even though the underlying mechanisms are still unclear (Mabrouk et al., 2008). Intracellular staining for FasL revealed a small but detectable induction by CD44^{hi} CD4 T cells compared with CD44^{lo} CD4 T cells analyzed directly ex vivo, and this was not affected by the deletion of *Foxo1* (Figure S3E). Further, the strong induction of FasL expression observed upon activation was also unaltered by the deletion of *Foxo1* (Figure S3F). Finally, FasL deficiencies in *gld* mutant mice or T cell-specific FasL-deficient mice are associated with the accumulation of an unconventional B220^{+}CD3^{+}CD8^{-}CD4^{-} T cells (Ramsdell et al., 1994; Mabrouk et al., 2008). Consistent with normal FasL expression, we did not observe this accumulation in *Cd4Cre Foxo1*$^{f/f}$ mice (Figure S3G and data not shown). Thus, Foxo1 is dispensable for FasL expression and FasL-dependent peripheral tolerance mechanisms.

To distinguish between cell-intrinsic and dominant-suppressive mechanisms, we produced bone marrow chimeras in which irradiated *Tcra*$^{-/-}$ mice were reconstituted with bone marrow from wild-type (*Foxo1*$^{f/f}$) mice, from *Cd4Cre Foxo1*$^{f/f}$ mice, or from a 1:4 mixture of the two. T cells from mice reconstituted with *Foxo1*$^{f/f}$ bone marrow possessed a typical distribution of naive and recently activated T cells (Figure 4A, column I), whereas mice reconstituted with *Cd4Cre Foxo1*$^{f/f}$ bone marrow developed a large population of effector memory T cells (Figure 4A, column II). Importantly, in the mice reconstituted with the mixture, the presence of cells derived from the *Foxo1*$^{f/f}$ bone marrow suppressed the emergence of effector memory *Cd4Cre Foxo1*$^{f/f}$ T cells (Figure 4A, column III). Furthermore, in the mixed bone marrow chimera the vast majority of Foxp3^{+} Treg cells were of wild-type origin (Figure 4B). These results were consistent with a cell-intrinsic deficiency in the development or survival of Foxo1 deficient Foxp3^{+} Treg cells and a defective dominant tolerance in *Cd4Cre Foxo1*$^{f/f}$ mice.

If Foxo1 is required for Treg cell function, then in irradiated mice reconstituted with *Foxp3*$^{-}$ bone marrow, the addition of wild-type, but not *Cd4Cre Foxo1*$^{f/f}$, bone marrow should restore quiescence to the T cell population that develops from Foxp3-deficient bone marrow (Fontenot et al., 2003). Mice were examined 6–7 weeks after irradiation before the appearance of dramatic pathology. As shown, the percentage of CD44^{lo} naive T cells averaged ~5% in *Tcra*$^{-/-}$ hosts reconstituted with bone marrow from *Foxp3* mutant mice (Figure 4C). The addition of

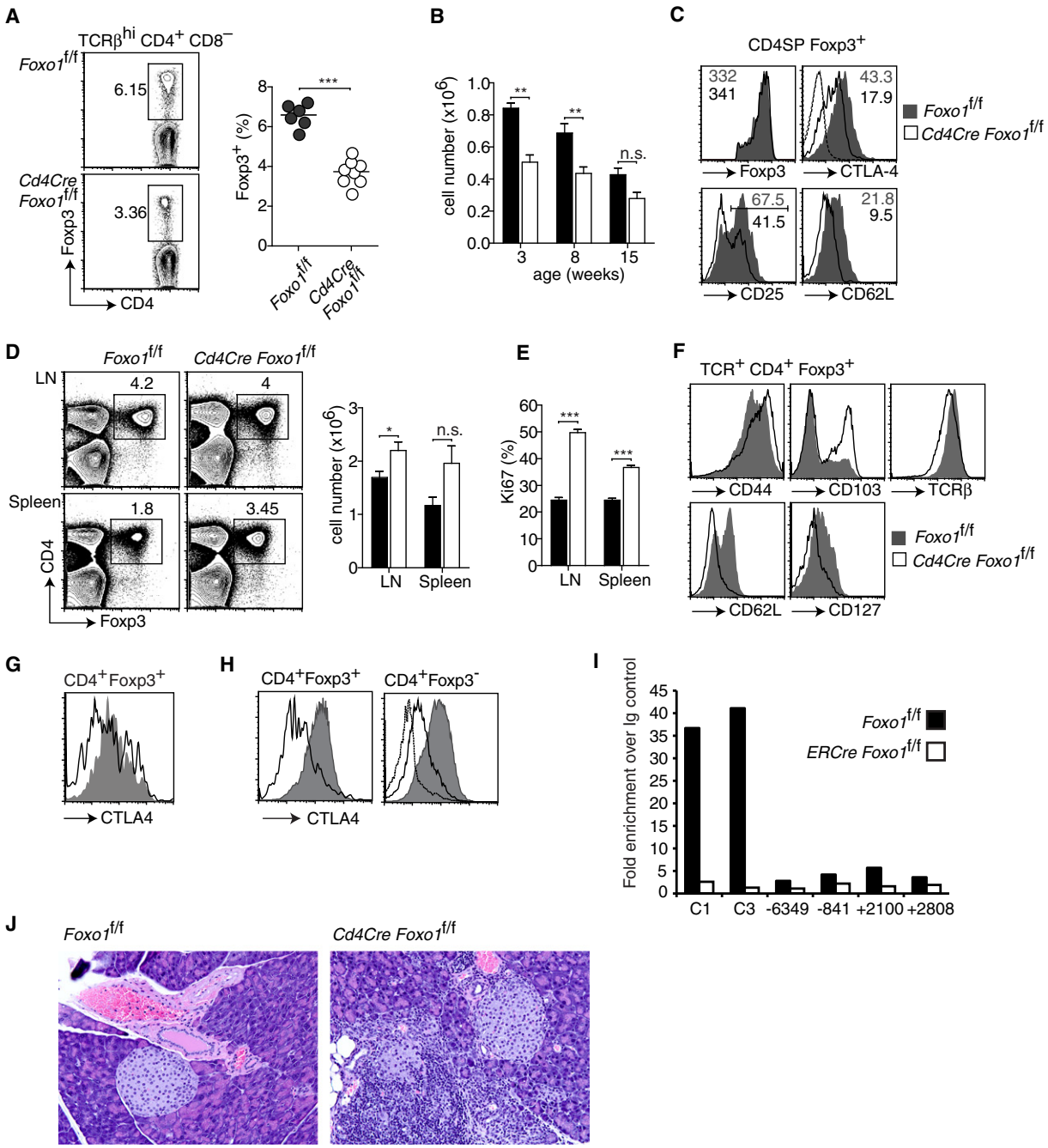

Figure 5. Foxo1 Controls nTreg Cell Homeostasis and Development

(A–I) Eight-week-old *Foxo1*$^{f/f}$ (filled bars, symbols or histograms) and *Cd4Cre Foxo1*$^{f/f}$ (open bars, symbols, or histograms) mice unless otherwise indicated.

(A) Analysis of Foxp3$^+$ cells within thymic CD4 SP cells (pooled results from twos experiments).

(B) Total thymic CD4 SP Foxp3$^+$ cells (mean + SEM; n = 3–7 mice per time point) with age.

(C) Profile of thymic CD4 SP Foxp3$^+$ cells. Numbers indicates geoMFI or percentages (when bar is present) in accordance with the color code.

(D) LN TCR-β$^+$ cells from 8-week-old mice (n ≥ 5 mice per genotype analyzed in two independent experiments). Enumeration of TCR-β$^+$ CD4$^+$ Foxp3$^+$ cells in peripheral lymphoid organs of 8- to 15-week-old mice mice (n ≥ 9 mice per genotype analyzed in three independent experiments).

(E) Analysis of Ki67 expression assessed by flow cytometry on gated TCRβ$^+$ CD4$^+$ Foxp3$^+$ LN cells (n ≥ 9 mice per genotype analyzed in three independent experiments).

(F) Phenotype of TCRβ$^+$ CD4$^+$ Foxp3$^+$ cells in peripheral lymphoid organs (n ≥ 9 mice per genotype analyzed in three independent experiments).

(G) CTLA-4 expression on CD4$^+$ TCRβ$^+$ Foxp3$^+$ LN cells.

(H) CTLA-4 expression after 3 days of stimulation in culture with anti-CD3 and anti-CD28 and TGF-β. Dotted line indicates isotype control.

Foxo1$^{f/f}$ bone marrow rescued an average of 55% naive *Foxp3*$^{-}$ T cells. By contrast, addition of bone marrow from *Cd4Cre Foxo1*$^{-/-}$ mice did not substantially rescue a naive T cell phenotype within the *Foxp3*$^{-}$ T cells (Figure 4C). In this cohort of mice (Figure 4C), a population of Foxo1-deficient Foxp3^{+} cells developed, and from this we infer that Foxo1-deficient Treg cells are functionally deficient in vivo.

Natural Treg Cell Development Is Foxo1 Dependent

The development of Foxo1-deficient Treg cells was further examined in the thymus. Young *Cd4Cre Foxo1*$^{f/f}$ mice exhibited a noticeable decrease in the proportion and number of Foxp3^{+} cells among thymic mature CD4 SP cells that diminished with age (Figures 5A and 5B). Phenotypic analyses revealed that in 8-week-old mice, despite near normal Foxp3 expression, Foxo1-deficient thymic Treg cells showed reduced CD62L expression, consistent with its Foxo1-dependent expression (Kerdiles et al., 2009), but they also exhibited diminished CD25 and CTLA-4 expression (Figure 5C). An implication is that Foxo1 is important for gene expression that is required for Treg effector function.

Normal or increased proportions and numbers of CD4^{+} Foxp3^{+} cells were observed in peripheral lymphoid organs of *Cd4Cre Foxo1*$^{f/f}$ mice when compared to wild-type mice (Figure 5D). Accordingly, we found significantly increased proportions of Ki67^{+} Treg cells in LN and spleen (Figure 5E), and peripheral Treg cells were mostly CD44^{hi}TCRβ^{lo} CD103^{+}, indicating an activated phenotype (Figure 5F). These data are consistent with the notion that a reduced number of Foxo1-deficient thymus-derived Treg cells develop but expand in the peripheral lymphoid organs.

Similar to thymocytes, both naive Foxp3^{+} T cells as well as culture-stimulated Foxp3^{+} or Foxp3^{-} lymph node T cells expressed lower amounts of CTLA-4 (Figures 5G and 5H). These results suggested the possibility that Foxo1 is directly required for *Ctla4* transcription, and as such, we analyzed the *Ctla4* gene for conserved Foxo-binding sites. One evolutionarily conserved Foxo-binding site was identified 193 bp upstream of the transcription start site (Perkins et al., 1996) (Figures S4A and S4B). In order to study the role of this site in *Ctla4* expression, we performed Foxo1-specific chromatin immunoprecipitation by using activated T cells from *Foxo1*$^{f/f}$ or *ERCre Foxo1*$^{f/f}$ mice treated with tamoxifen. As shown in Figure 5I, two different primer pairs flanking the putative site elicited a strong and specific signal in wild-type but not Foxo1-deficient T cells, whereas four other primer sets up and downstream did not (Figure 5I and Figure S4C).

These results suggested the possibility that the autoimmunity associated with a deficiency in Foxo1 arises from an incomplete program of gene expression in the extant Foxp3-positive T cells that includes an insufficiency of CTLA-4 expression. In fact, the autoimmune phenotype seen in mice with a T cell-specific Foxo1 deficiency exhibits exocrine pancreatitis (Figure 5J) reminiscent of that in *Ctla4* mutant mice with a TCR-β transgene (Ise et al., 2010).

Foxo1 Regulates TGF-β Responsiveness of CD4 T Cells

Because Foxo1 has previously been shown to act as a coactivator with Smad4 downstream of TGF-β signaling, we considered the possibility that it is necessary for TGF-β-induced differentiation of Treg cells. In vitro stimulation of CD25^{-}CD69^{-} CD4^{+} T cells in the presence of TGF-β resulted in the generation of Foxp3^{+}-induced regulatory T (iTreg) cells (Chen et al., 2003) (Figure 6A); however, iTreg cell induction was highly impaired when cells purified from tamoxifen-treated *ERCre Foxo1*$^{f/f}$ mice were stimulated under the same conditions (Figure 6A). This did not occur because of altered proliferation (Figure 6A), and it was maintained over a wide range of TGF-β concentrations (Figure 6B). Further, this defect resulted from cell intrinsic mechanisms given that coculture with wild-type CD45.1 cells did not rescue the Foxo1-deficient iTreg cell differentiation (Figure 6C), nor did the presence of Foxo1-deficient T cells diminish the differentiation of wild-type Treg cells. These results demonstrate that Foxo1 is necessary for induced-Treg cell differentiation and confirm the notion that increased LN Treg cells in *Cd4Cre Foxo1*$^{f/f}$ mice arise by homeostatic expansion of nTreg cells.

Analyses revealed a reduced expression of the TGFβRII chain in naive CD4 T cells from *Cd4Cre Foxo1*$^{f/f}$ (Figure S5A) or ERCre Foxo1$^{f/f}$ mice treated with tamoxifen (Figure S5B). Although these results suggest a proximal signaling defect, four observations argue against this possibility. One, activation in the presence of TGF-β caused the majority of Foxo1-deficient T cells to produce IFN-γ, as if they had been misdirected to become Th1 cells (Figure 6D). Two, TGF-β stimulation normally suppressed IL4 secretion in Foxo1$^{f/f}$ Cd4Cre CD4 cells (Figure S5C). Three, wild-type T cells progressively acquire a Foxp3^{+} phenotype over a 90-fold titration of TGF-β (Figure 6B), whereas Foxo1-deficient T cells, with a 2-fold decrease in TGFβRII expression, did not respond in this assay (Figure 6B). Four, TGF-β-mediated Smad2,3 phosphorylation was not altered by the acute deletion of Foxo1 (Figure S5D). Rather, these results indicate that Foxo1-deficient T cells responded to TGF-β, but the response was misdirected. The few Foxo1-deficient Foxp3^{+} iTreg cells induced, either cultured alone (Figure 6E) or together with wild-type CD45.1 cells (Figure 6F), had comparable expression of Foxp3 and the Foxp3 target genes, CD25 (Figure 6E), and GITR (Figure 6F). However, similar to nTreg cells, Foxo1-deficient iTreg cells expressed reduced amounts of CTLA-4 compared to wild-type iTreg cells (Figures 6E and 6F).

Previous work has shown that TGF-β normally suppresses Th1 cell development by preventing the IFN-γ induction of T-bet (Lin et al., 2005). Stimulation of T cells showed an expected increase

(I) Chromatin immunoprecipitation of Foxo1. Fold enrichment over Ig control indicates the difference between the real-time PCR signal with anti-Foxo1 versus that of the Ig control in the immunoprecipitation step. Primers are indicated by their position relative to the transcription start site (Figure S4). Results are representative of three experiments.

(J) Hematoxylin-and-eosin-stained sections of pancreata from 9-month-old *Foxo1*$^{f/f}$ and *Cd4Cre Foxo1*$^{f/f}$ mice. Inflammatory cells were absent (7/10) to minimal (3/10) in the periductal and interstitial areas of the pancreas in wild-type mice. In contrast, scattered foci with prominent lymphocytic infiltrates centered on periductal and perivascular connective tissues with an extension of inflammatory cell infiltrates into the exocrine pancreas were observed in *Cd4Cre Foxo1*$^{f/f}$ mice (11/11). No insulitis or islet involvement was noted other than bystander damage.

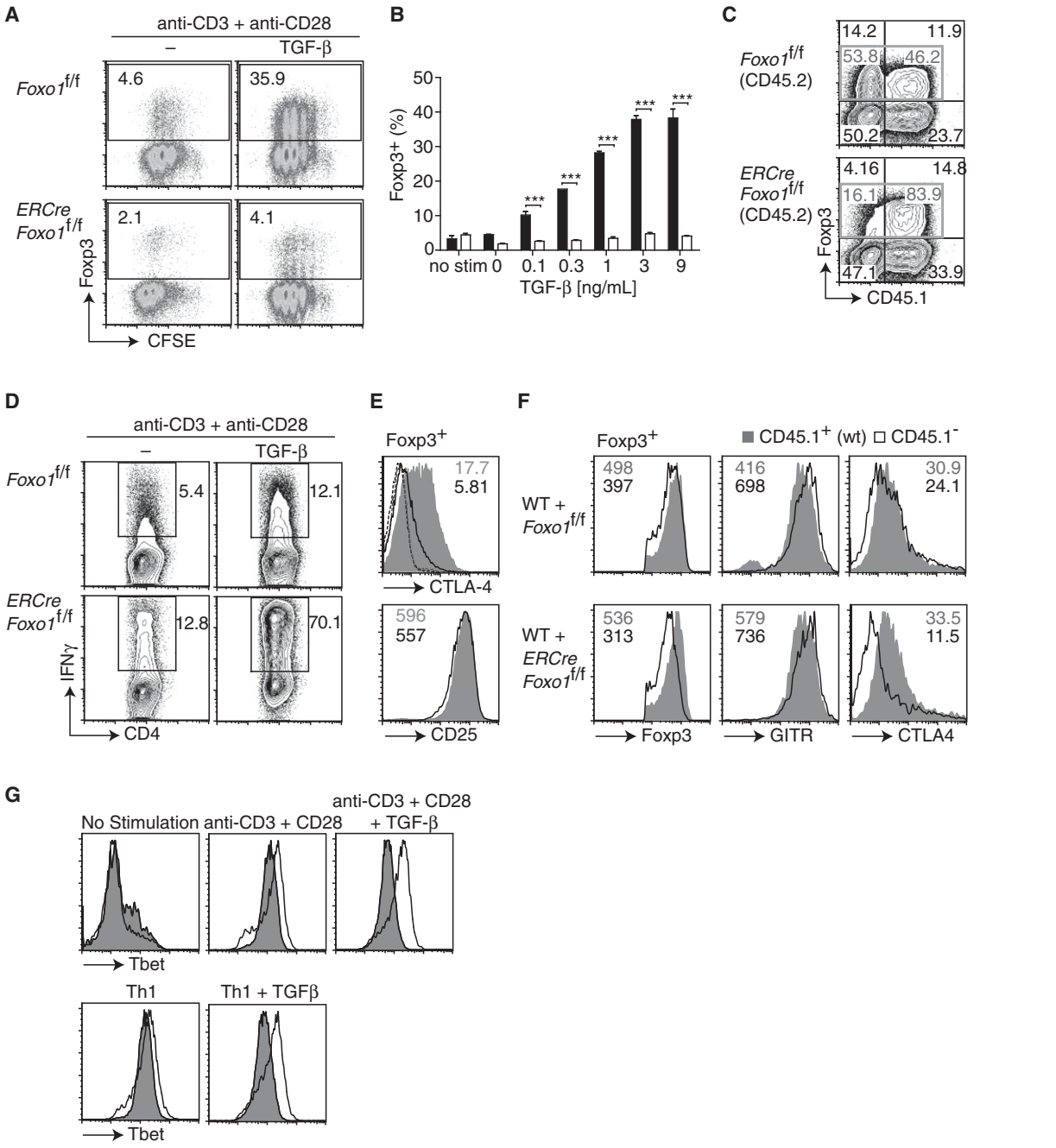

Figure 6. Foxo1 Directly Controls TGF-β Responsiveness

(A–G) Purified naive CD4 T cells ($CD4^+$ $CD69^-$ $CD25^-$) from *Foxo1*$^{f/f}$ (filled bars and histograms) and *ERCre Foxo1*$^{f/f}$ (open bars and histograms) mice were isolated and used for in vitro experiments.

(A) Flow cytometry profile of naive LN CD4 T cells stimulated for 3 days as indicated.

(B) $Foxp3^+$ cells from naive LN CD4 T cells stimulated for 3 days with anti-CD3 and anti-CD28 in the presence of various concentrations of rhTGF-β (mean ± SD of triplicates culture, one representative experiment out of three).

(C) Relative cell recovery of LN naive CD4 T cells cocultured with WT CD45.1 LN naive CD4 T cells. Percentages are indicated for each quadrant (outer numbers). Inner box indicates the relative proportion of $Foxp3^+$ cells (representative results of one out of two independent experiments).

(D) IFN-γ intracellular staining after PMA-ionomycin restimulation of naive LN CD4 T cells stimulated for 3 days as indicated (one representative experiment out of three).

(E) Analysis of $Foxp3^+$ cells recovered from culture containing anti-CD3, anti-CD28, and rhTGF-β. Numbers indicate geoMFI.

(F) Phenotype of LN naive CD4 T cells cocultured together with WT CD45.1 LN naive CD4 T cells and stimulated for 3 days with anti-CD3 and anti-CD28 with rhTGF-β (representative results of one out of two independent experiments). Numbers indicate geoMFI.

(G) Intracellular T-bet staining after culture for 3 days under conditions indicated in the figure. Representative results of one of two experiments are shown.

in T-bet expression in both wild-type and Foxo1-deficient T cells; however, the addition of TGF-β diminished T-bet expression in wild-type but not Foxo1-deficient T cells (Figure 6G). A similar effect was shown for T cells cultured in Th1 cell-inducing conditions including IL-12 and anti-IL-4. Altogether, these results show that Foxo1-deficient T cells respond to TGF-β despite reduced TGFβRII expression, but their differentiation to Treg cells is highly compromised and misdirected toward Th1 effector cells.

Overlapping Roles of Foxo1 and Foxo3

Although we did not find a T cell-intrinsic phenotypic alteration with a loss of Foxo3 (Dejean et al., 2009), we considered the possibility that a role for Foxo3 could be revealed in the absence of Foxo1. We first crossed *Cd4Cre Foxo1*$^{f/f}$ mice with *Foxo3*^{Kca} mice, a Foxo3-deficient strain created by insertional mutagenesis (Hosaka et al., 2004). Compared to the parental genotypes, *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{Kca/Kca}$ mice rapidly manifested signs of pathology (ruffled fur, hunching) and 50% of the mice died by the age of 15 weeks (data not shown). Double-mutant mice exhibited severe splenomegaly, lymphadenopathy, and multiorgan lymphocyte infiltrates (Figure 7A and data not shown), dsDNA-specific antibodies (Figure 7B), and immunoglobulin deposits in the kidney glomeruli (Figure 7C).

We previously reported that a deletion of Foxo3 in DCs increased their capacity to sustain T cell viability as a consequence of dysregulated IL-6 secretion (Dejean et al., 2009). Hence, we sought to determine whether the phenotype of *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{Kca/Kca}$ mice resulted from cooperative effects of a Foxo3 deficiency in dendritic cells and a Foxo1 deficiency in T cells or from cell-intrinsic redundant roles of Foxo1 and Foxo3 in T cells. To study this issue, we produced mice with a conditional deletion in both *Foxo1* and *Foxo3*. With deletion mediated by the *Cd4Cre* transgene, double-mutant mice rapidly developed signs of wasting disease and became moribund as early as 8 weeks of age, with half the mice deceased by 28 weeks (Figure 7D). Analysis of 8-week-old double-mutant mice revealed that the proportion and number of DP thymocytes were substantially decreased (Figure S6A) compared to controls, and although this distortion might arise from systemic inflammation, the same phenotype was seen in OTII *Rag1*$^{-/-}$ *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ mice (Figure 7E). Compared with wild-type, *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ thymuses had significantly increased numbers of TCR-β^{hi} HSA^{lo} single-positive thymocytes (Figure S6B) indicative of mature T cells, whereas the vast majority were still CD62L^{lo} and CD69^{+} (Figure S6C). In OTII *Rag1*$^{-/-}$ *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ mice, the thymic profile was also grossly normal, but the frequency of CD4 SP cells was dramatically increased along with the proportion of TCR-β^{hi} CD69^{+} cells (Figure 7E). This result suggests that double-deficient thymocytes do not egress expeditiously from the thymus, perhaps because of the Foxo dependence of KLF2 expression and the role of KLF2 in the expression of S1P receptors (Drennan et al., 2009) (Figure S6D).

By 10 weeks of age, more than 80% of CD4^{+} T cells from *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ mice exhibited an effector memory phenotype, most of them being CD69^{+} (Figure 7F). This arose from a reduction of naive CD4 T cells together with a highly expanded effector memory CD4 cell population (Figure 7F). Nonetheless, as shown for T cells from Foxo1-deficient mice, this expansion in *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ mice was not spontaneous but dependent upon antigen recognition given that OTII *Rag1*$^{-/-}$ *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ mice exhibited a 90% loss of T cells when compared with controls (Figure 7G). Similar to mice with a T cell-specific deletion of Foxo1, the total number of LN B cells in *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ mice was increased (Figure 7H), along with the numbers of GC and isotype-switched B cells in the LN and spleen (Figure 7I and data not shown).

Despite the mature thymocyte accumulation, there were almost no detectable Treg cells in the thymus of *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ mice (Figure 7J). However, similar to *Cd4Cre Foxo1*$^{f/f}$ mice, comparable numbers of cells expressing normal amounts of Foxp3 were recovered in the peripheral lymphoid organs of wild-type and *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ mice (Figures 7K and 7L). We conclude that Foxo1 and Foxo3 have critical overlapping roles in the development and function of thymic-derived Treg cells although they are not absolutely required for Foxp3 expression and maintenance.

DISCUSSION

The highly conserved role of Foxo transcription factors in cell-cycle inhibition and apoptosis has been extensively studied in the past decade; however, recent studies have challenged this view regarding T and B cells and revealed highly specialized roles in the regulation of the adaptive immune system development and homeostasis (Hedrick, 2009; Dejean et al., 2010). In this study, we uncovered mechanisms that underlie the critical role of Foxo transcription factors in T cell fate specification, especially with regard to Treg cell differentiation. An implication of this work is that Foxo transcription factors integrate signaling through the PI3K-mTORC2-AKT and TGF-β-SMAD signaling pathways in order to guide the differentiation of CD4^{+} T cells.

Mice with a T cell-specific deletion of Foxo1 exhibit T cell organ infiltration, Tfh cell differentiation, B cell proliferation, and autoantibody production. The additional T cell-specific deletion of Foxo3 dramatically aggravated this phenotype, leading to premature death. Because we previously showed that deletion of Foxo3 alone does not result in T cell-intrinsic defects (Dejean et al., 2009), these results imply that Foxo1 orchestrates a program of gene expression, whereas Foxo3 provides mainly redundant activity in T cells. This phenomenon contrasts with the observation that Foxo-dependent tumor suppressor activity in vivo can be rescued by the expression of a single allele of Foxo1, Foxo3, or Foxo4 (Paik et al., 2007).

The phenotypic characteristics resulting from T cell-specific Foxo deficiencies are associated with two critical and interrelated mechanisms of T cell tolerance: TGF-β responsiveness, as indicated by the impaired induction of iTreg in vitro, and thymic-Treg cell development, the latter being profoundly altered when both *Foxo1* and *Foxo3* were ablated. In accord with these results, recent evidence shows that the attenuation or inhibition of PI3K-Akt signaling in CD4^{+} T cells is required to allow proper Treg cell development both in vitro and in vivo. Initial studies reported that both human and mice peripheral Treg cells have altered AKT phosphorylation upon TCR or IL-2R stimulation (Bensinger et al., 2004; Crellin et al., 2007). Furthermore,

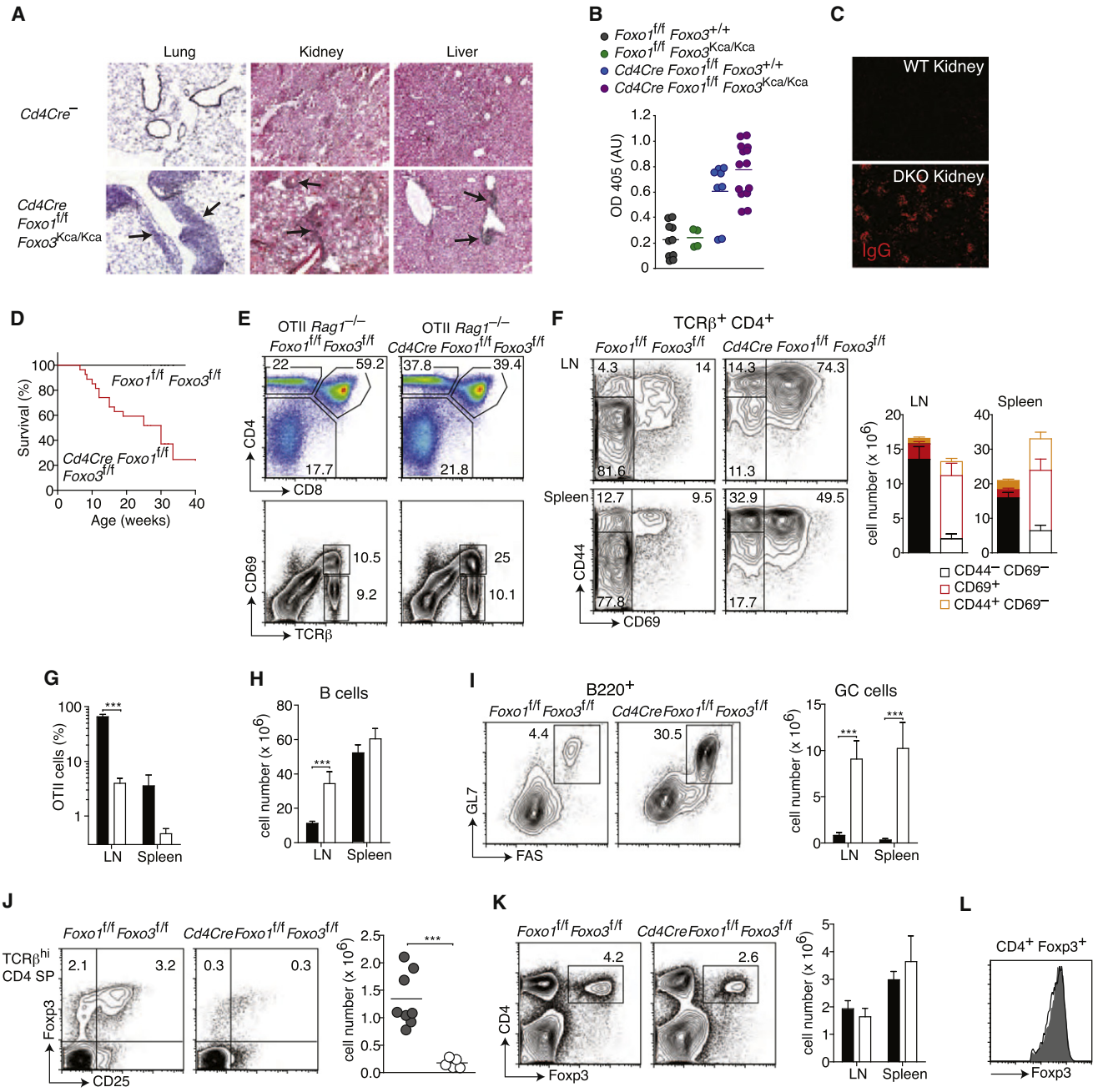

Figure 7. Partially Redundant Role of Foxo1 and Foxo3 in T Cells

(A–L) Eight-week-old *Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ (filled symbols) and *Cd4Cre Foxo1*$^{f/f}$ *Foxo3*$^{f/f}$ (open symbols).

(A) H&E staining of nonlymphoid tissues from 15-week-old mice (n = 6 mice per genotype).

(B) Anti-dsDNA ELISA quantification from 10-week-old mice (pooled results from two independent experiments).

(C) Immunofluorescence analysis of kidney sections from 10-week-old mice.

(D) Kaplan-Meier survival curve (n = 37, Cre$^-$; n = 27, Cre$^+$).

(E) Thymic subpopulations from 8-week-old mice (n ≥ 3 mice per genotype analyzed).

(F) Flow cytometry profile and enumeration of CD4$^+$ T cells from the peripheral lymphoid organs (n ≥ 5 mice per genotype analyzed in two independent experiments).

(G) Vα2$^+$ Vβ5$^+$ cell recovery among total leukocytes in peripheral lymphoid organs (n ≥ 3 mice per genotype analyzed).

(H) Enumeration of B220$^+$ cells from peripheral lymphoid organs (n ≥ 5 mice per genotype analyzed in two independent experiments).

(I) Flow cytometry profile and the number of LN B220$^+$ germinal center cells (n ≥ 5 mice per genotype analyzed in two independent experiments).

(J) Flow cytometry analysis and enumeration of Treg cells within thymic TCRβ^{hi} CD4 SP cells (pooled results from two experiments).

(K) Flow cytometry analysis (LN) and number of TCRβ$^+$ CD4$^+$ Foxp3$^+$ cells (pooled results from two experiments).

(L) Flow cytometry profile of Foxp3 expression in peripheral Treg cells.

inhibition of PI3Kα and δ, or Akt promotes Treg cell differentiation upon TCR stimulation in vitro in the absence of TGF-β signaling (Sauer et al., 2008). Conversely, constitutive expression of Akt impairs TGF-β-induced Foxp3 expression in vitro and selectively inhibits thymic Treg cell differentiation in vivo (Haxhinasto et al., 2008), whereas T cells lacking mTOR differentiate down a regulatory pathway by default (Delgoffe et al., 2009). These results are consistent with the thesis that Foxo is required for Treg cell development, and this depends upon counteracting the PI3K-, mTorc2-, Akt-dependent inactivation of Foxo. This could arise from TGF-β-induced inhibition of Akt phosphorylation or, in principle, by a TGF-β-induced nuclear localization of Foxo via one of the several posttranslational modifications known to override Akt-mediated nuclear exclusion (Hedrick, 2009).

Reports show that rapamycin treatment also enhances Foxp3$^+$ cell development (Haxhinasto et al., 2008; Sauer et al., 2008), and although it was thought to be specific for the inhibition of mTorc1 and not mTorc2 (Jacinto et al., 2004), more recent analyses have shown that long-term treatment with rapamycin does indeed affect mTorc2 (Sarbassov et al., 2006; Julien et al., 2010). Consistent with this notion, mTorc1-deficient cells do not spontaneously differentiate into iTreg cells in the absence of TGF-β (Delgoffe et al., 2009). In accord with the hypothesis that Foxo1 is the critical downstream target of PI3K-Akt signaling, we observed that PI3K inhibitors or rapamycin treatment does not rescue the defective differentiation of iTreg cells from Foxo1-deficient CD4 T cells (data not shown). The present results thus establish the PI3K-mTORC2-AKT-Foxo axis as the critical pathway in both thymic and induced Treg cell development.

The data presented suggest a link between downstream effectors of the PI3K pathway and the TGF-β-SMAD pathway. Consistent with this idea, PI3K-mTOR inhibition and TGF-β stimulation synergize to induce the development of iTreg cells in vitro (Sauer et al., 2008). Also, studies in nonlymphoid cells have shown that Foxo transcription factors directly interact with SMAD3 and SMAD4 and are required to regulate part of the TGF-β-induced gene expression program (Gomis et al., 2006; Seoane et al., 2004). Furthermore, TGF-β suppresses the expression of T-bet (Neurath et al., 2002), and the data presented here showed that TGF-β inhibition of T-bet required Foxo1.

As described in the introduction, extensive studies have shown that Foxp3 is not a lineage commitment factor, but rather there exists higher-order control of Treg cell lineage specification. We propose that Foxo transcription factors constitute one element of that higher order control. During the review of this manuscript, and consistent with this contention, two reports appeared showing that Foxo1 plays a role in the direct transcriptional control of *Foxp3* (Ouyang et al., 2010; Harada et al., 2010). We note, however, that within the population of Foxp3$^+$ Foxo1-deficient T cells, the amount of Foxp3 per cell was only marginally decreased.

One way that Foxo transcription factors could control Treg cell development is through TGF-β, but the relationship between thymic Treg cell development and TGF-β has not been entirely resolved. Mice with a deficiency in TGF-β or either one of its two receptors do not lack nTreg cells; however, T cell-specific loss of TGFβRI caused a deficiency in nTreg cells in neonates, and further deletion of the *Il2* gene caused a complete and permanent loss of nTreg cells (Liu et al., 2008). The authors concluded that heightened IL-2 expression in the absence of TGF-β signaling was responsible for expanding a diminished nTreg cell population. We also found a large population of peripheral Foxp3$^+$ cells in mice with a T cell-specific Foxo1, Foxo3 deficiency even though thymic Treg cell development was very severely compromised—the implication being that there exists a homeostatic mechanism that expands peripheral Treg cells in the absence of adequate thymic development. We propose that a Foxo and TGF-β-dependent genetic program controls aspects of Treg cell differentiation that are perhaps dispensable for the maintenance of Foxp3 expression and Foxp3$^+$ cell expansion in response to homeostatic or inflammatory cues. Consistent with this, constitutive AKT activation impairs de novo expression of Foxp3, but does not alter established Foxp3 expression in Treg cells (Haxhinasto et al., 2008). Interestingly, this phenomenon also mirrors the phenotype of mice deficient for a conserved noncoding DNA sequences in the *Foxp3* locus (CNS3-deficient mice), in which despite a normal level of Foxp3 expression, the proportion of Foxp3$^+$ cells is strongly decreased in the thymus but not in peripheral T cells (Zheng et al., 2010). A possibility is that Foxo factors are necessary to "open" the *Foxp3* locus, but the Foxo sites are not necessary elements for *Foxp3* transcription.

Although there is a larger-than-normal population of Foxp3$^+$ T cells in *Cd4Cre Foxo*$^{f/f}$ mice, these Treg cells are nonfunctional in vivo. This can be explained in part by reduced CTLA-4 expression in thymic, peripheral, and induced Treg cells. Importantly, this CTLA-4 phenotype is also observed in the Treg cells from mixed bone marrow chimeras, lacking chronic inflammation (data not shown). Underlying this loss of CTLA-4 expression is a proximal Foxo-binding element in the *Ctla4* promoter apparently required for full CTLA-4 expression. As such, we propose that the source of autoimmunity in Foxo-deficient mice derives from a highly inefficient production of Treg cells that are also ineffective as a consequence of deficient CTLA-4 expression.

Finally, in mice with a T cell-specific Foxo deficiency there appeared large numbers of Tfh cells. Such cells differentiate via a stepwise program of signaling that includes high-affinity interactions with peptide-MHC molecules, costimulation through CD28 and ICOS, further stimulation through CD30 and OX40, and antigen-specific cognate interactions with B cells (Linterman and Vinuesa, 2010; McHeyzer-Williams et al., 2009). The spontaneous formation of large numbers Tfh cells is thus unlikely to be simply a consequence of unrestricted T cell activation. One possibility is that PI3K-Akt-mTorc2-Foxo signaling constitutes a lineage commitment pathway that, in part, specifies alternative cell fates: Treg cells versus Tfh cells. Under stimulatory conditions that promote Foxo nuclear localization (e.g., the presence of TGF-β), T cells adopt a Treg cell fate, whereas under conditions that favor Foxo inactivation, such as signaling initiated by Icos activation of the potent p50α regulatory subunit of PI3K (Fos et al., 2008), T cells can adopt a Tfh cell phenotype. This implies that Foxo transcription factors are downstream of the *sanroque* mutation that promotes Tfh cell differentiation through exaggerated Icos expression (Yu et al., 2007).

Another possibility is that the paucity of Treg cell differentiation in the thymus produces a population of T cells preternaturally poised to assume a Tfh cell phenotype. Thymocytes with

a high-affinity receptor preferentially differentiate into nTreg cells (Josefowicz and Rudensky, 2009), and if this pathway is partly inactive, these higher-affinity T cells might be overrepresented in the secondary lymphoid organs. Because Tfh cells are also thought to preferentially differentiate from CD4$^+$ T cells with a high-affinity receptor (McHeyzer-Williams et al., 2009), a Foxo1 deficiency may increase the frequency of Tfh cell progenitors. This, combined with a loss of Foxo transcription factors mimicking Icos signaling and a paucity of Treg cell activity might be sufficient to promote Tfh cell differentiation and antibody-mediated autoimmunity. In conclusion, Foxo transcription factors play an essential role in T cell lineage commitment, minimally connecting the PI3K-AKT-mTOR and the TGF-β-SMAD pathways and potentially integrating this input with other aspects of organismal physiology to modify the induction and course of immunity.

EXPERIMENTAL PROCEDURES

Mice

Mice were maintained in a specific-pathogen free vivarium. All experiments were carried out in accordance to the Institutional Animal Care and Use Committee of University of California, San Diego. *Cd4Cre Foxo1*$^{f/f}$, *ERCre Foxo1*$^{f/f}$, *Foxo3*^{Kca}, *Foxo3*$^{f/f}$, *Cd4Cre Foxo1*$^{f/f}$. OTI *Cd4Cre Foxo1*$^{f/f}$, and OTII *Cd4Cre Foxo1*$^{f/f}$ *Rag1*$^{-/-}$ mice on mixed *FVB* and C57BL/6 strain backgrounds have been previously described (Kerdiles et al., 2009; Dejean et al., 2009). Mice were euthanized when they were no longer able to eat and drink and counted as deceased for the purposes of a Kaplan-Meier survival graph. *Cd2-Il7ra* transgenic mice were the generous gift of A. Singer (Yu et al., 2004) and crossed to *CD4Cre Foxo1*$^{f/f}$ mice. B10.A mice were bred to *Cd4Cre Foxo1*$^{f/f}$ mice for generating *CD4Cre Foxo1*$^{f/f}$ *H2*$^{b/k}$ mice. In all experiments using *ERCre Foxo1*$^{f/f}$ and *Foxo1*$^{f/f}$ controls, tamoxifen (Sigma) was administered i.p. daily for 5 days followed by 5 days' rest as previously described (Kerdiles et al., 2009). Spontaneous encephalitis was identified as described (Laouar et al., 2008). Bone marrow chimeras were produced in irradiated *Tcra*$^{-/-}$ mice as previously described (Kerdiles et al., 2009). The source of T cells was distinguished by CD45 alleles.

Flow Cytometry

Fluorochrome-labeled antibodies for FACS analysis were purchased from BD Biosciences, Biolegend, or eBioscience. Biotinylated antibodies specific for TGFβRII (R&D Systems) and CXCR5 (BD Biosciences) were used before SA-PE was used (eBioscience). PE-conjugated antibody reagent sets were used for Bcl2 and Ki-67 staining (BD Bioscience). Similarly, BrdU (Sigma) incorporation was detected with a FITC-conjugated BrdU antibody reagent kit (BD Biosciences). For intracellular Foxp3, CTLA-4, and T-bet staining, cells were first stained with antibodies to extracellular proteins then fixed, permeabilized with the Foxp3 Staining Kit (eBioscience), and stained with directly conjugated Foxp3-, CTLA-4-, or T-bet- (Santa Cruz Biotechnology) specific antibodies. For intracellular Foxo1 staining, after permeabilization, cells were treated with Foxo1-specific antibody (Cell Signaling) and then with a goat anti-rabbit PE antibody (Santa Cruz Biotechnology). For intracellular cytokine staining, cells were fixed with Cytofix/Cytoperm (BD Biosciences), permeabilized, and stained with Permwash (BD Biosciences). Data were analyzed with FlowJo Software (TreeStar).

Spinal cords were dissected from PBS-perfused mice, homogenized for 30 min at 37°C in HBSS plus 2% FCS supplemented with 1 mg/mL of Collagenase A (Sigma), filtered through a 70 μm nylon mesh, and centrifuged at 460 *g* for 5 min at 4°C. The pellet was then resuspended in 4 ml of Percoll 70% and overlaid with 4 ml 37% Percoll. The percoll gradient was centrifuged at 460 *g* for 20 min at 20°C. Cells were collected from the 37%/70% Percoll interface.

CD4$^+$ T Cell Purification, In Vitro Culture, and Proliferation

Naive CD4$^+$ T cells were isolated by magnetic depletion of cells labeled with biotinylated antibodies to Ter119, B220, MHCII, DX5, CD8, CD11b, CD25, and CD69 (eBioscience) and streptavidin-microbeads (Miltenyi Biotec). Splenocytes from *Tcra*$^{-/-}$ mice were used as antigen-presenting cells. Purified T cells were labeled with CFSE (Molecular Probes) as previously described (D'Souza et al., 2008). For T cell activation, 10^5 cells/well were cultured in U-bottom 96-well plates precoated with goat anti-Hamster IgG (Vector Labs) and supplemented with the indicated concentrations of anti-CD3 and anti-CD28 (Biolegend). iTreg cultures included 3 ng/mL recombinant human TGFβ1 (R&D Systems); Th1 cell-polarizing conditions included 10 μg/ml anti-IL4 (eBioscience) and 5 ng/ml IL-12 (eBioscience). Where indicated, IL-7 (eBioscience) was added at 10 ng/ml. For restimulation, cells were cultured for 4–5 hr with 100 ng/mL PMA (Calbiochem), 1 nM ionomycin (Sigma), and 2 μM GolgiStop solution (eBioscience) prior to intracellular cytokine staining. Cells were pulsed with 1 μCi [^{3}H]thymidine (PerkinElmer Life Sciences) for the last 8 hr of culture, and radioactivity was determined by scintillation counting. Secreted cytokines were quantified with the Th1, Th2, and Th17 cell cytometric bead array (BD Biosciences).

Quantitative PCR

Total RNA from sorted cells was extracted with TRIzol reagent (Invitrogen) and treated with the DNA-free kit (Ambion), and cDNA was synthesized with the Superscript III reverse transcription kit (Invitrogen). qPCR reactions were done with the Power SYBR Green PCR Master Mix (Applied Biosystems) and 30 nM reference dye (Stratagene). Data were collected by an Mx3005P (Stratagene) and analyzed with MxPro software (Stratagene). *Blimp1*, *Bcl6*, and *IL21* were amplified by PCR. Chromatin immunoprecipitation was carried out as described (Kerdiles et al., 2009; Lin et al., 2010). All primer sequences are available upon request.

Histology and Immunohistochemistry

For analysis of pathology and immune infiltration into nonlymphoid organs, 8–10 μm frozen sections were stained with hematoxylin and eosin. For germinal center identification, 8–10 μm frozen LNs sections were stained with anti-IgD-PE (Biolegend) and PNA-FITC (Vector Labs). IgG deposition in the kidney was identified by staining with anti-IgG-PE (BD Bioscience). Pancreata were fixed in formalin prior to sectioning.

Antibody Quantification

The quantification of each immunoglobulin isotype in sera was determined by use of the Mouse Immunoglobulin Isotype Panel Kit (Southern Biotech). Anti-dsDNA antibodies in sera were quantified with the mouse anti-dsDNA ELISA kit (Alpha Diagnostic Int.).

In Vitro and in Vivo Negative Selection

The in vitro OVA-peptide mediated thymocyte deletion assay was performed with *Tcra*$^{-/-}$ splenocytes for antigen presentation (Vasquez et al., 1992). Negative thymocyte selection in vivo was studied by the deletion of Vβ5$^+$, Vβ8$^+$, and Vβ11$^+$ T cells in blood of 8- to 12-week-old mice.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.immuni.2010.12.002.

ACKNOWLEDGMENTS

We thank A. Singer, NCI, Bethesda, MD, for providing *Cd2-Il7Ra* transgenic mice and D. Vignali (St. Jude's, TN) and A. Rudensky (Sloan-Kettering Memorial Hospital, New York, NY) for bone marrow from male *Foxp3*$^-$ mice. We thank Y. Lin (UC San Diego) for help with chromatin immunoprecipitation, I. Adams for expert mouse typing and technical support, and A. Morales and L. Lefler for mouse line maintenance. We especially thank P. Vogel, DVM, PhD, for the histological analysis and scoring of pancreata and the Veterinary Pathology Core at St. Jude for preparing, cutting, and staining tissue sections. This work was supported by PHS grant R01 AI073885 to S.M.H. and Juvenile Diabetes Research Foundation grant #2-2007-105 to M.A.M. E.L.S. is supported by an (K12) Institutional Research and Academic Career Development Award (L. Brunton, Director).

Received: May 11, 2010
Revised: September 3, 2010
Accepted: October 26, 2010
Published online: December 16, 2010

REFERENCES

Bensinger, S.J., Walsh, P.T., Zhang, J., Carroll, M., Parsons, R., Rathmell, J.C., Thompson, C.B., Burchill, M.A., Farrar, M.A., and Turka, L.A. (2004). Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. J. Immunol. *172*, 5287–5296.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857–868.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J. Exp. Med. *198*, 1875–1886.

Cooper, G.S., and Stroehla, B.C. (2003). The epidemiology of autoimmune diseases. Autoimmun. Rev. *2*, 119–125.

Crellin, N.K., Garcia, R.V., and Levings, M.K. (2007). Altered activation of AKT is required for the suppressive function of human CD4+CD25+ T regulatory cells. Blood *109*, 2014–2022.

D'Souza, W.N., Chang, C.F., Fischer, A.M., Li, M., and Hedrick, S.M. (2008). The Erk2 MAPK regulates CD8 T cell proliferation and survival. J. Immunol. *181*, 7617–7629.

Dejean, A.S., Beisner, D.R., Ch'en, I.L., Kerdiles, Y.M., Babour, A., Arden, K.C., Castrillon, D.H., DePinho, R.A., and Hedrick, S.M. (2009). Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells. Nat. Immunol. *10*, 504–513.

Dejean, A.S., Hedrick, S.M., and Kerdiles, Y.M. (2010). Highly specialized role of Foxo transcription factors in the immune system. Antioxid. Redox Signal. in press. Published online August 1, 2010.

Delgoffe, G.M., Kole, T.P., Zheng, Y., Zarek, P.E., Matthews, K.L., Xiao, B., Worley, P.F., Kozma, S.C., and Powell, J.D. (2009). The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity *30*, 832–844.

Drennan, M.B., Elewaut, D., and Hogquist, K.A. (2009). Thymic emigration: Sphingosine-1-phosphate receptor-1-dependent models and beyond. Eur. J. Immunol. *39*, 925–930.

Dutz, J.P., Ong, C.J., Marth, J., and Teh, H.S. (1995). Distinct differentiative stages of CD4+CD8+ thymocyte development defined by the lack of coreceptor binding in positive selection. J. Immunol. *154*, 2588–2599.

Fabre, S., Carrette, F., Chen, J., Lang, V., Semichon, M., Denoyelle, C., Lazar, V., Cagnard, N., Dubart-Kupperschmitt, A., Mangeney, M., et al. (2008). FOXO1 regulates L-Selectin and a network of human T cell homing molecules downstream of phosphatidylinositol 3-kinase. J. Immunol. *181*, 2980–2989.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. *4*, 330–336.

Fos, C., Salles, A., Lang, V., Carrette, F., Audebert, S., Pastor, S., Ghiotto, M., Olive, D., Bismuth, G., and Nunès, J.A. (2008). ICOS ligation recruits the p50alpha PI3K regulatory subunit to the immunological synapse. J. Immunol. *181*, 1969–1977.

Friedline, R.H., Brown, D.S., Nguyen, H., Kornfeld, H., Lee, J., Zhang, Y., Appleby, M., Der, S.D., Kang, J., and Chambers, C.A. (2009). CD4+ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance. J. Exp. Med. *206*, 421–434.

Gavin, M.A., Rasmussen, J.P., Fontenot, J.D., Vasta, V., Manganiello, V.C., Beavo, J.A., and Rudensky, A.Y. (2007). Foxp3-dependent programme of regulatory T-cell differentiation. Nature *445*, 771–775.

Gomis, R.R., Alarcón, C., He, W., Wang, Q., Seoane, J., Lash, A., and Massagué, J. (2006). A FoxO-Smad synexpression group in human keratinocytes. Proc. Natl. Acad. Sci. USA *103*, 12747–12752.

Gubbels Bupp, M.R., Edwards, B., Guo, C., Wei, D., Chen, G., Wong, B., Masteller, E., and Peng, S.L. (2009). T cells require Foxo1 to populate the peripheral lymphoid organs. Eur. J. Immunol. *39*, 2991–2999.

Guo, K., McMinn, J.E., Ludwig, T., Yu, Y.H., Yang, G., Chen, L., Loh, D., Li, C., Chua, S.J., Jr., and Zhang, Y. (2007). Disruption of peripheral leptin signaling in mice results in hyperleptinemia without associated metabolic abnormalities. Endocrinology *148*, 3987–3997.

Han, S., Dillon, S.R., Zheng, B., Shimoda, M., Schlissel, M.S., and Kelsoe, G. (1997). V(D)J recombinase activity in a subset of germinal center B lymphocytes. Science *278*, 301–305.

Harada, Y., Harada, Y., Elly, C., Ying, G., Paik, J.H., Depinho, R.A., and Liu, Y.C. (2010). Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells. J. Exp. Med.

Haxhinasto, S., Mathis, D., and Benoist, C. (2008). The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. J. Exp. Med. *205*, 565–574.

Hedrick, S.M. (2004). The acquired immune system: A vantage from beneath. Immunity *21*, 607–615.

Hedrick, S.M. (2009). The cunning little vixen: Foxo and the cycle of life and death. Nat. Immunol. *10*, 1057–1063.

Hill, J.A., Feuerer, M., Tash, K., Haxhinasto, S., Perez, J., Melamed, R., Mathis, D., and Benoist, C. (2007). Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. Immunity *27*, 786–800.

Hosaka, T., Biggs, W.H., 3rd, Tieu, D., Boyer, A.D., Varki, N.M., Cavenee, W.K., and Arden, K.C. (2004). Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc. Natl. Acad. Sci. USA *101*, 2975–2980.

Hsieh, C.S., Zheng, Y., Liang, Y., Fontenot, J.D., and Rudensky, A.Y. (2006). An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. Nat. Immunol. *7*, 401–410.

Ise, W., Kohyama, M., Nutsch, K.M., Lee, H.M., Suri, A., Unanue, E.R., Murphy, T.L., and Murphy, K.M. (2010). CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. Nat. Immunol. *11*, 129–135.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Rüegg, M.A., Hall, A., and Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat. Cell Biol. *6*, 1122–1128.

Josefowicz, S.Z., and Rudensky, A. (2009). Control of regulatory T cell lineage commitment and maintenance. Immunity *30*, 616–625.

Julien, L.A., Carriere, A., Moreau, J., and Roux, P.P. (2010). mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. Mol. Cell. Biol. *30*, 908–921.

Kerdiles, Y.M., Beisner, D.R., Tinoco, R., Dejean, A.S., Castrillon, D.H., DePinho, R.A., and Hedrick, S.M. (2009). Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. Nat. Immunol. *10*, 176–184.

Laouar, Y., Town, T., Jeng, D., Tran, E., Wan, Y., Kuchroo, V.K., and Flavell, R.A. (2008). TGF-beta signaling in dendritic cells is a prerequisite for the control of autoimmune encephalomyelitis. Proc. Natl. Acad. Sci. USA *105*, 10865–10870.

Lin, J.T., Martin, S.L., Xia, L., and Gorham, J.D. (2005). TGF-beta 1 uses distinct mechanisms to inhibit IFN-gamma expression in CD4+ T cells at priming and at recall: Differential involvement of Stat4 and T-bet. J. Immunol. *174*, 5950–5958.

Lin, W., Haribhai, D., Relland, L.M., Truong, N., Carlson, M.R., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cell development in the absence of functional Foxp3. Nat. Immunol. *8*, 359–368.

Lin, Y.C., Jhunjhunwala, S., Benner, C., Heinz, S., Welinder, E., Mansson, R., Sigvardsson, M., Hagman, J., Espinoza, C.A., Dutkowski, J., et al. (2010). A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. Nat. Immunol. *11*, 635–643.

Linterman, M.A., and Vinuesa, C.G. (2010). Signals that influence T follicular helper cell differentiation and function. Semin. Immunopathol. *32*, 183–196.

Liu, Y., Zhang, P., Li, J., Kulkarni, A.B., Perruche, S., and Chen, W. (2008). A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. Nat. Immunol. *9*, 632–640.

Mabrouk, I., Buart, S., Hasmim, M., Michiels, C., Connault, E., Opolon, P., Chiocchia, G., Lévi-Strauss, M., Chouaib, S., and Karray, S. (2008). Prevention of autoimmunity and control of recall response to exogenous antigen by Fas death receptor ligand expression on T cells. Immunity *29*, 922–933.

McHeyzer-Williams, L.J., Pelletier, N., Mark, L., Fazilleau, N., and McHeyzer-Williams, M.G. (2009). Follicular helper T cells as cognate regulators of B cell immunity. Curr. Opin. Immunol. *21*, 266–273.

Mueller, D.L. (2010). Mechanisms maintaining peripheral tolerance. Nat. Immunol. *11*, 21–27.

Neurath, M.F., Weigmann, B., Finotto, S., Glickman, J., Nieuwenhuis, E., Iijima, H., Mizoguchi, A., Mizoguchi, E., Mudter, J., Galle, P.R., et al. (2002). The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. J. Exp. Med. *195*, 1129–1143.

Ouyang, W., and Li, M.O. (2010). Foxo: In command of T-lymphocyte homeostasis and tolerance. Trends Immunol.. in press. Published online November 23, 2010.

Ouyang, W., Beckett, O., Flavell, R.A., and Li, M.O. (2009). An essential role of the Forkhead-box transcription factor Foxo1 in control of T cell homeostasis and tolerance. Immunity *30*, 358–371.

Ouyang, W., Beckett, O., Ma, Q., Paik, J.H., DePinho, R.A., and Li, M.O. (2010). Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. Nat. Immunol. *11*, 618–627.

Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. (2007). FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell *128*, 309–323.

Perkins, D., Wang, Z., Donovan, C., He, H., Mark, D., Guan, G., Wang, Y., Walunas, T., Bluestone, J., Listman, J., and Finn, P.W. (1996). Regulation of CTLA-4 expression during T cell activation. J. Immunol. *156*, 4154–4159.

Ramsdell, F., Seaman, M.S., Miller, R.E., Tough, T.W., Alderson, M.R., and Lynch, D.H. (1994). gld/gld mice are unable to express a functional ligand for Fas. Eur. J. Immunol. *24*, 928–933.

Rubtsov, Y.P., and Rudensky, A.Y. (2007). TGFbeta signalling in control of T-cell-mediated self-reactivity. Nat. Rev. Immunol. *7*, 443–453.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol. Cell *22*, 159–168.

Sauer, S., Bruno, L., Hertweck, A., Finlay, D., Leleu, M., Spivakov, M., Knight, Z.A., Cobb, B.S., Cantrell, D., O'Connor, E., et al. (2008). T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. Proc. Natl. Acad. Sci. USA *105*, 7797–7802.

Seoane, J., Le, H.V., Shen, L., Anderson, S.A., and Massagué, J. (2004). Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell *117*, 211–223.

Shiow, L.R., Rosen, D.B., Brdicková, N., Xu, Y., An, J., Lanier, L.L., Cyster, J.G., and Matloubian, M. (2006). CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature *440*, 540–544.

Sugimoto, N., Oida, T., Hirota, K., Nakamura, K., Nomura, T., Uchiyama, T., and Sakaguchi, S. (2006). Foxp3-dependent and -independent molecules specific for CD25+CD4+ natural regulatory T cells revealed by DNA microarray analysis. Int. Immunol. *18*, 1197–1209.

Tone, Y., Furuuchi, K., Kojima, Y., Tykocinski, M.L., Greene, M.I., and Tone, M. (2008). Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nat. Immunol. *9*, 194–202.

Torres-Borrego, J., Molina-Terán, A.B., and Montes-Mendoza, C. (2008). Prevalence and associated factors of allergic rhinitis and atopic dermatitis in children. Allergol. Immunopathol. (Madr.) *36*, 90–100.

Vasquez, N.J., Kaye, J., and Hedrick, S.M. (1992). In vivo and in vitro clonal deletion of double-positive thymocytes. J. Exp. Med. *175*, 1307–1316.

von Boehmer, H., and Melchers, F. (2010). Checkpoints in lymphocyte development and autoimmune disease. Nat. Immunol. *11*, 14–20.

Wan, Y.Y., and Flavell, R.A. (2009). How diverse–CD4 effector T cells and their functions. J. Mol. Cell Biol. *1*, 20–36.

Wing, K., and Sakaguchi, S. (2010). Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat. Immunol. *11*, 7–13.

Woodland, D.L., Happ, M.P., Gollob, K.J., and Palmer, E. (1991). An endogenous retrovirus mediating deletion of alpha beta T cells? Nature *349*, 529–530.

Yamamoto, A., Lucas, J.J., and Hen, R. (2000). Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell *101*, 57–66.

Yu, Q., Erman, B., Park, J.H., Feigenbaum, L., and Singer, A. (2004). IL-7 receptor signals inhibit expression of transcription factors TCF-1, LEF-1, and RORgammat: Impact on thymocyte development. J. Exp. Med. *200*, 797–803.

Yu, D., Tan, A.H., Hu, X., Athanasopoulos, V., Simpson, N., Silva, D.G., Hutloff, A., Giles, K.M., Leedman, P.J., Lam, K.P., et al. (2007). Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature *450*, 299–303.

Zheng, Y., Josefowicz, S., Chaudhry, A., Peng, X.P., Forbush, K., and Rudensky, A.Y. (2010). Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature *463*, 808–812.

Zhu, J., and Paul, W.E. (2010). Heterogeneity and plasticity of T helper cells. Cell Res. *20*, 4–12.

Ziegler, S.F. (2006). FOXP3: Of mice and men. Annu. Rev. Immunol. *24*, 209–226.