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Deltex1 Is a Target of the Transcription Factor NFAT that Promotes T Cell Anergy

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SUMMARY

The molecular process underlying T cell anergy is incompletely understood. Deltex1 (DTX1) is a Notch target with unknown physiological function. Here we show that *Dtx1* was a transcription target of nuclear factor of activated T cells (NFAT) and participated in T cell anergy. DTX1 protein was upregulated during T cell anergy, and transgenic expression of *Dtx1* attenuated T cell activation. DTX1 inhibited T cell activation by both E3-dependent and E3-independent mechanisms. In addition, DTX1 suppressed T cell activation in the absence of its Notch-binding domain. Importantly, DTX1 regulated the expression of two anergy-associated molecules, growth arrest and DNA-damage-inducible 45 β (Gadd45β) and Cbl-b. DTX1 interacted with early growth response 2 (Egr-2) for optimum expression of Cbl-b. Furthermore, deficiency of DTX1 augmented T cell activation, conferred resistance to anergy induction, enhanced autoantibody generation, and increased inflammation. DTX1 therefore represents a component downstream of calcium-NFAT signaling that regulates T cell anergy.

INTRODUCTION

T cell anergy is generated in vivo by stimulation of T cell receptors in the absence of any costimulatory molecule (Schwartz, 2003; Fathman and Lineberry, 2007; Saibil et al., 2007). The partial activation of T cells that follows, with new protein synthesis, leads to a state unresponsive to further stimulation through the antigen receptor. T cell anergy can also be produced in vitro by treating T cells with a calcium ionophore alone or through the activation of nuclear factor of activated T cells (NFAT) (Macián et al., 2002). Recent studies have revealed the involvement of specific E3 ubiquitin ligases, including Cbl-b, Itch, and the gene related to anergy in lymphocyte (GRAIL, ring finger protein 128 [Rnf128]), in T cell anergy (Anandasabapathy et al., 2003; Heissmeyer et al., 2004; Gao et al., 2004; Jeon et al., 2004; Fathman and Lineberry, 2007). T cell anergy is also mediated by molecules and processes independent of E3, such as LAT palmitoylation, $p27^{Kip1}$, and diacylglycerol kinase-α (Hundt et al., 2006; Li et al., 2006; Zha et al., 2006; Olenchock et al., 2006). In addition, a number of genes, including growth arrest and DNA-damage-inducible 45 β (*Gadd45b*), early growth response gene 2 (*Egr2*) and *Egr3*, are upregulated in anergic T cells (Safford et al., 2005). Interestingly, Cbl-b, Itch, GRAIL, Egr-2, Egr-3, and diacylglycerol kinase-α are downstream targets of calcium-NFAT signaling (Heissmeyer et al., 2004; Zha et al., 2006). Egr-2 and Egr-3 have been shown to regulate the expression of Cbl-b by a mechanism yet to be characterized (Safford et al., 2005).

Deltex was originally identified in *Drosophilia* by its genetic interaction with Notch (Matsuno et al., 1995) and is a gene transactivated by Notch (Matsuno et al., 1998). Deltex (DTX) contains two WWE domains at its N terminus, which account for its interaction with Notch (Zweifel et al., 2005), followed by a proline-rich motif and a RING finger domain at the C terminus. Several studies suggest that Deltex plays a critical role in the RBP-J-independent Notch signaling pathway (Ramain et al., 2001; Hu et al., 2003; Hori et al., 2004). Recent generation of a *Dtx* null mutant in *Drosophila*, however, suggests that Deltex is involved only in a fraction of Notch signaling that is RBP-J dependent (Fuwa et al., 2006). The function of Deltex in Notch signaling transduction in mammals remains mostly ambiguous. Overexpression of DTX1 also inhibits Notch signals, as shown by enhanced B cell lymphocyte development and suppressed T cell development (Izon et al., 2002; Yun and Bevan, 2003).

We recently demonstrated that overexpression of DTX1 suppresses T cell activation, whereas *Dtx1* downregulation enhances T cell activation (Liu and Lai, 2005). We found that, as an E3 ligase, DTX1 binds MAP kinase/ERK kinase kinase 1 (MEKK1) and stimulates its ubiquitination and degradation (Liu and Lai, 2005). However, lymphocyte development and antibody production are normal in mice with a DTX1 mutant lacking the RING finger domain (Storck et al., 2005), suggesting that E3 ligase activity of DTX1 is not absolutely required for lymphocyte development and function. In a second DTX1 gene-targeted study, deletion of the first WWE domain did not affect lymphocyte and myeloid cell development (Lehar and Bevan, 2006). The phenotypes found in these two $Dtx1^{\Delta/\Delta}$ mice are in sharp contrast to the profound effect of Notch deficiency in lymphocyte differentiation (Radtke et al., 2004; Maillard et al., 2005; Tanigaki and Honjo, 2007; Rothenberg et al., 2008). Because the Deltex family contains four members (DTX1, DTX2, DTX3,

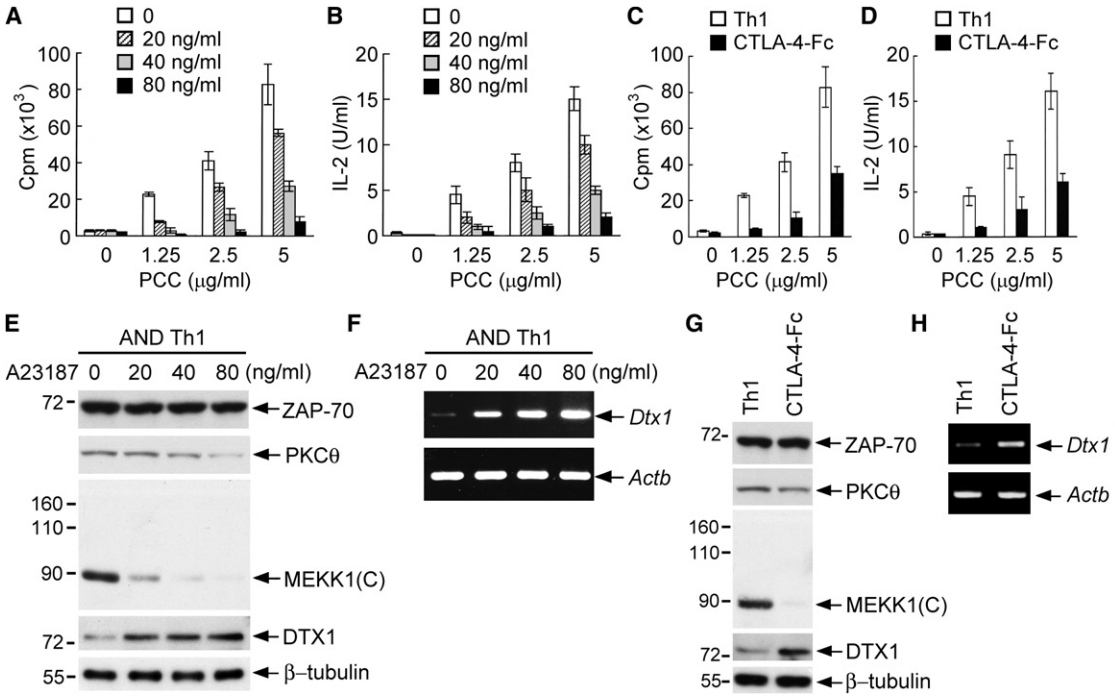

Figure 1. Upregulation of DTX1 in T Cell Anergy Induced by Calcium Ionophore and CTLA4-Fc

(A and B) A23817-triggered T cell anergy. T cells from AND transgenic mice were primed to Th1 cells, and then treated with indicated doses of A23187 for 16 hr. T cells were washed and restimulated with the specific antigen pigeon cytochrome c (PCC) and irradiated presenting cells. T cell proliferation (A) was determined by ^{3}H-thymidine incorporation 60 hr later, whereas IL-2 production (B) was quantified after 24 hr.

(C and D) T cell anergy induced by CTLA4-Fc. AND Th1 cells were stimulated by antigen and irradiated presenting cells in the absence or presence of CTLA4-Fc for 2 days. T cells were isolated and T cell proliferation (C) and IL-2 generation (D) were determined during restimulation with PCC-presenting cells.

(E and G) DTX1 was upregulated in anergic T cells triggered by A23187 or CTLA4-Fc. Lysates from T cells tolerized by A23187 or CTLA4-Fc were analyzed for expression of ZAP70, PKCθ, DTX1, and MEKK1 via immunoblots.

(F and H) Induction of *Dtx1* transcript in T cells tolerized by A23187 and CTLA4-Fc. The expression of *Dtx1* mRNA was determined 16 hr after A23187 (F) or 36 hr after CTLA4-Fc cotreatment (H) of AND T cells.

Values (A–D) are the mean ± SD of triplicate samples. Data shown are the representative of two independent experiments.

and DTX4) in mammals (Kishi et al., 2001; Storck et al., 2005), the lack of effect from targeting *Dtx1* could be due to compensation from other Deltex members for the physiological function of DTX1. Alternatively, either the RING finger or the WWE domain may be dispensable for the function of DTX1 in lymphocyte development.

In the present study, we generated DTX1-deficient mice without resorting to truncated *Dtx1* transcripts. We identified that DTX1 is an important player in T cell anergy: DTX1 is induced in T cell anergy, and *Dtx1* is a transcription target of NFAT. We showed that DTX1 suppressed T cell activation by both ubiquitin E3-dependent and E3-independent mechanisms. DTX1 also controlled the expression of Gadd45β and Cbl-b. Deletion of *Dtx1* promoted T cell activation, conferred resistance to tolerance induction, and led to increased inflammation and autoimmunity. Our results identify DTX1 as an NFAT target that promotes T cell anergy.

RESULTS

Dtx1 Is Upregulated in Anergic T Cells

We found that T cell anergy is associated with increased DTX1 expression. Anergy was induced in AN6.2 and 5C.C7-derived TCR (AND) Th1 cells by persistent calcium signaling (A23187 treatment) in the absence of other stimulation (Macian et al., 2002) or by blockage of costimulatory signaling via cytotoxic T lymphocyte antigen-4 (CTLA4)-Fc (Wells et al., 2001). The status of anergy was confirmed by attenuated T cell proliferation and diminished IL-2 production when stimulated by the specific antigen pigeon cytochrome c (PCC) (Figures 1A–1D), with the extent of anergy proportional to the concentration of A23187 used. Similar to previous observations in anergic T cells (Heissmeyer et al., 2004), A23187 or CTLA4-Fc treatment led to decreased PKCθ expression (Figures 1E and 1G). In addition, there was a prominent induction of DTX1 protein (Figures 1E and 1G). The upregulation of DTX1 in anergic T cells was accompanied with a proportional downregulation of MEKK1(C) (Figures 1E and 1G), the catalytically active form of MEKK1 (Liu and Lai, 2005). In contrast, expression of ZAP70 was not substantially altered in anergic T cells (Figures 1E and 1G), as reported by Jeon et al. (2004) and Hundt et al. (2006). T cell anergy was also induced in vivo in mice by intravenous administration of anti-CD3, as shown by the attenuated T cell responses upon restimulation (Figures S1A and S1B available online). In anti-CD3-triggered T cell anergy, there was also a profound induction of DTX1 protein, with nearly undetectable MEKK1(C) (Figure S1C).

The extent of DTX1 induction was proportional to the dose of A23187 used to tolerize T cells (Figure 1E). The increase in DTX1 protein correlated with an upregulation of *Dtx1* mRNA in anergic T cells (Figures 1F and 1H). The increase in DTX1 transcript and protein was also proportional to the duration of A23187 stimulation (Figures S2A and S2B). Abolition of calcium ionophore-mediated signaling by cyclosporin-A, previously shown to prevent T cell anergy, suppressed the induction of *Dtx1* mRNA and DTX1 protein by A23187 (Figures S2C and S2D). Therefore, expression of DTX1 is strongly associated with T cell anergy.

Dtx1 Is a Transcription Target of NFAT

Because *Dtx1* is induced in anergic T cells (Figures 1F and 1H) and NFAT dictates the expression of several T cell anergy-associated molecules (Macián et al., 2002; Heissmeyer et al., 2004), we went on to examine whether *Dtx1* expression is linked to NFAT. There is an NFAT-like binding sequence (GGAAAA) 88 bp upstream of the AUG site in the *Dtx1* promoter. Overexpression of NFATc and NFATp in T cells enhanced the expression of *Dtx1* mRNA and protein (Figures 2A and 2B), suggesting that *Dtx1* is a transcription target of NFATc and NFATp. This was further confirmed by the activation of the *Dtx1* promoter (−1300 to −1 bp) in T cells treated with A23187 or transfected with NFATc or NFATp (Figures 2C and 2D). A DNA affinity precipitation assay (DAPA) also illustrated that nuclear-localized NFATp and NFATc (Figures 2E and 2F, Input) were brought down by biotinylated NFAT-containing sequence from the *Dtx1* promoter in A23187-treated EL4 cells (Figures 2E and 2F, DAPA). The association of NFAT with *Dtx1* promoter in vivo was further examined by chromatin immunoprecipitation (ChIP) analysis. Anti-NFATp or anti-NFATc, but not mouse IgG control, pulled down DNA containing the NFAT-binding element on the *Dtx1* promoter (Figures 2G and 2H), indicating that NFAT is bound to the promoter of *Dtx1* in anergic T cells. Therefore, *Dtx1* is a direct transcription target of NFAT.

Transgenic Expression of *Dtx1* Suppresses T Cell Activation

To further delineate the role of DTX1 in T cell anergy in vivo, *Dtx1*-transgenic mice were generated with a *Cd4* promoter to direct T cell-specific *Dtx1* expression (Figure S3A). *Dtx1* transgene expression in T cells led to a reduction in MEKK1 expression, but did not interfere with T cell profiles in thymocytes and splenocytes (Figures S3B and S3C). A profound suppression of the proliferation and IL-2 production of thymocytes (Figures S3D and S3F) and splenic T cells (Figures S3E and S3G) from *Dtx1*-transgenic mice was found, compared to those of normal littermate controls (NLC) mice. Neither the $CD44^{lo}CD62L^{hi}$ naive T cell fraction nor the $CD44^{hi}CD62L^{lo}$ memory T cell population was affected by the DTX1 transgene (Figures S3H and S3I). Overexpression of DTX1 did not affect the $CD4^{+}CD25^{+}$ population, Foxp3 expression in $CD4^{+}CD25^{+}$ or $CD4^{+}CD25^{-}$ cells (Figure S3J), or production of IL-10 and TGF-β by regulatory T (Treg) cells (Figures S3K and S3L). Therefore, transgenic DTX1 inhibits T cell activation but does not alter T cell development.

DTX1 Also Inhibits T Cell Activation through an E3-Independent Mechanism

Two independent *Dtx1* gene-targeted mice have recently been generated, with no apparent immunological phenotype detected. Deletion of the RING finger domain from DTX1 did not affect either lymphocyte development or T cell-dependent B cell responses (Storck et al., 2005), whereas the removal of the first WWE domain from DTX1 (Supplemental Results, Figure S4) did not interfere with normal lymphocyte development (Lehar and Bevan, 2006).

We therefore examined the ability of DTX1 lacking either the RING finger domain (DTX1ΔRF) or the WWE domain (DTX1ΔN) to suppress T cell activation. In contrast to wild-type DTX1,

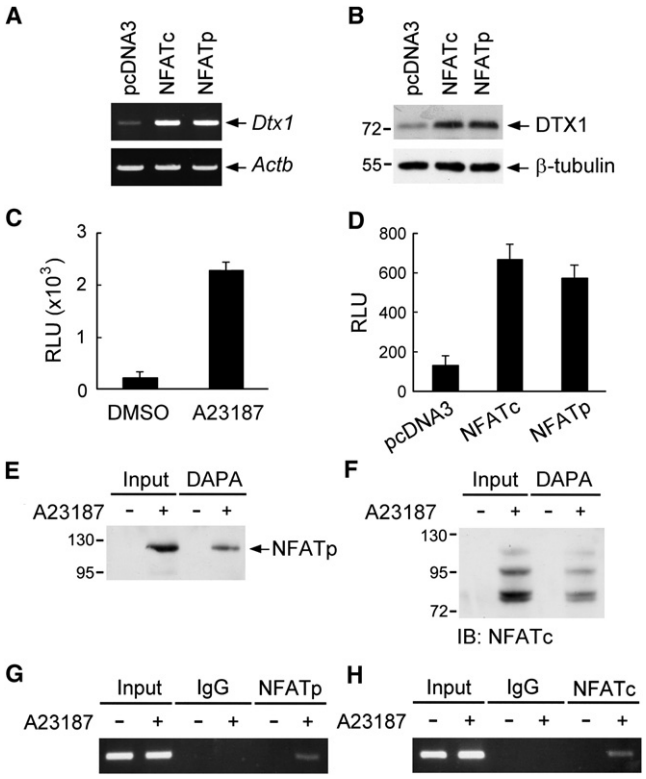

Figure 2. DTX1 Is a Target of NFAT

(A and B) *Dtx1* is a transcription target of NFAT. DO11.10 T cell hybridomas were transfected with pcDNA3 vector, NFATc, or NFATp, and the expression of *Dtx1* transcripts (A) and DTX1 protein (B) was determined 24 hr later.

(C) Activation of *Dtx1* promoter by A23187. Luciferase reporter driven by *Dtx1* promoter (−1300 to −1 bp) (*Dtx1*-Luc) was transfected into EL4 cells, and luciferase activity stimulated by A23187 or DMSO was determined after 24 hr.

(D) Direct activation of the *Dtx1* promoter by NFATc or NFATp. EL4 cells were transfected with *Dtx1*-Luc plus pcDNA3, NFATc, or NFATp, and luciferase quantified after 24 hr.

(E and F) Association of NFAT with *Dtx1* promoter sequences. Nuclear extracts were prepared in DO11.10 cells treated with DMSO (−) or A23187 for 1 hr (E) and 3 hr (F). Biotinylated oligonucleotides corresponding to *NFAT* element on *Dtx1* promoter were used in DNA affinity precipitation assay (DAPA). The precipitates were resolved by SDS-PAGE and probed with anti-NFATp (E) or anti-NFATc (F).

(G and H) Localization of NFATp or NFATc on *Dtx1* promoter. DO11.10 cells were treated with DMSO (−) or A23187 for 1 hr (G) and 3 hr (H), cross-linked with formaldehyde, and nuclei isolated. Fragmented chromatin was immunoprecipitated with anti-NFATp (4G6-G5), anti-NFATc (7A6), or control mouse IgG. The presence of NFAT-element-containing *Dtx1* promoter region was identified by PCR. Inputs are the PCR products from fragmented chromatins. Values (C, D) are the mean ± SD of triplicate samples. Data shown are the representative of two independent experiments.

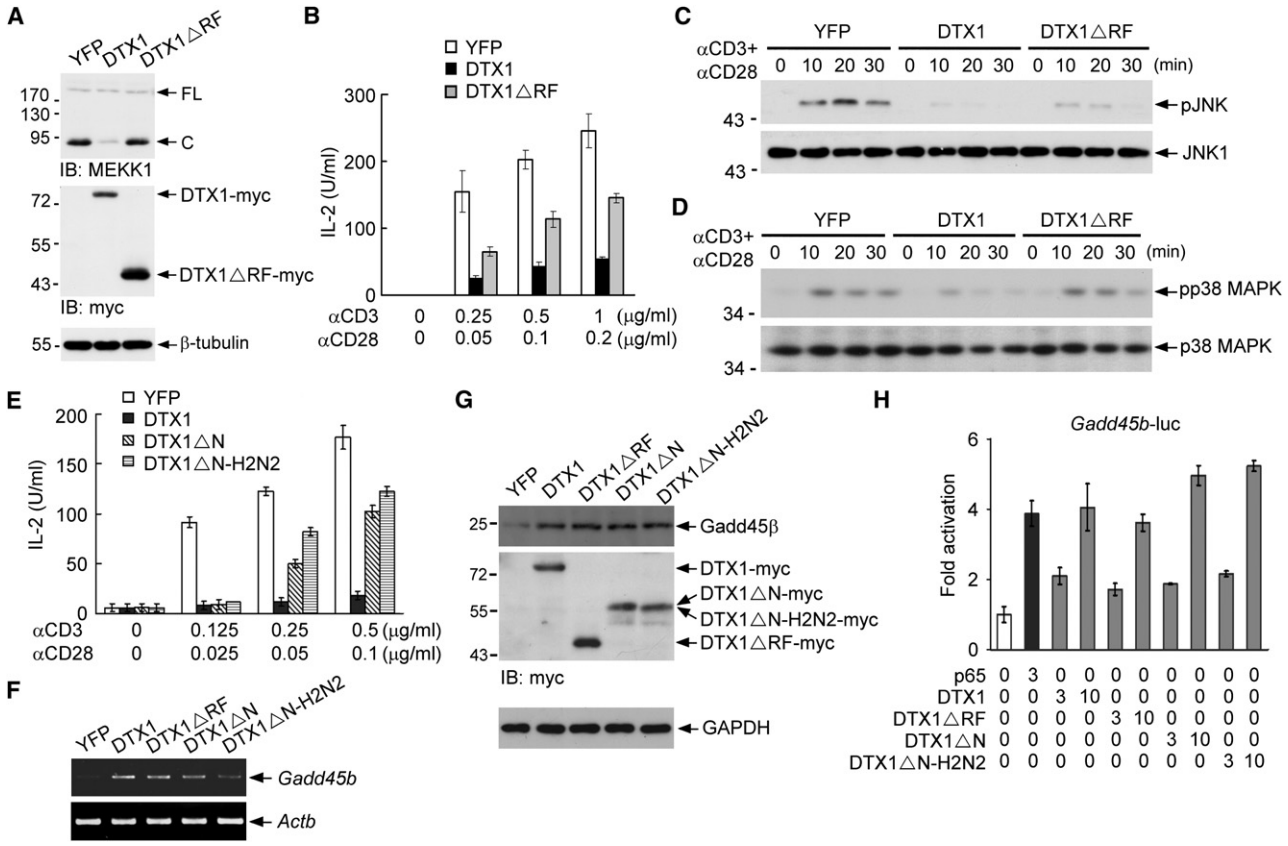

Figure 3. DTX1 Inhibits T Cell Activation also through E3- and WWE-Independent Mechanism

(A) RING finger (RF) domain deletion abolished the ability of DTX1 to downregulate MEKK1. DO11.10 cells were transduced with control retrovirus (YFP) or retrovirus containing either DTX1-Myc or DTX1ΔRF-Myc. The infected cells were sorted and the expression of DTX1-Myc, DTX1ΔRF-Myc, and MEKK1 was determined by anti-Myc. FL, full length MEKK1; C, C-terminal domain of MEKK1.

(B) RING finger domain deletion from DTX1 (DTX1ΔRF) led to a partial decrease in the inhibition of T cell activation. YFP-, DTX1-, or DTX1ΔRF-expressing DO11.10 T cells were stimulated with anti-CD3+anti-CD28 at the indicated concentrations, and IL-2 produced was quantified.

(C and D) DTX1ΔRF inhibited JNK, but not p38 MAPK. YFP control and DO11.10 cells expressing DTX1 or DTX1ΔRF were activated and total cell extracts prepared at the indicated time points. The activation of JNK was assessed with anti-phospho JNK (T183-Y185) and anti-JNK1 (C), whereas the activation of p38 MAPK was determined by anti-phospho p38 MAPK (T180-Y182) and anti-p38α (D).

(E) DTX1 mutants lacking WWE domain or with additional inactivated RF domain still inhibited T cell activation. YFP control and DO11.10 cells expressing DTX1, WWE domain-deleted DTX1 (DTX1ΔN), and DTX1 double mutant (DTX1ΔN-H2N2) were activated through TCR+CD28 stimulation, and IL-2 expression was determined.

(F and G) DTX1, DTX1ΔRF, DTX1ΔN, or DTX1ΔN-H2N2 promoted the expression of *Gadd45b* in T cells. DO11.10 T cells were transduced with YFP vector, DTX1, DTX1ΔRF, DTX1ΔN, or DTX1ΔN-H2N2 and total RNA (F) or total cell lysates (G) were prepared. The expression of *Gadd45b* transcript (F) was determined by RT-PCR. The expression of Gadd45β protein and various DTX1 constructs (G) were quantified by anti-Gadd45β and anti-Myc, respectively.

(H) DTX1 and its mutants activate *Gadd45b* promoter. The *Gadd45b* promoter (−1191 to +140) was subcloned into pGL3 and transfected into EL4 cells with DTX1, DTX1ΔRF, DTX1ΔN, DTX1ΔN-H2N2, or NF-κB p65 at the indicated doses (in μg) by electroporation. Luciferase activity was determined after another 24 hr.

Values (B, E, H) are the mean ± SD of triplicate samples. Data shown are the representative of two (A, C, D, F, G), three (E, H), or four (B) independent experiments.

DTX1ΔRF did not affect MEKK1 expression (Figure 3A). The removal of the RING finger domain modestly reduced the ability of DTX1 to attenuate T cell activation (Figure 3B), supporting a contribution of the E3 ligase activity of DTX1 to T cell inactivation. However, DTX1ΔRF still effectively suppressed T cell activation (Figure 3B), indicating that DTX1 also inhibits T cell activation in an E3-independent manner. In addition, DTX1ΔRF inhibited JNK activation in the presence of intact MEKK1 (Figure 3C). RING finger deletion did abolish the ability of DTX1 to suppress TCR-mediated p38 MAPK activation (Figure 3D). Therefore, both the E3 activity and RF-independent activities of DTX1 contribute to its inhibition of T cell activation. A similar observation was found for DTX1 lacking the WWE domain (DTX1ΔN), which produced an attenuated, but still effective, suppression of T cell activation (Figure 3E). Though less effective than DTX1, DTX1ΔN inhibited the activation of JNK and p38 MAPK (Figures S5A and S5B). In addition, the DTX1 double mutant (DTX1ΔN-H2N2) with inactive RF and deleted WWE still inhibited T cell activation (Figure 3E). The DTX1 double mutant did not inhibit p38 MAPK, but modestly antagonized the activation of JNK and ERK (Figures S6A–S6C), suggesting that DTX1 may antagonize activation signals in the absence of E3 ligase and Notch-binding abilities.

We further identified another suppressive mechanism whereby DTX1 blocks JNK activation. Overexpression of DTX1 in T cells led to expression of *Gadd45b* mRNA and Gadd45β protein (Figures 3F and 3G). Gadd45β is known to suppress JNK by inactivation of MKK7 (Papa et al., 2007), suggesting that DTX1 could inhibit JNK activation by transactivation of Gadd45β. DTX1ΔRF, DTX1ΔN, or DTX1ΔN-H2N2 induced Gadd45β transcript and protein expression to an extent comparable to the wild-type DTX1 (Figures 3F and 3G). We isolated the *Gadd45b* promoter (−1191 bp to +140 bp) and demonstrated that DTX1 alone, similar to NF-κB p65 (De Smaele et al., 2001), activated the *Gadd45b* promoter (Figure 3H). Inactivation of the RF domain, WWE domain, or both domains did not affect the ability of DTX1 to activate the *Gadd45b* promoter (Figure 3H). Together, DTX1 inhibits T cell activation also in E3- and Notch-binding-independent manners, with induction of Gadd45β as one of such mechanisms.

DTX1 Deficiency Does Not Affect T Cell Development

The observation that DTX1 may inhibit T cell activation in the absence of RF or WWE domains led us to produce a new line of DTX1 gene-targeted mice completely lacking the *Dtx1* transcript. A *loxP*-flanked (fl) *Dtx1* locus was generated by inserting a neomycin cassette (*Neo*) into intron 3 and lox P sites into introns 2 and 3 (Figure 4A). Exon 3 and *Neo* were systematically deleted by breeding $Dtx1^{fl/+}$ mice with *EIIa-Cre* transgenic mice, followed by intercrossing between F1 progeny. Deletion of *Dtx1* was confirmed by Southern blot analysis (Figure 4B). The absence of *Dtx1*-truncated coding transcripts was also confirmed with primers to detect exons 2–3, exons 8–10, and 3′-UTR of *Dtx1* in RT-PCR analyses (Figure 4C). Deletion of the exon 3 did not affect the expression of exons 1–2 of *Dtx1*. DTX1 protein was also undetectable in splenic lymphocytes by immunoblot analysis (Figure 4D). These results indicate that our *Dtx1*-targeted mutant was indeed a *Dtx1* null mouse line. We also produced $Dtx1^{fl/fl\Delta neo}$ mice with only *Neo* deleted by breeding $Dtx1^{fl/fl}$ mice with *ACT-FLPe* transgenic mice.

$Dtx1^{-/-}$ mice were viable and fertile. DTX1 deficiency did not apparently interfere with thymocyte development or bone marrow B cell development (Figures 4E and 4F), similar to mice with genetic deletion of either RING-finger or WWE domains from *Dtx1* (Storck et al., 2005; Lehar and Bevan, 2006). The total thymocyte and bone marrow cell numbers were comparable between control and $Dtx1^{-/-}$ mice (Figure S7A). Early T cell development and differentiation of γδ T cells were indistinguishable between normal and $Dtx1^{-/-}$ mice (Figure S7B). Populations of mature $CD4^+$ and $CD8^+$ T cells were similar between normal and $Dtx1^{-/-}$ mice (Figures S7C and S7D). DTX1 deficiency also did not alter the $CD4^+CD25^+$ population, the $CD4^+Foxp3^+$ population, or Foxp3 expression in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells (Figures S8A–S8C). The production of inhibitory cytokines by Treg cells was mostly not affected by DTX1 deficiency except for a moderate decrease of TGF-β (Figures S8D and S8E). A more detailed description of B cells in $Dtx1^{-/-}$ mice is described in Supplemental Results (Figures S9 and S10). Therefore, the majority of the lymphocyte populations we examined were not substantially affected by the absence of DTX1, suggesting that DTX1 is mostly dispensable for lymphocyte development. DTX1 deficiency did lead to increased MEKK1 expression and reduced Gadd45β expression (Figure S7E).

Increased T Cell Activation and Systemic Inflammation Diseases in $Dtx1^{-/-}$ Mice

Despite the relatively normal lymphocyte population, there was an increase in total mature peripheral lymphocyte numbers in 8-week-old $Dtx1^{-/-}$ mice (Figure 5A). Modest splenomegaly was always detected in $Dtx1^{-/-}$ mice (Figure 5A). The increase in total mature lymphocytes may be due to a reduced susceptibility of thymocytes to spontaneous T cell death (Figure S11). DTX1 deficiency led to a profound increase in T cell activation. The proliferation of $Dtx1^{-/-}$ thymocytes and splenic T cells doubled relative to those of NLC T cells, as measured by ^{3}H-thymidine incorporation (Figure 5B). Increased proliferation in DTX1 null T cells was also confirmed by CFSE halving (Figure S12). TCR-induced IL-2 production was similarly elevated in $Dtx1^{-/-}$ splenic T cells (Figure 5C). Production of IFN-γ and IL-4 increased by nearly 100% in DTX1-deficient T cells (Figures 5D and 5E). We also characterized the signaling abnormality in DTX1-deficient T cells. Activation of JNK, p38 MAPK, and ERK was prominently increased in $Dtx1^{-/-}$ T cells (Figures 5F–5H). As a control, T cell activation was not affected in $Dtx1^{fl/fl\Delta neo}$ mice (Figure S13).

We went on to investigate whether elevated activation of T cells was linked to T cells themselves or was due to accessory cells. We generated mice with T cell-specific deletion of *Dtx1* (CD4-$Dtx1^{-/-}$). The absence of DTX1 in splenic T cells was confirmed by immunoblots (not shown). T cell proliferation and IL-2 production were substantially higher for CD4-$Dtx1^{-/-}$ T cells than for control T cells (Figure S14). The increase in T cell activation was comparable between T cells from $Dtx1^{-/-}$ mice and T cells from CD4-$Dtx1^{-/-}$ mice, suggesting that the observed elevated T cell activation was chiefly due to DTX1 deficiency in T lymphocytes.

The deletion of the *Dtx1* gene altered the sensitivity of T cells to anergy induction. In T cell anergy triggered by calcium ionophore (Figure 6A) or CTLA4-Fc (Figure 6B), DTX1 deficiency decreased the extent of T cell tolerance, as measured by IL-2 production (Figures 6A and 6B), or T cell proliferation (not shown). The IC_{50} for A23187 to induce anergy was increased from 75 ng/ml in WT cells to 360 ng/ml in DTX1 null T cells (Figure 6C). A similar increase in IC_{50} in DTX1-deficient T cells was also found with CTLA4-Fc (Figure 6D). $Dtx1^{-/-}$ T cells were more resistant to anergy induction than T cells from control littermates.

We also found that deletion of the *Dtx1* gene increased the susceptibility of mice to autoimmune disease. Hematoxylin and eosin (H&E) staining of paraffin-embedded sections of lung and liver revealed mononuclear cell infiltrations in older DTX1-deficient mice (>9 months) (Figure 6E), in contrast to control littermates. Anti-DNA and anti-histone in the serum of $Dtx1^{-/-}$ mice were elevated (Figures 6F and 6G), predominantly of IgM and IgG2b isotypes (Figure S15). A modest increase in autoantibody titer was also found in CD4-$Dtx1^{-/-}$ mice (Figure S16), supporting the role of T cell in autoimmunity. There was no apparent abnormality in the morphology of kidney glomeruli of $Dtx1^{-/-}$ mice, yet immunoglobulin complex was detected in the kidney glomeruli of $Dtx1^{-/-}$ mice (Figure 6H). Taken together, these disparate findings suggest that DTX1 deficiency in T cells led to increased inflammation and autoimmunity.

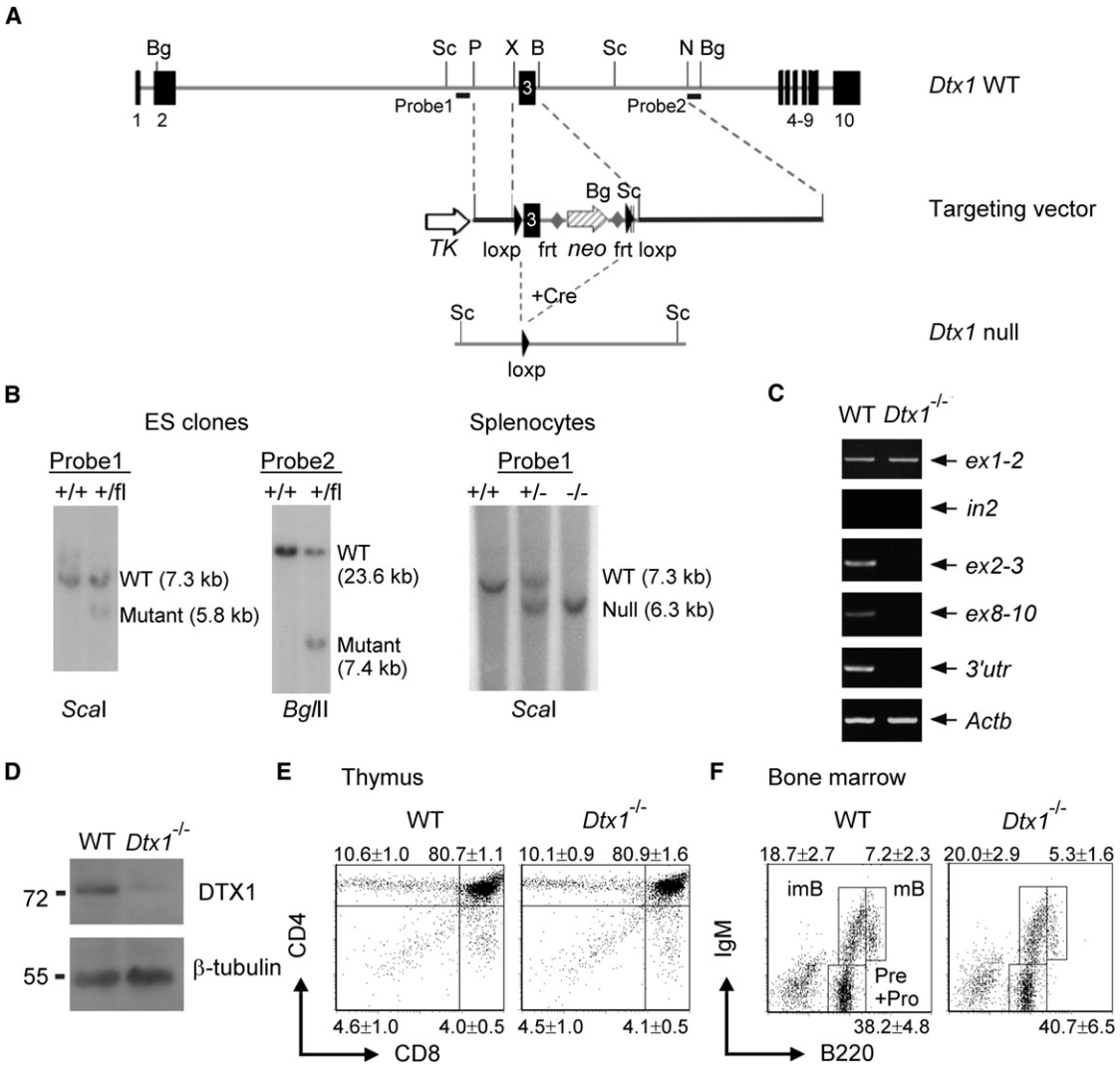

Figure 4. Targeted Disruption of *Dtx1*

(A) Structure of *Dtx1* genome and targeting construct. Upper panel (*Dtx1* WT) illustrates the genomic structure of *Dtx1*. The exons are numbered according to NCBI. The targeting construct is illustrated in the second panel, with genomic fragments corresponding to short arm (P to X) and long arm (B to N) indicated. The null allele is illustrated at bottom. B, BamHI; Bg, BglII; N, NheI; P, PmlI; Sc, ScaI; X, XmnI.

(B) Homologous recombination of the targeting construct and Cre recombination of the floxed allele. Left panels show Southern analysis of ScaI- or BglII-digested genomic DNA from wild-type and targeted R1 ES clones by probe 1 or 2 (indicated in A), respectively. Right panel shows Southern analysis of ScaI-digested genomic DNA from wild-type (+/+), +/−, and null (−/−) mice by probe 1.

(C) Absence of *Dtx1* transcript expression in *Dtx1*$^{-/-}$ mice. RNA isolated from splenocytes of control and *Dtx1*$^{-/-}$ mice was subjected to RT-PCR analysis with primers flanking exons 1–2, intron 2, exons 2–3, exons 8–10, or 3′-untranslated region. Primers for intron 2 were to screen hidden miRNA in that region.

(D) Absence of DTX1 protein in *Dtx1*$^{-/-}$ mice. Expression of DTX1 protein in *Dtx1*$^{-/-}$ and WT littermate in splenic lymphocytes was determined by immunoblot.

(E) Normal thymocyte development in DTX1-deficient mouse. Thymocytes from WT littermate and *Dtx1*$^{-/-}$ mice were stained with anti-CD4 and anti-CD8 to assess different thymocyte populations. n = 8.

(F) Normal bone marrow B cell development in DTX1-deficient mice. Bone marrow cells were stained with anti-IgM and anti-B220 to analyze the populations of developing B lymphocytes. imB, immature B; Pre+Pro, PreB plus ProB. n = 5. Number (E, F) indicates percentage and standard deviation of each subpopulation.

DTX1 Interacts with Egr-2 for Optimal Cbl-b Expression

The availability of a DTX1-deficient T cell enabled us to search for additional signaling defects caused by DTX1 deficiency. We examined the induction of *Cblb*, *Itch*, *Dgka*, *Rnf128*, *Egr2*, *Egr3*, and *Gadd45b* in T anergy generated by A23187 (Figure 7A). The expression of most anergy-associated molecules was not affected by the absence of DTX1, yet Ca^{2+}-mediated upregulation of Cbl-b and Gadd45β mRNA and protein were largely abolished in *Dtx1*$^{-/-}$ T cells (Figures 7A and 7B). The impaired induction of Cbl-b and Gadd45β in *Dtx1*$^{-/-}$ T cells was also found in T cell anergy generated through costimulatory blockade (Figure S17). Because Cbl-b expression is mediated by Egr-2 and Egr-3 (Safford et al., 2005), we examined whether DTX1 modulates the transactivation of Cbl-b by Egr-2. Overexpression

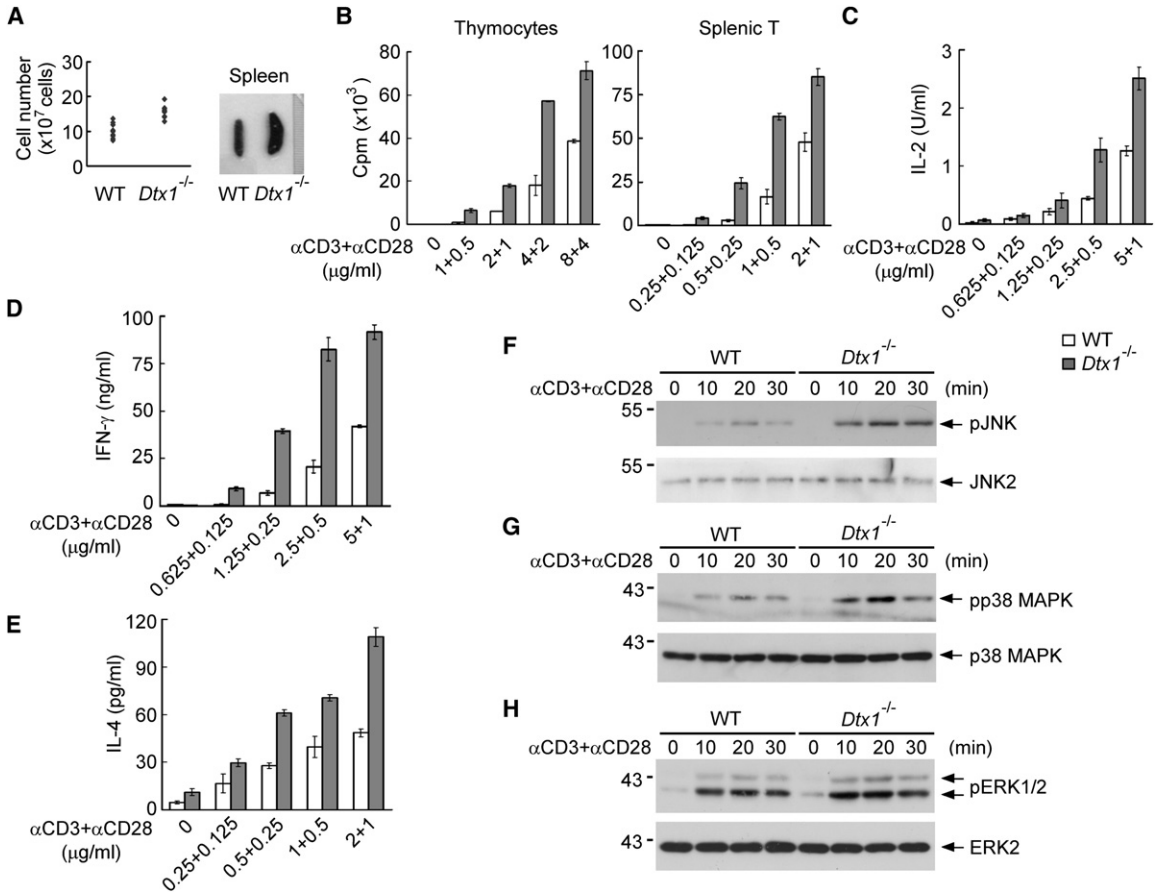

Figure 5. Enhanced Activation and Increased Cytokine Production in DTX1-Deficient T Cells

(A) Moderate splenomegaly in *Dtx1*$^{-/-}$ mice. The total splenocyte numbers for six pairs of *Dtx1*$^{-/-}$ and WT littermate mice were compared. Spleens from a pair of *Dtx1*$^{-/-}$ and WT control are shown (right).

(B and C) Enhanced T cell activation in *Dtx1*$^{-/-}$ mice. Thymocytes and splenic T cells from *Dtx1*$^{-/-}$ mice and WT littermates were stimulated with the indicated amount of anti-CD3+anti-CD28, and T cell proliferation (B) or splenic T cell IL-2 production (C) were determined 50 hr (B) or 24 hr (C) later.

(D and E) Both IFN-γ and IL-4 production were increased in *Dtx1*$^{-/-}$ T cells. IFN-γ production was measured in splenic T cells at same time as IL-2 in (C). For IL-4 production, splenic T cells were first activated with TPA (10 ng/ml) and A23187 (80 ng/ml) for 1 day, washed, and cultured with IL-2 for 2 additional days. T cells were restimulated with anti-CD3+anti-CD28, and IL-4 generated quantitated 24 hr later.

(F–H) Increased MAPK activation in DTX1-deficient T cells. Phosphorylation of JNK (F), p38 MAPK (G), and ERK (H) in splenic T cells after anti-CD3+anti-CD28 (5 μg/ml and 2.5 μg/ml) stimulation was determined.

Values (B–E) are the mean ± SD of triplicate samples. Data shown are the representative of three (F–H) or five (B–E) independent experiments.

of DTX1 alone in T cells did not induce the expression of Cbl-b, in contrast to a clear stimulatory effect of Egr-2 (Figures 7C and 7D). However, addition of DTX1 increased Egr-2-stimulated Cbl-b expression. The enhancement of Egr-2-mediated Cbl-b expression did not apparently involve the WWE or RF domains of DTX1: DTX1ΔN, DTX1ΔRF, and DTX1ΔN-H2N2 were as effective as DTX1 in promoting the expression of Cbl-b (Figure 7E). The synergistic effect of Egr-2 and DTX1 on Cbl-b expression was also observed on T cell activation. T cell activation was substantially attenuated by expression of either DTX1 or Egr-2, yet was nearly shut down by the simultaneous presence of DTX1 and Egr-2 (Figure 7F). In addition, there was an interaction between DTX1 and Egr-2-FLAG, demonstrated by the fact that DTX1 was pulled down by FLAG antibodies (Figure 7G). Therefore, Cbl-b is another anergy-associated molecule that is directly regulated by DTX1 through partnership with Egr-2.

DISCUSSION

Notch is well known for its pivotal role in dictating lymphocyte development (Radtke et al., 2004; Maillard et al., 2005; Tanigaki and Honjo, 2007; Rothenberg et al., 2008). Notch signaling is essential for T cell fate specification. Many Notch-mediated T cell development events are mediated by RBP-J (Tanigaki and Honjo, 2007). In contrast, a role for DTX in Notch signaling in T cell development has yet to be demonstrated. Two *Dtx1* gene targeting studies have illustrated that the RING finger domain or WWE domains of DTX1 are dispensable for normal lymphocyte development (Storck et al., 2005; Lehar and Bevan, 2006). Consistent with their finding, T cell development was nearly normal in our *Dtx1*$^{-/-}$ mice. Therefore, contrary to the profound effect of Notch1 absence in T cell development, DTX1 deficiency does not apparently affect T cell development.

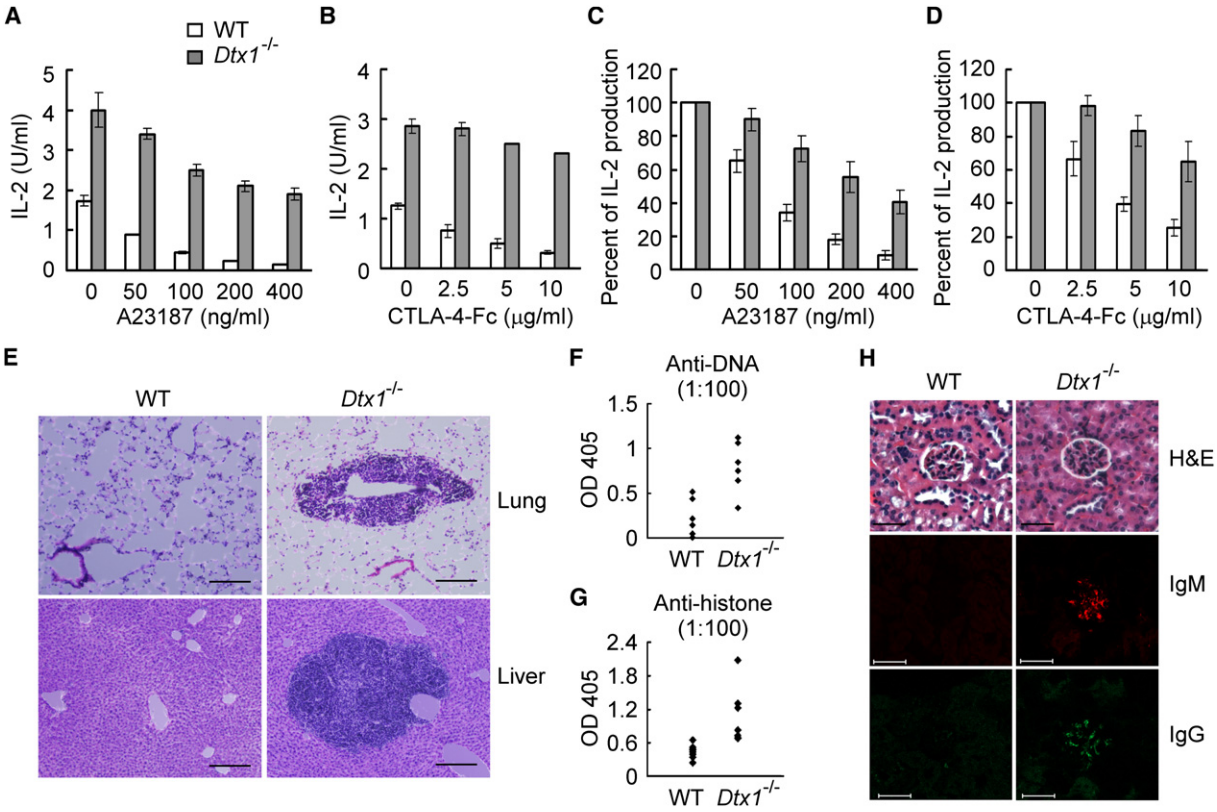

Figure 6. Deficiency in DTX1 Impairs T Cell Anergy Generation and Leads to Inflammation and Autoimmunity

(A–D) Absence of DTX1 impaired the induction of T cell anergy. Splenic T cells from WT littermate and *Dtx1*$^{-/-}$ mice were primed to Th1 and treated with A23187 for 16 hr (A) or CTLA4-Fc (B) to induce anergy. T cells were then stimulated with anti-CD3+anti-CD28 and IL-2 expression was determined after another 24 hr, and the shown results are the mean ± SD of triplicate samples from a single experiment (A, B). IL-2 production in the absence of anergy induction was used as 100% and the percentages of attenuation by treatment with A23187 (C) and CTLA4-Fc (D) shown are the mean ± SD from 3 independent experiments.

(E) Inflammation in DTX1-deficient mouse. Lung and liver from 9-month-old *Dtx1*$^{-/-}$ mice and normal littermate controls (WT) were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Scale bars represent 100 μm in lung sections and 200 μm in liver sections.

(F and G) Serum anti-DNA and anti-histone antibodies in *Dtx1*$^{-/-}$ mice. Sera (1:100 dilution) from *Dtx1*$^{-/-}$ mice and WT littermates older than 5 months were analyzed for anti-DNA and anti-histone antibodies. n = 6.

(H) Immunoglobulin deposits in kidney glomeruli in *Dtx1*$^{-/-}$ mice. Kidney frozen sections from *Dtx1*$^{-/-}$ mice and WT littermates were stained with Alexa Fluor 488-anti-mouse IgM or FITC-anti-mouse IgG. Scale bars represent 50 μm. The morphology of glomeruli was examined by H&E staining.

Micrographs (E, H) shown are the representative of five pairs of mice.

The lack of effect on T cell development by *Dtx1*$^{-/-}$ may be due to the presence of DTX2, DTX3, and DTX4, the three other DTX family members. Alternatively, enhanced Notch signaling is detected with deficiencies of DTX (Lehar and Bevan, 2006), suggesting that DTX1 does not mediate, but instead blocks, Notch1 signaling. We found that DTX1 deficiency led to increased IL-4 production in peripheral T cells, in direct contrast to the direct activation of IL-4 expression by Notch (Tanaka et al., 2006; Fang et al., 2007), suggesting that DTX1 may act in opposition to Notch. Additionally, given the complexity of Notch signaling, DTX1 may mediate Notch2 signals and inhibit Notch1 signaling. More work is required to establish the exact role of the DTX family in Notch signaling in T cells.

We had previously illustrated that DTX1 is an ubiquitin E3 ligase that directs MEKK1(C) for degradation (Liu and Lai, 2005). In this study we further found that DTX1 suppresses T cell activation, also in an E3-ligase-independent manner. Removal of the RF domain impaired the ability of DTX1 to suppress p38 MAPK, but DTX1 retained a partial capacity to attenuate T cell activation. We identified two additional mechanisms that DTX1 used to inhibit T cell activation, neither of which required the RF domain. First, DTX1 inhibits JNK activation through enhanced expression of Gadd45β. Gadd45β suppresses JNK signaling (De Smaele et al., 2001) by direct binding to MKK7 (Papa et al., 2007). The induction of Gadd45β is specifically relevant to anergy, as indicated by the fact that it is overexpressed in anergic T cells (Safford et al., 2005) and Gadd45β deficiency leads to autoimmunity (Liu et al., 2005). Identification of Gadd45β as a DTX1 target also illustrates the double-action of DTX1 to attenuate JNK activation by downregulation of MEKK1 and stimulation of GADD45β expression. There are similarities and differences in phenotype between the *Dtx1*$^{-/-}$ mouse and the *Gadd45b*$^{-/-}$ mouse. Hyperproliferation is observed in both *Gadd45b*$^{-/-}$ T cells (Liu et al., 2005) and *Dtx1*$^{-/-}$ T cells. Splenomegaly and IgM deposition in glomeruli are found in both *Gadd45b*$^{-/-}$ (Liu et al., 2005) and *Dtx1*$^{-/-}$ mice. Alternatively, TCR-induced IFN-γ production is

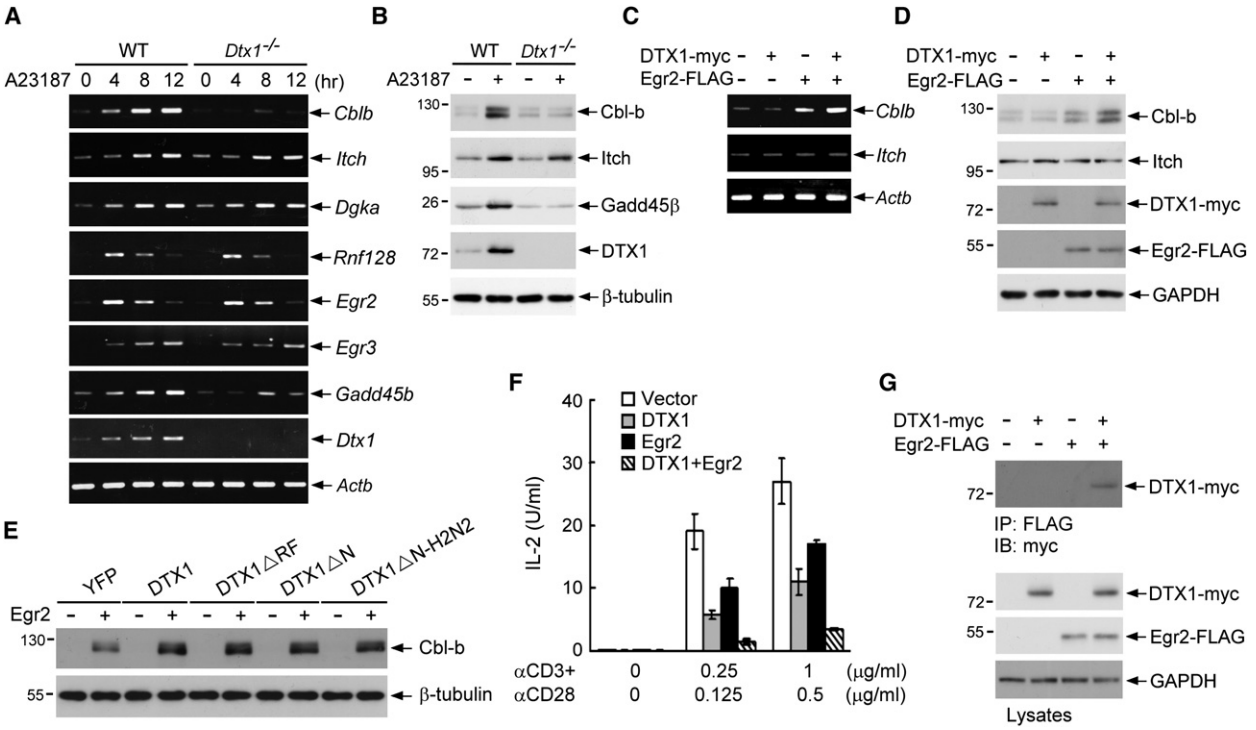

Figure 7. DTX1 Coordinates with Egr-2 for Cbl-b Expression

(A and B) Diminished expression of Cbl-b and Gadd45β in *Dtx1*$^{-/-}$ Th1 cells during anergy induction. T cells from control (WT) and *Dtx1*$^{-/-}$ mice and were primed to Th1 cells and were treated with A23187 (200 ng/ml) for 4, 8, 12 (A), or 16 (B) hr.

(A) RNA was isolated and the expression of *Cblb*, *Itch*, *Dgka*, *Rnf128*, *Egr2*, *Egr3*, *Gadd45b*, and *Actb* determined by RT-PCR.

(B) Cell lysates were prepared and the expression of Cbl-b, Itch, Gadd45β, DTX1, and β-tubulin analyzed by immunoblot.

(C and D) Egr-2-stimulated Cbl-b expression was enhanced by DTX1. DO11.10 T cells were transfected with DTX1-Myc and Egr-2-FLAG as indicated, and the expression of the mRNA (C) and protein (D) of Cbl-b and Itch determined.

(E) WWE or RF domains were not required for DTX1 to enhance Egr-2-mediated Cbl-b expression. DO11.10 cells expression YFP (Control), DTX1, DTX1ΔN, DTX1ΔRF, or DTX1ΔN-H2N2 were transfected with Egr-2 (+) or vector (–), and the expression of Cbl-b determined.

(F) Synergistic inhibitory effect of DTX1 and Egr-2 on T cell activation. DO11.10 T cells, transfected with the indicated constructs, were stimulated with anti-CD3+anti-CD28 24 hr after transfection. IL-2 was quantitated 18 hr after activation. Values are the mean ± SD of triplicate samples.

(G) Interaction between Egr-2 and DTX1. DTX1-Myc and Egr-2-FLAG were transfected into Jurkat cells, and cell lysates were prepared 24 hr later. Samples of 200 μg of cell lysates were precipitated with anti-FLAG, and DTX1-Myc was detected by anti-Myc by immunoblot. The expression of DTX1-Myc, Egr-2, and GAPDH in cell lysates was also determined.

Data (A–G) shown are the representative of two independent experiments.

impaired in *Gadd45b*$^{-/-}$ T cells (Lu et al., 2004) but is enhanced in *Dtx1*$^{-/-}$ T cells. TCR-mediated activation of ERK and p38 MAPK is reduced in *Gadd45b*$^{-/-}$ T cells (Lu et al., 2004) but is increased in *Dtx1*$^{-/-}$ T cells. The difference between Gadd45β null and DTX1 null mice can be partly attributed to the fact that DTX1 deficiency decreases, but does not completely abolish, the expression of Gadd45β. Furthermore, DTX1 regulates additional anergy-contributing molecules.

The second identified mechanism DTX1 uses to promote T cell anergy is the DTX1-dependent expression of Cbl-b. DTX1 deficiency diminished expression of Cbl-b in anergic T cells. We showed here that DTX1, similar to Egr-2 and Egr-3, is a downstream target of NFAT that regulates the transcription of *Cblb*. The regulatory effect of DTX1 is Cbl-b specific, as shown by the fact that Itch and GRAIL remained unaffected in DTX1-deficient T cells. DTX1 alone did not promote the expression of Cbl-b, yet DTX1 augmented Egr-2-mediated Cbl-b expression. *Dtx1*$^{-/-}$ and *Cblb*$^{-/-}$ mice share some phenotype similarities. T cell proliferation and IL-2 production are profoundly increased in *Dtx1*- and *Cblb*-deficient T lymphocytes (Chiang et al., 2000; Bachmaier et al., 2000). The susceptibility of *Cblb*$^{-/-}$ mice to acute lung inflammation (Bachmaier et al., 2007) may be analogous to the lung inflammation we observed in *Dtx1*$^{-/-}$ mice. However, there are also clear differences between *Cblb*$^{-/-}$ and *Dtx1*$^{-/-}$ T cells. The activation of JNK, ERK, and p38 MAPK is not affected in *Cblb*$^{-/-}$ T lymphocytes (Bachmaier et al., 2000), but was prominently enhanced in *Dtx1*$^{-/-}$ T cells. The production of IFN-γ in the primary stimulation of T cell is not altered in *Cblb*$^{-/-}$ T cells (Bachmaier et al., 2000), but here was elevated in *Dtx1*$^{-/-}$ T cells. Therefore, DTX1 regulates the amount of MEKK1, Gadd45β, and Cbl-b, yet the function of DTX1 cannot be attributed to any one of MEKK1, Gadd45β, or Cbl-b.

Another notable observation is this study is that the inhibitory activity of DTX1 on T cell activation was only weakly compromised in the absence of the WWE domain. Notch binding

therefore is not an essential component for DTX1 to suppress T cell signaling. DTX1ΔN stimulates Gadd45β expression, indistinguishably from DTX1 and DTX1ΔRF. In addition, DTX1 lacking the WWE domain, similar to DTX1ΔRF, enhanced Cbl-b expression. The observation that DTX1 controls the expression of Gadd45β and Cbl-b in the absence of WWE or RF domains demonstrates the diversified action mechanism of DTX1 in anergic T cells. There is a discrepancy between our DTX1 null mice and the previous two DTX1 gene-targeted mice (Storck et al., 2005; Lehar and Bevan, 2006): whereas inflammation and autoimmunity were detected in the present study, no apparent abnormality in lymphocyte function was found in the previous two DTX1 gene-targeted mice. We speculate that expression of the truncated DTX1 with deletion of either RING finger domain or WWE domains in these two gene-targeted mice still suppresses T cell activation. It may also be noted that we found autoimmune pathology in relatively old (>9-month-old) *Dtx1*$^{-/-}$ mice. Given the moderate effect of DTX1ΔN or DTX1ΔRF on T cell activation, some manifestation of autoimmunity at later stages cannot be excluded for the previous two DTX1 gene-targeted mice.

It may also be noted that in addition to a prominent role in T cell anergy, DTX1 is involved in the attenuation of B cell activation. The contribution of B cells to some of the autoimmune phenotypes in *Dtx1*$^{-/-}$ mice therefore cannot be excluded. We did not detect any change of Treg cell populations in *Dtx1*-transgenic and *Dtx1*$^{-/-}$ mice, but found a modest decrease of TGF-β production in *Dtx1*$^{-/-}$ Treg cells, suggesting that a role of Treg cells in T cell suppression cannot be completely ruled out. Future experiments shall help map the exact contribution of DTX1 from each cell population to prevent autoimmunity.

In summary, DTX1 is an addition to the list of molecules that contribute to T cell tolerance. *Dtx1* is a transcription target of NFAT that contributes to T cell anergy. NFAT binds to *Dtx1* promoter and promotes the expression of DTX1. It has previously been noted that T cell anergy is attained by the coordinated action of a complicated array of molecules (Saibil et al., 2007). Of the several E3 ligases that contribute to T cell anergy, each acts with a distinct mechanism: Cbl-b targets Vav-1, GRAIL binds RhoGDI, Itch promotes the degradation of JunB, and DTX1 stimulates the ubiquitination of MEKK1(C). Interestingly, similar to other tolerance-associated E3 ligases, DTX1 is localized in endosomes (Hori et al., 2004; Mukherjee et al., 2005). How the endocytic vesicle localization links to the action of DTX1 is currently being investigated. Among the E3 ligases associated with T cell anergy, DTX1 is unique in its ability to block T cell activation in both E3-dependent and E3-independent manners. Importantly, the identification of Gadd45β and Cbl-b as targets of DTX1 reveals a network in the regulation of T cell anergy by Ca^{2+}-NFAT signaling. Ca^{2+}-NFAT signaling stimulates the expression of DTX1, Egr-2, and Egr-3, and DTX1 in turn coordinates with Egr-2 and Egr-3 for the induction of Cbl-b. Whereas NFAT separately promotes the expression of Itch and GRAIL, the anergic function of NFAT is further extended by DTX1 through Gadd45β expression and MEKK1 downregulation. In addition, MEKK1, GADD45β, and Cbl-b are likely not the sole mediators of DTX1 in T cell anergy: DTX1ΔN is as effective as DTX1 in stimulating Gadd45β and Cbl-b expression, and DTX1ΔN downregulates MEKK1 via an intact RF domain, yet DTX1ΔN was less inhibitory than DTX1, indicating the presence of an additional suppressive mechanism of DTX1 that is WWE dependent. Moreover, DTX2, DTX3, and DTX4 may also regulate T cell tolerance, so the contribution from the whole DTX family to T cell anergy is likely higher than that which we report in the present study. Further work will help establish the full role of DTX family members in T cell anergy.

EXPERIMENTAL PROCEDURES

Reagents

Mouse *Dtx1* cDNA (Kishi et al., 2001) was a gift from H. Okano (Keiko University, Tokyo). DTX1ΔRF was generated by deletion of the C-terminal amino acids 347–627 from DTX1 (full-length 1–627 aa) by BamHI cleavage. DTX1ΔN was generated by deletion of the first 179 amino acid (containing both WWE domains) from DTX1 via PCR. The double mutant DTX1ΔN-H2N2 was generated from DTX1ΔN by inactivation of RF through mutation of H453 and H456 into N453 and N456. DTX1ΔRF, DTX1ΔN, and DTX1ΔN-H2N2 were subcloned into pcDNA4 and pGC-YFP for expression in T cells. The *Dtx1* promoter (−1300 bp to −1 bp) and *Gadd45b* promoter (−1191 to +140 bp) were isolated by PCR and subcloned into pGL2 and pGL3 luciferase reporter vector, respectively.

T Cell Anergy Induction

$CD4^+CD25^-$ T cells isolated from spleen were primed into Th1 cells as described in Supplemental Experimental Procedures. For anergy generated by CTLA4-Fc, T cells (2×10^5 per well) and γ-irradiated presenting cells (8×10^5 per well) were treated with anti-CD3 (5 μg/ml) and CTLA4-Fc for 2 days, washed, and restimulated with specific antigen or anti-CD3/anti-CD28.

Production of *Dtx1*-Transgenic Mice

Dtx1 was subcloned into *Cd4* promoter, a gift from D.R. Littman (New York University School of Medicine, NY). Transgenic mice were generated in the Transgene-Knockout Core of the Institute of Molecular Biology, Academia Sinica (Taipei, Taiwan). *CD4-Dtx1* was microinjected into the pronuclei of C57BL/6 zygotes. All transgenic mice were maintained in the SPF mice facility of the Institute of Molecular Biology, Academia Sinica. All mouse experiments were conducted with approval from the Experimental Animal Committee, Academia Sinica.

Production of *Dtx1*$^{-/-}$ Mice

The targeting construct was designed to delete exon 3 of *Dtx1*. A neomycin cassette with FLPe recombinase recognition sequence (Frt) at both ends was inserted into intron 3 of *Dtx1*. Two lox P sites were inserted into intron 2 and intron 3 so that exon 3 and *Neo* were flanked (fl) by lox P. The PmlI-XmnI fragment of intron 2 (1783 bp) was used as the short arm and *Herpes simplex virus thymidine kinase* (TK) gene was introduced into its 5′ end (Figure 4A). The BamHI-NheI fragment (6445 bp) of intron 3 was used as the long arm (Figure 4A). The construct was electroporated into R1 ES cells and G418- and gancyclovir-resistant clones were first screened by PCR. Only one positive clone was identified out of 1515 picked clones. The positive clone was confirmed by Southern blot analysis (Figure 4B) and was then injected into mouse blastocytes to generate chimera. Three mice with chimerism of 95% were then bred with C57BL/6 mice to confirm germline transmission, and the *Dtx1*$^{fl/+}$ mice were maintained by breeding with C57BL/6 mice. *EIIa-Cre* (B6.FVB-Tg(EIIa-Cre)C5379Lmgd/J) and *ACT-FLPe* (B6.Cg-Tg(ACTFLPe)9205Dym/J) transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME). *Cd4-Cre* mice were obtained from Taconic Farms (Hudson, NY). *Dtx1*$^{fl/+}$ mice were crossed with *EIIa-Cre* mice for systemic deletion of *Dtx1* (*Dtx1*$^{fl/+}$*EIIa-Cre*) and then intercrossed to generate mice with homozygous deletion of *Dtx1* (*Dtx1*$^{fl/fl}$*EIIa-Cre*, abbreviated as *Dtx1*$^{-/-}$). *Dtx1*$^{-/-}$ mice were backcrossed with C57BL/6 mice for 5 or more generations for this study. *Dtx1*$^{fl/fl\Delta neo}$ mice were obtained by breeding *Dtx1*$^{fl/fl}$ with *ACT-FLPe* mice to delete *Neo*. *Dtx1*$^{fl/fl\Delta neo}$ mice were then crossed with *Cd4-Cre* for T cell-specific deletion of *Dtx1* (*Dtx1*$^{fl/fl}$*Cd4-Cre*, abbreviated as *CD4-Dtx1*$^{-/-}$).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, and 17 figures and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00273-8.

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