

Control of the differentiation of regulatory T cells and T_H17 cells by the DNA-binding inhibitor Id3

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The molecular mechanisms that direct transcription of the gene encoding the transcription factor Foxp3 in CD4⁺ T cells remain ill-defined. We show here that deletion of the DNA-binding inhibitor Id3 resulted in the defective generation of Foxp3⁺ regulatory T cells (T_{reg} cells). We identify two transforming growth factor- β 1 (TGF- β 1)-dependent mechanisms that were vital for activation of *Foxp3* transcription and were defective in *Id3*^{-/-} CD4⁺ T cells. Enhanced binding of the transcription factor E2A to the *Foxp3* promoter promoted *Foxp3* transcription. Id3 was required for relief of inhibition by the transcription factor GATA-3 at the *Foxp3* promoter. Furthermore, *Id3*^{-/-} T cells showed greater differentiation into the T_H17 subset of helper T cells *in vitro* and in a mouse asthma model. Therefore, a network of factors acts in a TGF- β -dependent manner to control *Foxp3* expression and inhibit the development of T_H17 cells.

Regulatory T cells (T_{reg} cells) that express the transcription factor Foxp3 are instrumental in the induction and maintenance of peripheral immune tolerance and the regulation of tumor immunity and infection^{1–8}. The differentiation of Foxp3⁺ T_{reg} cells requires transforming growth factor- β (TGF- β) signaling^{9–13}; however, the molecular pathways that transduce this signal remain largely unknown. The TGF- β receptor-regulated adaptor molecules Smad2 and Smad3 and the common Smad protein Smad4 are involved in Foxp3 induction^{14–16}. However, the *Foxp3* promoter lacks Smad-binding sequences. In addition, the time lag between the activation of Smad2 or Smad3 (within minutes) and *Foxp3* mRNA expression (over 12 hours) after stimulation with TGF- β 1 suggests that there are intermediate factors between the TGF- β 1-mediated Smad signaling and *Foxp3* transcription. Thus, understanding this is not only essential for understanding the generation of T_{reg} cells but also important for the treatment of autoimmune diseases, infection and cancer¹⁷, especially given the reciprocal differentiation of TGF- β 1-induced Foxp3⁺ T_{reg} cells and interleukin 17 (IL-17)-producing helper T cells (T_H17 cells)^{18–23}.

Here we show that the DNA-binding inhibitor Id3, a transcription factor involved in T cell development^{24,25}, growth inhibition of a B cell progenitors²⁶ and protection of mice against autoimmune-like Sjögren's syndrome²⁷, regulated the TGF- β 1-mediated reciprocal differentiation of T_{reg} cells and T_H17 cells in mice. Deletion of *Id3* blocked the TGF- β 1-induced generation of Foxp3⁺ T_{reg} cells. This was attributed to a failure to enrich binding of the transcription factor E2A and an inability to suppress binding of the transcription factor GATA-3 at the *Foxp3* promoter in *Id3*^{-/-} T cells. These *Id3*^{-/-} T cells showed more differentiation into T_H17 cells *in vitro* and *in vivo*. Id3-dependent

reciprocal regulation of T_{reg} cells and T_H17 differentiation also occurred in an experimental model of house dust mite (HDM)-induced allergic asthma in mice.

RESULTS

Fewer CD4⁺Foxp3⁺ T_{reg} cells in *Id3*^{-/-} mice

Id3^{-/-} mice have been shown to develop a T cell-dependent autoimmune-like Sjögren's syndrome²⁷; we therefore reasoned that these mice might have a defect in the generation and/or function of CD4⁺Foxp3⁺ T_{reg} cells. Young *Id3*^{-/-} mice (3 weeks old) had a significantly lower frequency and absolute number of CD4⁺Foxp3⁺ T_{reg} cells in the spleen (Fig. 1a–d and Supplementary Fig. 1a) and peripheral lymph nodes (data not shown) than did wild-type C57BL/6 mice. These young *Id3*^{-/-} mice also had fewer CD4⁺Foxp3⁺ thymocytes (Fig. 1a–d). Helios has been used as a marker to distinguish natural T_{reg} cells from induced T_{reg} cells²⁸; we observed that both Foxp3⁺Helios⁺ and Foxp3⁺Helios⁻ T_{reg} cells were less abundant in *Id3*^{-/-} mice (Fig. 1b). Therefore, in terms of Helios expression, both natural T_{reg} cells and induced T_{reg} cells are affected by the absence of Id3.

In contrast to the lower frequency and number of T_{reg} cells in young *Id3*^{-/-} mice, the frequency of CD4⁺Foxp3⁺ T_{reg} cells in the spleen (Supplementary Fig. 1a), lymph nodes and thymus (data not shown) of older *Id3*^{-/-} mice was gradually restored by 6–7 weeks of age and became even greater after 3–4 months. The recovery of T_{reg} cells in older *Id3*^{-/-} mice was mainly due to expansion of the T_{reg} cell population, as there were substantially more Ki67⁺ (dividing) cells among *Id3*^{-/-} T_{reg} cells than among wild-type T_{reg} cells *ex vivo* (Supplementary Fig. 1). Even in young (3-week-old) *Id3*^{-/-} mice,

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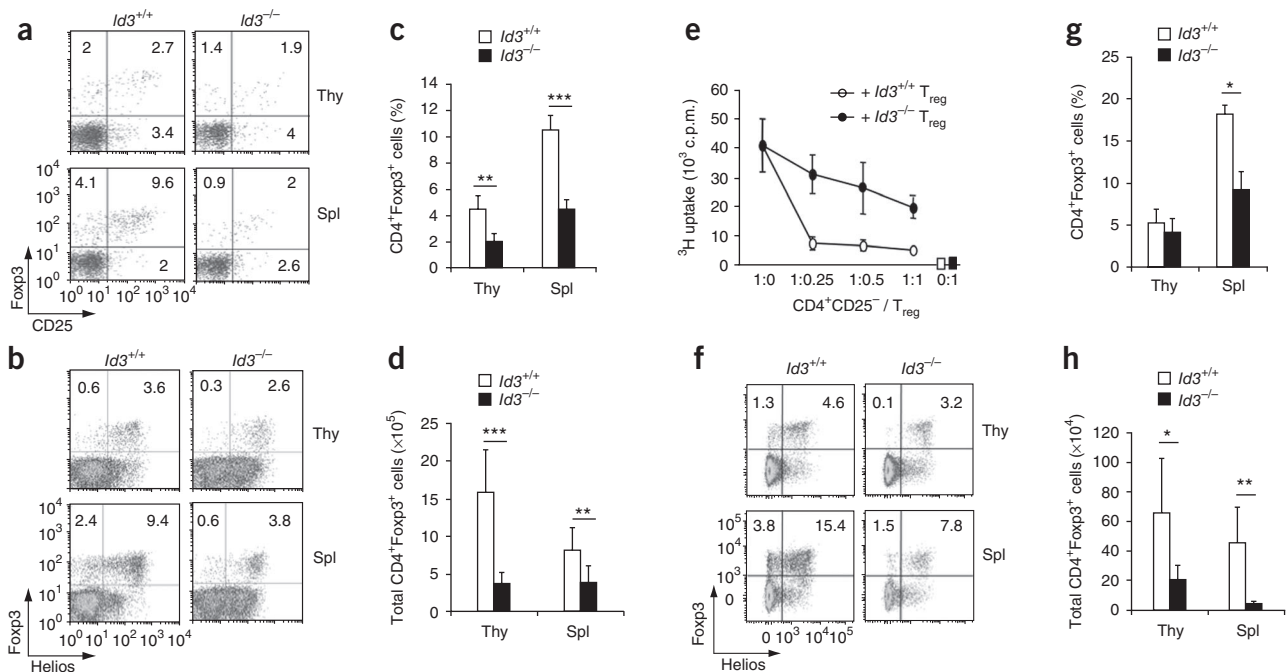


Figure 1 Id3 regulates the generation of Foxp3⁺ T_{reg} cells. (a,b) Flow cytometry of CD4⁺CD8⁻ T cells in the thymus (Thy) and spleen (Spl) of 3-week-old wild-type (*Id3*^{+/+}) and *Id3*^{-/-} mice. Numbers in quadrants indicate percent Foxp3⁺CD25⁻ cells (top left) or Foxp3⁺CD25⁺ cells (top right; a), or percent Foxp3⁺Helios⁻ cells (top left) or Foxp3⁺Helios⁺ cells (top right; b). (c,d) Frequency (c) and total number (d) of CD4⁺Foxp3⁺ T_{reg} cells in the mice in a,b. (e) Proliferation of wild-type CD4⁺CD25⁻ responder T cells cultured together at various ratios (horizontal axis) with *Id3*^{+/+} or *Id3*^{-/-} T_{reg} cells (key), assessed as uptake of [³H]thymidine. (f) Flow cytometry of gated CD4⁺CD8⁻ CD45.1⁻ T cells in the thymus and spleen of *Rag1*^{-/-} recipient mice 4 weeks after transfer of *Id3*^{+/+} or *Id3*^{-/-} (CD45.2⁺) bone marrow mixed at a ratio of 1:2 with C57BL/6 (CD45.1⁺) bone marrow. Numbers in quadrants indicate percent Foxp3⁺Helios⁻ cells (top left) or Foxp3⁺Helios⁺ cells (top right). (g,h) Frequency (g) and total number (h) of CD4⁺Foxp3⁺ T cells in the mice in f. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (Student's *t*-test). Data are representative of more than three experiments with four mice per group (a), three experiments (b), three experiments with seven mice (c,d; mean and s.d.), four independent experiments (e; mean ± s.d. of triplicate wells), two experiments with five mice per group (f) or two experiments (g,h; mean and s.d.).

there was already a much greater frequency of Ki67⁺ cells among T_{reg} cells than in wild-type mice (Supplementary Fig. 1), despite the significantly fewer T_{reg} cells in these young *Id3*^{-/-} mice (Fig. 1a–d).

Despite the recovery and even greater frequency of Foxp3⁺ T_{reg} cells in older *Id3*^{-/-} mice, old *Id3*^{-/-} mice showed signs of T cell activation and inflammation²⁷ (Supplementary Fig. 1e). This suggests that the initial deficiency in Foxp3⁺ T_{reg} cells in the neonatal *Id3*^{-/-} mice resulted in dysregulated activation that affected the homeostasis of T cells in *Id3*^{-/-} mice. In addition, the suppressive function of *Id3*^{-/-} CD4⁺CD25⁺ T_{reg} cells was also compromised in *in vitro* coculture assays (Fig. 1e and Supplementary Fig. 2), whether the CD4⁺CD25⁻ T responder cells being suppressed were wild-type (Fig. 1e) or *Id3*^{-/-} (Supplementary Fig. 2a). However, the expression of Foxp3 and other T_{reg} cell-associated molecules such as CD25, CTLA-4 and GITR was unaltered in the *Id3*^{-/-} mice (data not shown). T_{reg} cells from young *Id3*^{-/-} mice (3–5 weeks old; Fig. 1e and Supplementary Fig. 2) or older *Id3*^{-/-} mice (4–6 months old; data not shown) showed compromised suppressive activity.

Next we generated mixed-bone marrow chimeras to further confirm the function of Id3 in the generation of CD4⁺Foxp3⁺ T_{reg} cells. We injected a mixture of C57BL/6 (CD45.1⁺) bone marrow and *Id3*^{-/-} (CD45.2⁺) bone marrow or a mixture of C57BL/6 (CD45.1⁺) bone marrow and wild-type (CD45.2⁺) bone marrow into sublethally irradiated recipient mice deficient in recombination-activating gene 1 (*Rag1*^{-/-}). Both spleen and thymus reconstituted with *Id3*^{-/-} (CD45.2⁺) bone marrow contained fewer CD4⁺Foxp3⁺ T_{reg} cells than did those transplanted with wild-type (CD45.2⁺) bone marrow (Fig. 1f–h).

Similar to results obtained with intact *Id3*^{-/-} mice (Fig. 1b), both Foxp3⁺Helios⁻ and Foxp3⁺Helios⁺ T_{reg} cells were less abundant in mice reconstituted with *Id3*^{-/-} bone marrow (Fig. 1f). The deletion of Id3 therefore resulted in a deficiency in Foxp3⁺ T_{reg} cells.

Id3 is required for TGF-β1-induced Foxp3 expression

Next we determined whether Id3 deficiency affected the generation of Foxp3⁺ T_{reg} cells *in vitro*. We cultured peripheral naive CD4⁺CD25⁻Foxp3⁻ T cells with CD3- and CD28-specific antibodies in the presence of TGF-β1 to induce Foxp3⁺ T_{reg} cells⁹. TGF-β1 induced much less Foxp3 mRNA and Foxp3 protein in naive *Id3*^{-/-} CD4⁺CD25⁻ T cells than in naive wild-type CD4⁺CD25⁻ T cells (Fig. 2a–c) over a range of TGF-β1 concentrations, although the defect was partially reversed with high doses of TGF-β1 (Supplementary Fig. 3). The addition of exogenous IL-2 did not correct the defect (Fig. 2d), which eliminated the possibility of a lack of IL-2 as the cause. The inclusion of retinoic acid in the cultures resulted in an only slightly higher percentage of *Id3*^{-/-} Foxp3⁺ T cells, whereas it produced the reported synergistic effect on TGF-β1-dependent Foxp3⁺ T_{reg} differentiation of wild-type CD4⁺ T cells²⁹ (Fig. 2e). Analysis of proximal Smad activation downstream of TGF-β1 signaling showed there was no substantial difference between *Id3*^{-/-} and wild-type CD4⁺ T cells in TGF-β receptor-activated phosphorylation of Smad2 and Smad3 (assessed as phosphorylated Smad2) after TGF-β1 treatment (Supplementary Fig. 4a). Smad7, an inhibitory Smad that antagonizes Smad2- and Smad3-mediated TGF-β signaling³⁰, was less abundant in *Id3*^{-/-} CD4⁺ T cells than in wild-type CD4⁺ T cells after TGF-β1

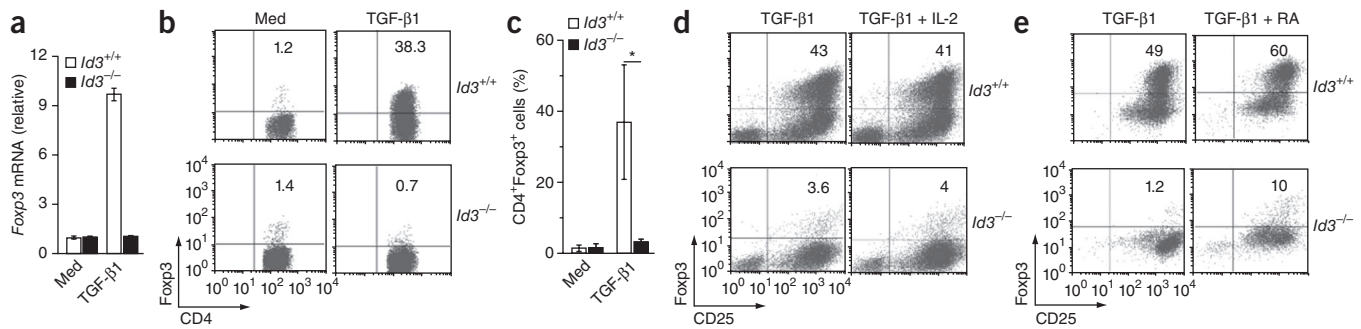


Figure 2 *Id3*^{-/-} T cells fail to generate Foxp3⁺ T_{reg} cells in response to TGF-β1. (a) Quantitative analysis of *Foxp3* mRNA expression in naive CD4⁺CD25⁻ T cells after TCR stimulation overnight in the presence (TGF-β1) or absence (Med) of TGF-β1, presented relative to the expression of *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase). (b) Flow cytometry of naive CD4⁺CD25⁻ T cells cultured for 3 d with TCR stimulation with or without TGF-β1. Numbers in plots indicate percent Foxp3⁺ T_{reg} cells. (c) Frequency of Foxp3⁺ T_{reg} cells among the cells in b. **P* < 0.01 (Student's *t*-test). (d,e) Flow cytometry of CD4⁺CD25⁻ T cells cultured for 3–5 d with TGF-β1 alone (left; d,e) or with TGF-β1 plus IL-2 (right; d) or retinoic acid (RA; right; e). Numbers in quadrants indicate percent CD25⁺Foxp3⁺ T_{reg} cells. Data are from one experiment representative of at least five (a; mean ± s.d. of duplicate wells) or at least two (d,e), one experiment representative of five (b) or five experiments (c; mean ± s.d.).

treatment (Supplementary Fig. 4b), which ruled out the possibility of Smad7 as a factor causing the diminished induction of Foxp3 in *Id3*^{-/-} cells. Id3 is therefore a critical component of TGF-β1-mediated generation of Foxp3⁺ T_{reg} cells, but it probably functions downstream of signal pathways mediated by phosphorylated Smad2 and Smad3.

E2A enrichment at the *Foxp3* promoter activates *Foxp3*

We next studied the molecular mechanisms by which Id3 is involved in the TGF-β1-induced generation of Foxp3⁺ T_{reg} cells. Freshly isolated CD4⁺CD25⁻ T_{reg} cells from the spleen of wild-type mice had more *Id3* mRNA and Id3 protein (Supplementary Fig. 5a,b) than did CD4⁺CD25⁻ T cells. We then investigated whether TGF-β signaling affected Id3 expression in naive CD4⁺ T cells after stimulation of the T cell antigen receptor (TCR). Treatment with TGF-β1 resulted in more *Id3* mRNA during the first 1–2 h than did stimulation of the TCR alone (Supplementary Fig. 5c), consistent with findings obtained with B cells²⁶. However, by 12–24 h, TGF-β1-treated T cells had less *Id3* mRNA than did T cells without TGF-β1 treatment (Supplementary Fig. 5c). We further verified a role for TGF-β1 in the regulation of *Id3* expression in mice with T cell-specific conditional deletion of TGF-β receptor I (with deletion of loxP-flanked (floxed) alleles encoding TGF-β receptor I (*Tgfbri*^{fl/f}) mediated by Cre recombinase driven by the *Cd4* promoter; *Tgfbri*^{fl/f}*Cd4*-Cre⁺ mice)¹², as CD4⁺CD25⁻ T cells from these mice showed no difference in *Id3* expression with or without TGF-β1 (data not shown). The regulation of *Id3* by TGF-β1 required Smad3 and/or Smad4, as CD4⁺CD25⁻ T cells either singly deficient in Smad3 (*Smad3*^{-/-}) (Supplementary Fig. 5d) or doubly deficient in Smad3 and Smad4 (from *Smad3*^{-/-}*Smad4*^{fl/f}*Lck*-Cre⁺ mice, in which deletion of *Smad4*^{fl/f} is mediated by Cre driven by the *Lck* promoter; data not shown) lost the aforementioned biphasic effects induced by TGF-β1. Thus, T_{reg} cells have higher expression of *Id3* and TGF-β1 regulates *Id3* expression in naive CD4⁺ T cells in a time-dependent manner, with a transient increase during the first 2 h, followed by considerable downregulation by 12–24 h.

Id3 lacks the basic region needed for DNA binding but retains the functional dimerization domain and therefore is regarded as an inhibitor of the binding of E2A to its target genes through the formation of inactive heterodimers²⁴. We therefore hypothesized that TGF-β1 might influence the binding of E2A to the *Foxp3* promoter and thereby regulate gene transcription. E2A proteins (E47 and E12) have a DNA-binding domain and bind the CANNTG (where 'N' indicates any

nucleotide; E-box) DNA motif as protein dimers to regulate gene transcription during the development of both T cells and B cells²⁴. We first analyzed *Foxp3* with the help of Genomatix MatInspector software and found that the sequence between -1.1 kb and +1.1 kb relative to the transcription initiation site (+1), contained many E-box elements with the canonical nucleotide sequence CANNTG, which are expected to bind members of the E-protein family of transcription factors^{31–34} (Fig. 3a). Using naive CD4⁺CD25⁻ T cells isolated from the spleens of wild-type mice, we analyzed whether TGF-β1 influenced the binding of E2A to the *Foxp3* promoter. We treated CD4⁺CD25⁻ T cells overnight with CD3- and CD28-specific antibodies and TGF-β1 and assessed binding of E2A to the *Foxp3* promoter by chromatin immunoprecipitation (ChIP)-coupled quantitative PCR. For this we used an antibody to E47, a major component of the E2A protein²⁴, to precipitate chromatin from CD4⁺CD25⁻ T cells, followed by quantitative PCR analysis of the precipitated DNA. To determine where E2A binds to *Foxp3*, we analyzed the following three different regions of *Foxp3*: the 5' promoter region (amplicon 1; position -995 to position -798); the proximal promoter plus a region consisting of the untranslated 5' mRNA (amplicon 2; position +327 to position +513); and the region located next to the enhancer containing the Smad-binding domain (amplicon 3; position +2335 to position +2532)¹⁵ (Fig. 3a). We observed more binding of E2A to the regulatory region of *Foxp3* comprising the proximal promoter and its untranslated 5' mRNA region (positions +327 to +513; Fig. 3b). We then quantitatively compared the binding of E2A to this region in *Foxp3* in T cells with stimulation of the TCR alone or with TCR stimulation plus treatment with TGF-β1 and found that TGF-β1 significantly enhanced the binding of E2A to the promoter relative to binding in the control condition without TGF-β1 treatment (Fig. 3c). TGF-β1-driven enhancement of the binding of E2A to the *Foxp3* promoter occurred 12–24 h after TGF-β1 stimulation, which correlated with the time of Foxp3 mRNA expression (>12 h) detected in TGF-β1-treated T cells (data not shown). This enrichment of E2A was probably not due to the change in protein amounts, as neither treatment substantially affected the expression of whole E2A protein (Supplementary Fig. 6a). More E2A binding was associated with binding of TBP (TATA-binding protein), a marker of active transcription, in the *Foxp3* promoter (Fig. 3d), which suggested that both factors were recruited to this *Foxp3*-regulatory region. We confirmed the essential role of TGF-β1 signaling in promoting binding of E2A

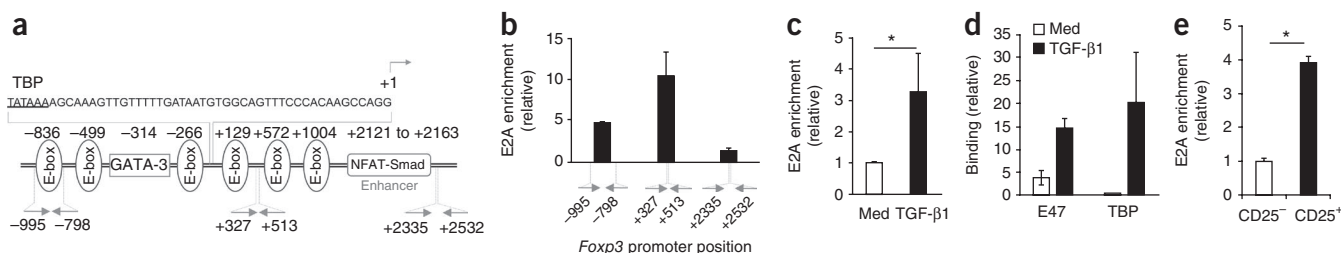


Figure 3 Enrichment of E2A at the *Foxp3* promoter. **(a)** E-protein-binding sites (E-boxes) at the *Foxp3* promoter. **(b)** ChIP-coupled quantitative PCR analysis of the enrichment of E2A at E-boxes at various positions along *Foxp3* (horizontal axis) in CD4⁺CD25⁻ T cells after TCR stimulation and treatment with TGF- β 1, assessed with antibody to E47 (anti-E47) and presented relative to results obtained with control IgG. **(c)** ChIP-coupled quantitative PCR analysis of the enrichment of E2A in the region between positions +327 and +513 of the *Foxp3* promoter in wild-type CD4⁺CD25⁻ T cells 12–24 h after TCR stimulation with TGF- β 1, assessed as in **b** and presented relative to results obtained without TGF- β 1 (Med), set as 1. **(d)** ChIP-coupled quantitative PCR analysis of the binding of E2A and TBP to the *Foxp3* promoter in wild-type CD4⁺CD25⁻ T cells as described in **c**, presented as the results obtained with anti-E47 or anti-TBP relative to those obtained with control IgG. **(e)** ChIP-coupled quantitative PCR analysis of E2A enrichment as in **c** in purified CD4⁺CD25⁺ T_{reg} cells (CD25⁺), presented relative to results obtained with purified CD4⁺CD25⁻ T_{reg} cells (CD25⁻), set as 1. * $P < 0.01$ (Student's *t*-test). Data are representative of four experiments (**b**; error bars, s.d.), four independent experiments (**c**; mean and s.d.) or one experiment representative of two (**d,e**; mean and s.d. of duplicate (**d**) or triplicate (**e**) wells).

to the *Foxp3* promoter by using *Tgfb1^{fl}Cd4-Cre⁺* CD4⁺CD25⁻ T cells, which lack TGF- β signaling. There was no upregulation of E2A binding in response to TGF- β 1 treatment (Supplementary Fig. 6b). In addition to TGF- β 1-treated naive CD4⁺ T cells, freshly isolated CD4⁺CD25⁺ T_{reg} cells from wild-type spleen also showed enrichment in E2A at the *Foxp3* promoter relative to the binding in CD4⁺CD25⁻ T cells (Fig. 3e). These data collectively support the idea of a positive role for E2A in the activation of *Foxp3* transcription in response to TGF- β 1 treatment, which can be explained by the direct binding of E2A to the E-box rich region in the regulatory region of the *Foxp3*.

To study whether binding of E2A to the *Foxp3* promoter has a causative role in *Foxp3* transcription, we challenged the ability of TGF- β 1 to

activate *Foxp3* transcription after decreasing E2A expression. We first did gene-knockdown experiments in EL4 mouse T lymphoma cells (subclone EL4 LAF), as treatment with TGF- β 1 and stimulation of the TCR induces *Foxp3* expression in these cells^{15,35}. Using short hairpin RNA, we knocked down E2A expression (Fig. 4a, inset). The presence of less E2A protein in EL4 LAF cells blocked the induction of *Foxp3* expression by TGF- β 1 (Fig. 4a). To confirm the finding that E2A binding had an intrinsic effect on *Foxp3* transcription, we used a reporter *Foxp3* construct containing the *Foxp3* proximal promoter and the *Smad* enhancer (which responds to TGF- β 1 directly¹⁵) to engineer mutated E-boxes (Fig. 4b, left) and challenged the ability of TGF- β 1 to activate *Foxp3* promoter activity. Mutation of the sequences of the E-boxes diminished activation of the *Foxp3* promoter in response to treatment with TGF- β 1 and stimulation of the TCR (Fig. 4b, right). Furthermore, we obtained naive CD4⁺CD25⁻ T cells from *E2a^{fl}Heb^{fl}Er-Cre⁺* mice, which have tamoxifen-induced conditional deletion of loxP-flanked alleles encoding E2A (*Tcf3*; called 'E2a' here) and the transcription factor HEB (*Tcf12*; called 'Heb' here), mediated by Cre

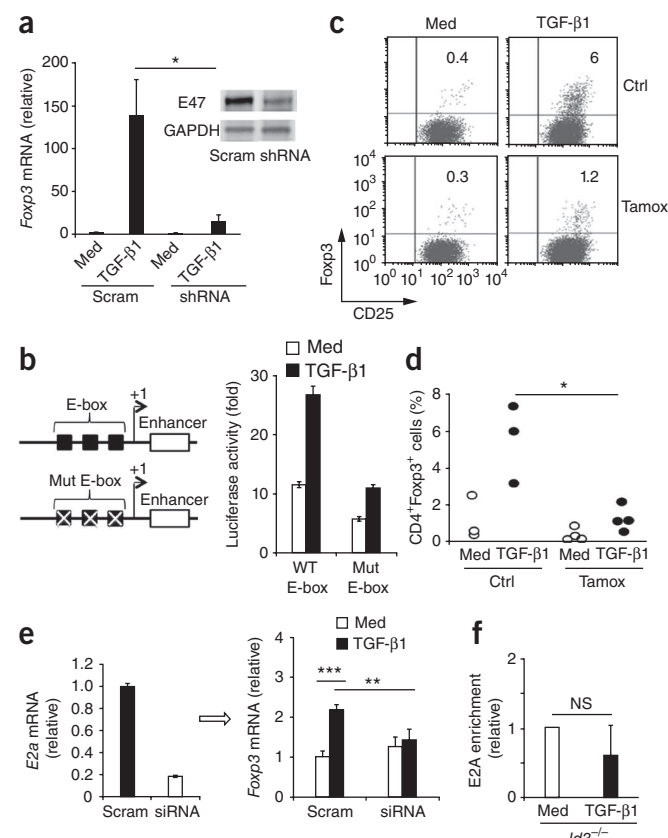


Figure 4 Binding of E2A to the *Foxp3* promoter is required for activation of *Foxp3* by TGF- β 1. **(a)** *Foxp3* mRNA expression in EL4 LAF cells treated with E2A-specific short hairpin RNA (shRNA) or scrambled (control) shRNA (Scram), assessed 12 h after activation with anti-CD3 and anti-CD28, with or without TGF- β 1, presented relative to *Hprt1* expression. Inset, immunoblot analysis of E47 and GAPDH (glyceraldehyde phosphate dehydrogenase; loading control). **(b)** Luciferase assay of TGF- β 1-induced *Foxp3* activity (right) in EL4 LAF cells with wild-type (WT) or mutated (Mut) *Foxp3* E-boxes (constructs, left), assessed after TCR stimulation with or without TGF- β 1 and presented relative to. **(c,d)** Flow cytometry of splenic CD4⁺ T cells (**c**) and frequency of CD4⁺Foxp3⁺ T_{reg} cells among splenic CD4⁺CD25⁻ T cells (**d**) obtained from *E2a^{fl}Heb^{fl}Er-Cre⁺* mice treated with sunflower seed oil (control (Ctrl)) or tamoxifen (Tamox) and cultured for 3 d with anti-CD3 plus anti-CD28, and TGF- β 1 (0.2 ng/ml). Numbers in plots (**c**) indicate percent Foxp3⁺CD25⁺ cells; each symbol (**d**) represents an individual mouse. **(e)** *Foxp3* mRNA (right) in naive CD4⁺CD25⁻ T cells treated with E2A-specific siRNA (siRNA) or scrambled siRNA (Scram), assessed after TCR stimulation with or without TGF- β 1 and presented relative to *Hprt1* expression. Left, *E2a* mRNA in cells treated with E2A-specific or scrambled siRNA; results are presented relative to *Hprt1* expression. **(f)** Enrichment of E2A at the *Foxp3* promoter in *Id3*^{-/-} CD4⁺CD25⁻ T cells with or without TGF- β 1. NS, not significant; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (Student's *t*-test). Data are representative of three experiments (**a**; mean and s.d.), two independent experiments (**c,d**), two experiments (**e**; mean and s.d. of four samples) or three independent experiments (**f**; mean and s.d.) or are from one experiment representative of three (**b**; mean $\pm$ s.d. of triplicate measurements).

driven by the promoter of the estrogen receptor gene (*Esr1*; called 'Er' here), and assessed *Foxp3* induction with CD3- and CD28-specific antibodies plus TGF- β 1 (ref. 36; (Supplementary Fig. 6d,e). We used these doubly deficient mice because they largely eliminated the possible compensatory effect of HEB in mice with conditional single deletion of E2A alone, and mice with T cell-specific double deficiency in E2A and HEB could not be used as they had an almost complete deficiency in CD4⁺ T cells, including T_{reg} cells³⁷. T cells with double deficiency in E2A and HEB induced by tamoxifen generated significantly fewer Foxp3⁺ T_{reg} cells than did control T cells without tamoxifen induction (Fig. 4c,d). The defect in Foxp3 induction was most profound at a low concentration of TGF- β 1 (0.2 ng/ml; Fig. 4c,d). Furthermore, we knocked down E2A with small interfering RNA (siRNA) in wild-type primary naive CD4⁺CD25⁻ cells and found that less E2A resulted in defective *Foxp3* induction in response to TGF- β 1 (Fig. 4e). Thus, our findings show that binding of E2A to the *Foxp3* promoter represents a key (enhancer) step in directly turning on *Foxp3* transcription in naive CD4⁺ T cells in response to TGF- β 1.

We then examined binding of E2A to the *Foxp3* promoter in *Id3*^{-/-} CD4⁺CD25⁻ T cell populations, which showed much lower frequencies of Foxp3⁺ T_{reg} cells in response to treatment with TGF- β 1 (Fig. 2). The *Id3*^{-/-} CD4⁺CD25⁻ T cells had slightly but reproducibly more basal binding of E2A to the *Foxp3* promoter in response to TCR stimulation alone than did wild-type control T cells (data not shown and presented below), which could indicate an inhibitory function for Id3 in the binding of E2A to E boxes on target genes²⁴. However, TGF- β 1 treatment failed to enhance the binding of E2A to the *Foxp3* promoter in *Id3*^{-/-} T cells over the cells treated with stimulation of the TCR alone (Fig. 4f). Freshly isolated CD4⁺CD25⁺ T_{reg} cells from the spleens of *Id3*^{-/-} mice showed no substantial enrichment of E2A at the *Foxp3* promoter relative to that of CD4⁺CD25⁻ T cells from the same mice (Supplementary Fig. 6c). These data were consistent with a positive function for the binding of E-protein to E-boxes at the *Foxp3* promoter but raised the question of why treatment with TGF- β 1 failed to upregulate the binding of E2A to the *Foxp3* promoter and consequent lack of Foxp3 expression in the *Id3*^{-/-} T cells.

A role for Id3 in regulating GATA-3 expression

We reasoned that in addition to enhancing positive regulators of *Foxp3* transcription (binding of E2A to the promoter), TGF- β 1 must also act to remove a negative regulator that represses *Foxp3* transcription, and we hypothesized that this would require Id3. We focused on GATA-3, a key transcription factor for T helper type 2 (T_H2) differentiation³⁸, reported to bind directly to the *Foxp3* promoter and inhibit its transcription³⁹. As TGF- β inhibits *Gata3* expression in CD4⁺ T cells^{39,40}, we first compared *Gata3* expression in *Id3*^{-/-} and wild-type CD4⁺CD25⁻ T cells. Although naive CD4⁺ T cells isolated from wild-type and *Id3*^{-/-} mice had undetectable *Gata3* (data not shown), *Id3*^{-/-} T cells had much more *Gata3* mRNA (Fig. 5a) and GATA-3 protein (Fig. 5b) after TCR stimulation than did wild-type T cells. Notably, whereas it almost completely eliminated *Gata3* expression in the control T cells, TGF- β 1 only partially inhibited *Gata3* expression in *Id3*^{-/-} T cells (Fig. 5a,b). The higher *Gata3* expression in *Id3*^{-/-} T cells was probably attributable to abundant production of IL-4 in these T cells after TCR stimulation (Fig. 5c and data not shown). TGF- β 1 failed to substantially inhibit IL-4 expression in *Id3*^{-/-} T cells (Fig. 5c). Although TGF- β 1 suppressed *Gata3* expression in *Id3*^{-/-} T cells, the overall larger amount of GATA-3 in *Id3*^{-/-} cells meant that TGF- β was unable to completely suppress GATA-3 to the amount in wild-type cells (Fig. 5a,b). To confirm that TGF- β 1 signaling had an

essential role in the suppression of *Gata3*, we examined its expression in *Tgfb1*^{fl/fl}CD4-Cre⁺ T cells. These cells had more GATA-3 than did control *Tgfb1*^{fl/fl}CD4-Cre⁺ T cells after TCR stimulation (Fig. 5d). In *Tgfb1*^{fl/fl}CD4-Cre⁺ T cells, the addition of TGF- β 1 had no inhibitory effect on GATA-3 expression and there was no Foxp3 induction¹² (Fig. 5d). Thus, Id3 is required for the regulation of *Gata3* in CD4⁺ T cells, which occurs mainly through control of IL-4 production.

To understand how Id3 regulates IL-4 production in wild-type CD4⁺ T cells, we hypothesized that Id3 forms a complex with E2A and prevents it from binding to the E-boxes at the promoter of *Il4*. Indeed *Il4* contains at least four E-boxes in a highly conserved region of its promoter (400 base pairs upstream of the transcription start site). We found that knockdown of E2A with siRNA in wild-type CD4⁺CD25⁻ T cells resulted in significantly less *Il4* induction in response to TCR stimulation (Fig. 5e). These data suggest that the 'extra' E2A in the absence of Id3 could induce large amounts of IL-4 that in turn drive GATA-3 expression.

Next we assessed whether Id3 deficiency affected the binding of GATA-3 to the *Foxp3* promoter. Consistent with the inhibition of *Gata3* expression (Fig. 5a,b), treatment with TGF- β 1 resulted in less binding of GATA-3 to the *Foxp3* promoter in wild-type CD4⁺ T cells, as determined by ChIP-coupled quantitative PCR (Fig. 5f). In line with the higher *Gata3* expression in *Id3*^{-/-} T cells (Fig. 5a,b), there was much more binding of GATA-3 to the *Foxp3* promoter after TCR stimulation in these cells than in wild-type cells (Fig. 5f). However, treatment with TGF- β 1 did not diminish the binding of GATA-3 in *Id3*^{-/-} T cells to the amount in wild-type cells (Fig. 5f). These data suggest that failure to remove GATA-3 from the *Foxp3* promoter acted as a 'brake' (silencer) on *Foxp3* expression. In the absence of Id3, this 'brake' rendered TGF- β 1 unable to mediate the enrichment of E2A at the *Foxp3* promoter, resulting in lack of *Foxp3* transcription in *Id3*^{-/-} T cells.

To confirm that hypothesis, we eliminated GATA-3 from TGF- β 1-treated *Id3*^{-/-} CD4⁺ T cells by administering an IL-4-neutralizing antibody. We had already found that naive *Id3*^{-/-} CD4⁺CD25⁻ T cells produced large amounts of IL-4 immediately after TCR stimulation (Fig. 5c) and that IL-4 is a potent GATA-3 inducer⁴¹. Indeed, neutralization of IL-4 in TGF- β 1-treated *Id3*^{-/-} CD4⁺ T cells abrogated their GATA-3 expression (Fig. 5a,b) and permitted the generation of Foxp3⁺ T_{reg} cells (Fig. 5b and Supplementary Fig. 7). Consistent with a positive role for E2A binding in *Foxp3* transcription, neutralization of IL-4 in the presence of TGF- β 1 restored the enrichment of E2A at the *Foxp3* promoter in *Id3*^{-/-} CD4⁺ T cells (Fig. 5g). However, inhibiting only GATA-3 in the absence of TGF- β 1 did not result in any generation of Foxp3⁺ T_{reg} cells from either *Id3*^{-/-} or wild-type CD4⁺ T cells (Fig. 5b), which suggested that downregulation of GATA-3 is only one element of the complex TGF- β 1-mediated program of *Foxp3* induction. That idea was further supported by the finding that elimination of GATA-3 via IL-4 neutralization in the presence of TGF- β 1 did not result in *Foxp3* induction in *Tgfb1*^{fl/fl}CD4-Cre⁺ T cells (Fig. 5d). In contrast to neutralization of IL-4, neutralization of IL-6, IL-23 or interferon- γ (IFN- γ) in TGF- β 1-treated *Id3*^{-/-} CD4⁺CD25⁻ T cells failed to restore the generation of Foxp3⁺ T_{reg} cells (Supplementary Fig. 7 and data not shown), which eliminated the possibility that signals mediated by these cytokines served as regulators in this process. Thus, our data showed that TGF- β 1 induced *Foxp3* expression through at least two indispensable and interdependent molecular events: promoting binding of E2A protein to the *Foxp3* promoter (enhancer), and inhibiting and/or removing negative factors bound to that promoter, such as GATA-3 (silencer). Both of these events were defective in *Id3*^{-/-} T cells.

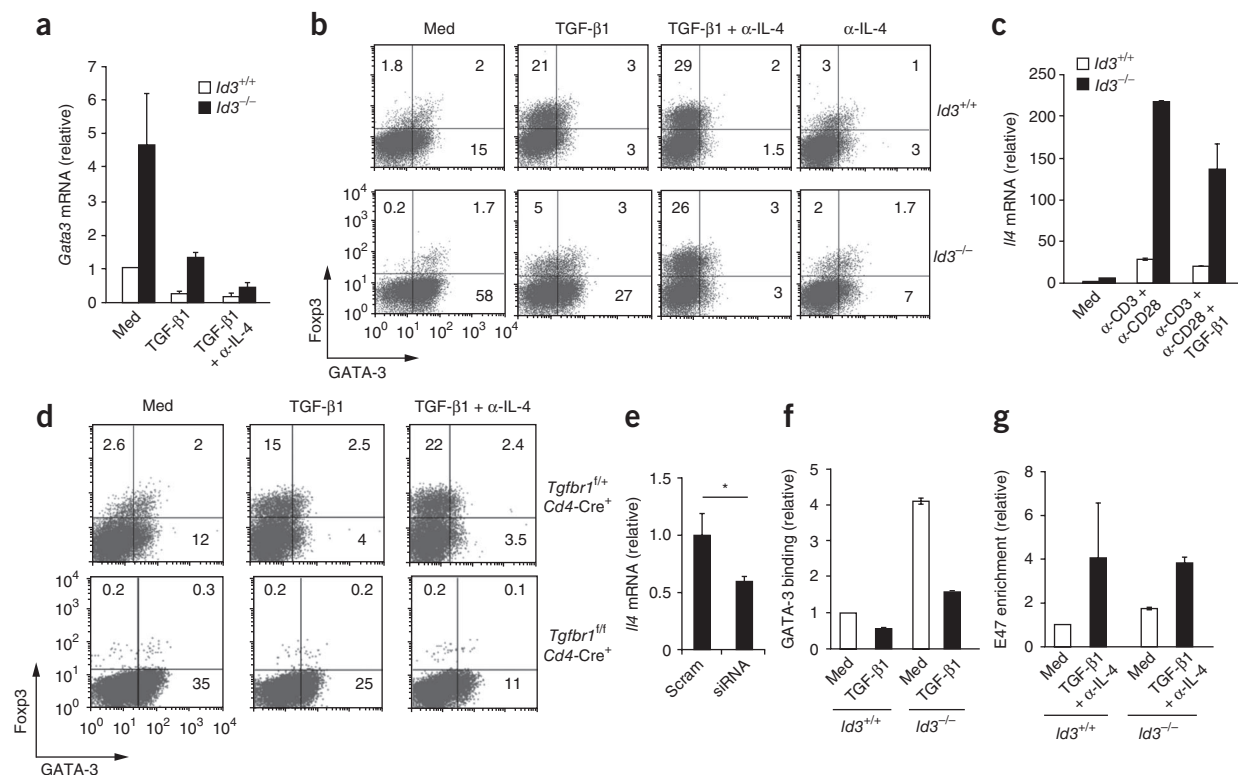


Figure 5 Id3 deficiency increases GATA-3. **(a)** *Gata3* mRNA expression in naive CD4⁺CD25⁻ T cells 12 h after activation of the TCR and treatment with TGF- β 1 alone or TGF- β 1 and anti-IL-4 (α -IL-4), presented relative to *Hprt1* expression. **(b)** Flow cytometry of CD4⁺CD25⁻ T cells at 24 h after activation of the TCR and treatment with various combinations of TGF- β 1 and anti-IL-4 (above plots). **(c)** *I/4* mRNA in CD4⁺CD25⁻ T cells at 2 h after activation and treatment (horizontal axis), presented relative to *Hprt1* expression. **(d)** Flow cytometry of *Tgfb1*^{+/+}*Cd4-Cre*⁺ and *Tgfb1*^{-/-}*Cd4-Cre*⁺ CD4⁺CD25⁻ T cells cultured for 24 h (treatment, above plots). **(e)** *I/4* mRNA expression in wild-type naive CD4⁺CD25⁻ T cells treated with E2A-specific or scrambled siRNA, presented relative to *Hprt1* expression. **P* < 0.05 (Student's *t*-test). **(f)** Binding of GATA-3 to the *Foxp3* promoter in *Id3*^{+/+} or *Id3*^{-/-} CD4⁺CD25⁻ T cells cultured for 24 h (treatment, horizontal axis), assessed with anti-GATA-3 and presented relative to results obtained with control IgG. **(g)** Enrichment of E2A at the *Foxp3* promoter in *Id3*^{+/+} or *Id3*^{-/-} CD4⁺CD25⁻ T cells cultured for 24 h with TCR stimulation and treatment with TGF- β 1 and anti-IL-4, presented relative to *Hprt1* expression. Numbers in quadrants **(b,d)** indicate percent Foxp3⁺GATA-3⁻ cells (top left), Foxp3⁺GATA-3⁺ cells (top right) or Foxp3⁻GATA-3⁺ cells (bottom right). Data are from one experiment representative of at least three **(a–d)** or two **(e–g)**; mean and s.d. of duplicate **(a,c,f,g)** or triplicate **(e)** measurements.

More T_H17 cells after deletion of *Id3*

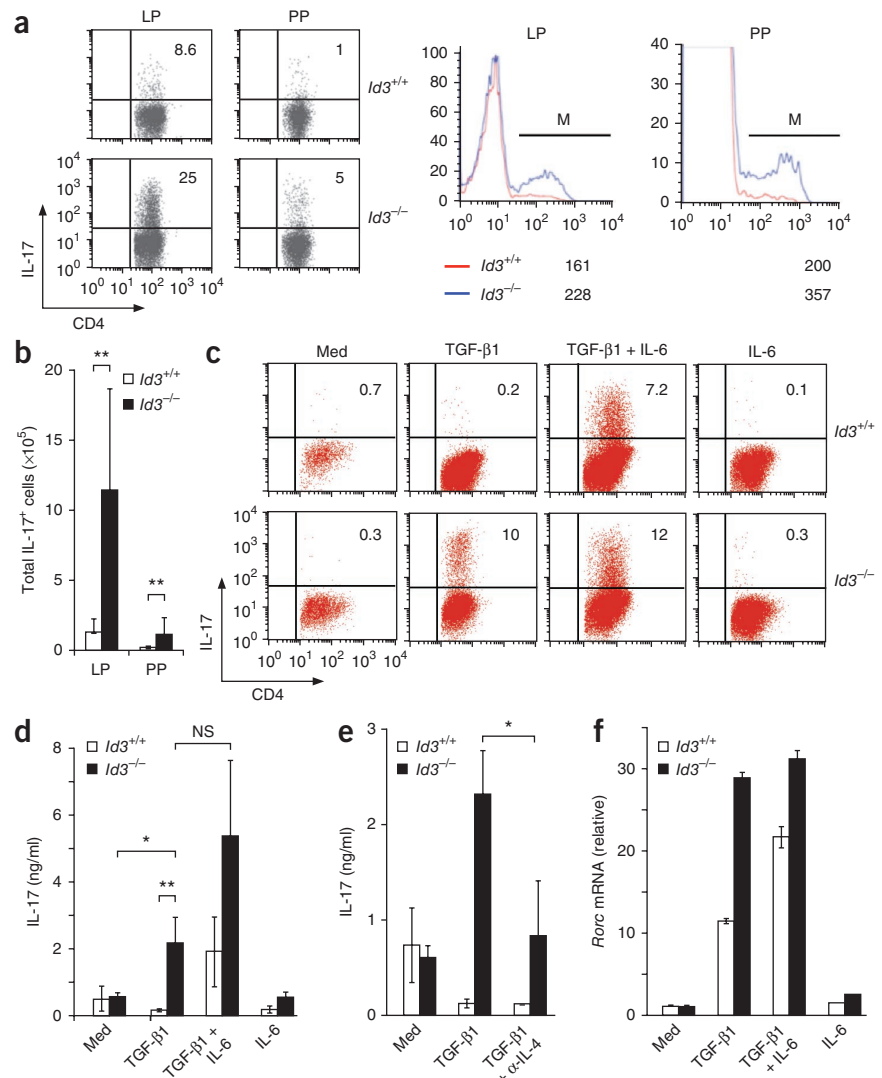
As the *Id3*^{-/-} T cells showed a defect in generating CD4⁺Foxp3⁺T_{reg} cells, and evidence indicates reciprocal control of T_{reg} and T_H17 differentiation by TGF- β 1 (refs. 18,22) and that Foxp3 directly binds to and inhibits the expression of the transcription factor ROR γ t (encoded by *Rorc*)²², we investigated the possible dysregulation of T_H17 differentiation of *Id3*^{-/-} T cells. We first examined the frequency of T_H17 cells in the gut of *Id3*^{-/-} mice, as T_H17 cells can be detected in normal unmanipulated mice¹⁹. *Id3*^{-/-} mice had a much greater frequency and absolute number of T_H17 cells in the lamina propria and Peyer's patches and among intraepithelial lymphocytes than did wild-type mice (Fig. 6a,b and data not shown). In addition, the amount of IL-17 per cell was also greater in *Id3*^{-/-} T_H17 cells than in wild-type T_H17 cells (Fig. 6a, right). T_H17 cells were almost undetectable in the spleen and peripheral lymph nodes of young and asymptomatic *Id3*^{-/-} mice, as in wild-type mice, but they were much more abundant in older *Id3*^{-/-} mice (>6 months) than in age-matched wild-type mice (data not shown). These data suggest that loss of Id3 in mice leads to more T_H17 cells.

To test whether Id3 is involved in T_H17 differentiation, we cultured naive CD4⁺CD25⁻ T cells with TGF- β 1 alone or with TGF- β 1 plus IL-6 (refs. 18,21,22). Treatment with TGF- β 1 plus stimulation of the

TCR resulted in significantly more IL-17-producing cells among *Id3*^{-/-} CD4⁺CD25⁻ T cells but not among wild-type naive CD4⁺CD25⁻ T cells (Fig. 6c–e). The addition of IL-6 to TGF- β 1-treated cultures failed to further enhance the abundance of T_H17 cells in *Id3*^{-/-} T cells but substantially increased the abundance of IL-17⁺ cells in wild-type cultures^{18,20–22} (Fig. 6c,d). Consistent with the effect on IL-17 production, treatment with TGF- β 1 plus stimulation of the TCR induced optimal expression of *Rorc* in *Id3*^{-/-} T cells, and the addition of IL-6 provided no substantial enhancement (Fig. 6f). In line with those results, neutralization of endogenous IL-6, IL-21 or IL-23 alone or in combination had no effect on the TGF- β 1-induced T_H17 differentiation of *Id3*^{-/-} CD4⁺CD25⁻ T cells (data not shown); however, neutralization of IL-4 abrogated the T_H17 polarization of *Id3*^{-/-} T cells induced by TGF- β 1 (Fig. 6e), possibly because *Foxp3* expression had been restored (Fig. 5b and Supplementary Fig. 7b).

We next investigated whether binding of E2A to the *Rorc* promoter would directly enhance transcription and IL-17 production in wild-type naive CD4⁺ T cells. There are four E-boxes in the promoter of *Rorc*^{42,43}, and binding of E-proteins to these is required for *Rorc* transcription in immature thymocytes during maturation⁴³. We first determined that in wild-type CD4⁺CD25⁻ T cells, treatment with TGF- β 1 substantially enriched E2A at the *Rorc* promoter relative

Figure 6 *Id3*^{-/-} CD4⁺ T cells differentiate into T_H17 cells in response to TGF- β 1 *in vitro*. (a) Flow cytometry of intracellular IL-17 in CD4⁺ T cells from the lamina propria (LP) and Peyer's patches (PP) of 7- to 8-week-old *Id3*^{+/+} and *Id3*^{-/-} mice. Numbers in plots (left) indicate percent IL-17⁺ cells; numbers below plots (right) indicate mean fluorescence intensity (M; bracketed lines above) of IL-17⁺ cells among gated CD4⁺ T cells. (b) Absolute number of IL-17⁺CD4⁺ cells in the mice in a. (c) Flow cytometry of intracellular IL-17 in naive CD4⁺CD25⁻ T cells obtained from mice 3–5 weeks of age and cultured for 3–5 d with TCR stimulation plus various combinations of TGF- β 1 and IL-6 (above plots). Numbers in quadrants indicate percent IL-17⁺CD4⁺ cells. (d) Enzyme-linked immunosorbent assay of IL-17 in supernatants of CD4⁺CD25⁻ T cells cultured for 72 h as in c. (e) IL-17 production in naive CD4⁺ T cells cultured with TCR stimulation alone (Med) or with the addition of TGF- β 1 alone or with TGF- β 1 and anti-IL-4. (f) Quantitative RT-PCR analysis of *Rorc* mRNA in CD4⁺CD25⁻ T cells 48 h after activation of the TCR and treatment with various combinations of TGF- β 1 and IL-6 (horizontal axis), presented relative to *Hprt1* expression. **P* < 0.05 and ***P* < 0.01 (Student's *t*-test). Data are representative of three independent experiments with three mice (a,b; mean and s.d.), one experiment representative of more than five (c), four independent experiments (d; except IL-6 treatment (two experiments); mean $\pm$ s.d.) or two independent experiments (e; mean $\pm$ s.d. of four samples), or are from one of three independent experiments (f; mean $\pm$ s.d. of duplicate wells).



to TCR stimulation alone (Supplementary Fig. 8a). We knocked down E2A expression in wild-type CD4⁺CD25⁻ T cells with siRNA and showed that less E2A resulted in much less *Rorc* transcription and IL-17 production in the CD4⁺ T cells in response to TGF- β 1 plus IL-6 (Supplementary Fig. 8b,c). These data suggest that E2A, in combination with as-yet-undetermined transcription factors downstream of IL-6, has a role in *Rorc* activation and the consequent T_H17 differentiation of normal T cells.

In addition to the *in vitro* studies reported above, we also determined that naive *Id3*^{-/-} CD4⁺ T cells preferentially differentiated into IL-17⁺ cells after transfer into syngeneic *Rag1*^{-/-} mice (Supplementary Fig. 8d). Although the frequency of Foxp3⁺ T cells was also greater among the transplanted *Id3*^{-/-} T cells than among wild-type T cells, the degree of increase in Foxp3⁺ T_{reg} cells was much less than that of T_H17 cells. Thus, the ratio of T_H17 cells to Foxp3⁺ T_{reg} cells was much greater for *Id3*^{-/-} T cells (Supplementary Fig. 8d). Together our data demonstrate that in the absence of Id3, CD4⁺ T cells preferentially differentiated into T_H17 cells.

Id3 regulates T_{reg} and T_H17 cells in HDM-induced asthma

We next examined whether the preferential differentiation of *Id3*^{-/-} cells into T_H17 cells might also occur during the course of disease in *Id3*^{-/-} mice. We chose a model of HDM-induced allergic asthmatic inflammation in the lung characterized by dominant T_H2 cytokines⁹. Although IL-17 has been found in experimental and human allergy and asthma, the exact function of T_H17 cells in the pathogenesis and

development of asthmatic lung inflammation is under debate^{44,45}. We found that although control mice developed massive inflammatory cell infiltrates in the lung with mucus obstruction of the airways⁹, *Id3*^{-/-} mice showed considerably less infiltration of inflammatory cells and less mucus production in the airways (Fig. 7a). Their bronchioalveolar lavage (BAL) fluid had significantly fewer inflammatory leukocytes, particularly eosinophils (Fig. 7b). In accordance with the diminished inflammation in their lungs, *Id3*^{-/-} mice had much less immunoglobulin E (IgE) in the BAL washes (Fig. 7c) and in the circulation (Fig. 7d) than did wild-type mice. There was a significantly greater frequency of T_H17 cells among CD4⁺ T cells isolated from the BAL fluid of *Id3*^{-/-} mice than among those from the BAL fluid of wild-type mice (Fig. 7e,f), whereas their CD4⁺IFN- γ ⁺ T cells were similar (Fig. 7g,h). There were considerably fewer IL-13⁺ cells (Fig. 7e,f) and IL-4⁺ cells (Fig. 7g,h) among CD4⁺ T cells in *Id3*^{-/-} BAL fluid than in wild-type BAL fluid. Analysis of IL-4 protein in BAL washes and plasma provided similar results (Supplementary Fig. 9). The BAL fluid of *Id3*^{-/-} mice had fewer CD4⁺Foxp3⁺ T_{reg} cells than did the BAL fluid of wild-type mice (Fig. 7i,j). The mediastinal draining lymph nodes and spleens of *Id3*^{-/-} mice also similarly had more T_H17 cells and fewer IL-13⁺ and IL-4⁺ T cells (Supplementary Fig. 10), whereas CD4⁺Foxp3⁺ T_{reg} cells were unchanged or greater in abundance in the same tissues of *Id3*^{-/-} mice (Supplementary Fig. 10). Nevertheless,

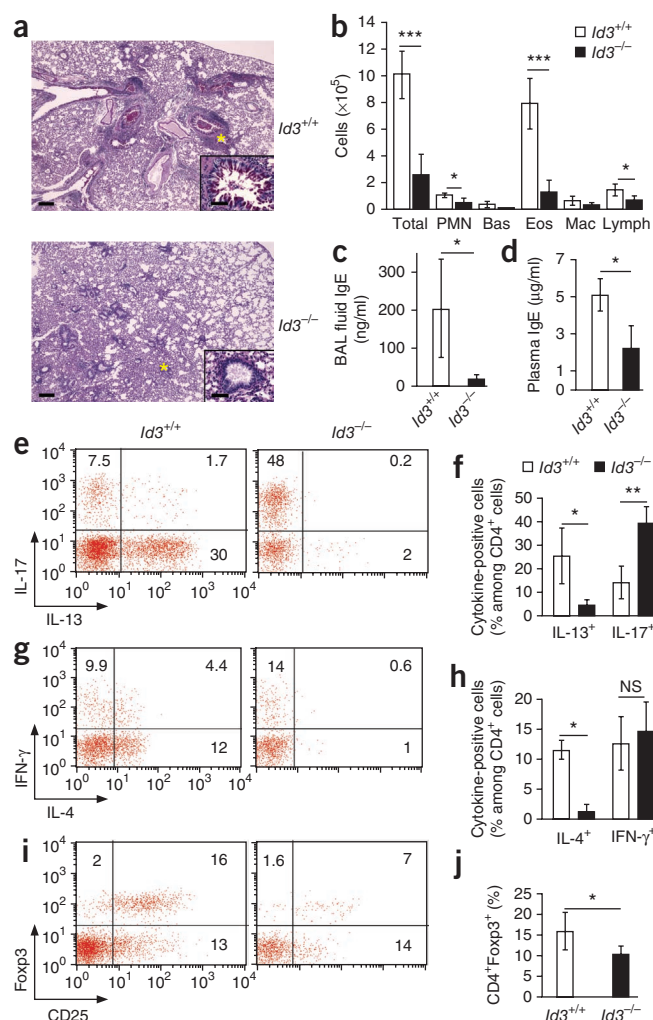


Figure 7 Id3 regulates T_H17 cells in HDM-induced asthma. (a) Mucus in the airways and inflammation in the lungs of *Id3*^{+/+} and *Id3*^{-/-} mice, assessed by periodic acid Schiff staining. Inset, enlargement of area indicated by yellow asterisk. Scale bars, 1,000 μm (main images) or 250 μm (insets). (b) Inflammatory cells in BAL fluid 4 d after the second of two intratracheal challenges of *Id3*^{+/+} and *Id3*^{-/-} mice with HDM. PMN, polymorphonuclear neutrophils; Bas, basophils; Eos, eosinophils; Mac, macrophages; Lymph, lymphocytes. (c,d) Enzyme-linked immunosorbent assay of IgE in BAL fluid (c) or plasma (d) of *Id3*^{+/+} and *Id3*^{-/-} mice. (e-h) Flow cytometry of the intracellular expression of IL-17 and IL-13 (e,f) or of IFN-γ and IL-4 (g,h) in CD4⁺ T cells in BAL fluid of *Id3*^{+/+} and *Id3*^{-/-} mice. (i,j) Flow cytometry of intracellular Foxp3 and surface CD25 in gated CD4⁺ T cells from *Id3*^{+/+} and *Id3*^{-/-} mice. Numbers in quadrants (e,g,i) indicate percent cells in each; results at right indicate frequency of cytokine-positive cells (f,h) or CD4⁺Foxp3⁺ cells (j) among CD4⁺ T cells. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (Student's *t*-test). Data are representative of three independent experiments with nine to ten mice per group (a); four mice per group (b; mean ± s.d.); four (c) or six to nine (d) mice per group (c,d; mean ± s.d.); ten *Id3*^{+/+} mice or nine *Id3*^{-/-} mice (e-h; mean ± s.d. in f,h); or nine to ten mice per group (i,j; mean ± s.d. in j).

This T_{reg} cell deficiency occurred in both the spleen and thymus in young *Id3*^{-/-} mice, although the effect was more profound and consistent in the former organ than in the latter. We found that the defective generation of Foxp3⁺ T_{reg} cell in *Id3*^{-/-} mice was T cell intrinsic, as there were fewer Foxp3⁺ T_{reg} cells among CD4⁺ T cells in chimeras reconstituted with *Id3*^{-/-} bone marrow than in wild-type controls. We excluded the possibilities of more cell death and less population expansion as causes of the T_{reg} cell deficiency in young *Id3*^{-/-} mice, because *Id3*^{-/-} T_{reg} cells did not undergo more apoptosis (data not shown) and had higher rates of proliferation *in vivo*. Greater population expansion of T_{reg} cells in the absence of *Id3* was responsible for the higher frequency of Foxp3⁺ T cells in older *Id3*^{-/-} mice. Although details of the mechanisms remain to be elucidated, the inflammation and autoimmune-like disease in older *Id3*^{-/-} mice can be attributed to at least two possibilities. First, the initial deficiency in Foxp3⁺ T_{reg} cells in the neonatal *Id3*^{-/-} mice resulted in lack of regulation of T cell activation. Alternatively, but not a mutually exclusively possibility, the defective suppressive function of *Id3*^{-/-} T_{reg} cells could explain how an inflammatory syndrome was accompanied by a greater frequency of T_{reg} cells in older *Id3*^{-/-} mice.

Notably, we have determined that Id3 has a crucial role in the correct execution of TGF-β1-mediated signals in T cells. Downstream of the TGF-β1 signal, we found that more binding of E2A had a key role in the activation of *Foxp3* transcription, which was defective in *Id3*^{-/-} T cells. This enrichment of E2A was mediated by the E-boxes detected in the regulatory region of the *Foxp3*. This is in agreement with published findings showing that this same region (+1 kb relative to the transcription start site) corresponds to an open chromatin status (trimethylated histone H3 Lys4) in a *Foxp3* gene that is being transcribed⁴⁶. Our results showed that after treatment with TGF-β1, TBP bound to the *Foxp3* promoter at the same time as E2A, which further suggested a positive role for E2A in the transcriptional regulation of *Foxp3*. This activity was regulated through TGF-β1 signaling; regardless of the method used to disrupt the binding of E2A to the *Foxp3* promoter, disruption impaired the ability of TGF-β1 to activate *Foxp3*.

Our data suggest that TGF-β1 mediates enrichment of E2A at the *Foxp3* promoter and that this is important in the activation of *Foxp3* transcription. However, the molecular mechanisms by which TGF-β1 enriches E2A remain unknown; there is no experimental evidence that Smad2 or Smad3 directly interacts with E2A proteins. Treatment with TGF-β downregulated *Id3* expression in wild-type

the overall ratio of T_H17 cells to Foxp3⁺ T_{reg} cells was considerably higher in *Id3*^{-/-} mice than in wild-type mice. As a control for HDM-induced lung inflammation, we also examined T cells in untreated *Id3*^{-/-} lungs. Unmanipulated *Id3*^{-/-} mice had many more IL-17 cells in the BAL fluid than did naive wild-type mice (Supplementary Fig. 11). The BAL fluid of naive wild-type mice CD4⁺ T cells showed a high frequency of IL-17⁺ cells (~18%; Supplementary Fig. 11), greater than that of wild-type mice with HDM-induced asthma (Fig. 7e). The BAL fluid of *Id3*^{-/-} mice had only slightly more IFN-γ⁺ or IL-4⁺ cells than did wild-type BAL fluid but had almost undetectable IL-13⁺ CD4⁺ T cells (Supplementary Fig. 11). Notably, the BAL fluid of untreated *Id3*^{-/-} mice contained considerably fewer CD4⁺ Foxp3⁺ T_{reg} cells than did that of wild-type mice (Supplementary Fig. 11). Thus, our data demonstrated that Id3 also regulated the reciprocal differentiation of T_{reg} cells and T_H17 cells *in vivo* in naive lungs as well as in response to an allergic allergen in experimental asthma. On the basis of the data we have presented here, we propose a model in which the delicate balance of converging enhancer and silencer effects at the *Foxp3* promoter results in correct *Foxp3* transcription. Id3 has a critical role in the regulation of this process (Supplementary Fig. 12).

DISCUSSION

We have shown here that Id3 is critical for promoting the generation of Foxp3⁺ T_{reg} cells and controlling T_H17 differentiation. A null mutation in *Id3* led to a considerable deficit in CD4⁺Foxp3⁺ T_{reg} cells in mice.

T cells at 12–24 h, which suggested potential release of E2A protein from Id3-E2A complexes, with E2A being able to bind to E-boxes at the *Foxp3* promoter. Additionally, proof that TGF- β 1-mediated suppression of *Gata3* has a direct effect on the enrichment of E2A at the *Foxp3* promoter also remains elusive, although inhibition of *Gata3* could restore the E2A enrichment in response to TGF- β 1 in *Id3*^{-/-} T cells. Notably, *E2a*-mutation experiments suggested that TGF- β 1 enhances the amount of E2A bound to the *Foxp3* promoter rather than changing the functional capacity of E2A. Finally, TGF- β 1 could influence *Foxp3* epigenetically by loosening the chromatin, allowing more access of E2A to the *Foxp3* promoter. The E-proteins have been shown to form complexes with the acetyltransferases p300 and CBP and to recruit histone acetyltransferases and RNA polymerase II (ref. 26), which may consequently promote *Foxp3* transcription⁴⁷. Thus, details of the mechanisms by which E2A binds to E-boxes at *Foxp3* promoter and how this promotes *Foxp3* transcription await further investigation.

We have further demonstrated that defective TGF- β 1-mediated enrichment of E2A at the *Foxp3* promoter in *Id3*^{-/-} T cells was mainly due to large amounts of GATA-3 in these cells. In *Id3*^{-/-} cells, uncontrolled IL-4 production promoted the expression of GATA-3, which occupied the *Foxp3* promoter and was not sufficiently suppressed by TGF- β 1; thus, enrichment of E2A at the *Foxp3* promoter was not possible. In support of that, neutralization of IL-4 in TGF- β -treated *Id3*^{-/-} T cells completely blocked *Gata3* expression and restored the binding of E2A to the *Foxp3* promoter. This consequently allowed the generation of Foxp3⁺ T_{reg} cells.

To further address the issues noted above, we attempted to knock down GATA-3 with siRNA in naive T cells. However, GATA-3 knockdown proved difficult in *Id3*^{-/-} T cells (a decrease of only 20–30%) but was easier in wild-type T cells (a decrease of 80–90%). Consequently, *Id3*^{-/-} T cells treated with GATA-3-specific siRNA had only slightly more TGF- β 1-mediated induction of *Foxp3* (data not shown). These data further support the idea that overexpression of GATA-3 was a key factor in defective Foxp3 induction in *Id3*^{-/-} T cells. One possible reason for the insufficient knockdown of GATA-3 by siRNA was uncontrolled IL-4 production in *Id3*^{-/-} CD4⁺ T cells; this would antagonize the GATA-3-specific siRNA by continuously inducing more GATA-3. How Id3 deficiency leads to uncontrolled IL-4 production in T cells remains an open issue. Here we found that E2A had an important role in switching on *Il4* in wild-type CD4⁺ T cells after TCR stimulation, which indicates a function for Id3 in inhibiting the binding of E2A to the *Il4* promoter.

On the basis of the data presented here, we propose that TGF- β 1 induces *Foxp3* expression via at least two indispensable and complementary molecular events: by promoting the binding of E-proteins to the *Foxp3* promoter (enhancer), and by inhibiting and/or removing negative factors such as GATA-3 bound to the *Foxp3* promoter (silencer). Although it is too simplistic to state that E2A and GATA-3 are the only enhancer and silencer in *Foxp3* gene transcription, our findings provide an example of dynamic balance between positive and negative factors as the key to correct gene transcription.

The inability of CD4⁺ T cells lacking Id3 to induce Foxp3 resulted in an intrinsic preference for T_H17 differentiation, as restoration of Foxp3 in *Id3*^{-/-} T cells abrogated their IL-17 production, consistent with the finding that Foxp3 interacts with *Rorc* and suppresses its expression²². In addition, we have shown that binding of E2A to the *Rorc* promoter may have directly enhanced its transcription and the consequent production of IL-17 in CD4⁺ T cells. Evidence in support of that conclusion included the following: TGF- β -enrichment of E2A at the *Rorc* promoter in wild-type T cells in which the TCR

was stimulated, and less *Rorc* transcription and IL-17 production in response to TGF- β and IL-6 after siRNA-mediated knockdown of E2A. Key issues that remain are how IL-6 signals crosstalk with TGF- β -mediated E2A enrichment to promote *Rorc* transcription, as well as identification of the missing link in this process. In contrast to the *Foxp3* promoter, the *Rorc* promoter contains no GATA-3-binding sites. Nevertheless, these findings provide clues to help decipher the mystery of T_H17 differentiation.

The demonstration that deletion of *Id3* enhanced T_H17 cells in an asthma model highlights a function for Id3 in the balance between T_{reg} and T_H17 differentiation *in vivo*. Notably, this preferential increase in T_H17 cells was not limited to allergic inflammation in *Id3*^{-/-} mice. It has also been observed in a model of experimental autoimmune encephalomyelitis (data not shown), which suggests generality of the role of Id3 in the reciprocal differentiation of T_H17 cells and T_{reg} cells. However, the findings of more IL-17⁺ cells and less-abundant T_H2 cytokines, as well as less allergic inflammation in *Id3*^{-/-} mice, were unexpected, especially given that *Id3*^{-/-} T cells produced large amounts of IL-4 *in vitro* and in naive *Id3*^{-/-} lungs.

The mechanisms that underlie the finding of a lower abundance of T_H2 cytokines remain undefined, but our data could suggest a critical role for HDM treatment in *Id3*^{-/-} mice. BAL fluid from naive *Id3*^{-/-} mice had a greater frequency of T_H17 cells than did BAL fluid from naive wild-type mice. HDM challenge resulted in fewer T_H17 cells and more T_H2 cells in the BAL fluid of wild-type mice but even more T_H17 cells and fewer T_H2 cells in the BAL fluid of *Id3*^{-/-} mice. Thus, it remains possible that T_H17 cells may inhibit T_H2 differentiation in asthmatic inflammation. Nevertheless, our data indicate at least a lack of a positive correlation between T_H17 cells and lung inflammation in asthma, consistent with published observations⁴⁴. However, this does not necessarily eliminate the possibility that T_H17 cells have a role in initiating the disease⁴⁵. Collectively, our data have shown that Id3 is not only a key regulator of TGF- β -dependent immune responses, able to promote Foxp3 induction and inhibit T_H17 differentiation, but also a crucial factor in diseases that involve immune dysregulation.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

T.M. and J.L. designed and did experiments, analyzed data and contributed to the writing of the manuscript; J.P.V. designed and did ChIP experiments, analyzed data and contributed to the writing of the manuscript; J.E.K. did experiments, analyzed data and contributed to the writing of the manuscript; Y.W. designed and W.W. and D.Y. did the luciferase, E2A-knockdown and Id3 immunoblot experiments and analyzed data; B.Z. and Y.Z. generated and identified mice doubly deficient in E2A and HEB and provided critical input; P.Z. and B.F.Z. did experiments; J.S.G. supervised and designed the ChIP study and contributed to the writing of the manuscript; and W.C. conceived of the research, directed the study, designed experiments, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 and *Rag1*^{-/-} mice were from The Jackson Laboratory. *Id3*^{-/-} mice²⁷ (on a C57BL/6 background), *Smad3*^{-/-} mice and their littermates (on a C57BL/6 × 129 background), *Tgfbri*^{fl/fl} *CD4*-Cre⁺ mice, *Tgfbri*^{fl/fl} *CD4*-Cre⁺ mice⁴⁸ and *E2a*^{fl/fl} *Heb*^{fl/fl} *ER*-Cre⁺ mice³⁶ were bred in our facilities (National Institute of Dental and Craniofacial Research and Duke University) under specific pathogen-free conditions. All animal studies were according to National Institutes of Health guidelines for use and care of live animals and were approved by the Animal Care and Use Committees of the National Institute of Dental and Craniofacial Research and Duke University.

Antibodies and reagents. The following antibodies (to mouse) were from BD Biosciences: phycoerythrin-conjugated anti-CD45RB (16A), anti-CD69 (H1.2F3), anti-CD62L (MEL14), anti-Ki67 (B56) and anti-IFN- γ (XMG1.2); allophycocyanin-conjugated anti-CD44 (IM7) and anti-IL-4 (11B11); purified anti-CD3 (no azide and low endotoxin; 145-2C11), anti-CD28 (no azide and low endotoxin; 37.51) and anti-Fc γ RII/III (2.4G2); fluorescein isothiocyanate-conjugated anti-CD45.1 (NDS58); fluorescein isothiocyanate-, phycoerythrin- or allophycocyanin-conjugated anti-CD25 (7D4 and PC61); peridinin chlorophyll protein-, Pacific blue- or fluorescein isothiocyanate-conjugated anti-CD4 (RM4-5) and anti-CD8 α (53-6.7) and their respective isotype-matched control antibodies (rat IgG2a (R35-95), rat IgG1 (R3-34), rat IgG2b (A95-1), rat IgM (R4-22), mouse IgG1 (MOPC-21) and mouse IgG2b (27-35)); and purified (no azide and low endotoxin) anti-IL-4 (11B11), anti-IL-6 (MP5-20F3) and anti-IFN- γ (XMG1.2). The following antibodies were from eBioscience: phycoerythrin-conjugated anti-GATA3 (TWAJ), anti-ROR γ t (AFKJS-9), anti-IL-13 (eBio13A) and isotype-matched control antibody (rat IgG2b; 12-4031); and fluorescein isothiocyanate-, phycoerythrin- or allophycocyanin-conjugated antibody to mouse or rat Foxp3 (staining set; FJK-16s). Allophycocyanin-conjugated anti-mouse IL-17 (TC11-18H10.1), phycoerythrin-conjugated antibody to mouse and human Helios (22F-6) and the isotype-matched control antibody (Armenian hamster IgG; HTK888) were from BioLegend. Anti-Id3 (B72-1), anti-IL-21 (AF594), anti-IL-23p19 (AF1619), recombinant mouse IL-6 (406-ML), IL-4 (404-ML) and IL-2 (1150-ML), and human TGF- β 1 (240-B) were from R&D Systems.

ChIP. A Red ChIP kit was used for ChIP assays according to the manufacturer's instructions (Diagenode). CD4⁺CD25⁻ T cells (10×10^6) or CD4⁺CD25⁺ T cells (7×10^6 to 10×10^6) were routinely used. CD4⁺CD25⁻ cells were stimulated for 12–24 h with plate-bound anti-CD3 (5 μ g/ml) and soluble anti-CD28 (2 μ g/ml) with or without TGF- β 1 (2 ng/ml). An equal amount of processed chromatin was used as an input control or was incubated with 4 μ g antibody to human and mouse E47. (G127-32; BD Biosciences), anti-TBP (Mab-002-100; Diagenode) or anti-GATA-3 (B-10; Santa Cruz,) or their respective isotype-matched control antibody (mouse IgG1; 107.3; BD Biosciences). Immunoprecipitated DNA and total input DNA were analyzed with a SYBR Green Supermix kit and a quantitative iCycler iQ Real-Time PCR Detection system (Bio-Rad). PCR primers for the detection of promoters were as follows: *Foxp3* amplicon 1, 5'-GGCCGCTATGTGTATGG TTT-3' (forward) and 5'-CTGCTGCGAGTCTCTGAGTG-3' (reverse); *Foxp3* amplicon 2, 5'-GCAACTCAAGATGCTGTCCA-3' (forward) and 5'-GGCTGG AAGAGACAGACAGG-3' (reverse); and *Foxp3* amplicon 3, 5'-GCGCTTATG TGGCTTCTTTC-3' (forward) and 5'-GCAGATGGATGGGTCTTTGT-3' (reverse). PCR primers for *Rorc* were 5'-GTGCAGATCTAAGGCTGAG GC-3' (forward) and 5'-CATTCCTACTTCTCATGACTG-3' (reverse).

T cell purification and culture. CD4⁺ T cell subsets were isolated from spleens and lymph nodes with magnetic beads (Miltenyi Biotec)^{12,49}. The T_{reg} cell suppression coculture assay was done as described⁴⁹.

T_{reg} and T_H17 differentiation in vitro. T_{reg} and T_H17 differentiation was achieved as reported before^{9,18}. Naive CD4⁺CD25⁻ T cells were cultured with plate-bound anti-CD3 (5 μ g/ml) and soluble anti-CD28 (2 μ g/ml), with the addition of TGF- β 1 (0.002–20 ng/ml), IL-6 (50 ng/ml), IL-21 (50 ng/ml), IL-2 (5 ng/ml) and/or retinoic acid (100 nM) where appropriate. In some cultures, anti-IL-4 (10 μ g/ml), anti-IFN- γ (10 μ g/ml), anti-IL-6 (10 μ g/ml) anti-IL-21 (10 μ g/ml) or anti-IL-23 (10 μ g/ml) was included. Culture supernatants were collected at 24 h (for IL-4) or 72 h (for IL-17) for cytokine enzyme-linked immunosorbent assay (eBioscience and BioLegend).

Flow cytometry. Flow cytometry was done as described¹².

Immunoblot analysis. Immunoblot analysis was done as described¹². The following antibodies were used: antibody to Smad2 and Smad3 (18), anti-E47 (G98-271), anti-Id3 (B72-1) and anti-GATA-3 (L50-823; all from BD Biosciences); antibody to phosphorylated Smad2 (3101S; Cell Signaling Technology); anti- α -tubulin (SAB3500023; Sigma); and anti- β -actin (C-11; Santa Cruz).

Real-time RT-PCR. Real-time RT-PCR was done as described^{12,49}. *Foxp3*, *Rorc*, *Id3*, *Il4*, *Smad7*, *Gata3* and *Hprt1* were analyzed with a TaqMan gene-expression assay kit. Primers for the following were from Applied Biosystems: *Foxp3*, NM_054039.1; *Il4*, NM_021283.1; *Hprt1*, NM_013556.2; *Smad7*, NM_001042660.1; *Gata3*, NM_008091.3. The primers and probe for *Rorc* have been described¹⁹.

RNA-mediated interference. EL4 LAF cells^{15,35} were incubated for 3 h with E2A short hairpin RNA lentiviral particles (1×10^6 ; transduction units per ml; Sigma) in complete DMEM. Puromycin was added to cell cultures at a concentration of 1 μ g/ml and 1.5 μ g/ml on day 2 and day 5, respectively. The efficiency of E2A knockdown was determined at day 9 with anti-E47. Five short hairpin RNA constructs (all from Sigma) were tested; the two that were most effective in the knockdown of E2A (XM_125750.3-438s1c1 (these results presented here) and XM_125750.3-2060s1c1) were used together with control particles (SHC003H, Sigma) for analysis of *Foxp3* induction with anti-CD3 and anti-CD28, plus TGF- β 1.

An Amara Mouse T cell Nucleofector kit was used for knockdown of E2A or GATA-3 via siRNA in naive CD4⁺ cells according to the manufacturer's instructions (Lonza). Wild-type naive CD4⁺CD25⁻ (1×10^6) cells were stimulated overnight with anti-CD3 and anti-CD28 and were transduced by electroporation with E2A- and/or GATA-3-specific siRNA (5 nM). After overnight culture, cells were restimulated for 24 h with anti-CD3 and TGF- β , followed by real-time PCR analysis of *Foxp3*, *Il4*, *Rorc* or *Il17* mRNA. Of the GATA-3-specific siRNAs (SI01009771, SI01009778, SI01009792 and SI02708615) and E2A-specific siRNAs (SI01444359, SI01444366, SI01444373 and SI01444380) tested (all from Qiagen), results obtained with SI01009778 (GATA-3) and SI01444373 (E2A) are presented. The control siRNA was 1027280.

Generation of E-box mutations and luciferase assay. Site-directed mutagenesis of all the consensus sequence of the E-box element (CANNTG to AGNNT) in the *Foxp3* promoter region was done by Genscript and was confirmed by DNA sequencing. EL4 LAF cells (2×10^6) were transfected with control *Foxp3* promoter¹⁵ or E-box-mutated *Foxp3* promoter with enhancer firefly reporter plasmid (3 μ g) in 100 μ l Nucleofector Solution L (Lonza) and were cotransfected with the pRL-Null renilla luciferase vector (Promega) as an internal control. Transfected cells were stimulated for 12 h with anti-CD3 and anti-CD28 plus TGF- β 1 (2 ng/ml). *Foxp3* promoter activity was measured by a dual-luciferase assay system (Promega)^{15,35} and results are presented as follows: (firefly luminescence – background) / (renilla luminescence – background).

Adoptive T cell transfer. CD4⁺CD25⁻CD62L⁺ cells from *Id3*^{-/-} or wild-type spleens were transferred intraperitoneally into *Rag1*^{-/-} mice; 4 weeks later, donor CD4⁺ T cells were analyzed by flow cytometry.

HDM-induced asthma. Asthma was induced with HDM as described⁹ in 6- to 10-week-old *Id3*^{-/-} or wild-type control mice.

Mixed-bone marrow chimeras. Bone marrow was isolated from 3-week-old *Id3*^{+/+} or *Id3*^{-/-} (CD45.2⁺) mice or 5- to 6-week-old C57BL/6 wild-type (CD45.1⁺) mice. Donor bone marrow from *Id3*^{+/+} or *Id3*^{-/-} mice (1.5×10^6 cells) was mixed with bone marrow from the wild-type mice (3×10^6 cells) and was injected intravenously into sublethally irradiated (450 rads) *Rag1*^{-/-} mice. Cells from thymus and spleen were analyzed by flow cytometry after 4 weeks.

Statistical analysis. Student's *t*-test (two-tailed) was used to analyze the significance of data comparison, except where indicated otherwise.

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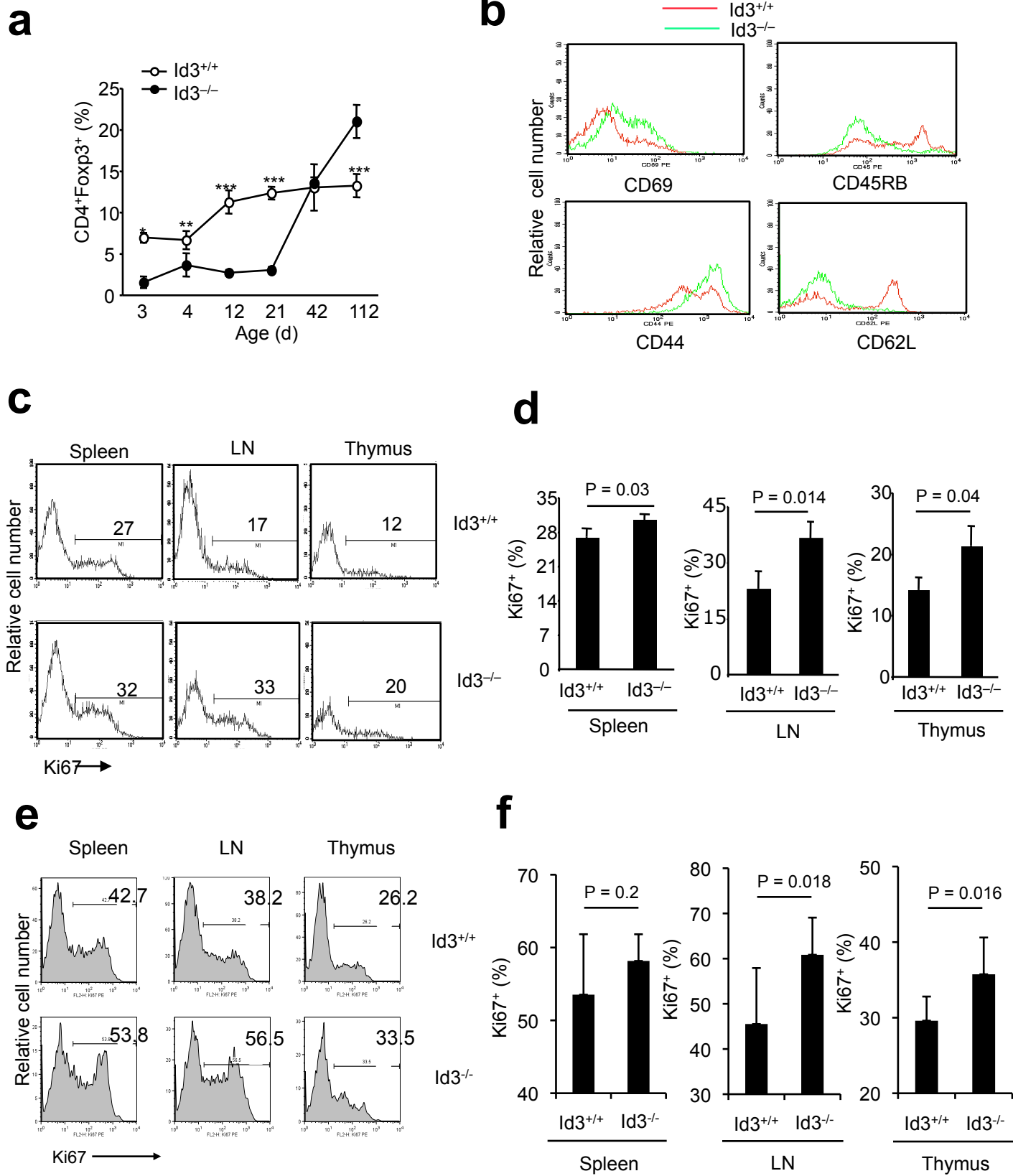
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Supplementary text and figures
Supplementary Figures 1-12

Control of regulatory T cell and T_h17 cell differentiation by inhibitory helix-loop-helix protein Id3

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Supplementary Fig. 1



Supplementary Fig. 1. Defective generation and suppressive function of T_{reg} cells in $Id3^{-/-}$ mice.

a, Kinetics of $CD4^{+}Foxp3^{+}$ T_{reg} cells in the spleens of mice. Data represent mean $\pm$ s.d. of T_{reg} cells in individual mice. Each time point contains three to five mice ($Id3^{+/+}$) or four to eight mice ($Id3^{-/-}$) except at day 42 with two mice in each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

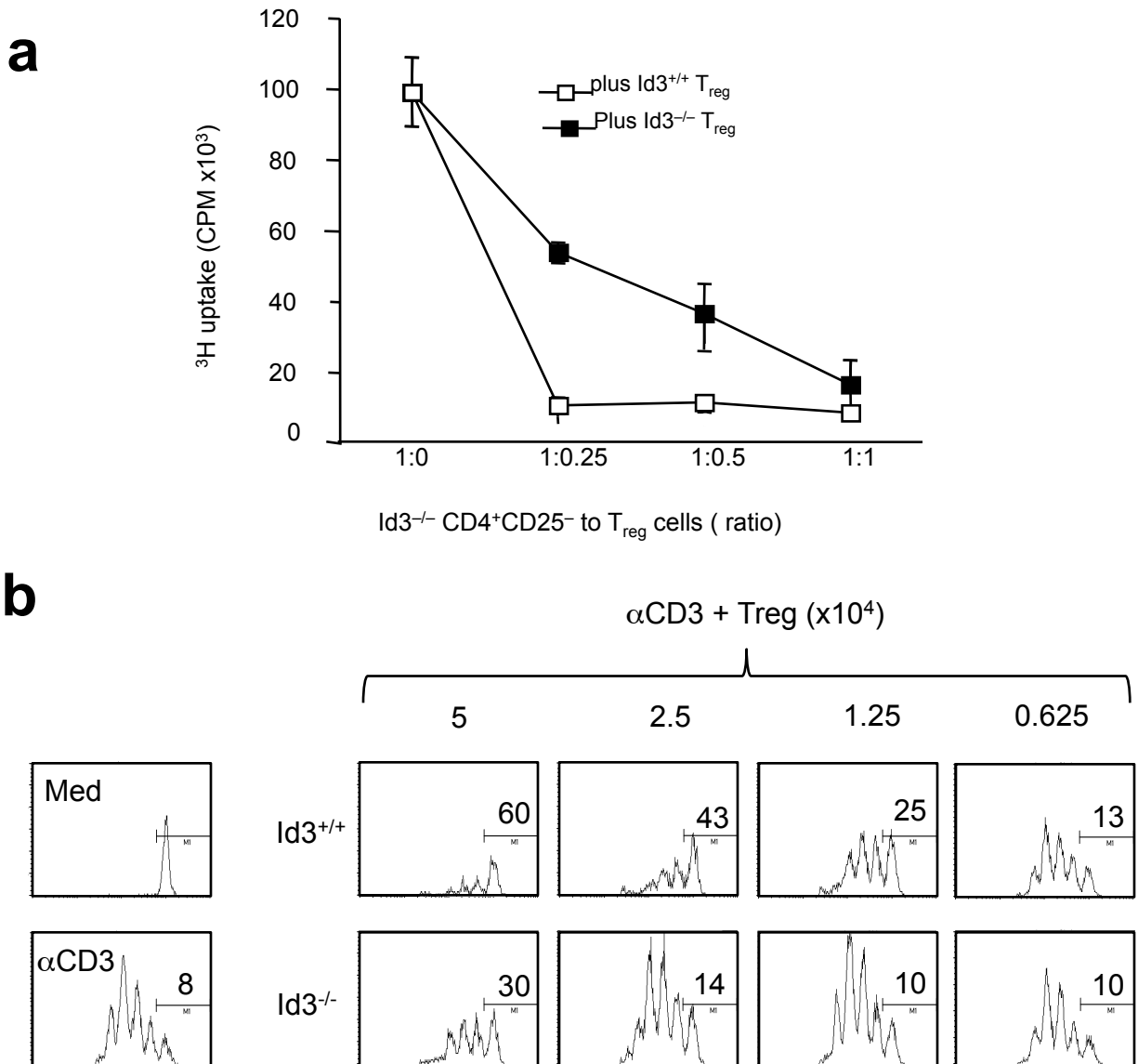
b, Flow cytometry analysis of surface markers (CD44, CD69, CD45RB and CD62L) on splenic T cells in symptomatic $Id3^{-/-}$ mice (>4-6 months) compared to age-matched WT mice. Data are shown as histograms of indicated markers in gated $CD4^{+}$ T cells and represent at least three experiments.

c,d, Frequency of $Ki67^{+}$ dividing $CD4^{+}Foxp3^{+}$ T_{reg} cells in the spleen, lymph nodes (LN) and thymus in 3-5-month-old mice. **c**, Flow cytometry of $Ki67^{+}$ frequency within gated $CD4^{+}Foxp3^{+}$ T_{reg} cells in each tissue in one mouse representative of four ($Id3^{+/+}$) and three ($Id3^{-/-}$); **d**, Percent $Ki67^{+}$ $Foxp3^{+}$ cells (mean $\pm$ s.e.m.) in the spleen, LN or thymus in the mice in **c**. $P < 0.05$ (t-test, one tail).

e, Flow cytometry analysis of $Ki67^{+}$ frequency within gated $CD4^{+}Foxp3^{+}$ T_{reg} cells in mice (three weeks-old). Data are shown as one mouse representative of seven.

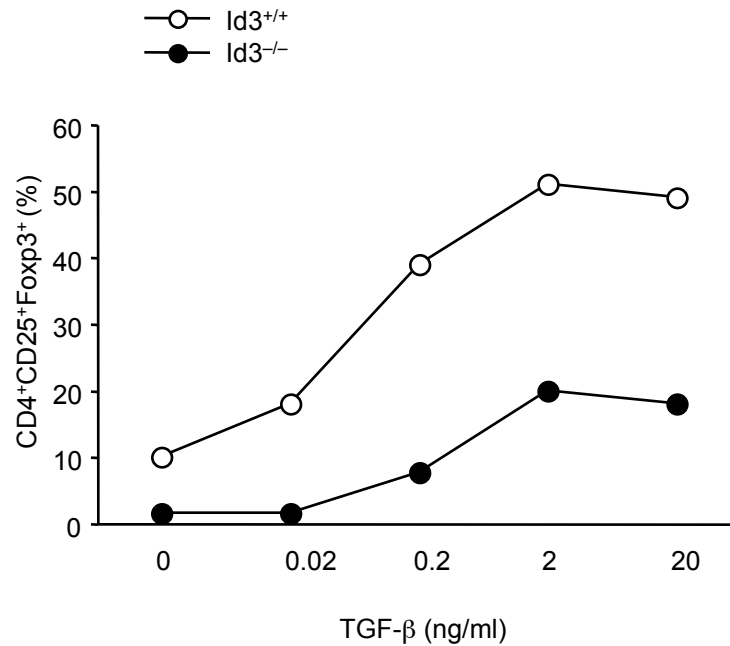
f, Percent $Ki67^{+}$ $Foxp3^{+}$ cells (mean $\pm$ s.e.m.) in the spleen, LN or thymus in the mice in **e**. $P < 0.05$ (t-test, one tail).

Supplementary Fig. 2



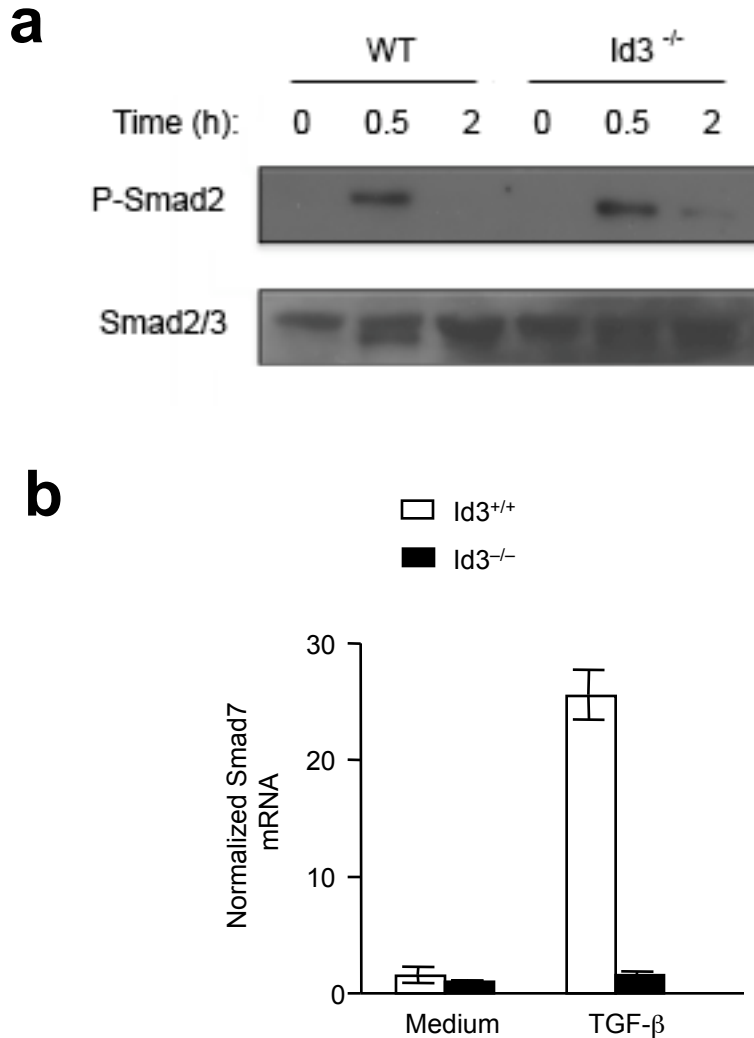
Supplementary Fig. 2. *Id3*^{-/-} T_{reg} cells show defective suppressive activity *in vitro*. **a**, *Id3*^{-/-} CD4⁺CD25⁻ T cells (5x10⁴) were co-cultured with indicated numbers of WT (*Id3*^{+/+}) or *Id3*^{-/-} CD4⁺CD25⁺ T_{reg} cells at the indicated ratios for 3 days. T cell proliferation was determined with ³H incorporation (c.p.m., mean ± s.d. of triplicate wells). Data shown are one experiment representative of three. **b**, CFSE labeled WT CD4⁺CD25⁻ cells (5x10⁴) were co-cultured with indicated numbers of *Id3*^{+/+} or *Id3*^{-/-} CD4⁺CD25⁺ T_{reg} cells (3-weeks old) in the presence of soluble anti-CD3 and WT splenic APCs for 3 days. The proliferation of CD4⁺ responder T cells was assessed by analyzing the CFSE dilution with flow cytometry.

Supplementary Fig. 3



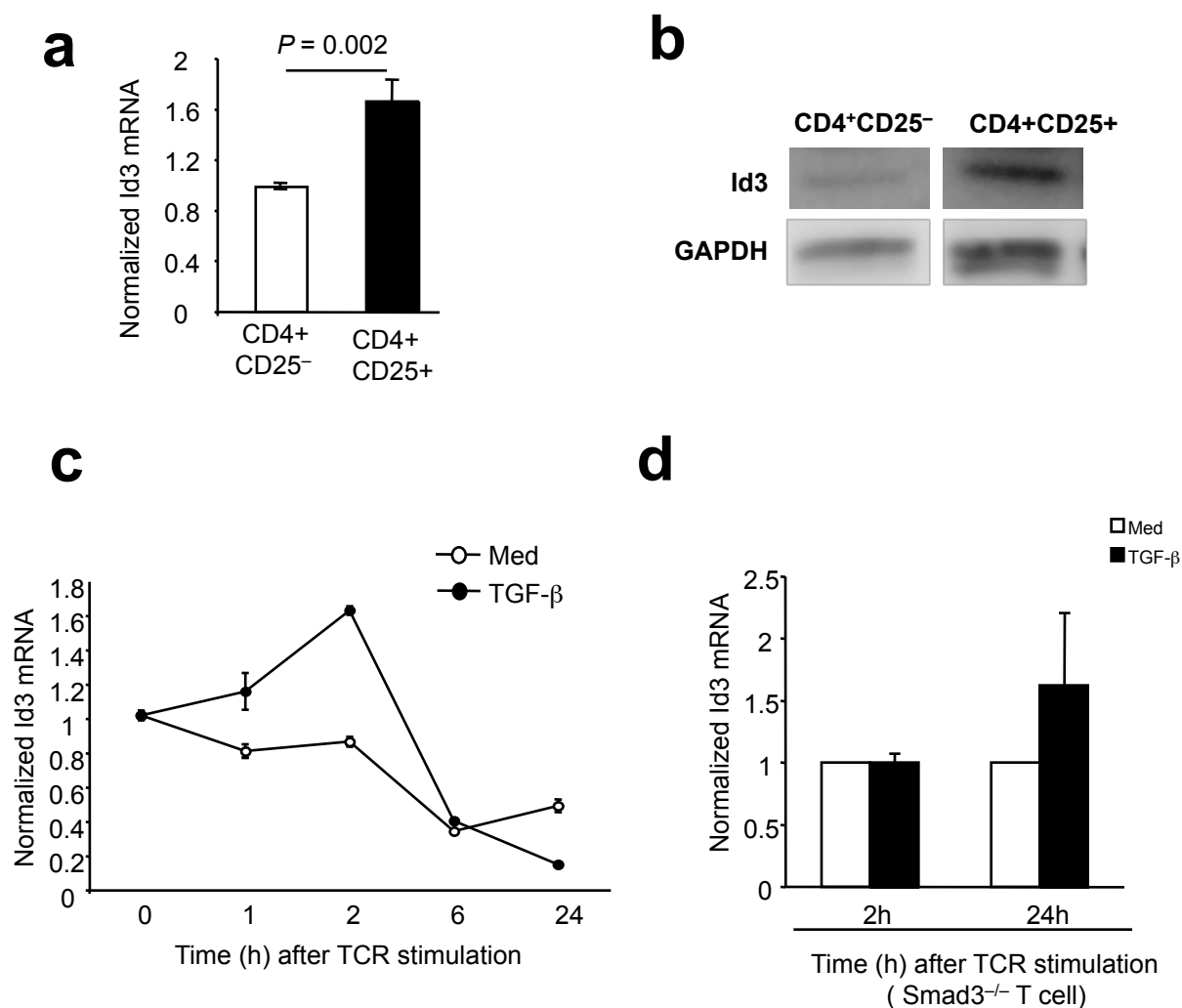
Supplementary Fig. 3. Foxp3⁺ T_{reg} generation in *Id3*^{-/-} T cells in response to different concentrations of TGF-β. Naïve CD4⁺CD25⁻ T cells were cultured with anti-CD3 and CD28 mAbs in the presence of indicated doses of TGF-β for 24 h. T_{reg} cells were determined by flow cytometry. Data are shown as percent CD4⁺Foxp3⁺ cells in one experiment representative of two.

Supplementary Fig. 4



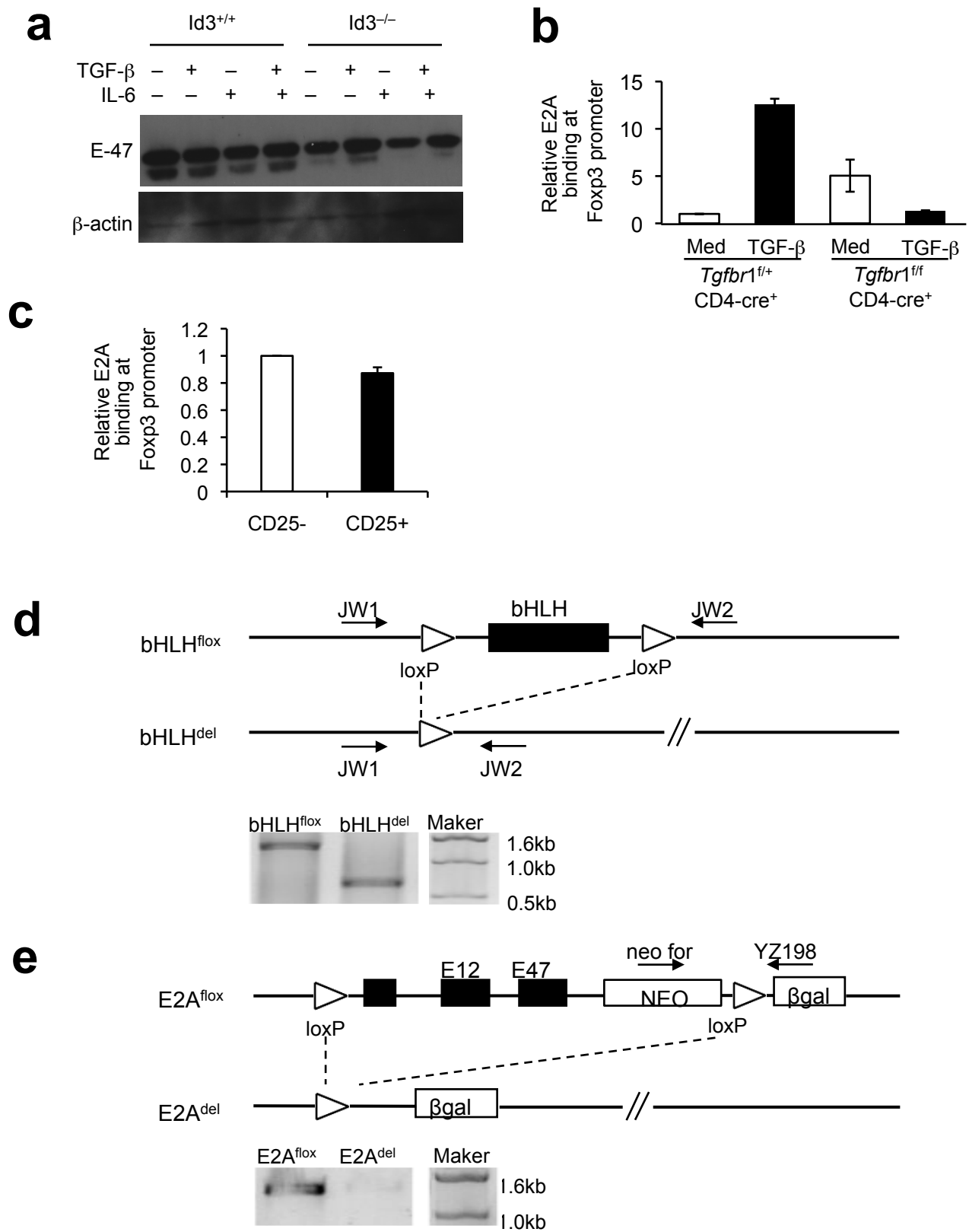
Supplementary Fig. 4. Analysis of P-Smad2 and Smad7 expression in *Id3*^{-/-} T cells. CD4⁺CD25⁻ T cells were cultured with anti-CD3-and CD28 mAbs in the absence or presence of TGF- β (2 ng/ml) for indicated times. **a**, Immunoblot analysis of P-Smad2 and Smad2 and 3. Data shown are one experiment representative of three. **b**, Quantitative RT-PCR analysis of Smad7, presented as mRNA expression relative to *Hprt* in CD4⁺CD25⁻ T cells at 2 h after activation with anti-TCR mAb in the absence or presence of TGF- β (2 ng/ml). Data are displayed as mean $\pm$ s.d of duplicate wells in one experiment representative of two.

Supplementary Fig. 5



Supplementary Fig. 5. Id3 expression in CD4⁺ T cells. (a), Quantitative RT-PCR analysis of Id3, presented as mRNA expression relative to *Hprt*, in freshly isolated CD4⁺CD25⁺ T_{reg} cells and CD4⁺ CD25⁻ naïve T cells in the WT spleen (mean $\pm$ s.d. of Id3 in three independent experiments). ** $P < 0.01$. (b), Immunoblot analysis of Id3 protein. (c), Kinetics of Id3 expression (Quantitative RT-PCR, presented as mRNA relative to *Hprt*) in CD4⁺CD25⁻ T cells cultured with anti-CD3 and CD28 mAb in the absence or presence of TGF- β (mean $\pm$ s.d. of duplicate measurements in one experiment representative of four). (d), Smad3 knockout (*Smad3*^{-/-}) CD4⁺CD25⁻ T cells were stimulated with anti-CD3 and CD28 mAb in the absence (white bar) or presence (black bar) of TGF- β at indicated time points. Id3 mRNA was analyzed with qPCR (ratio relative to *Hprt*). The values of Id3 mRNA in T cell without TGF- β were set as one at individual time points. Data are shown as mean $\pm$ s.e.m. of normalized Id3 mRNA in three independent experiments. No statistical significance was observed.

Supplementary Fig. 6



Supplementary Fig. 6. E2A binding at the *Foxp3* promoter in TGF- β receptor I deficient T cells and in freshly isolated *Id3*^{-/-} T_{reg} cells.

a, E47 protein (immunoblot) in CD4⁺CD25⁻ T cells 2h after activation with anti-CD3 and CD28 mAb and indicated cytokines.

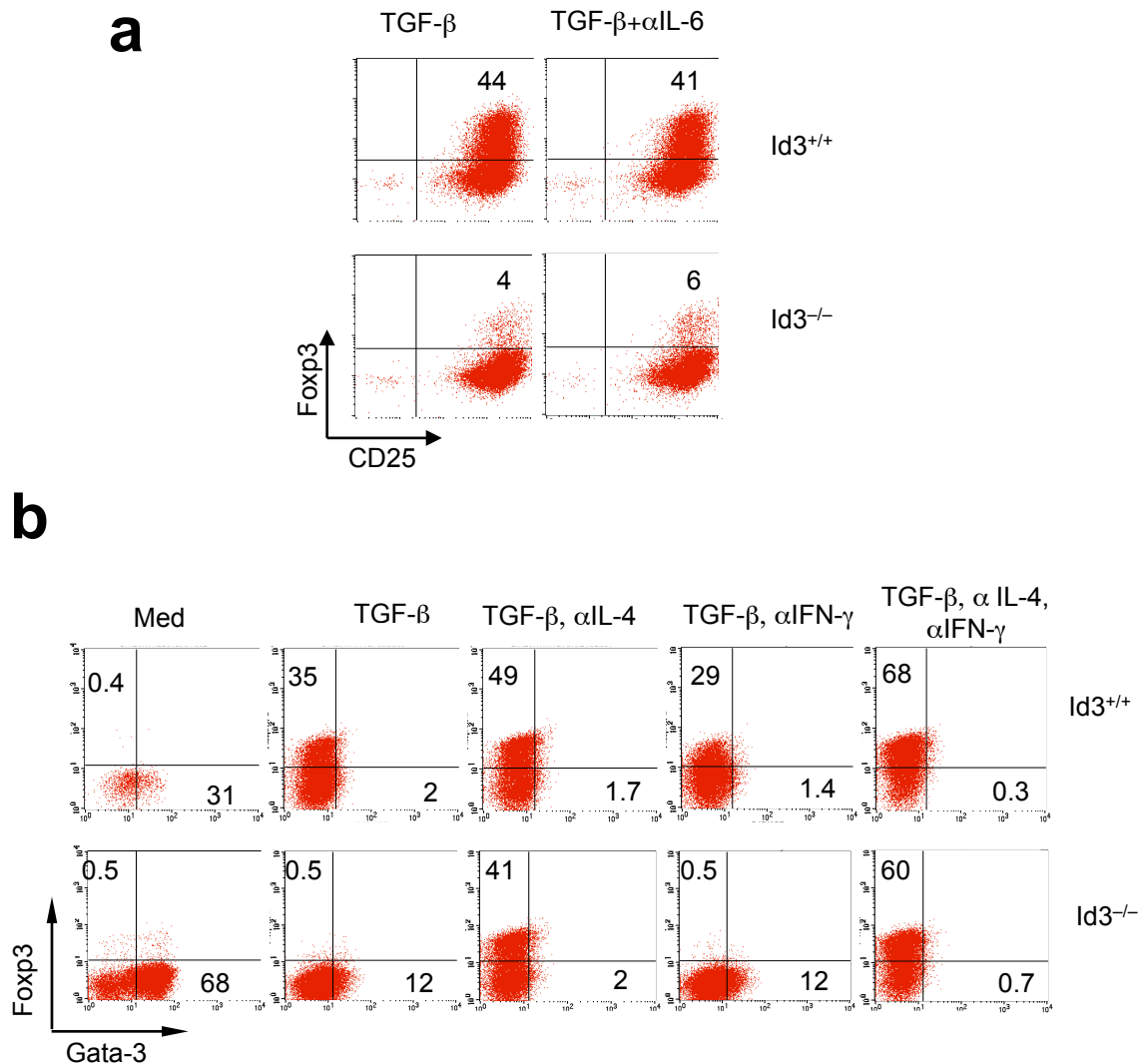
b, Analysis of relative E2A binding (E47/control IgG) to the *Foxp3* promoter in CD4⁺CD25⁻ T cells from TGF- β receptor I deficient (*Tgfb1*^{ff} *Cd4*-Cre⁺) or littermate control (*Tgfb1*^{ff/+} *Cd4*-Cre⁺) mice in response to TCR stimulation with or without TGF- β (24h). Data are displayed as mean $\pm$ s.d. of the values of triplicate wells in one experiments representative of two. The mean value of E47/control IgG in control T cells treated with anti-TCR mAb alone was set as 1.

c, Analysis of E2A binding (E2A/IgG control, ChIP-qPCR assay at +327/+513) to the *Foxp3* promoter in CD4⁺CD25⁻ (CD25⁻) or freshly isolated CD4⁺CD25⁺ T_{reg} (CD25⁺) cells isolated from spleen and lymph nodes in *Id3*^{-/-} mice (3 weeks-old, cells pooled from 11 mice). Data are displayed as mean $\pm$ s.d. of the values of triplicate wells. The mean value of E47/control IgG in control CD4⁺CD25⁻ T cells was set as 1.

d,e, Efficient depletion of HEB and E2A after tamoxifen treatment. HEB^{ff}E2A^{ff} ER-Cre mice were i.p. injected with tamoxifen (1mg/day) or sunflower seed oil for 5 consecutive days. Analysis of deletion level for splenocytes is determined by PCR. Primer design designated in above diagrams for flox and deleted alleles. **d**, HEB deletion. Primers JW1 and JW2 identify flox and deleted alleles (Wojciechowski, J. et al, *J Immunol* **178**, 5717-5726 (2007).

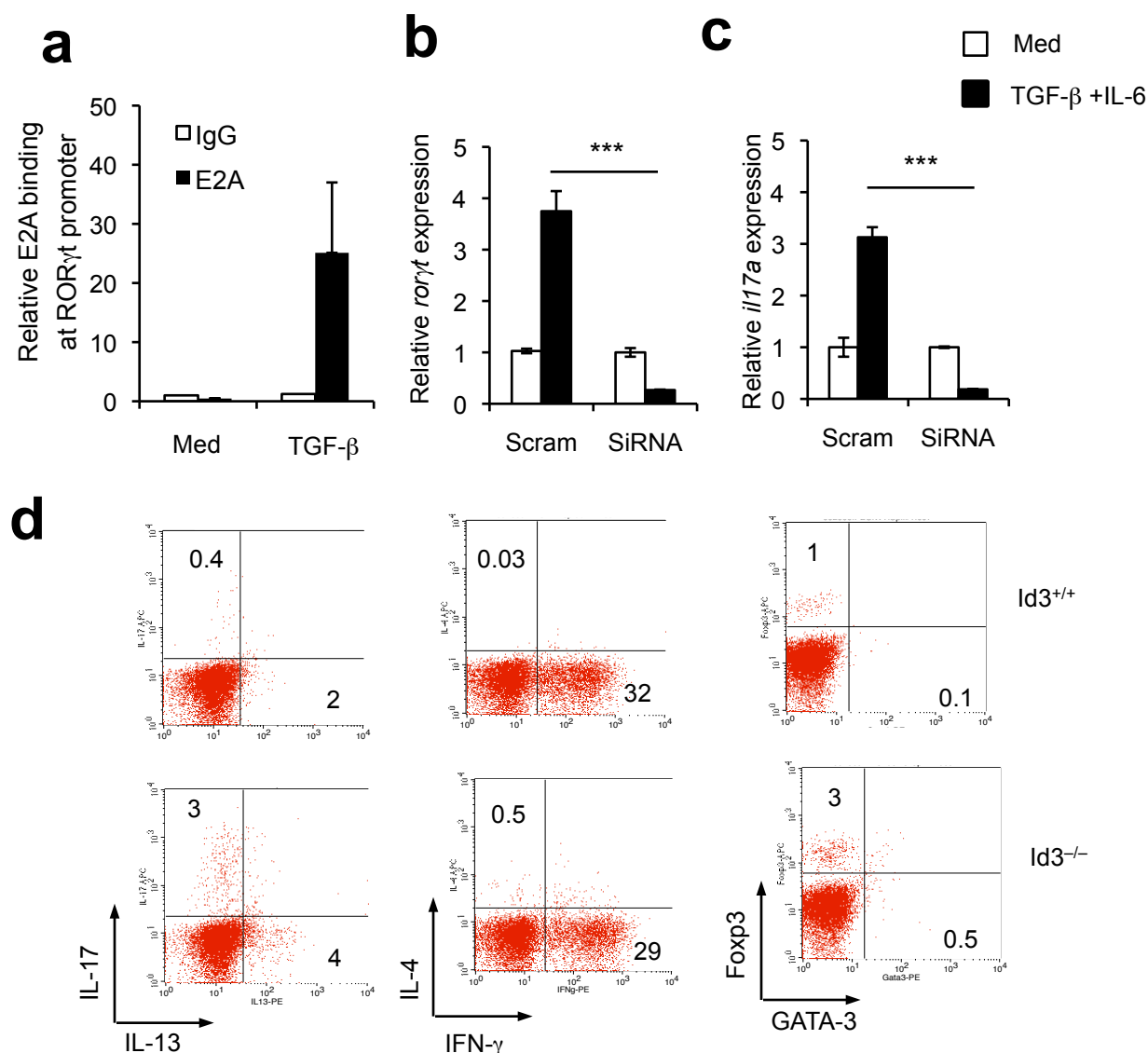
e, E2A deletion. Primers neo for and YZ198 detect the flox allele (Lazorchak, A.S., et al, *J Immunol* **177**, 2495-2504 (2006).

Supplementary Fig.7



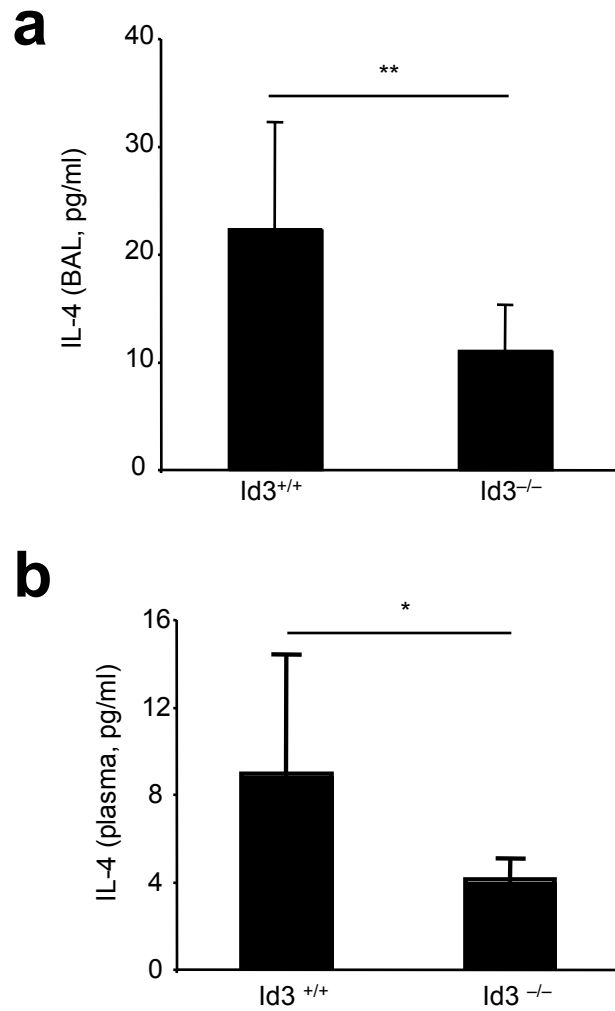
Supplementary Fig. 7. Effects of endogenous cytokines on Foxp3⁺ T_{reg} generation in *Id3*^{-/-} T cells. **a**, Flow cytometry analysis of Foxp3 versus CD25 in naïve CD4⁺CD25⁻ T cells at 3 d after activation with CD3- and CD28- antibodies and TGF- β in the absence or presence of anti-IL-6 antibody. **b**, Flow cytometry of Foxp3 versus GATA-3 in naïve CD4⁺CD25⁻ T cells at 3-5 d after activation with CD3- and CD28- antibodies and TGF- β in the presence of anti-IL-4 and/or anti-IFN- γ . Numbers in quadrants indicate percent positive. Data shown are one experiment representative of at least three.

Supplementary Fig. 8



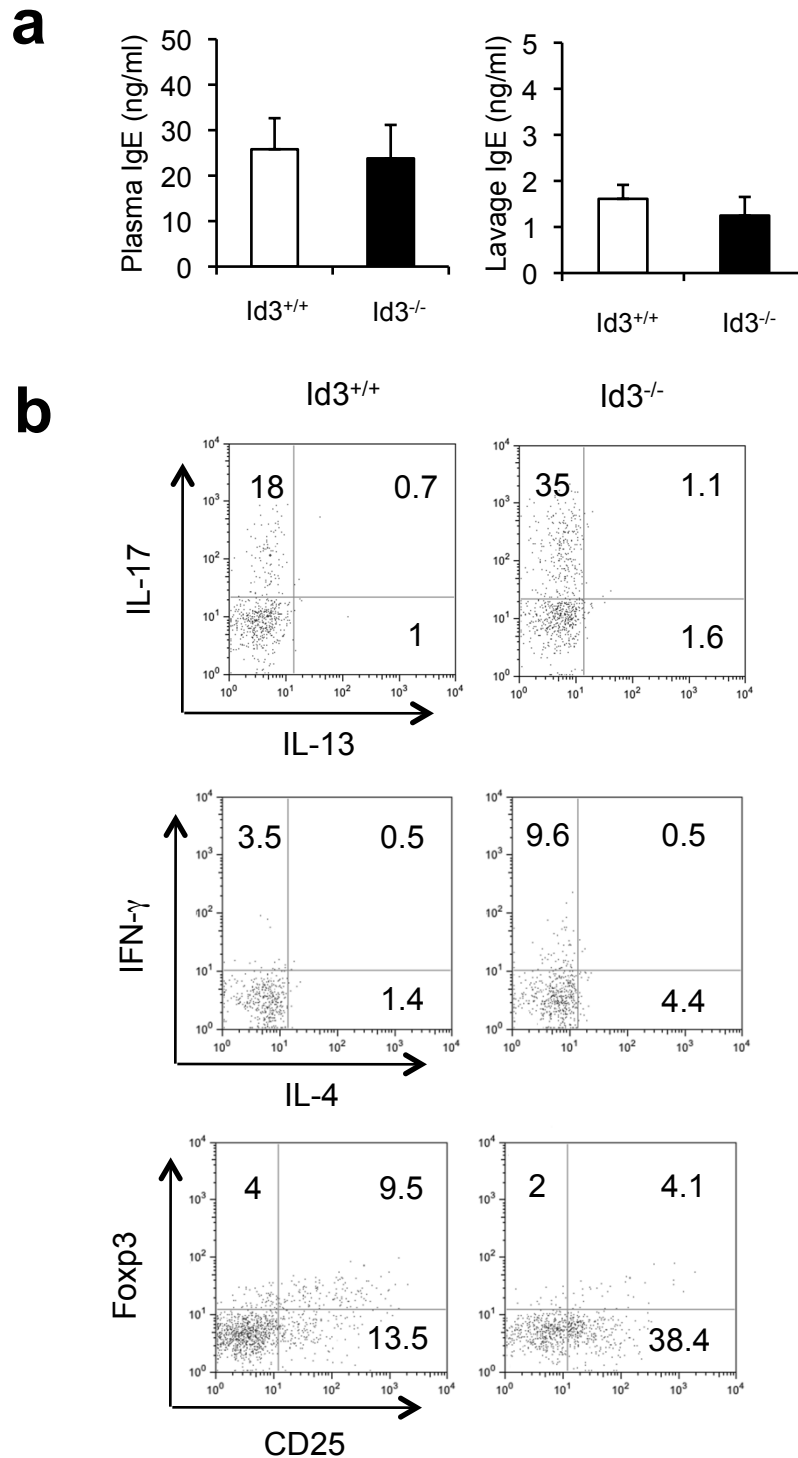
Supplementary Fig. 8. *Id3*^{-/-} T cells preferentially differentiate T_h17 cells *in vivo*. **a**, Analysis of relative E2A binding (E47/control IgG, ChIP-qPCR) to the *Rorγt* promoter in WT CD4⁺CD25⁻ T cells 24 h after TCR stimulation with or without TGF- β (mean $\pm$ s.e.m. of duplicate measurements). **b,c**, Knockdown E2A with SiRNA decreases *Rorγt* (**b**) and *Il17* (**c**) gene expression in WT CD4⁺CD25⁻ T cells 48 h after activation with TCR and TGF- β plus IL-6. *RORγt* and *IL-17* mRNAs expression relative to *HPRT* are shown (mean $\pm$ s.d. of triplicate measurements). The relative values in T cells cultured with TCR alone (Med) in Scram and SiRNA treatments are set as 1, respectively. Data shown represent one of two independent experiments. ***, $P < 0.001$. **d**, Flow cytometry of intracellular IL-17, IFN- γ , IL-13 and IL-4 and nuclear Foxp3 and GATA-3 in CD4⁺ T cells isolated from the spleens of *Rag1*^{-/-} mice at four weeks after adoptive transfer of naïve CD4⁺CD25⁻ T cells from *Id3*^{-/-} or *Id3*^{+/+} mice. The ratio of T_h17⁺ cells to Foxp3⁺ T_{reg} cells is higher in *Id3*^{-/-} T cells than in control cells. Each plot is of one mouse representative of two in each group. The experiment is representative of two.

Supplementary Fig. 9



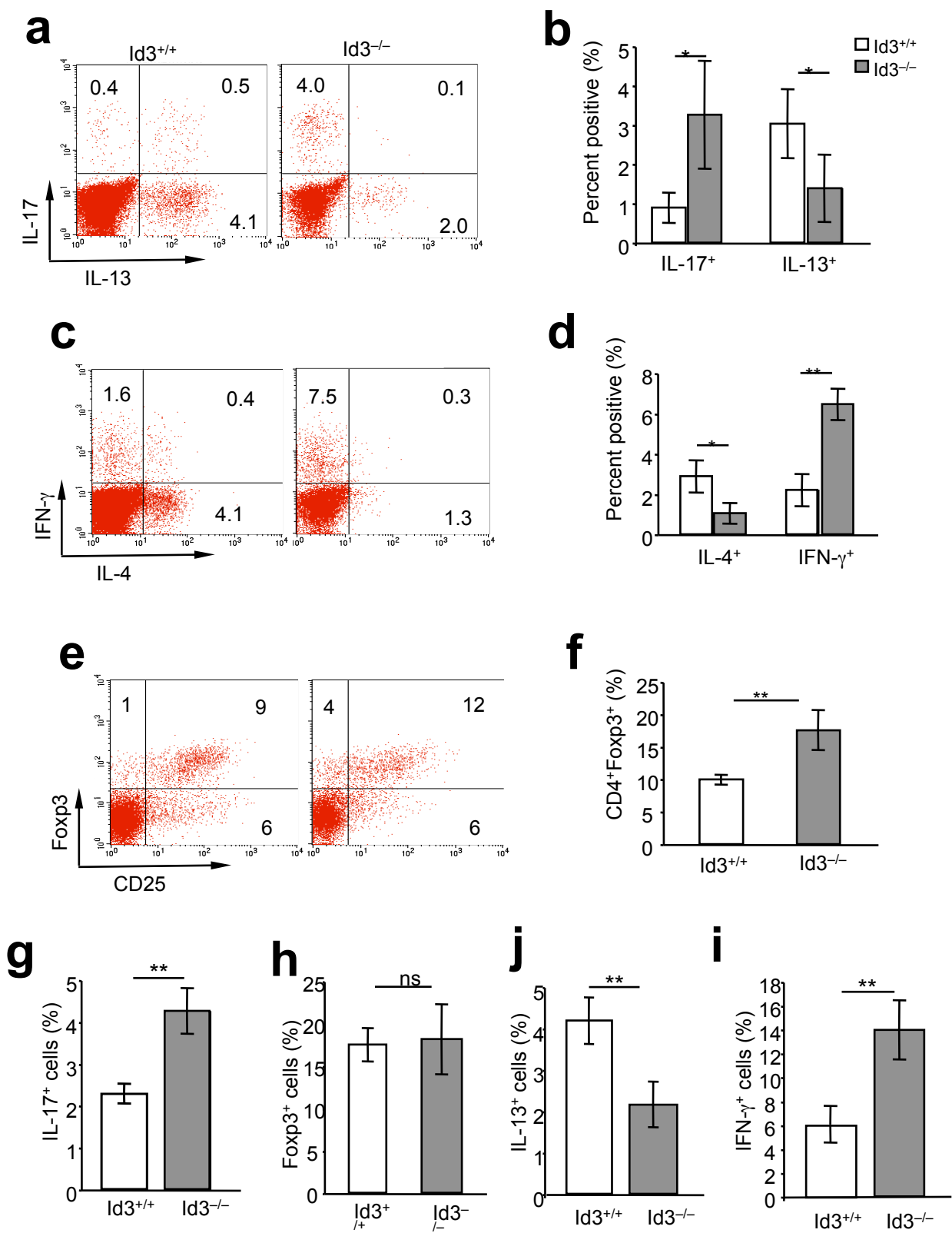
Supplementary Fig. 9. IL-4 in *Id3*^{-/-} mice during asthma. ELISA analysis of IL-4 levels in BAL (**a**, mean $\pm$ s.d of IL-4 in ten mice per group); and plasma (**b**, mean $\pm$ s.d. of IL-4 in seven *Id3*^{+/+} and eight *Id3*^{-/-} mice) in mice induced by HDM. Data represent three independent experiments. ** $P < 0.01$.

Supplementary Fig. 10



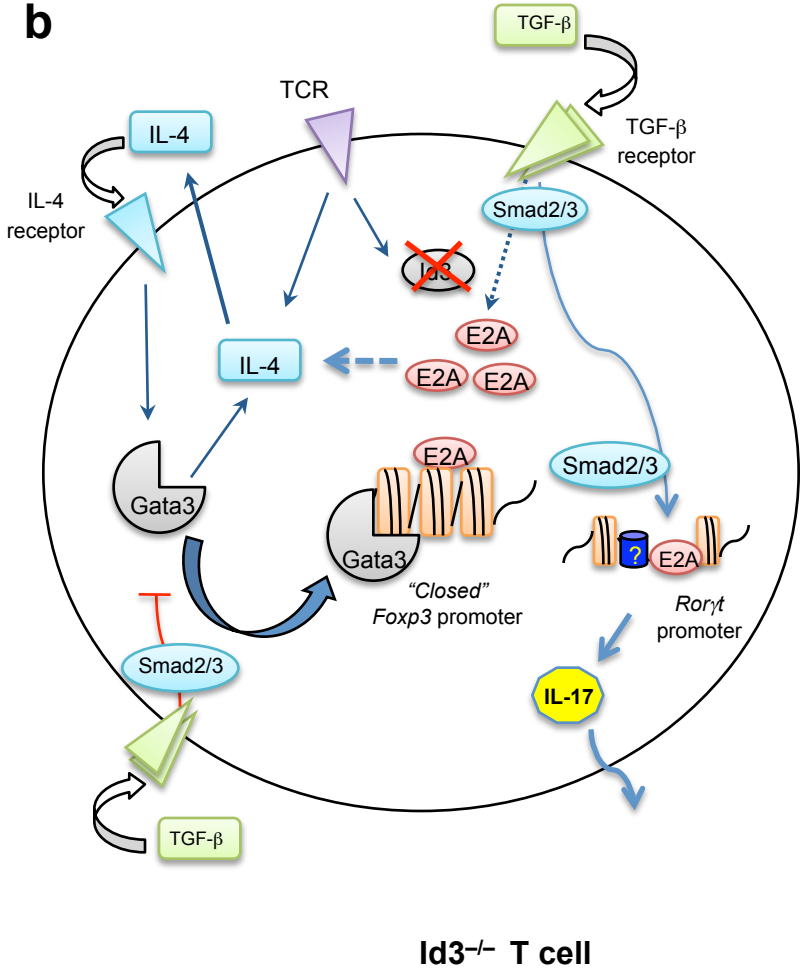
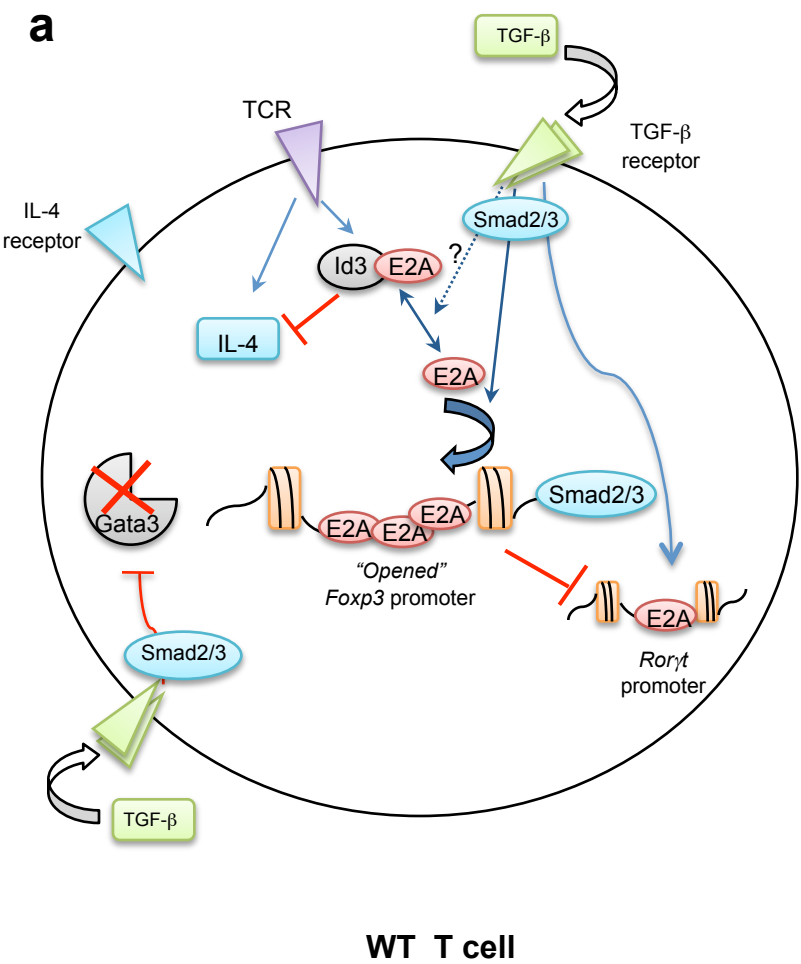
Supplementary Fig. 10. Analysis of lungs of naïve *Id3*^{-/-} mice. a, Total of IgE (ng/ml) in the plasma (left) or BAL (right). (Mean ± SD, n = 2 mice) b, Flow cytometry of intracellular IL-17, IL-13, IFN-γ, and IL-4, and Fop3⁺ T_{reg} cells in BAL in untreated *Id3*^{-/-} and *Id3*^{+/+} mice. Each dot plots represents pooled cells from 2 mice.

Supplementary Figure 11



Supplementary Fig. 11. T_h17, T_h1, T_h2 and Foxp3⁺ T_{reg} cells in spleen and LNs in asthmatic mice. **a-f**, Cells are from the draining LNs (Mediastinal). **a,c,e**, Flow cytometry analysis of intracellular IL-17, IL-13, IFN- γ and IL-4 cytokines or Foxp3⁺ T_{reg} cells in CD4⁺ T cells. Numbers in quadrants indicate percent positive cells. Each plot is of one mouse representative of ten (Id3^{-/-}) and nine (Id3^{+/+}) mice. **b,d,f**, Percent of positive cells (mean $\pm$ s.d.) in the mice in **a,c,e** respectively. **g-i**, Flow cytometry analysis of IL-17⁺ (**g**), IL-13⁺ (**j**) and IFN- γ ⁺ (**i**) positive cells and Foxp3⁺ cells (**h**) in CD4⁺ T cells in spleens in the same mice. *, $P < 0.05$; **, $P < 0.01$; ns, not statistically significant.

Supplementary Figure 12



Supplementary Fig.12. A proposed model for Foxp3 gene transcription. a, WT CD4⁺ T cells. TGF- β together with TCR stimulation induces Foxp3 expression through at least two indispensable and complementary molecular events: by promoting E2A protein binding to the Foxp3 promoter to positively regulate the gene transcription (*enhancer*) and by inhibiting/removing the negative factors bound to the Foxp3 promoter such as GATA-3 (*silencer*).

TGF- β enrichment of E2A binding at the *foxp3* promoter could be mediated by, **1**), a direct yet undetermined TGF- β effect; **2**), an indirect effect by suppression of Id3 expression at 12-24 h after TCR stimulation, which help break down the Id3-E2A complex; and/or **3**), an inhibition of GATA-3 expression.

TGF- β mediated GATA-3 suppression could be, **1**), a direct effect by TGF- β signaling; **2**), an inhibition of IL-4 production and/or **3**), an indirect effect by upregulation of Id3 expression at 1-2 h after TCR stimulation, which helps form the Id3-E2A complex that may inhibit IL-4 production.

The activated *Foxp3* promoter and consequent gene transcription could inhibit *Ror γ t* gene activation. Although TGF- β may induce some degree of *Ror γ t* promoter activity through enriching E2A, the overall balance is toward against the optimal *Ror γ t* gene activation and IL-17 production in the absence of proinflammatory cytokines (e.g. IL-6) in wild type mice.

b. *Id3*^{-/-} CD4⁺ T cells. In the absence of Id3, TCR stimulation causes extremely higher level of GATA-3 expression, and consequently large amounts of GATA-3 bind to the *Foxp3* promoter in CD4⁺ T cells. This high level of GATA-3 is attributed largely to the uncontrolled endogenous IL-4 production in these knockout T cells. IL-4 in turn induces GATA-3. Thus, Id3 is required for the control of IL-4 production and consequent GATA-3 expression. How Id3 deficiency results in uncontrolled IL-4 remains unknown, but it could involve a possible dysregulation of TCR signaling and/or the more Id3-E2A complex derived "free" E2A that could promote IL-4 gene activation. In addition, the upregulated GATA-3 may also enhance more IL-4 production forming a positive feedback loop. As a result, TGF- β signaling fails to inhibit GATA-3 expression to the levels of WT T cells. The extra GATA-3 preoccupies the *foxp3* promoter, which precludes/interferes with TGF- β mediated E2A binding at the *foxp3* promoter. Although *Id3*^{-/-} T cells may have slightly higher levels of basal E2A binding at the *foxp3* promoter due to the reported feature of Id3 as an inhibitor for E2A binding to its target genes, E2A binding to the *Foxp3* promoter cannot be enhanced enough to reach the threshold to initiate *Foxp3* gene transcription in response to TGF- β , and *Foxp3* gene cannot be transcribed (inactive).

Along with defective Foxp3 induction, *Id3*^{-/-} T cells, show optimal *Ror γ t* gene transcription and IL-17 production in response to TGF- β in the absence of exogenous IL-6. Although the exact molecular mechanisms remain to be elucidated, at least two possibilities can be participated. Firstly, the lack of Foxp3 expression could allow *Ror γ t* gene activation in *Id3*^{-/-} T cells; Secondly, TGF- β mediated E2A binding at the *ROR γ t* promoter, together with other yet determined factors (molecules) that normally require IL-6 signal, may directly promote the gene transcription of *Ror γ t* and *Il17* in *Id3*^{-/-} T cells.

Solid arrows (dark blue) indicate positive regulation; red lines indicate negative regulation; Dotted lines indicate no experimental evidence available; question markers indicate to be determined.