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TGF-β induces the expression of Nedd4 family-interacting protein 1 (Ndfip1) to silence IL-4 production during iT_{reg} cell differentiation

Allison M. Beal[1], **Natalia Ramos-Hernández**[2], **Chris R. Riling**[2], **Erin A. Nowelsky**[1], and **Paula M. Oliver**[1,2,3]

[1] The Children's Hospital of Philadelphia, Cell Pathology Division

[2] University of Pennsylvania, School of Medicine

[3] Department of Pathology and Laboratory Medicine

Abstract

Mice deficient for the adaptor Ndfip1 develop inflammation at sites of environmental antigen exposure. We show here that these animals contain fewer inducible regulatory (iT_{reg}) cells. *In vitro*, Ndfip1-deficient T cells express normal levels of the transcription factor Foxp3 during the first 48 hours of iT_{reg} cell differentiation, however this cannot be sustained. Abortive Foxp3 expression is because *Ndfip1*$^{-/-}$ cells produce interleukin 4 (IL-4). We demonstrate that Ndfip1 is transiently unregulated during iT_{reg} cell differentiation in a transforming growth factor-β (TGF-β) dependent manner. Once expressed Ndfip1 promotes Itch-mediated degradation of the transcription factor JunB, thus preventing IL-4 production. Based on these data, we propose that TGF-β signaling induces Ndfip1 expression to silence IL-4 production, thus permitting iT_{reg} cell differentiation.

Once in peripheral lymphoid compartments, T cells are poised to activate the immune system in an effort to destroy invading pathogens. These responses play essential roles in pathogen clearance; however, mechanisms exist to ensure that T cell responses are directed towards harmful pathogens while remaining tolerant to self. Furthermore, T cells must remain tolerant not only to self, but also to non-pathogenic (or harmless) environmental antigens. One mechanism that prevents T cells from directing immune responses towards self or environmental antigens is suppression by T regulatory (T_{reg}) cells [1,2].

Correspondence: Paula M. Oliver, Cell Pathology Division 816F/ARC, Children's Hospital of Philadelphia, 3615 Civic Center Blvd., Philadelphia, PA 19104; paulao@mail.med.upenn.edu.

Author Contributions

A.M.B. designed and performed experiments and wrote the manuscript; N.R.-H., C.R.R. and E.A.N. did experiments and contributed data; P.M.O designed experiments and wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

T_{reg} cells are a specialized subset of T cell that can be generated in one of two ways. Natural T_{reg} cells (nT_{regs}) develop in the thymus and bear T cell receptors (TCR) that primarily recognize self-peptides, whereas iT_{reg} cells, also known as adaptive T_{reg} cells) differentiate from naïve T cell precursors in peripheral lymphoid tissues such as mesenteric lymph nodes that drain the gastrointestinal (GI) tract[1,2]. These two T_{reg} cell subsets differ in their expression of certain genes and their plasticity, but both subsets express a transcription factor known as Foxp3[1,2]. The essential role of Foxp3 in T_{reg} cell development was revealed by genetic mutations leading to the loss of Foxp3 function. The spontaneous Scurfy (*sf*) mutation in mice results in a loss of function mutation in *Foxp3* and death of the mice by 3-4 weeks of age[3], while the loss of Foxp3 function in humans leads to IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome) [4,5]. In these cases, the result of the genetic mutation is loss of Foxp3 expression and a consequent lack of functional T_{reg} cells[6-8].

Foxp3 expression in T_{reg} cells relies on both TGF-β and IL-2 receptor (IL-2R) signaling[9-11]. T_{reg} cells constitutively express CD25, the IL-2Rα component of the high affinity IL-2 receptor complex[12]. Signaling by IL-2 is important for T_{reg} cell differentiation and maintenance[9,10]. In addition to IL-2, both nT_{reg} and iT_{reg} cells need TGF-β to induce Foxp3 expression[9,11]. Stimulation of naïve T cells by TGF-β promotes the induction of Foxp3 expression and iT_{reg} cell differentiation[13-18]. Additionally, TGF-β dampens IL-4 production and thus suppresses T_H2 differentiation[19,20]. Both of these TGF-β mediated outcomes depend on Smad proteins. For example, Smad3 binds to the *Foxp3* gene and activate its transcription[21]. In addition to directly regulating *Foxp3* transcription, Smad activation downstream of TGF-β signaling also induces the expression of TGF-β induced early gene 1 (TIEG1)[22]. TIEG1 is a transcription factor that binds the *Foxp3* gene and induces its transcription[23,24]. Thus, Smad proteins induce *Foxp3* expression by both direct and indirect mechanisms. Following TGF-β signaling, TIEG1 is monoubiquitylated by the E3 ubiquitin ligase known as Itch[23]. This monoubiquitylation allows TIEG1 to induce Foxp3 transcription[23] and is proposed to explain why *Itch*-deficient T cells are defective at differentiating into iT_{reg} cells *in vitro*[23].

We have identified an adaptor protein, known as Ndfip1, that is required for Itch polyubiquitylation of transcription factors of the Jun family[25]. Jun family members can act with NFAT to induce expression of IL-4[26,27]. Thus, in the absence of either Itch or Ndfip1, levels of Jun family members, such as JunB and c-Jun, accumulate and promote the transcription of IL-4 and T_H2 polarization[25,28] leading to T_H2-mediated inflammation in the skin, lung, and GI tract[25,28,29] in these mice. Knowing that Ndfip1 is required for Itch polyubiquitylation of JunB, we hypothesized Ndfip1 may also promote Itch mono-ubiquitylation of TIEG1. Indeed, T cells lacking Ndfip1 were much less likely to become iT_{reg} cells *in vitro* than their wild-type (WT) counterparts. However, we did not see a defect in TIEG1 binding to the Foxp3 promoter in either *Ndfip1*$^{-/-}$ or *Itch*-deficient T cells within the first 48 hours of iT_{reg} cell induction. Rather our results demonstrate that their defect in iT_{reg} cell induction was due to overproduction of IL-4. Our data indicate that Ndfip1 is highly expressed in a TGF-β-dependent manner, peaking after 24 hours of iT_{reg} cell induction, to prevent the accumulation of JunB and IL-4 production. Based on these results,

we propose that Ndfip1 and Itch dampen IL-4 production and thus provide a window of opportunity for iT_{reg} cell lineage commitment.

RESULTS

Mice lacking Ndfip1 have fewer iT_{regs} *in vivo*

Ndfip1-deficient mice develop a severe atopic inflammatory disease, characterized by hyperproliferative T cells that produce T_H2 cytokines and eosinophilia[25,29]. The disease is reminiscent of the T_H2 aspects of the pathology that occurs in Scurfy mice[3] and IPEX patients[4,5], suggesting that *Ndfip1*$^{-/-}$ mice might have defects in Foxp3$^+$ T_{reg} cells. Thus, we sought to determine whether *Ndfip1*$^{-/-}$ mice had a block in the development of Foxp3$^+$ nT_{reg} cells in the thymus. We first analyzed numbers and percentages of CD4$^+$CD25$^+$Foxp3$^+$ cells in the thymi of 4-6 week old mice **(Figure 1a, b and Supplementary Figure 1a)**. This revealed a significant increase in the percentages of Foxp3$^+$ T_{reg} cells in *Ndfip1*$^{-/-}$ thymi. In contrast, no difference was seen when comparing the total numbers of these cells, possibly due to a decrease in the total number of thymocytes harvested from *Ndfip1*$^{-/-}$ mice (data not shown). Inflammation can cause both an increase in thymic Foxp3$^+$ cells[30] as well as thymic involution. To test whether the increased percentages were due to inflammation or increased nT_{reg} cell differentiation, we analyzed 9-day old neonatal mice, when T_{reg} cell numbers are increasing[31], and 2.5-week old mice, prior to histologic evidence of inflammation[29]. Flow cytometric analysis of Foxp3$^+$ T cells in the thymi of 9-day old mice revealed, based on both percentages and total numbers, that nT_{reg} cells in mice lacking Ndfip1 are similar to their Ndfip1$^{+/+}$ littermates (**Supplementary Fig. 1b, c**). Analysis of 2.5-week old Ndfip1-deficient mice showed slightly increased numbers and percentages of these cells **(Supplementary Fig. 1d, e**). From these data, we conclude that the increase in the percentages of nT_{reg} cells in *Ndfip1*$^{-/-}$ mice at 4-6 weeks is consistent with changes caused by inflammation. Supporting this, when we analyzed thymi from mixed bone marrow chimeras, the percentages of WT nT_{reg} cells were increased to amounts comparable with *Ndfip1*$^{-/-}$ cells (**Supplementary Fig 1f**). Moreover, while Foxp3 staining of the lymph nodes and spleens showed similar percentages (data not shown**)**, there was an increase in the absolute numbers of Foxp3$^+$ T_{reg} cells in *Ndfip1*$^{-/-}$ over *Ndfip1*$^{+/+}$ littermates **(Supplementary Fig. 1g**). Taken together, these results demonstrate that there is an age-dependent increase in the percentages of nT_{reg} cells in the thymi of Ndfip1$^{-/-}$ mice due to inflammation. Additionally, since nT_{reg} cells still develop in the absence of Ndfip1, Ndfip1 is not required for the development of Foxp3$^+$ cells in the thymus.

The small bowel is a major site of iT_{reg} cell accumulation[32-34]. Importantly, the small bowel is a major site of inflammation in *Ndfip1*$^{-/-}$ mice[29], suggesting that iT_{reg} cell differentiation in the GI tract may be defective. Flow cytometry analysis of cells isolated from the small bowel of *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ littermates revealed a significant decrease in the percentages and numbers of Foxp3$^+$ T_{reg} cells at this site (**Fig. 1c-e**). Recently, the transcription factor Helios was described as a marker to differentiate thymically derived nT_{reg} cells from peripherally induced iT_{reg} cells[35]. However, the use of Helios as a marker for iT_{reg} cells remains controversial. While Helios expression is not a useful marker for iT_{reg} cells differentiated *in vitro*[36], most *in vivo* models support the original report[37]. Therefore,

we sought to determine whether the decrease in Foxp3$^+$ T_{reg} cell in the small bowel was due to a decrease of the Helios^{lo} iT_{reg} cell population. Helios staining of the cells described in **Fig 1c**, showed a significant decrease in the percentages of Helios^{lo} Foxp3$^+$ population (**Fig. 1f and g**) while the percentages of Helios^{hi} cells were lower but not statistically different from those in *Ndfip1*$^{+/+}$ littermates (**Fig. 1h**). These results suggest that in the absence of Ndfip1 there is a defect in iT_{reg} cell differentiation *in vivo*.

Ndfip1$^{-/-}$ T cells are defective in iT_{reg} cell differentiation

To test whether Ndfip1 is required for iT_{reg} cell differentiation, we next sought to determine whether *Ndfip1*$^{-/-}$ T cells could differentiate into Foxp3$^+$ iT_{reg} cells *in vitro*. To do this, we first needed to eliminate T_H2-effector T cells from our cultures as these cells can inhibit iT_{reg} cell differentiation[19,38,39]. Thus, we sorted naïve (CD25$^-$, CD44^{low}, CD62L^{hi}) CD4$^+$ T cells from 5-7 week old *Ndfip1*$^{-/-}$ and *Ndfip1*$^{+/+}$ littermates. To ensure that IL-4-producing effectors were removed, we tested the cells before and after sorting for IL-4 production by ELISA. Although IL-4 was not detectable in cultures of *Ndfip1*$^{+/+}$ cells, prior to sorting *Ndfip1*$^{-/-}$ cells produced 4.1 ng/ml IL-4 after overnight stimulation. Sorting reduced IL-4 production by *Ndfip1*$^{-/-}$ cells to 0.02 ng/ml. Knowing this, we cultured naïve *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells under iT_{reg} cell differentiation conditions for 5 days and then assessed their expression of CD25 and Foxp3. We found that Ndfip1-deficient T cells were severely impaired in their ability to induce Foxp3 even when given a concentration TGF-β sufficient to induce Foxp3 expression in nearly all of the WT T cells (**Fig. 2a**).

We next assessed whether defective iT_{reg} conversion in *Ndfip1*$^{-/-}$ T cells could also be observed *in vivo*. For this, we adopted a recently described model of Ovalbumin (Ova) - induced iT_{reg} cell conversion of Ova-specific (OTII transgenic) T cells[33]. To generate *Ndfip1*$^{-/-}$ Ova-specific T cells, we crossed *Ndfip1*$^{-/-}$ mice to *Rag1*$^{-/-}$ OTII. As with *Ndfip1*$^{+/+}$*Rag1*$^{-/-}$OTII$^+$ T cells, T cells from *Ndfip1*$^{-/-}$*Rag1*$^{-/-}$ OTII$^+$ mice were naïve and Foxp3$^-$ when isolated and analyzed directly *ex vivo* (**Supplementary Fig 2a,b**). To test iT_{reg} cell conversion *in vivo*, we transferred Ova-specific T cells into congenic recipients and fed animals a low dose of Ovalbumin (Ova) for 5 consecutive days. We found that approximately 13% of transferred WT T cells isolated from the Peyer's Patches, and the mesenteric lymph nodes (mLN) had differentiated into Foxp3+ iT_{reg} cells in response to oral antigen (**Fig. 2b-e**). In contrast, fewer *Ndfip1*$^{-/-}$ T cells became Foxp3$^+$ during this period, resulting in slightly reduced percentages in the small bowel (**Supplementary Fig 2c**) and significantly reduced percentages of *Ndfip1*$^{-/-}$ iT_{reg} cells in the mLN and Peyer's Patches (**Fig. 2b-e**). These results demonstrate that *Ndfip1*$^{-/-}$ T cells are defective at converting into iT_{reg} cells both *in vitro* and *in vivo*.

Impaired conversion by Ndfip1- and Itch-deficient cells

Ndfip1 is an adaptor protein that promotes the Itch-mediated ubiquitylation and consequent degradation of JunB and cJun[25]–transcription factors involved in T_H2 development. Thus both *Itchy* mutant and *Ndfip1*$^{-/-}$ T cells are T_H2 biased. *Itchy* mutant T cells are also impaired in iT_{reg} cell conversion[23]. Considering this, we sought to test whether the defect in iT_{reg} cell differentiation in *Ndfip1*$^{-/-}$ T cells was due to Ndfip1 regulation of Itch function. We thus compared the iT_{reg} cell differentiation capacity of Ndfip1- and Itch-deficient T

cells, using the same sorting and *in vitro* culture conditions described above. Consistent with what was shown previously[23], *Itchy* mutant T cells are impaired at converting into iT_{reg} cells (**Fig. 3 a, b**). We found that *Ndfip1*$^{-/-}$ T cells are even less likely to differentiate into iT_{regs} *in vitro* than Itch-deficient counterparts (**Fig. 3a, b**). Combining data from these experiments, we calculated that *Ndfip1*$^{-/-}$ T cells would need approximately 29 fold more TGF-β for a half-max conversion to Foxp3^{+} iT_{reg} cells than WT cells, whereas *Itchy* mutant T cells would need about 2 fold more TGF-β (**Fig. 3b**). This is unlikely to be due to background differences between the two strains as both have been backcrossed more than 9 generations onto C57BL6. Nonetheless, both *Itchy* mutant and *Ndfip1*$^{-/-}$ T cells are defective in iT_{reg} cell conversion.

It has been suggested that Itch promotes iT_{reg} cell differentiation via monoubiquitylation of TIEG1[23], a transcription factor that promotes Foxp3 expression. Monoubiquitylation of TIEG1 appeared to promote the association of TIEG1 with DNA elements in the Foxp3 locus[23]. TIEG1 binds two sites in the Foxp3 locus, one within the Foxp3 proximal promoter region[24], and the other in an enhancer region known as CNS2[23]. In *Itchy* mutant T cells, TIEG1 did not bind to the CNS2 enhancer region[23], but binding of TIEG1 to the proximal promoter region was not described. However, the CNS2 region was recently shown to be irrelevant for iT_{reg} cell differentiation[40]. Thus, to test whether Ndfip1 regulates TIEG1 binding to Foxp3 sequences, we used chromatin immunoprecipitation (ChIP) to analyze TIEG1 association with the Foxp3 proximal promoter region in T cells lacking either Ndfip1 or Itch. For this analysis, cells were analyzed for binding after both 18 and 42 hours of iT_{reg} cell conversion. This was based on data that TGF-β signaling is particularly important during this period[41]. The location of the primers used to detect Foxp3 DNA bound to TIEG1 is illustrated in **Supplementary Fig. 3a**. TIEG1 associated with the Foxp3 proximal promoter region in WT, *Itchy* mutant and *Ndfip1*$^{-/-}$ T cells (**Supplementary Fig. 3b**). Supporting these results, TIEG1 was also bound to the CNS2 region as determined using previously published primers (data not shown). These results show that impaired iT_{reg} cell differentiation in Ndfip1- and Itch- deficient T cells cannot be explained by a lack of TIEG1 binding to the Foxp3 locus at early time points during iT_{reg} differentiation cell.

Abortive Foxp3 expression in T cells lacking Ndfip1

Based on our results thus far, TIEG1 is bound to the Foxp3 promoter 48 hours after iT_{reg} cell induction. However, these cells do not express Foxp3 after 5 days in these same culture conditions. To resolve this apparent contradiction, we decided to test whether *Ndfip1*$^{-/-}$ T cells express Foxp3 during the time points tested by ChIP, namely two days after stimulation. Using the same protocol described in **Fig. 2a**, we tested Foxp3 expression by flow cytometry analysis at day 2 and again at day 5 during iT_{reg} cell differentiation. Using this approach, we found that on day 2, *Ndfip1*$^{-/-}$ T cells express comparable levels of Foxp3 to those in WT cells (**Fig. 4a, b**). In contrast, but consistent with our previous results, Foxp3 expression is diminished by day 5 in *Ndfip1*$^{-/-}$ T cells, while it continues to increase in WT T cells. In addition, we see a similar trend in Foxp3 expression with Itch-deficient T cells (**Supplementary Fig. 4a**). Recently, it was shown that IL-2 can stabilize Foxp3 expression[42]. Thus, we sought to determine whether increased amounts of IL-2 can rescue the loss of Foxp3 expression in *Ndfip1*$^{-/-}$ T cells that occurred between day 2 and day 5.

However, increasing the concentration of IL-2 in our cultures to 100U/ml did not rescue the defect (**Supplementary Fig. 4b**). Another possible explanation for the decline in Foxp3$^+$ T cells from day 2 to day 5 could be that Foxp3$^+$ T cells lacking Ndfip1 die during this culture. Thus, we assessed the percentage of 7AAD$^+$ cells at day 2 and day 5 of iT$_{reg}$ cell differentiation. While we observed a slight increase in the percentage of *Ndfip1*$^{-/-}$ T cells that are 7AAD$^+$ at day 2, at day 5 the percentages of 7AAD$^+$ cells are reduced compared to controls (**Supplementary Fig. 4c**). These data suggest that other mechanisms must account for the loss of Foxp3$^+$ cells in the *Ndfip1*$^{-/-}$ cultures. Consistent with Foxp3 protein expression, Foxp3 mRNA was induced, albeit to a lesser extent, in cells lacking Ndfip1 (**Supplementary Fig. 5a**). *Ndfip1*$^{-/-}$ T cells showed reduced Foxp3 mRNA levels beginning at day one while Itch-deficient T cells began to show a reduction in mRNA induction after 2 days of iT$_{reg}$ cell induction (**Supplementary Fig. 5b**). These data indicate that Foxp3 expression, and by inference iT$_{reg}$ cell induction, is initiated in Ndfip1- and Itch-deficient T cells, but then is aborted. Knowing that IL-4 can block Foxp3 expression and that *Ndfip1*$^{-/-}$ T cells are prone to produce IL-4 under other culture conditions, we hypothesized that IL-4 production by Ndfip1-deficient T cells could be aborting the iT$_{reg}$ cell differentiation process.

To begin to test this, we first wanted to determine the amount of IL-4 that inhibits iT$_{reg}$ cell differentiation by adding IL-4 into cultures of WT cells undergoing iT$_{reg}$ cell conversion. Using this approach, we found that iT$_{reg}$ conversion was inhibited by small amounts of IL-4. Graphing this on a logarithmic scale, we could quantify the half maximal inhibitory concentration of IL-4 as 190 pg/ml (**Fig. 4c**). Knowing this, we next sought to determine whether *Ndfip1*$^{-/-}$ T cells were producing amounts of IL-4 that would block iT$_{reg}$ cell differentiation. To do this, we measured the amount of IL-4 in cultures of *Ndfip1*$^{-/-}$ and *Ndfip1*$^{+/+}$ cells undergoing iT$_{reg}$ cell differentiation using ELISA. While we saw little IL-4 produced from sorted naïve *Ndfip1*$^{-/-}$ T cells cultured for 24 hours, we found that the amount of IL-4 detected in supernatants increased after 48 hours of stimulation to levels sufficient to inhibit iT$_{reg}$ cell differentiation (**Fig. 4d**). Interestingly, while the amount of IL-4 produced by *Ndfip1*$^{-/-}$ T cells at 24 hours was not different regardless of whether the cells were stimulated in the presence or absence of TGF-β (data not shown), the amount of IL-4 produced by *Ndfip1*$^{-/-}$ T cells at 48 hours of iT$_{reg}$ cell culture was lower than *Ndfip1*$^{-/-}$ T cells stimulated in the absence of TGF-β (**Supplementary Fig. 5c, 5d**). This is consistent with previous data showing TGF-β can attenuate IL-4 production[19,20]. Furthermore, in agreement with the less severe defect in *Itchy* mutant T cells undergoing iT$_{reg}$ cell differentiation (**Fig. 3**), T cells lacking Itch produced much less IL-4 than Ndfip1-deficient counterparts (**Supplementary Fig. 5c, 5e**).

To test whether cells were able to detect IL-4 from their environment, we used flow cytometry to analyze levels of the IL-4 receptor (IL-4R). After day 1 in culture IL-4R expression was only slightly elevated compared to levels on naïve T cells (data not shown). In contrast, by day 2 of iT$_{reg}$ cell differentiation, IL-4R had increased (**Fig. 4e**). This elevated expression of IL-4R at day 2 was seen in cells stimulated in the presence or absence of TGF-β, likely due to IL-2R signaling[43]. This implies that there is a 'window of opportunity' in iT$_{reg}$ cell differentiation during which T cells express IL-4R to sense cues

from their environment. Signals they receive through these receptors likely impact how they proceed in the differentiation process. Furthermore, these data suggest that the impaired iT_{reg} cell differentiation in Ndfip1- and Itch-deficient T cells may be due to IL-4 produced by these cells.

Knowing that *Ndfip1*$^{-/-}$ T cells cultured under iT_{reg} cell differentiation conditions *in vitro* produced high levels of IL-4 and were defective in iT_{reg} cell differentiation we tested IL-4 production in *Ndfip1*$^{-/-}$ T cells using the *in vivo* model described in **Fig. 2a**. Consistent with our *in vitro* results **(Supplementary Fig. 5c, 5d**), Ova-specific *Ndfip1*$^{-/-}$ T cells induced to become iT_{reg} cells *in vivo* also produced IL-4, suggesting that the *in vivo* and *in vitro* iT_{reg} cell defects worked via a similar mechanism (**Supplementary Fig. 6**).

IL-4 blocks iTreg differentiation in *Ndfip1*$^{-/-}$ T cells

To determine whether IL-4 was inhibiting iT_{reg} cell differentiation in Ndfip1- and Itch-deficient T cells, we performed iT_{reg} cell conversion assays in the presence or absence of antibodies that block the binding of IL-4 to its receptor. While addition of anti-IL-4 had no impact on WT cells (data not shown), when IL-4 blocking antibodies were added to the Ndfip1- and Itch-deficient T cells, iT_{reg} cell differentiation was restored to that seen in the WT (**Fig. 5a, b**). Thus, production of IL-4 is sufficient to explain why both *Ndfip1*$^{-/-}$ and *Itchy* mutant T cells are poor at differentiating into iT_{reg} cells *in vitro*. Additionally, if the *Ndfip1*$^{-/-}$ T cells were converted into iT_{reg} cells in the presence of anti-IL-4 blocking antibodies, the cells could suppress just as well as WT iT_{reg} cells (**Supplementary Fig. 7**).

Furthermore, it is unlikely that other T_H2 cytokines, such as IL-5, that are also detectable in the supernatants of Ndfip1- and Itch-deficient T cells undergoing iT_{reg} cell differentiation (data not shown) lead to impaired iT_{reg} cell differentiation since the addition of IL-5 to WT T cells undergoing iT_{reg} cell conversion had no effect on their ability to become Foxp3^{+} (**Supplementary Fig. 8**).

To confirm that IL-4 production by *Ndfip1*$^{-/-}$ T cells was inhibiting iT_{reg} cell differentiation, we generated mice lacking both Ndfip1 and IL-4. As shown (**Fig. 5c, d**), iT_{reg} cell differentiation in T cells from *Ndfip1*$^{-/-}$*Il4*$^{-/-}$ mice was similar to T cells from *Ndfip1*$^{+/+}$*Il4*$^{-/-}$ littermates. These data show that IL-4 produced by Ndfip1- or Itch-deficient T cells prevents iT_{reg} cell differentiation, since blocking either IL-4 production or the binding of IL-4 to its receptor restores iT_{reg} cell differentiation in these cells *in vitro*.

As described above, IL-4 production increases in *Ndfip1*$^{-/-}$ T cells during iT_{reg} cell differentiation between 24-48 hours at a time when the cells are upregulating IL-4R expression. This suggests that there is an early ‘window’ during Foxp3 induction following the initial stimulation when cells are sensing their environment and that IL-4 signaling during this time would lead to abortive iT_{reg} cell differentiation. To test whether IL-4 was indeed mediating the abrogation of Foxp3 expression during this ‘window’, we repeated iT_{reg} cell conversion assays, blocking IL-4 signaling at various times following the initial stimulation. The delayed addition of IL-4 blocking antibodies after 24 hours restored iT_{reg} cell differentiation in *Ndfip1*$^{-/-}$ T cells (**Fig. 5e**) and this did not occur if the antibodies were added after 48 or 72 hours. These data show that IL-4 can abrogate Foxp3 expression during

this early stage of iT_{reg} cell differentiation and that there is a 'window' between 24-48 hours following initial stimulation where Foxp3 expression is unable to be rescued by neutralization of IL-4.

IL-4 produced by the Ndfip1-deficient T cells could act preferentially on the cells producing the cytokine, and/or it could act on neighboring cells, preventing their expression of Foxp3. To test which of these occurred in iT_{reg} cell differentiation cultures, we mixed *Ndfip1*$^{-/-}$ and congenic WT cells together at various ratios prior to initiating iT_{reg} cell differentiation. Using these mixed cultures, we found that IL-4 produced by the *Ndfip1*$^{-/-}$ T cells was able to inhibit iT_{reg} cell differentiation of WT cells at all ratios tested (**Fig. 5f**). Thus, IL-4 can prevent iT_{reg} cell differentiation in trans. However, *Ndfip1*$^{-/-}$ T cells were more defective at iT_{reg} cell differentiation than their WT counterparts, particularly when co-cultured at low ratios (25:1) (**Fig. 5f**). This indicates that while IL-4 can act in both an autocrine and paracrine manner, it has a more profound effect on *Ndfip1*$^{-/-}$ cells. This implies that the there is something intrinsic to T cells lacking Ndfip1 that makes them more sensitive to IL-4 than their WT counterparts. This could be due to enhanced IL-4 receptor signaling in T cells lacking Ndfip1. To test this, we added IL-4, at the half maximal inhibitory concentration (based on **Fig. 4c**) to *Ndfip1*$^{+/+}$*Il4*$^{-/-}$ and *Ndfip1*$^{-/-}$*Il4*$^{-/-}$ T cells undergoing iT_{reg} cell differentiation (to eliminate the confounding effects of IL-4 production by the cells). As predicted, we saw approximately 50% inhibition of iT_{reg} differentiation in *Il4*$^{-/-}$ cells and a similar level of inhibition in *Ndfip1*$^{-/-}$*Il4*$^{-/-}$ cells (**Fig. 5g**). Interestingly, the *Ndfip1*$^{-/-}$ cells showed a modest (but not statistically significant) increase in sensitivity to IL-4. This may be due to the slight increase in IL-4R levels we observe in *Ndfip1*$^{-/-}$ T cells (data not shown). This might also explain why the *Ndfip1*$^{-/-}$ cells were more inhibited than their WT counterparts in the co-culture experiments. Nonetheless, this appears to play a minor role in the defective iT_{reg} cell differentiation as this difference is not as profound as their difference in IL-4 production.

Normal frequency of iT_{reg} cells in *Ndfip1*$^{-/-}$ *Il4*$^{-/-}$

To test whether IL-4 production accounts for the reduced numbers of iT_{reg} cells in *Ndfip1*$^{-/-}$ animals, we analyzed the percentages of Foxp3^{+}Helios^{lo} cells in the small bowel from mice lacking both Ndfip1 and IL-4. Whereas mice lacking Ndfip1 have reduced percentages of iT_{reg} cells (Foxp3^{+}Helios^{lo}) in the small bowel, mice lacking both Ndfip1 and IL-4 showed percentages comparable to WT and IL-4-deficient mice (**Fig. 6a**). Thus, similar to what we observed with iT_{reg} cell differentiation *in vitro*, iT_{reg} cell differentiation in *Ndfip1*$^{-/-}$ mice *in vivo* appears to be due to overproduction of IL-4.

Mice lacking Ndfip1 have increased percentages of activated T cells in their peripheral lymphoid organs[25, 29]. These activated T cells could be the direct or indirect consequence of aborted iT_{reg} cell differentiation *in vivo*. Thus, having shown that iT_{reg} cell induction was restored in mice lacking both Ndfip1 and IL-4, we next wanted to determine whether the percentages of activated T cells were reduced in mice lacking both Ndfip1 and IL-4. While mice lacking Ndfip1 had twice as many CD44^{hi} cells as *Ndfip1*$^{+/+}$ controls, the percentages of these cells in mice lacking both Ndfip1 and IL-4 were comparable to controls (**Fig. 6b**). Supporting this, fewer CD4^{+} T cells were found in the small bowel of mice lacking both

Ndfip1 and IL-4 than in mice lacking only Ndfip1 (**Fig. 6c**). Furthermore, mice lacking both Ndfip1 and IL-4 had reduced GI pathology, as evidenced by reduced eosinophil infiltration, compared to mice lacking only Ndfip1 (**Fig. 6d-i**). Additionally, mice lacking both Ndfip1 and IL-4 have longer life-spans than their *Ndfip1*$^{-/-}$ counterparts and have reduced inflammation in their lungs, as evidenced by decreased infiltrating leukocytes (manuscript in preparation). This would be expected, since eosinophil infiltration is likely the result of T_H2 cytokine production in this model[29]. Nonetheless, these *in vivo* data suggest that when *Ndfip1*$^{-/-}$ T cells cannot make IL-4, more T cells differentiate into iT_{reg} cells, fewer T cells have an activated phenotype, and GI pathology is reduced.

Ndfip1 limits JunB levels during iT_{reg} cell commitment

Taken together, our data show that as WT cells begin to differentiate into iT_{reg}, cells they upregulate both Foxp3 and their IL-4R. This implies that during this time they are acutely sensitive to cues from their environment, such as the presence of IL-4. It seems likely that Ndfip1 is acting at this stage since *Ndfip1*$^{-/-}$ T cells express Foxp3 early during iT_{reg} cell differentiation and then fail to fully differentiate into iT_{reg} cells. Thus, we analyzed the expression of Ndfip1 at different time points during iT_{reg} cell differentiation. To do this, we cultured naïve T cells under iT_{reg} cell differentiation conditions and extracted mRNA on days 1, 2 and 3, and expression of Ndfip1 was determined using Quantitative real-time PCR (qRT-PCR). Ndfip1 mRNA expression peaked in cells cultured 1 day in the presence of TGF-β (**Fig. 7a**). Expression of Ndfip1 peaks at approximately the time when cells are expressing both Foxp3 and IL-4R, and committing to the iT_{reg} cell lineage. This may explain why Foxp3 expression fails between day 2 and day 5 in *Ndfip1*$^{-/-}$ T cells. It is worth noting that the induction of Ndfip1 expression was TGF-β-dependent since stimulation without TGF-β showed lower levels of Ndfip1 expression (data not shown). Taken together these data indicate that, in the first 24-48 hours of iT_{reg} cell differentiation, T cells upregulate Ndfip1 in an effort to dampen IL-4 and allow iT_{reg} cell differentiation.

To identify a transcription factor that could account for the increased IL-4 production in Ndfip1- and Itch-deficient T cells we assessed the expression levels of factors that are known to promote early IL-4 production, namely Gata3 and Jun family members. Whereas Gata3 mRNA expression is increased at day 5 after iT_{reg} cell induction, Gata3 expression is comparable to *Ndfip1*$^{+/+}$ T cells at day 2 in both Ndfip1- and Itch-deficient T cells (**Supplementary Fig. 9a**). We next looked at JunB, c-Jun, and JunD levels during iT_{reg} cell differentiation (**Fig. 7b-7e**). We found a considerable increase in JunB protein in *Ndfip1*$^{-/-}$ T cells (**Fig. 7b, c**). *Itchy* mutant T cells also show elevated JunB but Ndfip1-deficient T cells had higher JunB than both Itch-deficient and *Ndfip1*$^{+/+}$ T cells (**Fig. 7b, c**). This is consistent with the increased production of IL-4 by *Ndfip1*$^{-/-}$ T cells during iT_{reg} cell conversion (**Supplementary Fig. 5c, e**). Elevated JunB protein expression in *Ndfip1*$^{-/-}$ T cells was evident as early as day 2 following iT_{reg} cell induction and increased further over that seen in the control cells at day 3 (**Supplementary Fig. 9b**). In *Ndfip1*$^{+/+}$ cells, JunB protein increased from day 1 to day 2 but then stayed relatively constant at day 3 (**Supplementary Fig. 9c**) when Ndfip1-deficient T cells had increased amounts (**Supplementary Fig. 9b**). Taken together, these data show that the elevated amounts of IL-4 produced by Ndfip1- and

Itch-deficient T cells during iT_{reg} cell differentiation is likely due to the accumulation of JunB in these cells.

To determine whether the elevated amounts of JunB in *Ndfip1*$^{-/-}$ T cells could be a cause or consequence of the IL-4 production during iT_{reg} cell differentiation, we next tested if there was an increase in JunB levels in *Ndfip1*$^{-/-}$ T cells in the presence or absence of anti-IL-4. We found that JunB protein was still elevated in *Ndfip1*$^{-/-}$ T cells undergoing iT_{reg} cell differentiation in the presence of anti-IL-4, suggesting elevated JunB was not a consequence of IL-4R signaling (**Supplementary Fig. 9d**). Thus, we next tested whether JunB might be the cause of IL-4 production. Supporting that JunB causes IL-4 production, we detected JunB binding to the IL-4 promoter in *Ndfip1*$^{-/-}$ T cells (**Fig. 7f**). However, we did not observe any binding of JunD to this region (**Supplementary Fig. 9e**).

Knowing that JunB could bind the IL-4 promoter and was not downstream of IL-4 production, we next sought to determine the signals leading to JunB expression in T cells undergoing iT_{reg} cell differentiation. To do this, we cultured cells under iT_{reg} cell conditions in the presence or absence of TGF-β or after removal of TCR stimulation. TGF-β induced an increase in JunB protein in both *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells (**Fig. 7g**). Thus, as has been seen previously in other cell types, TGF-β signaling can induce JunB expression. Furthermore, consistent with previously published data[44], we found that TCR signals are necessary for JunB expression (**Fig. 7h**). When we cultured *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells for 24 hours under normal iT_{reg} cell conditions and then removed TCR signaling for the duration of the culture, JunB protein was undetectable. Having found a scenario under which JunB was not expressed, we next tested whether TCR withdrawal affected iT_{reg} cell differentiation in *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells. We found that the withdrawal of TCR signals during iT_{reg} cell differentiation had no discernible impact on *Ndfip1*$^{+/+}$ T cells. Importantly, TCR signal withdrawal resulted in a loss of IL-4 production (data not shown) and restored iT_{reg} cell differentiation in *Ndfip1*$^{-/-}$ cells (**Supplementary Fig. 9f**). Thus, iT_{reg} cell differentiation can be rescued by removal of initial TCR signals concomitant with loss of JunB expression. Taken together these results show that overexpression of JunB is not a consequence of IL-4R signaling and that JunB is likely an active participant leading to IL-4 overproduction in *Ndfip1*$^{-/-}$ T cells during iT_{reg} cell differentiation.

Previous data has shown that JunB levels are increased in T_H2 cells lacking Ndfip1 and that this was due to impaired degradation of JunB[25]. To test whether the elevated levels of JunB were the result of impaired degradation or increased production, we assessed JunB mRNA levels and protein stability during iT_{reg} cell differentiation. JunB mRNA expression in *Ndfip1*$^{-/-}$ T cells was comparable to that in WT T cells (**Supplementary Fig. 10a**). In contrast, and consistent with it's known role as an adaptor for E3 ubiquitin ligases, *Ndfip1*$^{-/-}$ T cells showed impaired degradation of JunB (**Supplementary Fig. 10b**). Thus, the increased levels of JunB in T cells lacking Ndfip1 were a result of increased stability of JunB. Given these results, we propose a model in which TGF-β induces expression of Ndfip1 to dampen IL-4 production during iT_{reg} cell differentiation (**Supplementary Fig. 11**).

DISCUSSION

iT_{reg} cells, generated from naïve T cell precursors in peripheral lymphoid compartments, can attenuate immune responses to either self or environmental antigens[1,2]. These regulatory T cells are characterized by expression of Foxp3[2]. However, expression of Foxp3 is not sufficient to define a regulatory T cell, as activated T cells can transiently upregulate Foxp3[45] along with transcription factors that dictate other T cell fates. This has lead to the proposal that transcription factors compete in the early differentiation phase of T cells, potentially integrating environmental signals that ultimately allow cells to commit towards a particular T cell lineage. Here we report that Ndfip1 helps to regulate this process by dampening T_H2 cytokine production during the decision making phase of iT_{reg} cell differentiation.

In contrast to the apparent defect in iT_{reg} cell differentiation in *Ndfip1*$^{-/-}$ mice, we find elevated percentages of nT_{reg} cells in the thymi of *Ndfip1*$^{-/-}$ mice, thus nT_{reg} cells do develop in the absence of Ndfip1. This increase in nT_{reg} cells was likely a result of the inflammatory cytokines present in these mice. However, a more precise analysis of nT_{reg} cells in mice 3 to 9 days old would be required to entirely rule out a role for Ndfip1 in nT_{reg} cell development. TGF-β is important for both nT_{reg} cell differentiation and iT_{reg} cell differentiation [9,13,16,17,30]. That we see defects only during iT_{reg} cell differentiation could reflect a reduced capacity of developing thymocytes to produce or respond to IL-4 compared to peripheral naïve T cells. Also, whether IL-4 can affect nT_{reg} cell development in *Ndfip1*$^{-/-}$ mice is not clear. We do not detect significant amounts of IL-4 in the serum of *Ndfip1*$^{-/-}$ even when they present with overt signs of inflammation when IL-4 production is detectable in splenocytes (unpublished observation). Thus, it will be important to determine whether IL-4 is produced locally by *Ndfip1*$^{-/-}$ thymocytes and whether developing nT_{reg} cells respond to IL-4. While it is clear that there are circumstances under which CD4 -single positive (SP) cells in the thymus can make IL-4, it is possible that IL-4 is not produced in the thymi of young *Ndfip1*$^{-/-}$ mice since the majority of CD4 SP thymocytes in neonatal mice are not functionally competent and do not respond the same as peripheral T cells[46]. These will be the focus of future studies.

Preventing IL-4 production is a particular challenge for cells undergoing iT_{reg} cell differentiation. While iT_{reg} cells are dependent on IL-2R signaling[9,10], these signals are known to promote both IL-4 production and IL-4R expression[47]. Thus, as iT_{reg} cells differentiate, they receive IL-2R signals, increase expression of their IL-4R[43], and inhibit their own IL-4 production to seek cues from their environment. If IL-4 production is not silenced during this period, it could prevent iT_{reg} cell differentiation in both an autocrine and paracrine manner. This could result in enhanced and/or prolonged immune responses with damaging consequences.

Although it is known that iT_{reg} cell differentiation is remarkably sensitive to effector cytokines such as IL-4[19,38,39], the mechanisms that prevent IL-4 production by T cells during iT_{reg} cell differentiation are only partially understood. For example, it is known that TGF-β receptor signaling dampens IL-4 production in WT T cells[19]. In part, this is because TGF-β receptor signaling reduces Gata3 expression[20]. We show here that in the absence of

Ndfip1, T cells produce IL-4 at levels that inhibit their own iT_{reg} cell differentiation, and iT_{reg} cell differentiation of other T cells in their vicinity.

Paradoxically, JunB was increased in a TGF-β-dependent manner. While this was surprising, data in other non-immune cell types has shown that TGF-β can induce JunB via a Smad-dependent pathway[48]. Why and how TGF-β induces JunB expression in T cells is not clear. However, JunB expression in WT cells plateaus at day 2 during iT_{reg} cell differentiation. In contrast, in *Ndfip1*$^{-/-}$ T cells, JunB expression continues to increase and JunB was bound to the IL-4 promoter in *Ndfip1*$^{-/-}$ T cells undergoing iT_{reg} cell differentiation, demonstrating a causal role for JunB in IL-4 production. Supporting this, in one scenario under which JunB is not expressed in *Ndfip1*$^{-/-}$ T cells, IL-4 is not produced and iT_{reg} cell differentiation is restored.

TGF-β also induces increased expression of Ndfip1, an adaptor protein that promotes the ubiquitylation and degradation of Jun-family proteins by the E3 ligase Itch. Ndfip1 is particularly important in the first 24-48 hours of iT_{reg} cell differentiation, as cells are increasing expression of Foxp3 and IL-4R. In the absence of Ndfip1, T cells initially increase Foxp3, but also aberrantly express IL-4. This ultimately aborts the iT_{reg} cell differentiation process in these cells. Given these results, we suggest that Ndfip1 promotes Itch ubiquitylation and degradation of JunB to prevent IL-4 production and allow iT_{reg} cell differentiation. While these data support a role for Ndfip1 regulation of Itch, it is also clear that Ndfip1 also regulates iT_{reg} cell differentiation via an Itch-independent mechanism.

Interestingly, it seems that Ndfip1 is not needed once Foxp3^{+} T_{reg} cells are fully differentiated since cells that had already committed to the T_{reg} lineage have lower Ndfip1 expression than their naïve T cell counterparts (data not shown). Supporting this, *Ndfip1*$^{-/-}$ cells that differentiate into iT_{reg} cells (in the presence of anti-IL-4) suppress proliferation as well as WT iT_{reg} cells.

Here we define an early ‘window’ where Ndfip1 is expressed to dampen IL-4 production during iT_{reg} cell differentiation. The kinetics of Ndfip1 expression and inhibition of iT_{reg} cell differentiation by IL-4 are consistent with data showing that optimal iT_{reg} cell conversion occurs when TGF-β was added within 1-2 days[41]. Thus, environmental cues received by the T cell early during this time can alter the ability of these cells to differentiate into Foxp3 expressing T_{reg} cells. Supporting this, it is known that Foxp3 (induced by TGF-β receptor signaling) can bind directly to Gata3 (induced by IL-4R signaling) to prevent the induction of T_H2 cytokines[19]. On the other hand, if Gata3 levels increase (due to IL-4R signaling) and outcompete Foxp3, iT_{reg} cell differentiation is prevented [38,39]. Based on the data we have presented, we propose that Ndfip1 dampens IL-4 production during TGF-β stimulation to provide a ‘window of opportunity’ for iT_{reg} cell differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Methods

Mice

CD45.1^{+} (C57BL6.SJL-Ptprc^{a} Pepc^{b}/BoyJ), *Il4*$^{-/-}$ (B6.129P2-Il4^{tm1Cgn}/J), OT-II (B6.Cg-Tg (TcraTcrb) 425Cbn/J) and *Rag1*$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}/J) mice were purchased from the Jackson Laboratory. T cell transgenic OT-II Rag1$^{-/-}$ mice were obtained by crossing OT-II transgenic mice with Rag1$^{-/-}$ mice. Ndfip1$^{-/-}$ and *Itchy* mutant (also referred to as Itch-deficient) mice were previously described[25, 28] and have been backcrossed to C57BL/6 mice for more than 9 generations. *Ndfip1*$^{-/-}$ mice were bred from heterozygous parents since Ndfip1$^{-/-}$ mice die prematurely. *Ndfip1*$^{+/+}$ littermates were used as controls. *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ were 4-8 weeks of age unless otherwise noted. For data presented in figure 7e-h, T cells lacking Ndfip1 were derived from both *Ndfip1*$^{-/-}$ mice and *Cd4*-Cre *Ndfip1*$^{fl/fl}$ mice. T cells from these Cd4-Cre *Ndfip1*$^{fl/fl}$ mice lack Ndfip1 (data not shown) and respond similarly to T cells from *Ndfip1*$^{-/-}$ mice (for these data T cells from at least one *Ndfip1*$^{-/-}$ mouse were used for comparison). Cd4-Cre mice will be described elsewhere (manuscript in preparation). All mice used were maintained in a barrier facility at the Children's Hospital of Philadelphia and all animal experiments were approved and in accordance with guidelines established by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee.

Isolation of cells from the small bowel and thymus

The small bowel was dissected and the Peyer's Patches were excised. The lumen of the small bowel was cleaned by flushing with PBS. A section of the small bowel was then minced in DMEM media with 0.9mg/mL of collagenase A (Sigma), 0.8mg/mL collagenase 1A (Sigma), and 20μg/ml of DNase I (Sigma). Minced tissues were then incubated for 1hr at room temperature, with end over end mixing. The resulting cell suspension was passed through 100μm and then 40μm filters after which FBS was added to a final concentration of 10%. Thymi were harvested, and passed through 70μm filters to obtain cell suspensions.

Antibodies and Flow cytometry

Antibodies used for flow cytometry analysis include anti-CD4 (GK1.5, Biolegend) and anti-CD8 (53-6.7, Biolegend), anti-CD45.2 (104, Biolegend), anti-CD62L (MEL-14, eBioscience), anti-CD44 (IM7, Biolegend) anti-CD25 (PC61.5, eBioscience), anti-IL-4Rα/CD124 (mIL4R-M1, BD Biosciences), anti-Foxp3 (FJK-16s, eBioscience), or anti-Helios (22F6, Biolegend). Additionally, some experiments used streptavidin Alexa Fluor 647 or 488 conjugates (Invitrogen). Data was collected using a FACSCalibur (BD Biosciences) and analyzed by FlowJo (TreeStar).

In vitro iT_{reg} cell cultures

Spleens and lymph node cells were sorted for naïve T cells ($CD4^+$ $CD25^-CD62L^{hi}$ $CD44^{lo}$) using a FACS Aria (BD biosciences) or MoFlo (Beckman Coulter). 0.5 -1×10^6 naïve T cells were stimulated with 5 μg/ml plate-bound anti-CD3 (145-2C11, BD biosciences) and anti-CD28 (37.51, BD biosciences) in complete media (DMEM, 10% FCS, 50 U/ml IL-2) with or without TGF-β (PeproTech) at the indicated concentrations. We noticed that *Ndfip1*$^{+/+}$ cells stimulated without the addition of exogenous TGF-β displayed a small percentage of $Foxp3^+$ cells on day 5 due to the presence of TGF-β in the media, therefore in some experiments we added anti-TGF-β antibodies (1D11, R and D systems). Where indicated, either 20μg/ml anti-IL-4 antibodies (11B11, Biolegend) were added to block IL-4 or exogenous murine IL-4 (PeproTech) was added at the indicated concentrations to the indicated cells. T cells were incubated at 37°C 5% CO_2 and then analyzed for Foxp3 expression on day 2 and/or 5. While we noted variability in the percent converted cells even among WT mice from experiment to experiment, the trend was always the same between the samples from the various mouse strains.

In vivo iT_{reg} cell model

2×10^6 OT-II T cells from either *Ndfip1*$^{-/-}$*Rag1*$^{-/-}$$OTII^+$ or *Ndfip1*$^{+/+}$*Rag*$^{-/-}$$OTII^+$ mice were enriched by either sorting for $Thy1.2^+$ cells or depleting MHC class II^+ cells and then transferred intravenously into $CD45.1^+$ mice. Recipient mice were fed 1.5% OVA (grade III, Sigma) in the drinking water for 5 consecutive days[33] after which the mesenteric lymph nodes, Peyer's Patches and small bowel were harvested and processed.

ELISA

ELISA was performed using supernatants from cultured cells as described in the supplementary methods.

RNA isolation and Q PCR

RNA was isolated and analyzed by Q PCR as described in the supplementary methods. Ndfip1 primer and probe sequences are as follows: forward-TCCACCATACAGCAGCATCACT; reverse-AGAGTGCAGCATATTT; and probe-TTTGGAAATCCAGATTCATCTTTG.

Immunoblot

T cells were stimulated under iT_{reg} cell conditions as described above or under TCR withdrawal conditions (described in supplementary methods). Cells were then harvested after 2-3 days following the initial stimulation, counted and washed with cold Dulbecco's Phosphate-Buffered Saline. Harvested cells were lysed and prepared for SDS-PAGE as noted in the supplementary methods. For blotting, PVDF membranes were blocked for 1 hour at room temperature with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) and then immunoblotted with anti-JunB (mouse monoclonal antibody C-11, Santa Cruz), anti-c-Jun (rabbit monoclonal antibody, 60A8, Cell Signaling), anti-JunD (rabbit polyclonal

antibodies, Santa Cruz), or anti-GAPDH (mouse monoclonal antibody 6C5, Millipore). Secondary antibodies were either Alexa Fluor 680 or IRdye 800 conjugated. Immunoblots were imaged using the Odyssey Imager system (LI-COR Biosciences, Lincoln, NE).

ChIP for JunB binding to the *Il4* promoter

4.5×10^6 naïve T cells were stimulated as described for *in vitro* iT_{reg} cell cultures in the presence of 1ng/ml TGF-β for the indicated times. Cells were then harvested and fixed as described in the supplementary materials. IP was performed with anti-JunB (mouse monoclonal antibody C-11, Santa Cruz) antibodies and protein-G beads blocked with sheared salmon sperm DNA (Millipore). Primers for the *Il4* promoter were forward, 5'-GAGCCAGTGGCAACCCTACGCTGATAAG-3' and reverse, 5'-CTGCCAGCATTGCATTGTTAGC-3' [49] and surround the AP-1 site described in Li et al.[50]

Statistics

All statistical analysis was performed by student T-tests. A P value ≤ 0.05 was the threshold used to determine statistical significance. Error bars represent standard deviation of the mean unless otherwise noted.

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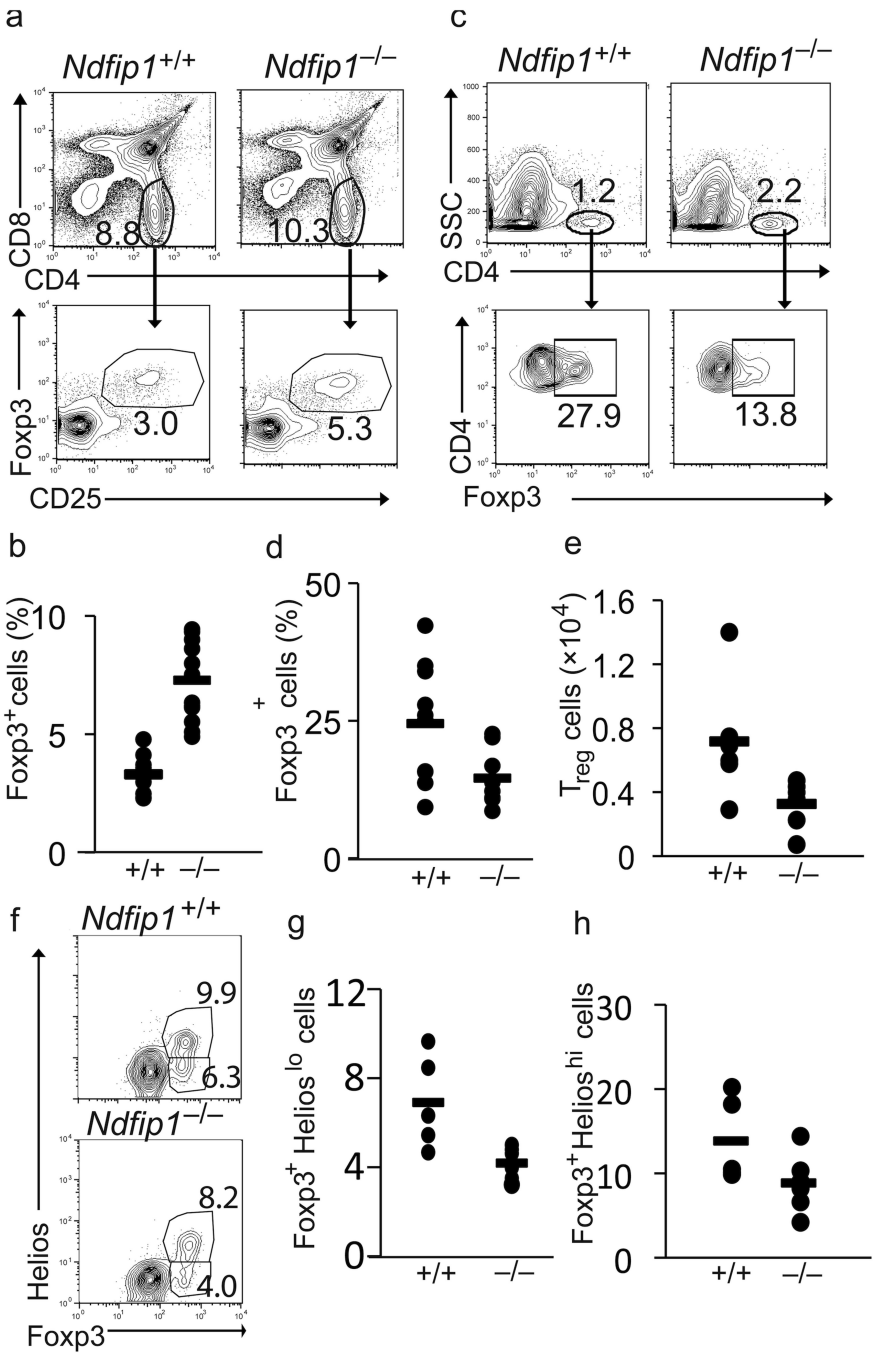

Figure 1. Reduced frequency of iT$_{reg}$ cells in *Ndfip1*$^{-/-}$ mice

(a-e) Thymus and small bowel samples from 4 to 8 week old *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ mice were analyzed for percentages of Foxp3^{+} regulatory T cells by flow cytometry. Representative contour and dot plots of T$_{reg}$ cells from the thymi and small bowels are depicted in **a and c,** respectively**. (b)** Percentages of CD25^{+} Foxp3^{+} T$_{reg}$ cells among the CD4 SP population from the thymus and **(d)** the percentages of Foxp3^{+} cells among the CD4^{+} population in the small bowel. n≥10 **(b and d)**. **(e)** The number of CD4^{+} Foxp3^{+} T cells in the small bowels of *Ndfip1*$^{-/-}$ or *Ndfip1*$^{+/+}$ mice. n=6. **(f-h)** The small bowels from 4

to 8 week old *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ mice were analyzed for the percentage of iT_{reg} cells (Foxp3^{+}Helios^{lo}) by flow cytometry. Representative plots are depicted in **f** and the percentage of Foxp3^{+} cells in individual mice is plotted in **g** and **h**. *P<0.05 **P<0.01 (two-tailed student T-test). Each dot represents one mouse, n≥5. The bar indicates the mean.

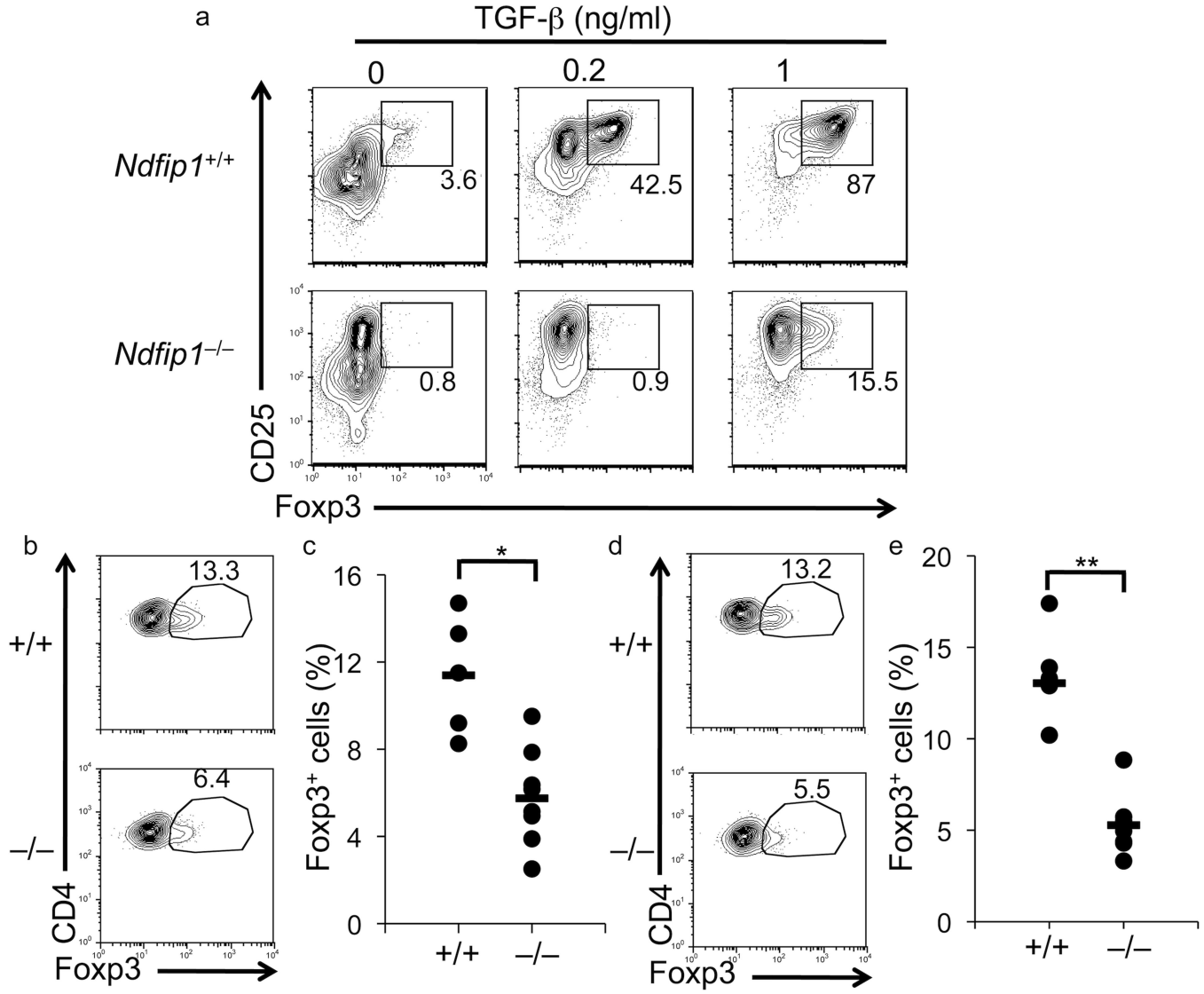

Figure 2. Impaired iT_{reg} cell conversion in naïve T cells lacking Ndfip1

(a) Naïve ($CD25^{-}CD62L^{hi}CD44^{lo}$) $CD4^{+}$ T cells were cultured for 5 days with IL-2, anti-CD3, anti-CD28 and varying concentrations of TGF-β. Cells were then analyzed by flow cytometry. Contour and dot plots of *Ndfip1*$^{-/-}$ and *Ndfip1*$^{+/+}$ (littermate control) T cells are representative of 6 independent experiments. **(b-e)** 2×10^{6} $CD4^{+}$ T cells ($CD45.2^{+}$) from either *Ndfip1*$^{-/-}$*Rag1*$^{-/-}$OTII^{+} or *Ndfip1*$^{+/+}$*Rag1*$^{-/-}$OTII^{+} mice were transferred intravenously into WT $CD45.1^{+}$ recipient mice. Recipient mice were then fed Ova for 5 days. Analyses of iT_{reg} cells in the mLN **(b and c)** and Peyer's Patches **(d and e)** was analyzed by flow cytometry. Representative dot plots are shown in **b** and **d**, while percentages of $CD25^{+}Foxp3^{+}$ cells among the $CD45.2^{+}$ T cells are indicated **(c and e)**. *Ndfip1*$^{+/+}$ n≥5, *Ndfip1*$^{-/-}$ n=8. Data are representative of two experiments. *P=0.002 and **P=0.00002 (two-tailed student T-test).

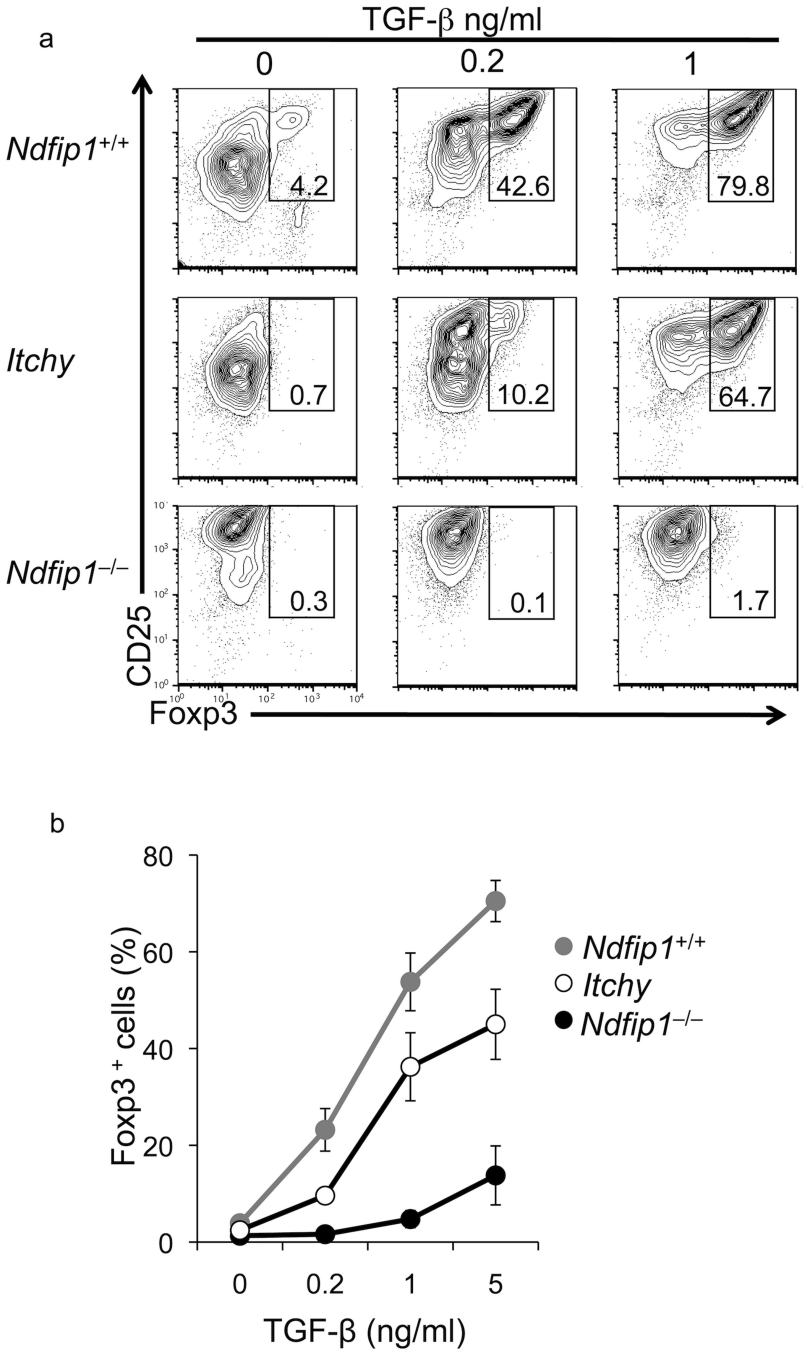

Figure 3. Both Ndfip1$^{-/-}$ and *Itchy* mutant T cells are defective in iT$_{reg}$ cell conversion

(a) Naïve T cells were stimulated with various concentrations of TGF-β as described in Figure 2. After incubation, cells were analyzed for iT$_{reg}$ cell conversion by flow cytometry. Analyses of WT, *Ndfip1*$^{-/-}$, and *Itchy* mutant T cells are shown. Data are representative of at least 6 independent experiments. (**b**) The mean frequency of Foxp3^{+} cells in the cultures of WT (gray circles), *Itchy* mutant (open circles), and *Ndfip1*$^{-/-}$ (black circles) cells is plotted over the indicated concentrations of TGF-β. (mean + SEM from 8-12 mice).

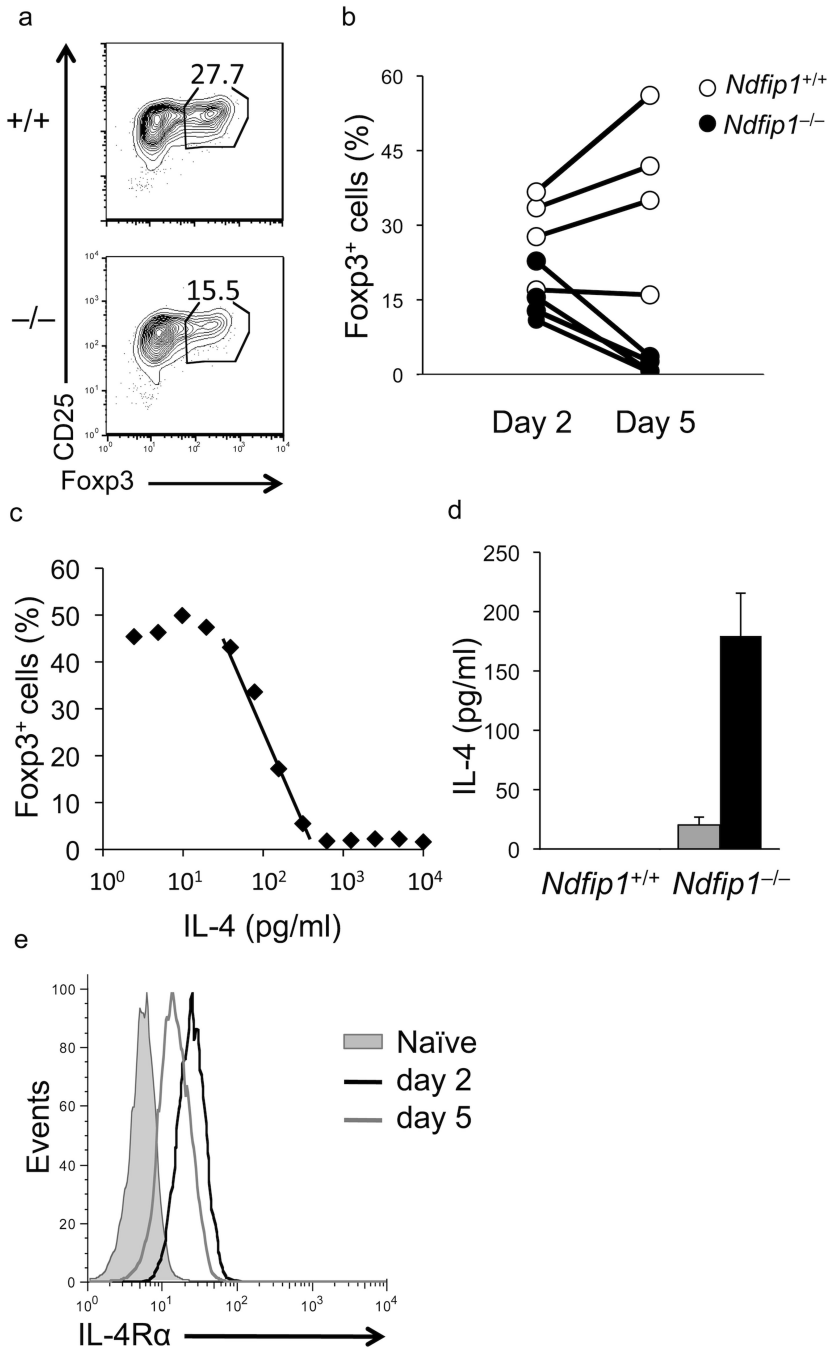

Figure 4. Foxp3 expression declines in *Ndfip1*$^{-/-}$ T cells after day 2

(a and b) Foxp3 expression was assessed in *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells at day 2 and day 5 during iT$_{reg}$ cell differentiation. **(a)** Representative dot plots from *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells after 2 days of stimulation in the presence of 1ng/ml TGF-β are shown. **(b)** Graph depicts the combined data from 4 *Ndfip1*$^{+/+}$ (open circles) and 4 *Ndfip1*$^{-/-}$ (closed circles) mice. Each dot represents a single mouse and samples from the same mouse are connected by a line. **(c)** The amount of IL-4 needed to inhibit iT$_{reg}$ cell differentiation was assessed by adding IL-4 to *Ndfip1*$^{+/+}$ cells in iT$_{reg}$ cell cultures. The percentage of cells that acquire

Foxp3^{+} during culture is plotted on log scale against the amount of IL-4 added. Data are representative of three independent experiments. An IC_{50} of 190 pg/ml was calculated by performing regression analysis of the linear region of the plot (average value of the three experiments). **(d)** ELISA analysis of IL-4 concentrations in the supernatants of T cells stimulated for 1 (gray bars) or 2 (black bars) days under iT_{reg} cell conditions (1ng/ml TGF-β) (mean + S.D. of three mice) **(e)** Analysis of IL-4Rα expression on naïve *Ndfip1*$^{+/+}$ T cells (gray filled) and T cells that were activated for 2 (black line) or 5 (gray line) days in the presence of 1ng/ml TGF-β. Data are representative of 2 independent experiments.

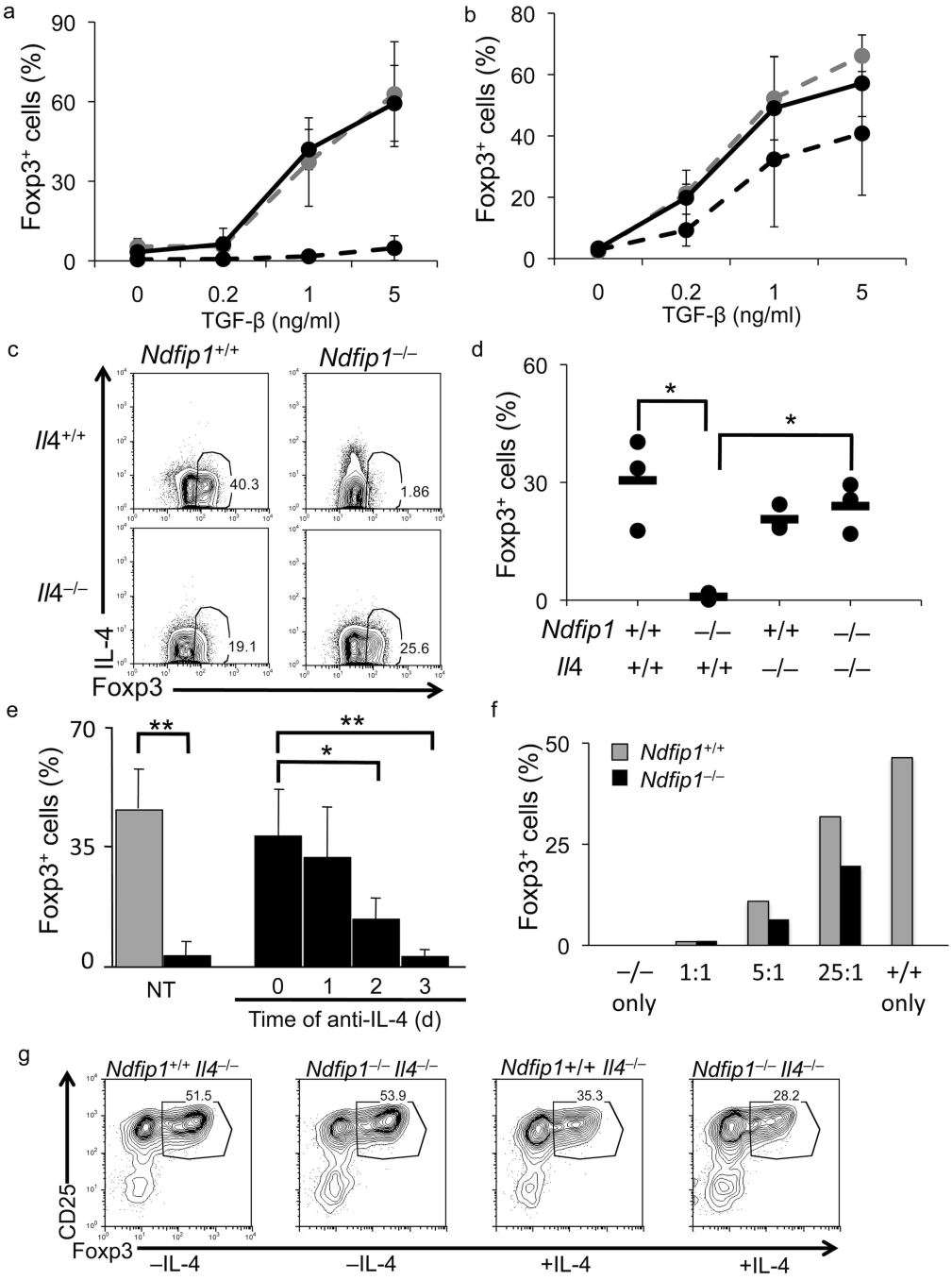

Figure 5. Depletion of IL-4 restores iT$_{reg}$ cell conversion in Ndfip1- and Itch-deficient T cells

(a-g) iT$_{reg}$ cell conversion assays were performed in the presence of 1ng/ml TGF-β (unless otherwise noted) and Foxp3 expression was analyzed on day 5. **(a and b)** The percentage of Foxp3^{+} cells was analyzed following iT$_{reg}$ cell conversion with (solid black line) and without (dashed black line) anti-IL-4 using Ndfip1$^{-/-}$ **(a)** and *Itchy* mutant **(b)** T cells. Conversion of *Ndfip1*$^{+/+}$ cells is shown (gray dashed line). (mean + s.d.; n≥4 mice; 2-4 independent experiments) **(c and d)** iT$_{reg}$ cell conversion of naïve T cells from mice of indicated genotypes. Data are representative of 3 mice of each genotype. **(d)** Analyses of

experiments described in **c** are shown (dot represents each mouse; dash shows the mean). *P<0.05 (two-tailed student T-test). **(e)** Percentages of Foxp3^{+} cells were analyzed among *Ndfip1*$^{+/+}$ (gray bar) or *Ndfip1*$^{-/-}$ (black bars) T cells cultured under iT$_{reg}$ cell conditions either without (NT) or with the addition of anti-IL-4 blocking antibodies at time points indicated. The percentage of Foxp3^{+} *Ndfip1*$^{+/+}$ T cells did not change with addition of anti-IL-4 (not shown). Bars show the mean + s.d. at least 3 mice. *P=0.01, **P<0.005 (two-tailed student T-test). **(f)** *Ndfip1*$^{+/+}$ *CD45.1*$^{+}$ or *Ndfip1*$^{-/-}$ *CD45.2*$^{+}$ naïve T cells were stimulated alone or in mixed cultures at the indicated ratios (*Ndfip1*$^{-/-}$=1 at all ratios). The graph depicts one representative experiment of two. **(g)** *Ndfip1*$^{+/+}$ *IL4*$^{-/-}$ or *Ndfip1*$^{-/-}$ *Il4*$^{-/-}$ T cells were differentiated into iT$_{reg}$ cells with or without the addition of 200pg/ml IL-4 (the half maximal inhibitory concentration of IL-4).

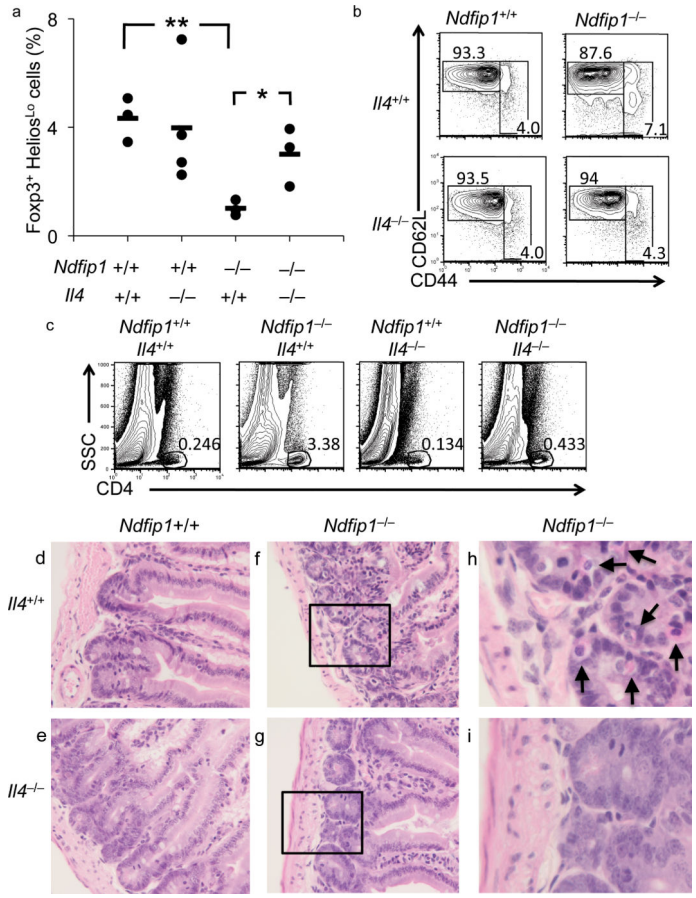

Figure 6. Reduced inflammatory disease in *Ndfip1*$^{-/-}$ mice lacking IL-4

(a) Small bowels from *Ndfip1*$^{+/+}$, *Ndfip1*$^{-/-}$, *Ndfip1*$^{+/+}$*Il4*$^{-/-}$, and *Ndfip1*$^{-/-}$*Il4*$^{-/-}$ mice were analyzed for the frequency of iT$_{reg}$ cells (Foxp3^{+}Helios^{lo}) by flow cytometry. The frequency of iT$_{reg}$ cells among the CD4^{+} population from the small bowel is shown. Each dot represents a single mouse and the mean is shown by lines. *P=0.04, **P=0.003. **(b)** CD4^{+} T cells from the lymph nodes were analyzed for activation by staining with anti-CD62L and anti-CD44. Representative plots from mice 6-7 weeks are shown. **(c)** The percentage of CD4^{+} T cells in the small bowel was assessed by flow cytometry. The plots shown are representative of 3-4 mice of each genotype. **(d-i)** H&E staining of 40X magnified samples from the small bowel of *Ndfip1*$^{+/+}$ **(d),** *Ndfip1*$^{-/-}$ **(f)**, *Ndfip1*$^{+/+}$ *Il4*$^{-/-}$ **(e)**, and *Ndfip1*$^{-/-}$ *Il4*$^{-/-}$**(g)** mice are shown. Images are typical of 3-4 mice from each genotype. **(h and i)** Inset of the boxed areas in **f and g**, respectively. Arrows in **h** indicate representative eosinophils in the field. Note the disorganized architecture of the *Ndfip1*$^{-/-}$ bowel that is not observed in *Ndfip1*$^{-/-}$ *Il4*$^{-/-}$ mice.

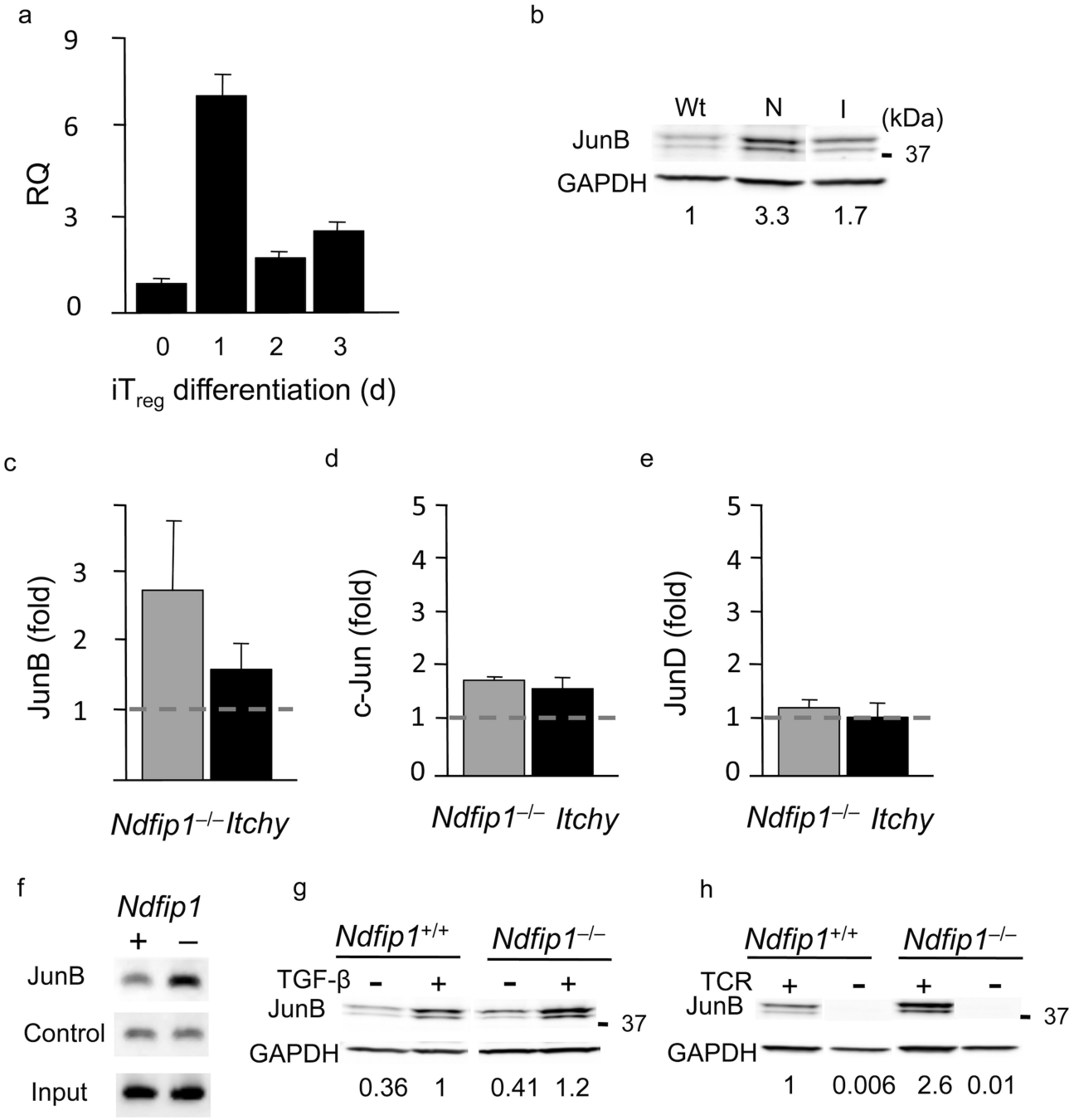

Figure 7. Ndfip1 expression peaks early during iT$_{reg}$ cell differentiation to attenuate JunB expression

(a) qRT-PCR analysis of Ndfip1 expression. Results presented are relative to the expression of Ndfip1 in naïve T cells (RQ) and show one representative plot (n=7, 3 independent experiments) (mean + s.d. of triplicate samples). **(b and c)** JunB protein (normalized to GAPDH) at day 3 of iT$_{reg}$ cell conversion. Values show fold expression over WT (set to one and indicated by the dashed gray line) **(b)** Representative blot and **(c)** graph of cumulative data from *Ndfip1*$^{+/+}$ (Wt), *Ndfip1*$^{-/-}$ (N) and *Itchy* mutant (I) iT$_{reg}$ cells (mean + s.d., n≥3). **(d and e)** c-Jun **(d)** and JunD **(e)** are shown as described above for JunB. **(f)** ChIP analysis

of T cells containing (+) or lacking (-) Ndfip1. PCR using primers specific for the *Il4* promoter was performed on chromatin DNA obtained before (Input) and after immunoprecipitation (IP) with anti-JunB or control IP. (n=5 $Ndfip1^{+/+}$ and n=4 $Ndfip1^{-/-}$. 2 independent experiments) **(g-h)** Representative blot of JunB expression in T cells containing (+/+) or lacking (–/–) Ndfip1 after approximately 2 days of stimulation **(g)** under normal iT_{reg} conditions (+) or in the absence (-) of TGF-β and **(h)** under normal iT_{reg} cell conditions (TCR+) or under TCR withdrawal conditions (TCR-) described in supplementary materials. Representative blot of JunB expression is shown. Values shown are adjusted for GAPDH and normalized to $Ndfip1^{+/+}$ T cells (set to 1) stimulated +TGF-β **(g)** or normal iT_{reg} cell conditions (+TCR) **(h)**.