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Ndfip1 Enforces a Requirement for CD28 Costimulation by Limiting IL-2 Production

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Although the pathways that permit IL-2 production and the full activation of T cells upon Ag encounter are fairly well defined, the negative regulatory circuits that limit these pathways are poorly understood. In this study, we show that the E3 ubiquitin ligase adaptor Ndfip1 directs one such negative regulatory circuit. T cells lacking Ndfip1 produce IL-2, upregulate IL-2Rα, and proliferate, in the absence of CD28 costimulation. Furthermore, T cells in mice lacking both Ndfip1 and CD28 become activated, produce IL-4, and drive inflammation at barrier surfaces. Ndfip1 constrains T cell activation by limiting the duration of IL-2 mRNA expression after TCR stimulation. Ndfip1 and IL-2 have a similar expression pattern, and, following TCR stimulation, expression of both Ndfip1 and IL-2 requires the activity of NFAT and Erk. Taken together, these data support a negative regulatory circuit in which factors that induce IL-2 expression downstream of TCR engagement also induce the expression of Ndfip1 to limit the extent of IL-2 production and, thus, dampen T cell activation. The Journal of Immunology, 2013, 191: 1536–1546.

Upon TCR stimulation, various signaling cascades are initiated that instruct T cells toward the appropriate response. For example, when T cells see their cognate Ag in the presence of costimulation, they produce and secrete IL-2 (1, 2). Autocrine IL-2R signaling initiates a positive feedback loop that further increases IL-2 and IL-2Rα expression and triggers proliferation (3). Costimulatory signals are key to this by complementing the signals received from the TCR, thus boosting IL-2 production.

In contrast, T cells that receive signals only through their TCR produce poor amounts of IL-2 and do not proliferate (4, 5). This is partly due to a lack of coreceptor signals that supplement the production of IL-2. This is also because, in the absence of costimulation, T cells activate mechanisms that actively suppress IL-2 expression (6–8). Whereas the pathways downstream of T cell activation that promote IL-2 production have been characterized, less is known about pathways that actively repress IL-2 production.

One way to repress IL-2 production and secretion is by reducing the levels or functions of signaling proteins by E3 ubiquitin ligases. E3 ubiquitin ligases that restrain T cell activation include Casitas B cell lymphoma-b (Cbl-b), gene regulating anergy in lymphocytes (Grail), and Itch (6, 9). These factors can dampen signaling downstream of the TCR by blocking protein–protein interactions or by ubiquitylating and degrading signaling proteins (9–12). For example, Itch and Cbl-b have been shown to increase the rate of degradation of protein kinase C0 and phospholipase CyI in effector T cells stimulated in the absence of costimulation (9).

Itch is homologous to the E6-AP C terminus-type E3 ubiquitin ligase of the Neural-precursor cell expressed and developmentally downregulated 4 (Nedd4) family. Nedd4 family E3 ubiquitin ligases have intrinsic catalytic activity and can directly mediate the transfer of ubiquitin to substrate proteins (13). Whereas Itch, WWP2, and Nedd4 have known functions in T cells (9, 14–16), a role for the other six Nedd4 family members in T cells has yet to be defined. In vitro, most members of this family have been shown to associate with the membrane-tethered adaptor Nedd4 family interacting protein 1 (Ndfip1) and its only relative Ndfip2 (17–19).

 Whereas Ndfip1 has been shown to bind most Nedd4 family members in vitro (18), to date it has only been shown to interact with Itch in primary T cells (17). Both Itchy-mutant (mice lacking Itch) and Ndfip1−/− mice develop Th2-mediated inflammation at barrier surfaces, including the skin, gastrointestinal (GI) tract, and lung (14, 17). This is in part because, in Ag-experienced T cells, both Ndfip1 and Itch are required for ubiquitylation and degradation of JunB, a transcription factor that promotes IL-4 and IL-5 production (14, 17). Accumulation of JunB in these cells leads to excessive IL-4 production and promotes the differentiation of T cells into Th2 cells (17). Moreover, IL-4 production by Itch- or Ndfip1-deficient T cells leads to defective inducible T regulatory cell (iTreg) differentiation (20). These findings may help explain why both Ndfip1−/− and Itchy-mutant mice develop Th2-mediated inflammation.

In contrast to Itchy-mutant mice, which exhibit inflammation at 5 mo of age, Ndfip1−/− mice develop inflammation by 6 wk of age and do not survive beyond 13 wk of age. Furthermore, T cells from 4- to 6-wk-old Ndfip1−/− mice display markers characteristic of activation (21), whereas T cells from Itchy-mutant mice do not (our unpublished observations). This suggests that Ndfip1 might regulate other Nedd4 family members in T cells. Due to the increased frequency of T cells with an activated phenotype in Ndfip1−/− mice, we hypothesized that Ndfip1-deficient T cells lack a negative regulatory circuit that limits...
T cell activation. In this study, we show that naive Ndfip1 \(-/-\) T cells are hyperactive in response to TCR stimulation due to a T cell–intrinsic defect. Loss of Ndfip1 leads to increased IL-2 production, elevated levels of CD25 expression, and proliferation in the absence of CD28 costimulation. Our data provide evidence that NFAT and Erk, which are essential for the expression of IL-2, also drive the expression of Ndfip1. Once expressed, Ndfip1 regulates the duration of IL-2 production and, thus, prevents T cells from becoming fully activated in the absence of costimulation.

Materials and Methods

**Mice**

Ndfip1 \(-/-\) and Itchy-mutant mice have been described previously (14, 17). CD45.1 (C57BL6.SJL-Ptprc \(^{a}\)Pepcb/BoyJ mice; 002014), IL-4 \(-/-\) (B6.129P2-Il4m1Jas/J; 002253), CD28 \(-/-\) (B6.129S2-Cd28m1Jas/J; 002666), OT-II \(+\) (B6.Cg-Tg (TcraTcrb) 425Cbn/J; 004194), and Rag1 \(-/-\) (B6.129S7-Rag1m1Mon/J; 002216) mice were purchased from the Jackson Laboratory. CD4-crc transgenic (Tg) mice (B6.Cg-Tg [CD4-crc] 1Cwi N9, 4196) were purchased from Taconic. Ndfip1\(^{+/-}\)CrkI mice were generated, as described in Fig. 2. All mice were housed in a barrier facility at the Children’s Hospital of Philadelphia in accordance with the Institutional Animal Care and Use Committee protocol. For genotyping, DNA from tail biopsies was amplified by PCR using the following primers: Ndfip1 wild-type (WT) forward, 5’-TACAGGAAAGCTTGGGCCTTT-3’; Ndfip1 WT reverse, 5’-AGAGGTTGGTCAACAGTGG-3’; Ndfip1 knockout forward, 5’-CGATCGATTCTAACATACG-3’; Ndfip1 knockout reverse, 5’-GTTCTGTGTGCGCAGTATAGC-3’. Primers for IL-4 \(-/-\), CD28 \(-/-\), Rag1 \(-/-\), and CD4-crc Tg mice are available on The Jackson Laboratory Web site (www.jaxmice.jax.org).

**Tissue processing and cell isolation**

Spleen and lymph nodes were harvested and mashed through 70-mm filters in cold HBSS. Cell suspensions from spleens were treated with ACK lysis buffer to lyse RBCs.

Esophagus and a 3–4” section of small bowel were flushed with cold PBS. Peyer’s patches were removed from small bowel. Organs were minced with scissors and treated with DNase (20 μg/ml; Sigma–Aldrich D5025), collagenase type I (0.8 mg/ml; Sigma–Aldrich C0130), and collagenase type Ia (0.9 mg/ml; Sigma–Aldrich C2674) in DMEM for 1 h in end-over-end rotation at room temperature. Cell suspensions were filtered through type Ia (0.9 mg/ml; Sigma-Aldrich C2674) in DMEM for 1 h in end-over-end rotation at room temperature. Cell suspensions were filtered through 100-mm filter, then a 40-mm filter, and 10% FCS was added. Cells were incubated for 10 min at 4°C with Fc block (2.4G2; BD Biosciences) prior to Ab staining.

**Flow cytometry, cell sorting, and Abs**

Flow cytometry was performed using a FACSCalibur or a BD LSR II (BD Biosciences). CD4 (GK1.5) and CD44 (IM7) Abs were obtained from eBiosciences. CD25 (PC61.5) and CD62L (MEL-14) Abs were obtained from BD Biosciences. CD28 (PT-28.2); Ndfip1 WT reverse, 5’-GTCTGTTGTGCCCAGTCATAGC-3’; Ndfip1 knockout reverse, 5’-GTCTGTTGTGCCCAGTCATAGC-3’; Ndfip1 knockout reverse, 5’-GTCTGTTGTGCCCAGTCATAGC-3’; Ndfip1 knockout reverse, 5’-GTCTGTTGTGCCCAGTCATAGC-3’. Primers for IL-4 \(-/-\), CD28 \(-/-\), Rag1 \(-/-\), and CD4-crc Tg mice are available on The Jackson Laboratory Web site (www.jaxmice.jax.org).

**Histology**

Esophagus and sections of small bowel were dissected and fixed in 10% formalin for at least 24 h. All organs were then embedded in paraffin, sectioned, and stained with H&E.

**ELISA**

IL-2 and IL-4 ELISAs were performed on supernatants harvested at the indicated times from in vitro cell cultures. Assays were performed using Ready-Set-Go ELISA kits (eBiosciences) in Nunc-Immuno MicroWell 96-well solid plates (Thermo Scientific). Results were analyzed using a Synergy HT Microplate Reader (Bio Tek).

**Cocultures, stimulation, and CFSE**

Naive sorted CD4 T cells were stimulated with plate-bound anti-CD3 (145-2C11; BD Biosciences) with or without anti-CD28 (37.51; BD Biosciences) Abs (as indicated) (5 μg/ml) for time points, as indicated. Percentage of live cells was determined using flow cytometry by live-cell gating of events on forward scatter by side scatter. For Fig. 7A, CD4 T cells (not sorted for naive) were stimulated with plate-bound anti-CD3 and anti-CD28 (5 μg/ml) for 3 d and left resting for 2 d in IL-2 (50 U/ml) (23-6019; Hoffman-LaRoche). Cells were then stimulated with ionomycin (0–3 μM) in 16 h, rested for 4 h, and restimulated with anti-CD3 and CD28 Abs (5 μg/ml). Culture supernatants were collected 24 h after restimulation. For Fig. 8, the following inhibitors were used: cyclosporine A (NFAT inhibitor) (239385; EMD Millipore), PI3K inhibitor (LY294002) (PEL144, Invitrogen), INK inhibitor (55567; Sigma–Aldrich), and Erk inhibitor (13001; Calbiochem). Inhibitors were added to cultures after the first 24 h of stimulation.

**CFSE labeling**

Cells were resuspended at a 1 × 10\(^{7}\) ml concentration in PBS at room temperature and mixed at a 1:1 ratio with CFSE (C-1157; Invitrogen) in PBS for 4 min with constant agitation. Labeling process was quenched with FCS.

**Coculture assays**

CD45.1 and CD45.2 cells were mixed in a 1:1 ratio and CFSE labeled, as described above. Cells were cultured in the presence of anti-IL-2 (5486; BD Biosciences) and IL-4 (11811; BioLegend) Abs, where specified.

**Quantitative PCR**

RNA from harvested cells was isolated with the RNeasy Mini Kit (Qiagen). RNA-to-cDNA reactions were done using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). For quantitative PCR (qPCR) reactions, TaqMan Gene Expression Master Mix was used (4370048; Applied Biosystems). The Ndfip1 primer/probe set has been previously described (20). FAM dye, MGB primer/probe sets for IL-2 (Mm00434256_m1), IL-2Rα (Mm01340213_m1), and ACTB (437933E) were obtained from Applied Biosystems. Samples were amplified in triplicate using the 7500 Real-Time PCR system (Applied Biosystems). Data were analyzed using the 7500 software v2.0 (Applied Biosystems).

**Statistical analysis**

All statistical analyses were performed using Student’s t tests, unless stated otherwise. A p value ≤0.05 was used to determine statistical significance.

**Results**

**T cells lacking Ndfip1 require Ag exposure to become CD44\(^{high}\) in vivo**

We have shown previously that Ndfip1 \(-/-\) mice develop inflammation at sites of environmental Ag exposure and die prematurely (17). In part, this is because Ndfip1 regulates JunB degradation in vivo (22–25). Under these conditions, T cells will respond to Ag exposure to become activated. Mice lacking Ndfip1 have increased percentages of T cells that are CD44\(^{high}\) (17, 21), suggesting that these cells have been activated by APCs. However, lymphopenic conditions, particularly cytokine signals, and the absence of factors that maintain quiescence can also cause T cells to acquire this activated phenotype (22–25). Under these conditions, T cells will display increased CD44 levels even in the absence of cognate Ag. To determine whether T cells lacking Ndfip1 require Ag exposure to acquire elevated CD44 levels, we generated Ndfip1 \(-/-\) mice that contain T cells specific for a peptide of OVA in the context of MHC class II. These mice, referred to as OTII, are Rag1 deficient and OTII TCR Tg. When compared with control Ndfip1 \(+/-\) OTII animals, Ndfip1 \(-/-\) OTII mice have similar life spans (data not shown). Additionally, Ndfip1 \(-/-\) OTII mice do not develop the eosinophilic inflammation that is observed in Ndfip1 \(-/-\) animals with a polyclonal T cell repertoire (data not shown). We analyzed CD44 levels from T cells isolated from the spleens of Ndfip1 \(-/-\) OTII mice and Ndfip1 \(+/-\) OTII control animals that had not been exposed to OVA. For comparison, we have included data showing this same analysis on Ndfip1 \(-/-\) and Ndfip1 \(+/-\) T cells. As shown previously (21), T cells from Ndfip1 \(-/-\) mice were more likely to have an activated phenotype than T cells from Ndfip1 \(+/-\) control.
animals (Fig. 1, upper right versus upper left panel, Fig. 1B) as long as these mice are maintained on a Rag1 \(^{-/-}\) background. Importantly, T cells from both OTII Ndfip1\(^{+/+}\) and Ndfip1\(^{-/-}\) mice remain CD44\(^{low}\) (Fig. 1A, lower panel, Fig. 1B). Compared with Ndfip1\(^{-/-}\) mice with a polyclonal T cell repertoire, T cells from Ndfip1\(^{-/-}\) OTII mice show significantly reduced percentages of CD44\(^{high}\) T cells. Therefore, T cells lacking Ndfip1 do not acquire an activated phenotype (CD44\(^{high}\)) in the absence of Ag. By inference, these data suggest that T cells from Ndfip1\(^{-/-}\) mice are CD44\(^{high}\) due to Ag-mediated activation.

**T cell–specific deletion of Ndfip1 leads to increased percentages of activated T cells and eosinophilic inflammation**

Having shown that activation of Ndfip1\(^{-/-}\) T cells did not occur in OTII Tg T cells in the absence of Ag, we next sought to determine the basis of the T cell activation in Ndfip1\(^{-/-}\) mice. Increased numbers of activated T cells in vivo could be due to cell-intrinsic or cell-extrinsic defects such as stimulation by innate cells (26). To determine whether these defects were T cell intrinsic, we generated mice lacking Ndfip1 only in cells of the T cell lineage (Ndfip1\(^{CD4-CKO}\)). To delete Ndfip1 in these cells, we inserted loxP sites on either side of exon 2 of the Ndfip1 gene. Cre-mediated recombination of these sites results in a deletion of exon 2 as well as a frame shift mutation for the remaining exons (Fig. 2A). Ndfip1-floxed mice were crossed to CD4-Cre Tg animals to generate mice lacking Ndfip1 in T cells. The resulting progeny were intercrossed, and offspring were analyzed for both the presence of the floxed Ndfip1 alleles as well as the Cre transgene (Fig. 2B). To analyze the effectiveness of Cre-mediated deletion of Ndfip1, T cells from mice homozygous for the floxed Ndfip1 and positive for Cre (i.e., Fig. 2B, lane 3) were tested for expression of Ndfip1 by qPCR (Fig. 2C). Stimulation of WT CD4\(^{+}\) T cells induced expression of Ndfip1 by 24 h. Ndfip1 mRNA expression in Ndfip1\(^{CD4-CKO}\) mice was similar to levels in Ndfip1\(^{-/-}\) T cells, indicating that Ndfip1\(^{CD4-CKO}\) mice lack expression of Ndfip1 in T cells.

Constitutive Ndfip1 knockout mice contain increased percentages of activated T cells and develop inflammation in the esophagus, characterized by an influx of both CD4\(^{+}\) T cells and eosinophils, by 6 wk of age (21). To determine whether Ndfip1-deficient T cells could drive these phenotypic changes, we first compared the activation status of the T cells from Ndfip1\(^{CD4-CKO}\) and Ndfip1\(^{-/-}\) mice. As described previously, spleens of Ndfip1\(^{-/-}\) animals have increased percentages of activated (CD44\(^{high}\)) CD4\(^{+}\) T cells compared with Ndfip1\(^{+/+}\) littermates (Fig. 2D, upper right). Importantly, we found that the frequency of activated T cells in spleens from Ndfip1\(^{CD4-CKO}\) mice was comparable to the frequency observed in Ndfip1\(^{-/-}\) mice (Fig. 2D, lower right). This shows that the activation of the Ndfip1-deficient T cells in vivo results from a T cell–intrinsic defect.

We next analyzed inflammation in the GI tract of Ndfip1\(^{CD4-CKO}\) mice. Histological analysis of the esophagus revealed increased percentages of CD4\(^{+}\) T cells and eosinophils (Fig. 2F). Increased percentages of eosinophils were also observed in the small bowel and lung of Ndfip1\(^{CD4-CKO}\) mice (data not shown). Interestingly, skin inflammation was less evident in the Ndfip1\(^{CD4-CKO}\) mice. Whereas these mice do develop inflammation of the skin, evidence of skin lesions occurs at ~9 wk of age, several weeks later than lesions observed in Ndfip1\(^{-/-}\) animals (data not shown). Furthermore, the extent of eosinophilia was reduced in Ndfip1\(^{CD4-CKO}\) mice compared with Ndfip1\(^{-/-}\) animals. Thus, the loss of Ndfip1 in cells other than T cells most likely contributes to the severity of the inflammation in Ndfip1\(^{-/-}\) mice. Nonetheless, these data show that loss of Ndfip1 specifically in T cells leads to increased T cell activation, infiltration of T cells into tissues, and eosinophilic inflammation.

**Ndfip1\(^{-/-}\) T cells are less dependent on CD28 costimulation than Ndfip1\(^{+/+}\) counterparts**

Data described to date show that the loss of Ndfip1 leads to increased frequency of activated T cells in the mice due to a T cell–intrinsic defect. Based on this, we hypothesized that Ndfip1\(^{-/-}\) T cells are

![FIGURE 1. Activated phenotype of T cells in mice lacking Ndfip1 is Ag dependent. (A and B) Spleen cells were isolated from mice and analyzed by flow cytometry. Representative plots (A) or combined data (B) of CD4\(^{+}\) T cells from three to seven mice per genotype. (A) Gate shown in plot illustrates how we define the percentage of activated T cells. Numbers represent percentage of cells in gate. (B) Graph representing activated phenotype cells illustrated in (A). Each dot represents the percentage of cells in the spleen of a single mouse. Lines represent mean for each sample population, and p values are for samples connected by the line. ***p < 0.001. n.s., Nonsignificant, with a p value >0.05 based on a paired t test. This analysis was performed in at least three independent experiments.](http://www.jimmunol.org/DownloadedFrom/)
representative of mice from at least three independent experiments. At least five mice of each genotype were analyzed. (A) Activation profiles of CD4+ gated T cells from spleens of mice of the indicated genotypes are shown. Gates indicate activated phenotype cells, and numbers indicate the percentage of cells falling within that gate. Data are representative of mice from at least three independent experiments using a total of four mice per genotype. (B) Sections of the esophagus of 6-wk-old mice were analyzed by histology using H&E stains. Images show representative sections from at least three different mice of each genotype. Bar illustrates p, percentages of cells from a single mouse; bars represent the mean. At least four mice were analyzed from each genotype. *p < 0.05.

FIGURE 2. Activation of T cells in the absence of Ndfip1 is T cell intrinsic. (A) Model illustrating T cell–specific deletion of Ndfip1. The targeting vector was designed to introduce loxP sites on either side of exon 2 of the gene encoding Ndfip1. Correct insertion of the vector into the Ndfip1 locus is shown as targeted Ndfip1 locus. Selection cassettes were removed using flp recombinase. Mice harboring the floxed exon 2 were crossed to CD4-cre mice to generate mice with T cells lacking Ndfip1, as illustrated at bottom of panel. In this Ndfip1 locus, Cre-mediated deletion results in loss of exon 2, and the remaining exons are out of frame (illustrated by frameshift). Ndfip2 is encoded on a separate chromosome and not directly affected by our knockout strategy. (B) Mice harboring the floxed allele were analyzed by PCR, and representative results are shown in upper panel. The upper (slower-migrating) band shows the WT locus, and the faster-migrating band shows the floxed locus. Presence of Cre was also determined by PCR and is shown in the lower panel. The slower-migrating band is an internal positive control, whereas the faster-migrating band reflects the presence of Cre. Lane 3 shows the results indicating a mouse with a T cell–specific deletion of Ndfip1 (designated by Ndfip1(CD4-CKO)). (C) Loss of Ndfip1 expression in Ndfip1(CD4-CKO) T cells was analyzed using qPCR. WT and Ndfip1(CD4-CKO) CD4+ T cells were stimulated with anti-CD3 and anti-CD28 for 24 h and analyzed using primers for Ndfip1. Ndfip1 mRNA levels were normalized to an internal control, and data shown are relative to WT unstimulated levels. Bars represent the mean, and lines show SD of triplicate samples. Data are representative of two different experiments using a total of four mice per genotype. (D) Activation profiles of CD4+ gated T cells from spleens of mice of the indicated genotypes are shown. Gates indicate activated phenotype cells, and numbers indicate the percentage of cells falling within that gate. Data are representative of mice from at least three independent experiments. At least five mice of each genotype were analyzed. (E) Sections of the esophagus of 6-wk-old mice were analyzed by histology using H&E stains. Images show representative sections from at least three different mice of each genotype. Bar illustrates 100 μm. (F) Cells were isolated from the esophagus and analyzed by flow cytometry for eosinophils (SiglecF+) or T cells (CD4+). Each dot represents percentages of cells from a single mouse; bars represent the mean. At least four mice were analyzed from each genotype. *p < 0.05.

hyperresponsive to TCR stimulation. To test this, we isolated naive CD4+ T cells from Ndfip1−/− mice and Ndfip1+/+ littermate controls and stimulated them ex vivo through their TCR (anti-CD3) in the presence or absence of CD28 costimulation (anti-CD28). We then measured their levels of CD44 (data not shown) and IL-2Rα (Fig. 3A, 3B). We found that Ndfip1−/− T cells increased their levels of IL-2Rα on day 1 when stimulated with anti-CD3, similar to the presence of CD28 costimulation (Fig. 3A), and this still occurred, albeit to a lesser extent, in the absence of CD28 costimulation (Fig. 3B). However, by day 3 in the absence of costimulation, the levels of IL-2Rα diminished. By day 5, Ndfip1+/+ cells that did not receive costimulation were mostly dead (data not shown). In contrast, Ndfip1−/− cells that were stimulated in the presence of CD28 costimulation continued to display high levels of IL-2Rα and survived well over the course of the experiment. Supporting previously published results, these data show that in vitro CD28 costimulation is required to maintain levels of IL-2Rα and promote survival of cells in vitro (27).

Levels of IL-2Rα on T cells lacking Ndfip1 looked strikingly similar to Ndfip1+/+ counterparts when stimulated with both anti-CD3 and anti-CD28 (Fig. 3A). Additionally, after 1 d of stimulation by anti-CD3 only, IL-2Rα levels on Ndfip1−/− T cells were equivalent to those on Ndfip1+/+ cells. However, after 3 d of stimulation with anti-CD3 only, Ndfip1−/− T cells showed increased levels of IL-2Rα, and by day 5 these cells looked similar to cells that received CD28 costimulation (Fig. 3B). These data suggest that T cells lacking Ndfip1 are hyperresponsive to TCR stimulation and thus less dependent on CD28 costimulation.

In Ndfip1−/− T cells, IL-2Rα levels increased following TCR signaling even in the absence of CD28 costimulation. IL-2Rα expression levels are known to increase further after IL-2R signaling, due to a positive feedback loop (3). The further upregulation of IL-2Rα on Ndfip1−/− T cells between days 1 and 3 after anti-CD3 stimulation suggested that these cells were producing IL-2 despite the lack of costimulation. Therefore, we measured the levels of IL-2 in the culture supernatants (Fig. 3C). Although Ndfip1+/+ T cells stimulated through their TCR alone produced little IL-2 over the course of the assay, T cells lacking Ndfip1 showed significant levels of IL-2 by days 3 and 5 (Fig. 3C). Additionally, by day 3 after anti-CD3–only treatment, T cells lacking CD44.
Ndfip1 were proliferating, as indicated by their loss of CFSE (Fig. 3D). In contrast, no proliferation was observed in the Ndfip1+/+ cultures during this period.

The increased levels of IL-2 could be due to enhanced IL-2 production or increased cell number due to improved survival. To determine whether the IL-2 production at day 3 could be accounted for by increased survival of Ndfip1+/− T cells, we analyzed the percentage of live cells in the cultures described in Fig. 3A and 3B. At day 3, the frequency of live cells did not differ significantly between Ndfip1+/+ and Ndfip1+/− cells regardless of whether the cells were stimulated in the presence or absence of anti-CD28 (data not shown). However, by day 5, the Ndfip1+/+ cells stimulated with anti-CD3 only were mostly dead, whereas a significantly higher percentage of the Ndfip1+/− cells survived (data not shown). This is most likely due to the well-characterized effects of IL-2 on T cell survival (27).

The hyperresponsiveness of T cells lacking Ndfip1 might only occur following a certain threshold of stimulation, or it could occur over a range of strengths of TCR stimulation. Thus, we analyzed naive Ndfip1+/+ and Ndfip1+/− T cells stimulated with different amounts of anti-CD3 in the absence of CD28 costimulation. To ensure that these cells were devoid of all CD28 costimulatory signals, we crossed Ndfip1+/− mice to CD28−/− animals and used T cells from the double-knockout animals (Ndfip1−/− CD28−/−), or mice lacking only CD28 (Ndfip1+/+CD28−/−). T cells were stimulated with increasing amounts of anti-CD3 and analyzed for IL-2 production and IL-2Rα levels on day 3. We found that, at all concentrations of anti-CD3 tested, T cells lacking Ndfip1 produced more IL-2 (Fig. 3E) than controls. Furthermore, the frequency of cells expressing high levels of IL-2Rα increased in cultures of T cells lacking Ndfip1 (data not shown). Interestingly, whereas both Ndfip1+/+ and Ndfip1+/− T cells showed peak IL-2 production at 5 µg/ml anti-CD3, the amount of IL-2 produced by Ndfip1−/− T cells far exceeded that of Ndfip1+/+ cells at all concentrations of anti-CD3 tested (Fig. 3E). Together, these data suggest that Ndfip1 negatively regulates IL-2 production following T cell activation.

Ndfip1−/− T cells become activated and differentiate into IL-4–producing cells in the absence of CD28 costimulation in vivo

Having demonstrated that Ndfip1−/− T cells were less dependent on CD28 costimulation in vitro, we sought to test whether this was also true in vivo. Hence, we analyzed Ndfip1−/− CD28−/− mice for signs of T cell activation and pathology. By 8 wk of age, mice lacking both Ndfip1 and CD28 contained significantly increased percentages of CD4+ T cells that were CD44high (Fig. 4A). When splenocytes isolated from the Ndfip1−/− CD28−/− animals were stimulated with anti-CD3, they produced significant amounts of IL-4 (Fig. 4B). In contrast, little or no IL-4 was detectable in
supernatants from \textit{Ndfip1\textsuperscript{+/+}CD28\textsuperscript{−/−}} cells stimulated in the same manner. Furthermore, elevated frequencies of T cells were evident in both the esophagi and lungs of mice lacking both Ndfip1 and CD28 compared with CD28-deficient controls (Fig. 4C and data not shown).

Increased T cell activation and effector differentiation correlated with increased tissue inflammation in the esophagus and lung (Fig. 4C, 4E and data not shown). \textit{Ndfip1\textsuperscript{+/−}CD28\textsuperscript{−/−}} mice showed histologic evidence of epithelial hypertrophy in the esophagus and showed increased infiltration of inflammatory cells. Whereas eosinophils were not significantly increased in the esophagus (Fig. 4C), these cells could be readily detected in the small bowel by histology and flow cytometry (Fig. 4D, 4F). Although we observed a delay in the onset of inflammation by ∼3 wk, the pathology that developed in \textit{Ndfip1\textsuperscript{+/−}CD28\textsuperscript{−/−}} mice was comparable to that in \textit{Ndfip1\textsuperscript{−/−}} mice. Thus, T cells lacking Ndfip1 can become activated in vivo in the absence of CD28 costimulation, differentiate into IL-4–producing effector cells, migrate into tissues, and promote the recruitment of eosinophils and inflammation.

\textbf{Negative regulation of IL-2 production by Ndfip1 is not dependent on the E6 ubiquitin ligase Itch.}

We have shown previously that Ndfip1 interacts with the homologous to the E6-AP C terminus-type E3 ubiquitin ligase known as Itch, and that this helps to promote the ubiquitylation and degradation of JunB to dampen IL-4 production by T cells (17, 20). Thus, Ndfip1 might promote other functions of Itch to dampen IL-2 production. This seemed particularly likely because \textit{Itchy}-mutant T cells have been shown to produce more IL-2 under certain conditions (14, 28). To test this, we analyzed IL-2 production by naïve T cells lacking either Ndfip1 or Itch following anti-CD3 stimulation. As shown previously, T cells lacking Ndfip1 produce more IL-2 at days 3 and 5 following stimulation with anti-CD3 alone (Fig. 4A). T cells lacking Itch produced no more IL-2 than their WT counterparts. These data were surprising considering previously published data showing that upon TCR-only stimulation, Itch-deficient T cells become activated (28). However, these prior experiments were performed using cells that had been previously activated in vitro or using differentiated effector cells. Thus, we performed experiments as the ones described by Heissmeyer et al. (9) using \textit{Ndfip1\textsuperscript{−/−}} or \textit{Itchy}-mutant cells differentiated in vitro. As shown in Fig. 5B, WT T cells differentiated in vitro produced less IL-2 upon TCR-only stimulation with ionomycin. In contrast, effector cells lacking either Ndfip1 or Itch were still able to make IL-2 after ionomycin treatment (Fig. 5B).

Taken together, these data suggest that there are different mechanisms that negatively regulate T cell activation in naïve and ef-

\textbf{FIGURE 4.} T cells in mice lacking both Ndfip1 and CD28 become activated, make IL-4, and migrate into the GI tract. (A) Percentages of activated phenotype cells among the CD4\textsuperscript{+} cells in the spleens of 5- to 10-wk-old \textit{Ndfip1\textsuperscript{+/−}CD28\textsuperscript{−/−}} mice or \textit{Ndfip1\textsuperscript{+/+}CD28\textsuperscript{−/−}} littermate controls. Each dot represents a single mouse, and data are representative of four independent experiments. Dots connected by lines show age-matched littermate pairs in each experiment. *p < 0.05 based on a paired \textit{t} test. (B) Cells were isolated from spleens of mice and stimulated overnight with anti-CD3. IL-4 in the supernatants was determined using ELISA. Data are representative of three independent experiments from three mice of each genotype. Bars represent the mean, and error bars indicate SD of three triplicate samples. (C and D) Cells isolated from esophagus (C) and small bowel (D) following collagenase treatment were analyzed by flow cytometry for eosinophils (SiglecF\textsuperscript{+}) or T cells (CD4\textsuperscript{+}). Representative plots from at least three independent experiments are shown. (E-H) Sections of esophagus from \textit{Ndfip1\textsuperscript{+/−}CD28\textsuperscript{−/−}} mice (E and G) or \textit{Ndfip1\textsuperscript{−/−}CD28\textsuperscript{−/−}} mice (F and H). (E and F) These were taken at original magnification ×20, whereas (G and H) are \textit{insets} depicted by boxed regions in (E and F). Epithelial thickening can be seen in (G and H). (L) This indicates the lumen for visual reference. Representative of three mice of each genotype from three independent experiments. (I-L) Sections of small bowel from \textit{Ndfip1\textsuperscript{+/−}CD28\textsuperscript{−/−}} mice (I and K) or \textit{Ndfip1\textsuperscript{−/−}CD28\textsuperscript{−/−}} mice (J and L). (I and J) These were taken at original magnification ×20, whereas (K and L) are \textit{insets} depicted by boxed regions in (I and J). Eosinophils can be seen in \textit{insets} and are indicated by arrows. Representative of three mice of each genotype from three independent experiments.
Ndfip1 restricts T cell activation by limiting the expression of IL-2

Following TCR stimulation, T cells increase the levels of expression of IL-2Rα, a subunit of the high-affinity IL-2R. When cells also receive CD28 costimulation, IL-2 production increases and acts in an autocrine manner via the IL-2R to further increase IL-2 production and IL-2R expression in a positive regulatory loop that results in proliferation. T cells lacking Ndfip1 show both increased IL-2 production as well as elevated levels of IL-2Rα. Thus, the hyperresponsive phenotype of Ndfip1+/− T cells could be directly due to increased IL-2 production or a consequence of increased IL-2R signaling. To test this, we cocultured naive CD4+ T cells from either Ndfip1−/− or Ndfip1+/+ littermates (both express CD45.2) together with equal numbers of WT T cells (expressing CD45.1) and analyzed IL-2Rα levels and proliferation. Supporting data are shown in Fig. 2D; T cells lacking Ndfip1 proliferate more than Ndfip1+/+ counterparts (Fig. 6A, upper left versus lower left panel). Interestingly, WT T cells cultured with Ndfip1−/− T cells proliferated more than cells cultured with Ndfip1+/+ cells (Fig. 6A, upper right versus lower right panel), suggesting that Ndfip1-deficient T cells secrete a soluble factor that drives proliferation of WT cells. We therefore repeated the experiment, but added anti–IL-2 to the cultures to block the binding of IL-2 to the IL-2R. In the presence of anti–IL-2, proliferation of Ndfip1+/+ T cells was abrogated, as was proliferation of the WT T cells in the same cultures (Fig. 6B, upper left and upper right panels). Furthermore, the proliferation of WT T cells cultured in the presence of Ndfip1−/− T cells was comparably diminished in the presence of anti–IL-2 (Fig. 6B, lower right panel). Regardless of whether IL-2 blocking Abs were present, Ndfip1−/− T cells proliferate more than the WT cells in the same cultures (Fig. 6A, 6B, lower left versus lower right, respectively).

This difference between Ndfip1−/− and WT T cells was not limited to proliferation, but was also evidenced by IL-2Rα levels.
expression data show that this is most likely due to the downstream consequences of increased IL-2 production. Thus, these data support a model in which Ndfip1 prevents the full activation of T cells by limiting IL-2 production at the transcriptional level.

**NFAT and Erk induce the expression of Ndfip1 to limit IL-2 production in the absence of costimulation**

Having shown that Ndfip1 limits the duration of IL-2 production following initial IL-2 expression (Fig. 7A), we next wanted to determine how Ndfip1 expression is regulated in T cells. Thus, we stimulated Ndfip1+/+ T cells through the TCR and analyzed expression of Ndfip1 at different time points. Prior to stimulation of naive T cells, little, if any, Ndfip1 was expressed. However, expression of Ndfip1 was upregulated after 12 h of TCR stimulation (Fig. 8A), dropped after 24 h of TCR signaling, and continued declining by 36 h. Interestingly, the expression pattern of Ndfip1 was strikingly similar to that of IL-2 in TCR-stimulated T cells (Fig. 7A). The similarity between the transcriptional patterns of Ndfip1 and IL-2 suggested that factors that induce IL-2 expression upon TCR stimulation might also play a role in regulating the expression of Ndfip1, to limit IL-2 transcription.

TCR signaling promotes IL-2 expression through the cooperation of various factors, including Jnk, NFAT, Erk, and PI3K (reviewed in 29). Although costimulatory signals, such as those delivered from CD28, can significantly enhance signaling, TCR stimulation alone can support IL-2 expression to some extent (30). It is not known, however, how Ndfip1 expression is affected by TCR signaling and whether the factors that promote IL-2 expression also play a role in its expression.

To determine whether Jnk, NFAT, Erk, or PI3K also regulates Ndfip1 expression, we stimulated naive Ndfip1+/+ T cells through the TCR in the presence of inhibitors for these different factors. We then analyzed Ndfip1 mRNA levels after overnight stimulation. Ndfip1 expression increased following TCR stimulation (Fig. 8B), but this was somewhat reduced when either Jnk or PI3K was inhibited. Importantly, the expression of Ndfip1 was almost completely abrogated in the presence of inhibitors of either NFAT or Erk. Thus, NFAT and Erk are required for Ndfip1 expression. Taken together, these data suggest that two key factors that induce IL-2 production, NFAT and Erk, are also inducers of Ndfip1, a factor that attenuates IL-2 expression. This suggests that NFAT and Erk induce Ndfip1 upon T cell stimulation to create a negative feedback loop that restricts IL-2 transcription. Supporting this, comparing the region within 5 kb of the mouse and human Ndfip1 promoter, we found several conserved noncoding sequences with NFAT and AP-1 binding sites (Fig. 8C).

**Increased IL-2 production by Ndfip1−/− T cells is independent of IL-4**

We have shown previously that Ndfip1−/− T cells aberrantly produce IL-4 after T cell activation (20, 31) and that these cells are biased toward Th2 differentiation (17). Whereas IL-4 signaling has not been shown to directly impact IL-2 production, IL-4 could increase cell survival and thus alter IL-2 production indirectly. To
test whether the increased IL-2 was due to IL-4 production by Ndfip1−/− cells, we analyzed T cells from mice lacking both Ndfip1 and IL-4. Naive T cells from Ndfip1−/− IL-4−/− mice or IL-4−/− littermate controls were stimulated with anti-CD3, and we analyzed the amount of IL-2 in the supernatants by ELISA. We found that IL-2 production by Ndfip1−/− IL-4−/− T cells was significantly greater than in IL-4−/− controls (Fig. 9A), suggesting that exposure to elevated IL-4 signals cannot account for the hyperresponsiveness of these cells in vitro.

We recently showed that T cells lacking Ndfip1 were defective in iTreg cell differentiation and that this was due to increased IL-4 production (20). Although mice lacking Ndfip1 showed fewer Foxp3+ T cells in their small bowel, mice lacking both Ndfip1 and IL-4 contained normal numbers of these cells. Based on the data in Fig. 9A, we hypothesized that Ndfip1−/− T cells would still be activated in vivo under conditions in which iTreg cell differentiation was restored (i.e., in Ndfip1−/− IL-4−/− animals). Thus, we analyzed Ndfip1−/− IL-4−/− mice for signs of T cell activation, T cell migration into tissues, and inflammation. Ndfip1−/− IL-4−/− animals do not show signs of inflammation at 6 wk of age, a time when Ndfip1−/− animals show pathology developing in the skin, lung, and GI tract (20). However, by 12 wk of age, Ndfip1−/− IL-4−/− mice begin to develop disease, and these mice ultimately die prematurely of inflammatory consequences (data not shown). Histological examination of the esophagus and lungs from Ndfip1−/− IL-4−/− mice revealed epithelial hyperplasia and infiltration of inflammatory cells (Fig. 9B, 9C). Supporting this, mice lacking both Ndfip1 and IL-4 showed increased percentages of T cells in mucosal tissues, such as esophagus and lung (Fig. 9D, 9E). These mucosal barrier sites also showed increased percentages of eosinophils and neutrophils (data not shown). Additionally, whereas we saw a trend toward increased percentages of activated T cells in the spleens of these mice, it did not reach statistical significance (data not shown). This may be because these cells emigrated to tissues following activation. Thus, although IL-4 overproduction clearly increases the number of activated T cells in Ndfip1−/− mice and exacerbates disease, even in the absence of IL-4 and with restored iTreg cell differentiation, T cells become activated, move into tissues, and drive inflammation, leading to premature death. Taken together, our data support that T cell hyperresponsiveness is most likely underlying the inflammation in Ndfip1−/− IL-4−/− mice.

**Discussion**

In this study, we show that Ndfip1, an adaptor for E3 ligases of the Nedd4 family, negatively regulates IL-2 production, thereby preventing the activation of T cells in the absence of CD28 costimulation. T cells lacking Ndfip1 produce IL-2, increase surface expression of the high-affinity IL-2Rα subunit, and proliferate in the absence of CD28 costimulation in vitro. Additionally, activation in the absence of this negative regulator has severe pathologic consequences in vivo, because mice lacking both Ndfip1 and CD28 develop a Th2-mediated inflammation at barrier surfaces much like mice lacking only Ndfip1. These pathologic consequences are due to intrinsic defects in T cells lacking Ndfip1 because mice lacking Ndfip1 only in T cells (Ndfip1CD4−/−CKO) show a similar expression profile of activation markers.

We have shown previously that Ndfip1 promotes Itch-mediated ubiquitination and degradation of JunB, thus dampening IL-4 production (17). Overproduction of IL-4 explains the Th2 bias of cells lacking Ndfip1; however, this mechanism does not account for the increased IL-2 production. Supporting this, Ndfip1−/− T cells lacking IL-4 produce IL-2 following TCR stimulation in the absence of CD28 costimulation. Additionally, in the absence of IL-4, Ndfip1−/− mice develop a delayed, yet ultimately fatal, inflammatory disease. We propose that hyperactive IL-2–producing T cells promote this inflammation.

Increased IL-2 production is not seen in naive T cells lacking the E3 ubiquitin ligase Itch. Thus, Ndfip1 regulates IL-2 production by naive T cells independent of its ability to promote Itch function. In contrast, when T cells are differentiated into effectors in vitro, both Ndfip1−/− and Itch-deficient cells show more IL-2 production than their WT counterparts. Thus, Itch and Ndfip1 both regulate IL-2 production in effector differentiated T cells. Based on our prior studies, we propose that Itch and Ndfip1 collaborate in this task. Additionally, these results suggest that there are different mechanisms used by Ndfip1 to control IL-2 expression in naive and Ag-experienced T cells. This is not surprising, as naive cells have
different requirements for costimulatory signals than their Ag-


experienced counterparts (32). As Ndfip1 can modulate the ac-
tivity of many E3 ligases of the Nedd4 family in vitro, future
studies will be needed to identify other Nedd4 family members
whose function is regulated by Ndfip1 after TCR stimulation of
na"ve T cells.

Our data show that Ndfip1 limits IL-2 expression. Interestingly,
Ndfip1 does not regulate initial expression of IL-2; rather, Ndfip1
restricts the duration of expression. Calcium-induced NFAT
and signaling via Erk induce both IL-2 and Ndfip1 expression following
TCR stimulation. Given that IL-2 and Ndfip1 have similar ex-
pression patterns after TCR signaling and that they rely on common
transcription factors suggests a negative feedback loop through
which TCR signaling activates IL-2 production, but also limits IL-2
expression via Ndfip1.

It is known that, in T cells stimulated in the absence of coreceptor
signaling, NFAT opposes T cell activation. It does so by inducing
the expression of genes that promote unresponsiveness, such as
Grail (8) and Ikaros (33), among others. Our results suggest that,
in addition to these factors, NFAT interferes with T cell activation
by increasing expression of Ndfip1. The Ndfip1 promoter contains
multiple putative sites for NFAT binding (Fig. 8C), supporting that
NFAT may directly regulate Ndfip1 expression.

CD28 costimulation can supplement TCR signaling, and thus
can enhance the signaling cascade downstream of the TCR that
allows for IL-2 production. However, the activation of both NFAT
and Erk pathways in T cells is less dependent on CD28 costim-
umulatory signals than that of other pathways such as NF-κB (32, 33).

Based on these data, we propose that the negative regulatory factor
Ndfip1 limits IL-2 production to enforce a requirement for co-
stimulation, thereby preventing T cells from responding to low-
affinity signals such as those coming from self-peptides or envi-
ronmental Ags. Such factors are prime candidates for therapeutic
strategies designed to either augment or dampen T cell signaling
to promote tumor rejection or prevent autoimmune or allergic
disorders, respectively.

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