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The E3 Ubiquitin Ligase Adaptor Ndfip1 Regulates Th17 Differentiation by Limiting the Production of Proinflammatory Cytokines

Hilda E. Ramon,*‡ Allison M. Beal,†,‡ Yuhong Liu,§ George Scott Worthen,§ and Paula M. Oliver*‡

Ndfip1 is an adaptor for the E3 ubiquitin ligase Itch. Both Ndfip1- and Itch-deficient T cells are biased toward Th2 cytokine production. In this study, we demonstrate that lungs from Ndfip1−/− mice showed increased numbers of neutrophils and Th17 cells. This was not because Ndfip1−/− T cells are biased toward Th17 differentiation. In fact, fewer Ndfip1−/− T cells differentiated into Th17 cells in vitro due to high IL-4 production. Rather, Th17 differentiation was increased in Ndfip1−/− mice due to increased numbers of IL-6–producing eosinophils. IL-6 levels in mice that lacked both Ndfip1 and IL-4 were similar to wild-type controls, and these mice had fewer Th17 cells in their lungs. These results indicate that Th2 inflammation, such as that observed in Ndfip1−/− mice, can increase Th17 differentiation by recruiting IL-6–producing eosinophils into secondary lymphoid organs and tissues. This may explain why Th17 cells develop within an ongoing Th2 inflammatory response. The Journal of Immunology, 2012, 188: 000–000.

CD4 T cells can differentiate into different Th subsets according to cues from their immediate cytokine environment. Th17 cells are a distinct group of CD4 Th cells that have been shown to protect against specific bacterial and fungal pathogens by producing proinflammatory cytokines that mediate neutrophil recruitment (1). Although they can play protective roles, Th17 cells can also worsen the pathogenesis of inflammatory diseases such as multiple sclerosis, arthritis, inflammatory bowel disease, and asthma (2, 3).

Th17 differentiation can be either promoted or prevented by various cytokines. For example, CD4 Th cells can differentiate into Th17 cells in response to TGF-β and the proinflammatory cytokine IL-6 (4, 5). Although TGF-β and IL-6 are sufficient for their differentiation, IL-23 signaling promotes long-term maintenance of the Th17 cell lineage, allowing Th17 cells to fully differentiate and proliferate (6). Additionally, IL-1 together with IL-6 can facilitate Th17 differentiation by increasing expression of retinoic-related orphan receptor γt and IFN response factor-4 and inducing higher levels of IL-17 production (7). In contrast, Th17 differentiation is inhibited by IL-2 as well as the Th2 and Th1-specific cytokines IL-4 and IFN-γ (8–10). Although cytokines made by Th1 and Th2 cells can directly inhibit Th17 differentiation, Th17 cells commonly occur alongside Th1 and Th2 cells in inflammatory settings such as in inflammatory bowel disease and asthma.

Atopic asthma is characterized by Th2-mediated inflammation in the lung and is often accompanied by eosinophilia, high serum IgE levels, and airway hyperreactivity (11). Although this disease is defined by the hallmark Th2-mediated inflammation, high levels of IL-17, as well as Th17 cells, are present in the lungs of asthmatic patients (12–14). Furthermore, high levels of IL-17 and neutrophil accumulation are normally seen in more severe cases of asthma (15, 16), including steroid-resistant asthma (17, 18). Th17 cells have been shown to contribute to lung inflammation in mouse models of asthma through the recruitment of neutrophils (19–22) that can promote tissue damage and by inducing lung stromal cell and epithelial cells to produce proinflammatory cytokines and chemokines (23–26).

Nedd4 family interacting protein 1 (Ndfip1) is an adaptor protein that binds to and augments the function of the E3 ligase Itch (27). Itch has been shown to ubiquitylate and cause the degradation of JunB (28), a transcription factor that promotes the expression of IL-2, IL-4, and IL-5 (29). In the absence of Ndfip1, activated CD4 T cells accumulate high levels of JunB and become Th2 polarized (27). We recently reported that Ndfip1−/− mice naturally develop Th2-mediated inflammation in the skin, gastrointestinal tract, and lungs and die prematurely (27, 30). These mice have increased percentages of activated CD4 T cells in their spleens and lymph nodes that are Th2 biased, as well as eosinophilia. Additionally, they have high circulating levels of IgE and IL-5 in the serum. Histological analysis revealed infiltrating inflammatory cells in the perivascular regions in the lung and goblet cell hyperplasia (27). Importantly, neutrophils were also evident in the perivascular regions of the lung, as well as within the alveolar space. Given the reported role of Th17 cells in lung inflammation and in the recruitment of neutrophils into the airways, we sought to determine whether Ndfip1−/− regulates Th17 differentiation.

In this report, we show that mice lacking Ndfip1 have increased numbers of Th17 cells. This finding suggested a scenario in which...
Ndfip1 acts to prevent Th17 differentiation, much like it does for Th2 differentiation. On the contrary, in vitro, Ndfip1<sup>−/−</sup> T cells were defective in becoming Th17 cells. This is because increased IL-4 production by Ndfip1<sup>−/−</sup> T cells inhibit Th17 differentiation. Thus, Ndfip1 promotes Th17 differentiation by inhibiting production of IL-4. In addition, our data show that although IL-4 directly inhibits Th17 differentiation in vitro, the Th2 inflammatory environment in Ndfip1<sup>−/−</sup> mice promotes the differentiation of Th17 in vivo by increasing the numbers of IL-6–producing eosinophils. Together, these data suggest that Th2 responses can promote Th17 responses in vivo. This might help to explain why Th17 cells develop in the setting of inflammatory diseases such as asthma.

Materials and Methods

Mice

Ndfip1<sup>−/−</sup> mice have been previously described (27). They have been backcrossed to the C57BL/6 background for more than nine generations. All mice were kept in a semi-barrier facility at the Children’s Hospital of Philadelphia. All experimentation was approved and followed guidelines established by the Children’s Hospital of Philadelphia Institutional Animal Care and Use Committee. Ndfip1<sup>−/−</sup> mice were bred from heterozygous parents, and their wild-type (WT) littersmates were used as controls. The IL-4<sup>−/−</sup> mice (31) were purchased from The Jackson Laboratory and bred with Ndfip1<sup>−/−</sup> mice in our mouse facility. Mice 4–8 wk old were used for experiments.

Analysis of lung bronchoalveolar lavage fluids and tissue homogenates

Lung tissue homogenates were prepared by perfusing the lungs and removing either a piece or whole lung, which was then digested with DNase (20 μg/ml), collagenase 1 (0.9 mg/ml), and collagenase 1A (0.8 mg/ml; Sigma-Aldrich) in 20–25 ml DMEM, shaking for 1 h at room temperature.

Collection of BAL

At designated time points, the animals were euthanized, the diaphragm was incised, and the trachea isolated and cannulated with a 20-gauge catheter, which was immobilized with 4–5% silk suture. BAL fluid was collected from the whole lung using four instillation/washout passes of 0.8 ml PBS containing 100 nM diethylenetriaminepentaacetic acid (Sigma-Aldrich), as previously described (32). BAL fluid (0.17 ml) was centrifuged and placed on glass cytosin slides, which were then stained by Diff-Quick reagents (Fisher Scientific) to enumerate leukocyte subtypes based on their cellular and nuclear morphological features.

Flow cytometry and Abs

Unstimulated cells were harvested from lung tissue homogenates (described above) and splenies and then stained with LIVE/DEAD Fixable Dead Cell Blue stain as per the manufacturer’s instructions (Invitrogen). Cells were then preincubated with Fc Block (anti-CD16/32, 2.4G2; BD Biosciences) prior to surface staining. Cells were stained for surface markers for 30 min at 4°C, washed, and then fixed with the Foxp3 Fixation/Permeabilization Kit from eBioscience. Samples were analyzed the next day on an LSRII Fortessa (BD Biosciences). The Abs used to stain for leukocyte cell subsets in the lung tissue homogenates and spleen samples were anti-CD11c (N418), anti-CD4 (GK1.5), anti-Ly6G (1A8), anti-CD11b (M1/70), and anti-c-Kit (2B8) from BioLegend; anti-Foxp3 (FJK-16s) and anti-FcRγI (MAR-1) from eBioscience; and anti-SiglecF (E502440) from BD Biosciences.

Th17 differentiation

Naive CD4<sup>+</sup> T cells were sorted by gating out the CD25<sup>+</sup> cells and selecting the CD44<sup>low</sup>CD62L<sup>high</sup> population of CD4<sup>+</sup> T cells. Cells were cultured in 48-well plates using 5 × 10<sup>5</sup> cells/well and activated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml; BD Pharmingen). Th17-differentiating conditions included TGF-β (5 ng/ml; PeproTech) and IL-6 (20 ng/ml; R&D Systems) or TGF-β (0.5 ng/ml), IL-6 (20 ng/ml), IL-1 (20 ng/ml; PeproTech), and IL-23 (50 ng/ml) (R&D Systems). Cells were cultured for 5 d and analyzed for IL-17 and IL-4 production by flow cytometry by intracellular cytokine staining. Supernatants were collected at 20–24 h to measure IL-2 production by ELISA. In some of the experiments, IL-4 (BioLegend) and IL-2 (BD Biosciences) blocking Abs were added at the initiation of the cultures (20 μg/ml).

Measurement of cytokines by ELISA

ELISA was performed to measure cytokines from either serum or spleen cultures. For serum, mice were bled, and serum was collected using minicollect tubes from Greiner bio-one and stored at −80°C until used for ELISA. Spleen cultures were set up using 4 × 10<sup>5</sup> splenocytes/well in a round-bottom 96-well plate. Soluble anti-CD3 (5 μg/ml) was used to activate T cells. Supernatants were collected at 24 h after stimulation and placed at −80°C until used. For IL-6 production from spleen cultures, splenocytes were stimulated with PMA (1 μM) and ionomycin (30 μg/ml) for 4.5–5 h, and then supernatants were collected and placed at −80°C until used. ELISA was performed using the eBioscience kit for the respective cytokine. Based on the manufacturer’s protocol, the limit of sensitivity for the ELISA measuring IL-6 was 4 pg/ml; however, our extrapolated results showed linear detection of positive controls as low as 2 pg/ml. All results shown are within the linear range of extrapolated data.

Measurement of serum IgE by ELISA

Serum was collected as described above. The amount of IgE from the serum of the indicated mice was analyzed using the Mouse IgE ELISA kit from BioLegend as per the instructions provided in the kit. Plates were analyzed using a Synergy HT Microplate Reader (BioTek).

Intracellular cytokine staining

For intracellular cytokine staining, the cells from lung tissue homogenates or Th17-differentiation cultures were restimulated with PMA (1 μM) and ionomycin (30 μg/ml) in the presence of GolgiStop for 4.5 h (BD Biosciences). Cells were fixed either using the BD Biosciences kit for intracellular cytokine staining or the Foxp3 Fixation/Permeabilization Kit from eBioscience. For Th17-differentiation cultures, cells were stained with anti–IL-17 (eBio17B7; eBioscience) and anti–IL-4 (11B11; BD Biosciences) and analyzed by flow cytometry. For analysis of Th subsets from lung and spleen samples, cells were stained with the LIVE/DEAD Fixable Cell stain (Invitrogen) and blocked with Fc Block (BD Biosciences) as described above. The cells were stained for intracellular cytokines using the following Abs: IL-4 (11B11; BioLegend), IL-9 (RM9A4; BioLegend), IFN-γ (XMG1.2; BioLegend), IL-5 (TRFK5; BD Biosciences), IL-6 (MP5-20F3; BD Biosciences), and IL-10 (JES5-16E3; eBioscience).

Anti–IL-6 Ab treatment

Ndfip1<sup>−/−</sup> mice were treated with IL-6 blocking Ab (R&D Systems) using 1 mg in 250 μl of PBS or with 1 mg/250 μl isotype control Abs per injection. Intraperitoneal injections were performed weekly, at 4 and 5 wk of age, and mice were analyzed at 6 wk of age.

Statistics

All statistical analysis was performed by Student’s t tests. A p value ≤0.05 was considered to determine statistical significance. Error bars represent SD of the mean.

Results

Ndfip1<sup>−/−</sup> mice have an increase in lung neutrophils and Th17 cells

Previous work from our group has shown that Ndfip1<sup>−/−</sup> mice develop Th2-mediated pulmonary inflammation characterized by goblet cell hyperplasia and increased numbers of perivascular lymphocytes and eosinophils. Neutrophils, leukocytes that are associated with Th17 rather than Th2-mediated inflammation, were also evident within the perivascular space. To quantify the
neutrophils from Ndfip1<sup>−/−</sup> and Ndfip1<sup>+/−</sup> littermates, we analyzed cells from BAL by both morphological and flow cytometric approaches. Examination of cytospin preparations revealed high numbers of neutrophils among cells isolated from BAL of Ndfip1<sup>−/−</sup> mice, but not Ndfip1<sup>+/−</sup> littermate controls (Fig. 1A, Supplemental Fig. 1A). This result was confirmed using flow cytometry of cells isolated from BAL. Abs against Ly6G and CD11b, markers used to identify neutrophils (Fig. 1B), revealed a significant increase in the number of neutrophils in the airspace of lungs from Ndfip1<sup>−/−</sup> animals. This increase in neutrophils in the lungs of Ndfip1<sup>−/−</sup> mice suggested that Th17 cells might be present among the Th2 cells that have been reported previously.

Th17 cells are known to recruit neutrophils into the lung during inflammatory conditions (33). To test whether Ndfip1<sup>−/−</sup> mice contained increased numbers of Th17 cells, we isolated cells from the lungs of Ndfip1<sup>−/−</sup> and Ndfip1<sup>+/−</sup> littermates and used flow cytometry to determine the percentage of CD4<sup>+</sup> T cells that expressed intracellular IL-17. Analysis of lung tissue homogenates from 6-wk-old Ndfip1<sup>−/−</sup> mice or Ndfip1<sup>+/−</sup> littermates showed a significant increase in the percentage of Th17 cells in the lungs of Ndfip1<sup>−/−</sup> mice (Fig. 1C). As shown in Fig. 1D, the percentage of cells in the lung tissue homogenates that were CD4<sup>+</sup>, and IL-17<sup>+</sup> was increased ~7-fold in Ndfip1<sup>−/−</sup> mice compared with Ndfip1<sup>+/−</sup> littermate controls.

We next sought to analyze the percentages of Th2 cytokine-producing cells in the lung and spleen of Ndfip1<sup>−/−</sup> and Ndfip1<sup>+/−</sup> mice, as well as with other cytokine-producing CD4<sup>+</sup> subsets. The most frequent cytokine-producing subsets in both lung and spleens of Ndfip1<sup>−/−</sup> animals were those producing IL-4 or IL-5 (Supplemental Fig. 1B). The percentages of these Th2 cytokine-producing cells in Ndfip1<sup>−/−</sup> mice were, on average, 12-fold higher than those in Ndfip1<sup>+/−</sup> littermates. Other cytokine-producing subsets were also increased, but to a lesser extent.

Together, these data show that Ndfip1<sup>−/−</sup> mice have an ongoing Th17 immune response in addition to their previously described Th2-mediated inflammation.

Ndfip1<sup>−/−</sup> T cells have a defect in Th17 differentiation in vitro

Ndfip1<sup>−/−</sup> T cells are more likely to become Th2 cytokine producers in vivo and in vitro (27). This is because Ndfip1 is required for Itch-mediated degradation of JunB, a transcription factor that promotes IL-4 and IL-5 transcription (29). Thus, one function of Ndfip1 is to prevent IL-4 production and Th2 differentiation. Given the increased percentages of Th17 cells in the lungs of Ndfip1-deficient mice, we hypothesized that Ndfip1 might also inhibit Th17 differentiation. To test this, we analyzed Th17 differentiation of Ndfip1<sup>+/−</sup> and Ndfip1<sup>−/−</sup> T cells in vitro. Naive, sorted CD4 T cells from Ndfip1<sup>−/−</sup> mice or Ndfip1<sup>+/−</sup> littermate controls were activated with anti-CD3 and anti-CD28 in the presence of TGF-β and IL-6, conditions shown previously to induce Th17 differentiation (4, 5). Consistent with these reports, ~11% of the Ndfip1<sup>+/−</sup> T cells became IL-17–producing cells under these conditions. Contrary to our expectations, Ndfip1<sup>−/−</sup>/CD4 T cells showed a reduced capacity to differentiate into Th17 cells (Fig. 2A, 2B), as ~4% of the cells were IL-17 producers. Thus, after 5 d in culture, Ndfip1<sup>+/−</sup>/CD4 T cells showed a significant decrease in IL-17 production under these conditions. It has been shown that low levels of TGF-β in conjunction with IL-6, IL-1, and IL-23 induce higher percentages of IL-17–producing T cells in culture than TGF-β and IL-6 alone (7). Supporting this, using WT cells, we obtained the highest level of Th17 differentiation using 0.5 μg/ml TGF-β together with IL-6, IL-1, and IL-23 (data not shown). We therefore tested the ability of naive Ndfip1<sup>−/−</sup>/CD4 T cells to differentiate into Th17 cells under these conditions. After 5 d of culture, naive Ndfip1<sup>−/−</sup>/CD4 T cells again showed a reduced capacity for IL-17 production compared with CD4<sup>+</sup> T cells isolated from Ndfip1<sup>+/−</sup>/littermate controls (Fig. 2C, 2D). Approximately 40% of Ndfip1<sup>+/−</sup>/CD4 T cells differentiated into Th17 cells, whereas >10% of the Ndfip1<sup>−/−</sup>/T cells became IL-17 producers. The combined data from five to six separate experiments are shown in Fig. 2B and 2D. Taken together, these data show that Ndfip1<sup>−/−</sup> T cells are defective at differentiating into Th17 cells in vitro.

IL-4 and IL-2 are cytokines that have been previously shown to inhibit Th17 differentiation (8–10). Ndfip1<sup>−/−</sup> T cells can produce increased levels of IL-2 and IL-4 under certain conditions (27, 34). To test whether Ndfip1<sup>−/−</sup> T cells produce higher levels of IL-2 than their Ndfip1<sup>+/−</sup> counterparts, we measured IL-2 produced by Ndfip1<sup>−/−</sup> or Ndfip1<sup>+/−</sup> T cells after activation with anti-CD3 and anti-CD28 in the presence or absence of Th17-polarizing conditions (Supplemental Fig. 2A). When cultured in the presence of anti-CD3 and anti-CD28, Ndfip1<sup>−/−</sup> T cells produced a similar amount of IL-2 compared with Ndfip1<sup>+/−</sup> T cells. Furthermore, under Th17-polarizing conditions, IL-2 production was also similar between the two genotypes. These data suggest that differences in IL-2 production are unlikely to ac-
the IL-4 production in these cells. Importantly, Ndfip1<sup>−/−</sup> T cells that produced IL-4 were not also producing IL-17 (Fig. 3). These data suggest that Ndfip1<sup>−/−</sup> T cells can become Th2 cytokine-producing cells even under Th17-polarizing conditions. Th17 differentiation is unlikely to occur in these IL-4–producing cells. Furthermore, IL-4 production by these cells could prevent Th17 differentiation by other cells in the cultures.

**Blocking IL-4 restores Th17 differentiation of Ndfip1<sup>−/−</sup> T cells**

Given the capacity of IL-4 and IL-2 to inhibit Th17 differentiation, we next tested whether IL-4 production or a combination of both IL-4 and IL-2 production by Ndfip1<sup>−/−</sup> T cells could prevent Th17 differentiation in vitro. To test this, we cultured naive Ndfip1<sup>+/+</sup> and Ndfip1<sup>−/−</sup> CD4<sup>+</sup> T cells under Th17-polarizing conditions in vitro in the presence or absence of IL-4– and IL-2–blocking Abs. We then measured the percentages of these CD4<sup>+</sup> T cells that differentiated into Th17 cells. As shown above, when activated in the presence of TGF-β, IL-6, IL-1, and IL-23, Ndfip1<sup>−/−</sup> CD4<sup>+</sup> T cells were defective in differentiating into Th17 cells when compared with Ndfip1<sup>+/+</sup> CD4<sup>+</sup> T cells. Importantly, in the presence of IL-4–blocking Abs, the ability of Ndfip1<sup>−/−</sup> T cells to differentiate into Th17 cells was restored (Fig. 3A, 3B). Both the percentage of cells making IL-17 and the amount of intracellular IL-17 being made by the Th17 cells reached levels similar to those of Ndfip1<sup>+/+</sup> CD4<sup>+</sup> T cells. Furthermore, T cells from Ndfip1<sup>−/−</sup> IL-4<sup>−/−</sup> mice do not have a defect in Th17 differentiation (Supplemental Fig. 2C). These data indicate that IL-4 produced by Ndfip1<sup>−/−</sup> CD4<sup>+</sup> T cells prevents their differentiation into Th17 cells.

It has been shown previously that blocking IL-2 can enhance Th17 differentiation (8, 35). Consistent with these observations, we saw an increase in the percentage of Ndfip1<sup>+/+</sup> cells that produced IL-17 in the presence of IL-2–blocking Abs. Ndfip1<sup>−/−</sup> CD4<sup>+</sup> T cells also showed an increased percentage of Th17-producing cells in the presence of the IL-2–blocking Ab. Ndfip1<sup>−/−</sup> IL-4<sup>−/−</sup> mice showed an average of a 2-fold increase compared with a 6-fold increase for Ndfip1<sup>−/−</sup> cells. However, in the presence of IL-2–blocking Abs, the levels of IL-4 produced by Ndfip1<sup>−/−</sup> T cells, and to some extent, Ndfip1<sup>−/−</sup> T cells, also decreased significantly (Fig. 3A). This suggests that in these cultures, blocking IL-2 is promoting Th17 differentiation of Ndfip1<sup>−/−</sup> T cells by reducing IL-4 production. Furthermore, when used together, anti–IL-4 and anti–IL-2 blocking Abs restored Th17 differentiation to levels seen in Ndfip1<sup>−/−</sup> cultures (Fig. 3A, 3B). Taken together, these data indicate that IL-4 produced by Ndfip1<sup>−/−</sup> T cells prevents their differentiation into Th17 cells. Furthermore, these results show that Ndfip1 facilitates Th17 differentiation by inhibiting the production of IL-4.

**Ndfip1<sup>−/−</sup> mice have elevated levels of IL-6**

Our results show that Ndfip1<sup>−/−</sup> mice have an increase in Th17 cells in the lung. Surprisingly, however, Ndfip1<sup>−/−</sup> CD4<sup>+</sup> T cells are less likely to differentiate into Th17 cells in vitro. This suggests that conditions exist in Ndfip1<sup>−/−</sup> mice that support Th17 differentiation. One possible factor required for Th17 differentiation in vivo is IL-6. Thus, we analyzed the levels of IL-6 in the serum of Ndfip1<sup>+/+</sup> and Ndfip1<sup>−/−</sup> mice. Analysis of IL-6 levels in the serum of 6-wk-old mice by ELISA showed higher amounts of IL-6 in Ndfip1<sup>−/−</sup> mice than Ndfip1<sup>+/+</sup> littermate controls (Fig. 4A). Thus, increased IL-6 in vivo could promote the Th17 differentiation of CD4<sup>+</sup> T cells observed in Ndfip1<sup>−/−</sup> mice. To test whether the increased level of IL-6 seen in Ndfip1<sup>−/−</sup> mice could account for the increased percentages of Th17 cells in the lungs, we treated Ndfip1<sup>−/−</sup> mice with an anti–IL-6 blocking Ab. Starting at 4 wk of...
age, IL-6–blocking Ab was administered for 2 wk. Following this treatment, we analyzed BAL fluid for the numbers of neutrophils and lung tissue homogenates for the presence of Th17 cells. Ndfip1−/− mice treated with isotype control showed similar numbers of neutrophils in their BAL fluid compared with untreated Ndfip1−/− mice (Fig. 4B versus Supplemental Fig. 1A); however, treatment with IL-6–blocking Abs led to a significant reduction in neutrophil numbers (Fig. 4C). Eosinophil numbers were also reduced (Fig. 4C). Although isotype control-treated controls showed similar percentages of Th17 cells to untreated mice, Ndfip1−/− mice that received IL-6–blocking Ab had significantly fewer Th17 cells than isotype control–treated animals (Fig. 4D). Although the percentages of Th17 cells were reduced, the percentages of other cytokine-producing CD4+ T cells were less affected (Supplemental Fig. 3). Importantly, the reduced percentages of Th17 cells correlated with decreased lung inflammation (Fig. 4E). These results indicate that mice treated with anti–IL-6 Abs have lower levels of Th17 cells in the lungs, supporting our hypothesis that the high levels of IL-6 facilitate Th17 differentiation in Ndfip1−/− mice.

Together our data show that Ndfip1−/− CD4+ T cells are partially defective in Th17 differentiation. This is because these cells produce too much IL-4, even in the presence of TGF-β. Furthermore, we propose that the high levels of IL-6 in Ndfip1−/− animals helps to promote Th17 differentiation in vivo and explains why we observe increased percentages of Th17 cells in the Ndfip1−/− mice compared with healthy Ndfip1+/+ littermates.

Eosinophil numbers are increased in the spleens of Ndfip1−/− mice and these cells are producing IL-6

As noted above we detect more IL-6 in the serum of mice lacking Ndfip1. It is known that CD4+ T cells, macrophages, eosinophils, mast cells, and neutrophils can make IL-6 (36, 37). To determine which cells in Ndfip1−/− mice were making IL-6 and thus driving Th17 differentiation, we restimulated cells from the lungs and spleens and assessed IL-6 production by intracellular cytokine staining. We detected more cells producing IL-6 in the spleens than in the lungs of Ndfip1−/− mice (Fig. 5A, 5B and data not shown). Although we could detect IL-6 production in CD4+ cells, the majorit (∼86%) of the splenocytes making IL-6 were CD4− negative cells (Fig. 5B). Among the CD4-negative cells, we did not detect a significant frequency of IL-6− cells in the Ly6G+ neutrophil or c-Kit+ mast cell populations (data not shown). In contrast, most of the IL-6+ cells were found among the SiglecF+ popula- tion, suggesting that the cells making IL-6 in Ndfip1−/− mice were eosinophils. We have previously shown that mice lacking Ndfip1 have increased numbers of eosinophils in the esophagus (30); however, the spleen was not investigated in this previous report. Therefore, we assessed the frequency of eosinophils in the spleens of mice lacking Ndfip1. We found that Ndfip1−/− have increased percentages and numbers of eosinophils in the spleen (Fig. 5D, 5E). Moreover, more of the eosinophils in Ndfip1−/− mice are activated as indicated by FceRIα expression and are making IL-6 (Fig. 5F, 5G). Based on these data, eosino- phils are the source of IL-6 that promotes Th17 differentiation in vivo in mice lacking Ndfip1.

The Th2 inflammatory environment in Ndfip1-deficient mice promotes Th17 differentiation

Our in vitro data show that IL-4 can directly decrease Th17 differentiation, whereas our in vivo data suggests that Th2-mediated inflammation may promote Th17 differentiation indirectly by increasing the levels of IL-6. It is possible that even in the presence of IL-4–producing Th2 cells, the inflammatory conditions in Ndfip1−/− mice promote the differentiation of Th17 cells. To test this, we crossed Ndfip1−/− mice to IL-4−/− animals and analyzed the percentages of Th17 cells in the lungs, IgE levels in the serum (Supplemental Fig. 4A), and IL-6 production from splenocytes in the resulting Ndfip1−/−, IL-4−/−, and Ndfip1−/−IL-4−/− progeny.

As illustrated in Fig. 6, Ndfip1−/−IL-4−/− double-knockout (DKO) mice showed a lower level of IL-6 in the serum compared with Ndfip1−/−IL-4−/− littermate controls (Fig. 6A) as well a decreased IL-6 production by ex vivo splenocytes (Fig. 6B), indicating that the Th2 response in Ndfip1−/− mice is contributing...
to the high levels of IL-6 in these mice. Supporting this, eosinophils in the spleens of \textit{Ndfip1}^{-/-} \textit{IL-4}^{-/-} DKO mice showed reduced intracellular IL-6 (Fig. 6C). Furthermore, \textit{Ndfip1}^{-/-} \textit{IL-4}^{-/-} DKO mice contained fewer neutrophils in the BAL fluid (Supplemental Fig. 4B) and, in addition to the decrease in Th2 cytokine-producing cells (Supplemental Fig. 4C), also showed lower percentages of Th17 cells in the lungs compared with \textit{Ndfip1}^{-/-} \textit{IL-4}^{+/+} controls (Fig. 6D). Additionally, there is much less inflammation in the lungs of \textit{Ndfip1}^{-/-} \textit{IL-4}^{-/-} DKO mice compared with \textit{Ndfip1}^{-/-} mice (N. Ramos and A. Beal, unpublished observations). These results indicate that the Th2 inflammatory environment seen in \textit{Ndfip1}^{-/-} mice contributes to the differentiation of Th17 cells (Fig. 7). We propose that this is, at least in part, due to an increase in production of IL-6, which occurs as a consequence of the Th2-mediated inflammation.

Discussion

A previously published report from our laboratory shows that mice deficient in Ndfip1 develop Th2-mediated inflammation (27). This phenotype can be explained by the role of Ndfip1 in the regulation of Itch-mediated ubiquitination and degradation of JunB, a transcription factor that regulates the expression of the Th2 cytokines IL-4 and IL-5 (29). The inflammation seen in these mice is characterized by high levels of IgE and IL-5 in serum and an influx of immune cells in the lung and at other mucosal surfaces. In the lungs of \textit{Ndfip1}^{-/-} mice, infiltrating inflammatory cells, including eosinophils, were observed in the perivascular regions. Goblet cell hyperplasia is also evident (27). We have now shown that in addition to this Th2 response, \textit{Ndfip1}^{-/-} mice have elevated numbers of neutrophils and increased percentages of Th17 cells in the lungs.

The increase in lung Th17 cells in these mice led us to hypothesize that Ndfip1 might prevent Th17 differentiation and that its absence would cause an increase in Th17 cells in vitro and in vivo. Thus, we tested the role of this adaptor protein in Th17 differentiation in vitro. Unexpectedly, our results showed that \textit{Ndfip1}^{-/-} CD4 T cells are defective in differentiating into the Th17 subset in vitro. \textit{Ndfip1}^{-/-} CD4 T cells are more likely to become Th2 cells compared with WT counterparts in the presence of Th2-driving conditions. Our results further show that even under neutral or Th17-driving conditions, \textit{Ndfip1}^{-/-} CD4 T cells make IL-4 after activation. IL-4 has been previously shown to inhibit Th17 differentiation (9, 10, 38). Consistent with these results, the IL-4 produced by \textit{Ndfip1}^{-/-} CD4 T cells significantly
inhibited their Th17 differentiation in vitro. This is not necessarily due to a direct inhibition of Th17 differentiation by JunB, as these results indicated that the defect in Th17 differentiation seen in Ndfip1−/− cells is dependent on signaling through the IL-4R. Given the role of Ndfip1 in the negative regulation of Th2 cytokine expression, our results show that this adaptor protein acts to promote Th17 differentiation by preventing IL-4 production.

It has been reported that TGF-β plays an important role in the inhibition of Th2 cytokine expression during Th17 differentiation as well as during induced regulatory T cell differentiation (39). We showed recently that TGF-β dampens IL-4 production by Ndfip1-dependent as well as Ndfip1-independent mechanisms (34). We show in this study that this is true during Th17 differentiation as well. Supporting this, in Ndfip1−/− CD4 T cells, TGF-β is not sufficient to completely prevent IL-4 production and allow differentiation into the Th17 subtype. Thus, CD4 T cells have at least two mechanisms that prevent IL-4 production during Th17 differentiation, one of which depends on Ndfip1.

Our in vitro experiments show that Ndfip1−/− CD4 T cells are defective in differentiating into Th17 cells; however, in vivo, Ndfip1−/− mice show increased numbers in lung Th17 cells. Given that IL-6 plays a major role in the differentiation of Th17 cells (4, 5), we analyzed the levels of IL-6 in the serum of Ndfip1−/− mice. The levels of IL-6 were elevated in the serum of Ndfip1−/− mice and ex vivo analysis of splenocytes from these mice revealed high percentages of IL-6–producing eosinophils. Blocking IL-6 in vivo resulted in a decrease in lung Th17 cells in Ndfip1−/− mice, indicating that this cytokine promotes Th17 differentiation in our system. Therefore, although Ndfip1−/− CD4 T cells are less likely to undergo Th17 differentiation in vitro, the presence of IL-6 promotes Th17 differentiation in vivo, leading to an increase in lung Th17 cells and neutrophilia as compared with healthy Ndfip1+/+ mice.

The inflammatory environment within Ndfip1-deficient mice contains factors that promote and inhibit Th17 differentiation. Our results indicate that high levels of IL-6 in Ndfip1−/− mice are conducive to Th17 differentiation. In contrast, Ndfip1−/− mice have a highly Th2-polarized cytokine environment, where IL-4 could prevent Th17 differentiation. To test the effect of the observed Th2 environment on Th17 differentiation in vivo, we crossed Ndfip1−/− to IL-4−/− mice. Analysis of mice lacking both Ndfip1 and IL-4 revealed a lower level of IL-6 in serum and fewer IL-6–producing eosinophils compared with mice lacking only Ndfip1, indicating that the Th2 cytokine production in Ndfip1−/− mice might lead to higher IL-6 expression. Furthermore, Ndfip1−/−/IL-4−/− mice contained fewer lung Th17 cells compared with Ndfip1−/− mice.

The inflammatory environment within Ndfip1-deficient mice promotes Th17 differentiation. Although IL-4 can directly decrease IL-6–producing eosinophils, which increases Th17 differentiation. Therefore, a balance between the direct in-
Inhibition of IL-4 and that of inflammation and IL-6 can determine the outcome of an accompanying Th17 response. Our results indicate that even in the presence of IL-4, the inflammatory environment and the increase in IL-6 expression observed in Ndfip1<sup>2/2</sup> mice can tilt the balance toward Th17 differentiation (Fig. 7). Furthermore, our results help to resolve the apparent discrepancy between in vitro data showing that IL-4 can directly impair Th17 differentiation (9, 10, 38) and ex vivo data showing elevated levels of Th17 cells in asthmatic patients despite a predominant Th2 response (12–14). Based on the results presented in this study, we propose that the missing link connecting the Th2 and Th17 responses in vivo is eosinophil production of IL-6.

In this study, we show that Ndfip1<sup>1/−/−</sup> mice have an ongoing Th17 response in the lung in addition to their previously described Th2-mediated inflammation. This is also seen in atopic asthma, because high levels of IL-17, as well as an increase in Th17 cells, have been observed in the lungs of asthmatic patients (12–14). Using mouse models of asthma, it has been shown that Th17 cells induce the recruitment of neutrophils into the lungs through their production of IL-17, which can induce the expression of neutrophil chemoattractants by bronchial fibroblasts (24). In addition, several reports have shown that a Th17 response can promote a Th2 response and increase lung eosinophilia (20–22), which is mediated by an increase in the expression of eotaxin (40). In this study, we show that a Th2 response can further promote Th17 differentiation through the production of the Th17-driving cytokine IL-6 by activated eosinophils. Based on previous data and our data, it is therefore possible that during lung inflammation, there is cross talk between the Th2 and Th17 responses that ultimately leads to amplification of both responses and significantly elevated levels of inflammation.

IL-6 is a cytokine that is normally expressed during both acute and chronic inflammation. IL-6 is elevated in cases of Th1-mediated autoimmune disease such as rheumatoid arthritis or Crohn’s disease (41), but also expressed during Th2-mediated diseases such as asthma (42). Although there are cases in which an immune response is directly guided toward a Th17 response, it is possible that inflammation and its consequent tissue damage generally promote Th17 differentiation, explaining why Th17 cells are found along with both Th1 and Th2 responses. The results presented in this paper support the idea that inflammation, even if it is highly Th2 polarized, can lead to the differentiation of Th17 cells through the induction of the proinflammatory cytokine IL-6.
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Disclosures

The authors have no financial conflicts of interest.

References


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Supplementary Figure Legends

Supplementary Figure 1. *Ndfip1*<sup>−/−</sup> mice have increased numbers of neutrophils in the BAL fluid and increased TH2 cytokine producing cells in lung and spleen. A) BAL fluid from *Ndfip1*<sup>+/+</sup> and *Ndfip1*<sup>−/−</sup> mice was collected and the number of the indicated cells was determined by cytospin. A representative pie chart is shown for each genotype n=3. B) Splenocytes and lung tissue homegenates from the indicated mice were restimulated with PMA and ionomycin and then stained and analyzed by flow cytometry as described in materials and methods. Representative flow plots for T<sub>H</sub>2 (staining for IL-4 and IL-5), T<sub>H</sub>1 (IFN-γ<sup>+</sup>), T<sub>H</sub>9 (staining for IL-10 and IL-9), and T<sub>reg</sub> cells (Foxp3<sup>+</sup>) are shown (gated on live CD4<sup>+</sup> cells), n=4 mice per genotype.

Supplementary Figure 2. *Ndfip1*<sup>−/−</sup> CD4 T cells produce IL-4 under T<sub>H</sub>17-driving conditions. (A) Sorted naïve CD4 T cells were activated with plate-bound anti-CD3 and anti-CD28 in the absence (-) or presence (+) of TGF-β, IL-6, IL-1, and IL-23. IL-2 production was analyzed by ELISA from culture supernatants at 18-20 hours of culture. (B) Sorted naïve CD4 T cells were activated with anti-CD3 and anti-CD28 +/- TGF-β, IL-6, IL-1, and IL-23. The percentage of IL-4-producing cells was analyzed by flow cytometry 5 days after culture. C) Naïve CD4 T cells were activated in the presence of TGF-β, IL-6, IL-1, and IL-23. On day 5, intracellular IL-17 and IL-4 expression was analyzed by flow cytometry. This data is representative of three different experiments.

Supplementary Figure 3. Anti-IL-6 effect on other T helper cell subsets in *Ndfip1*<sup>−/−</sup> mice. Splenocytes and lung tissue homegenates from Ndfip1<sup>−/−</sup> treated with anti-IL-6 or
isotype control (IC) were restimulated with PMA and ionomycin and then stained and analyzed by flow cytometry as described in materials and methods. Representative flow plots for T_{h}2, T_{h}1, T_{h}9, and T_{reg} cells are shown (gated on live CD4^{+} cells), n=3 mice per genotype.

Supplementary Figure 4. Ndfip1^{−/−} mice lacking IL-4 have a significantly decreased frequency of T_{h}2 cells, lower IgE and reduced eosinophilia. A) Reduced serum IgE levels in Ndfip1^{−/−} IL-4^{−/−} mice compared to Ndfip1^{−/−} IL-4^{+/+} counterparts. The amount of IgE detected in the serum from the indicated mouse genotypes was analyzed through ELISA. Bar graphs depict the mean ± s. e. m. from 4 mice. * p<0.05. B) BAL fluid from Ndfip1^{+/+} IL-4^{−/−} and Ndfip1^{−/−} IL-4^{−/−} mice was collected and the number of the indicated cells was determined by cytospin. A representative pie chart is shown for each genotype, n=3 per genotype. C) Splenocytes and lung tissue homegenates from the indicated mice were restimulated with PMA and ionomycin and analyzed by flow cytometry as described in materials and methods. Representative flow plots for T_{h}2, T_{h}1, T_{h}9, and T_{reg} cells are shown (gated on live CD4^{+} cells), n=3 mice per genotype.
Supplementary Figure 1

A

Ndfip1+/+

5200 1730

16700

Ndfip1-/-

18300 17100

17100

168800

Neutrophils
Eosinophils
Macrophages
Lymphocytes

B

Lung

Ndfip1+/+

1.1%

0.5%

Ndfip1-/-

1.1%

8.3%

Th2

IL-4

IL-5

Th1

5.4

8.0

Th9

IL-10

IL-9

Threg

FoxP3

CD4

CD4

Spleen

Ndfip1+/+

0.6%

10.6%

Ndfip1-/-

2.3%

6.6%