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The E3 ubiquitin ligase adaptor Ndfip1 regulates T_H17 differentiation by limiting the production of pro-inflammatory cytokines1

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Abstract

Ndfip1 is an adaptor for the E3 ubiquitin ligase Itch. Both Ndfip1- and Itch-deficient T cells are biased towards T_H2 cytokine production. Here we demonstrate that lungs from *Ndfip1*$^{-/-}$ mice showed increased numbers of neutrophils and T_H17 cells. This was not because Ndfip1−/− T cells are biased towards Th17 differentiation. In fact, fewer *Ndfip1*$^{-/-}$ T cells differentiated into T_H17 cells *in vitro* due to high IL-4 production. Rather, T_H17 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 WT controls and these mice had fewer T_H17 cells in their lungs. These results indicate that T_H2 inflammation, such as that observed in Ndfip1−/− mice, can increase T_H17 differentiation by recruiting IL-6 producing eosinophils into secondary lymphoid organs and tissues. This may explain why T_H17 cells develop within an ongoing T_H2 inflammatory response.

INTRODUCTION

CD4 T cells can differentiate into different helper (T_H) subsets according to cues from their immediate cytokine environment. T_H17 cells are a distinct group of CD4 T_H cells that have been shown to protect against specific bacterial and fungal pathogens by producing pro-inflammatory cytokines that mediate neutrophil recruitment.[1] While they can play protective roles, T_H17 cells can also worsen the pathogenesis of inflammatory diseases such as multiple sclerosis, arthritis, inflammatory bowel disease (IBD), and asthma.[2,3]

T_H17 differentiation can be either promoted or prevented by various cytokines. For example, CD4 T_H cells can differentiate into T_H17 cells in response to transforming growth factor- β (TGF-β) and the pro-inflammatory cytokine IL-6.[4,5] While TGF- β and IL-6 are sufficient for their differentiation, IL-23 signaling promotes long-term maintenance of the T_H17 cell lineage allowing T_H17 cells to fully differentiate and proliferate.[6] Additionally, IL-1 together with IL-6 can facilitate T_H17 differentiation by increasing expression of retinoic-related orphan receptor γt (RORγt) and interferon response factor (IRF-4) and inducing higher levels of IL-17 production.[7] In contrast, T_H17 differentiation is inhibited by IL-2 as well as the T_H2 and T_H1-specific cytokines IL-4 and IFN-γ.[8–10] Although cytokines made

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by T_H1 and T_H2 cells can directly inhibit T_H17 differentiation, T_H17 cells commonly occur alongside T_H1 and T_H2 cells in inflammatory settings such as in inflammatory bowel disease (IBD) and asthma.

Atopic asthma is characterized by T_H2-mediated inflammation in the lung, and is often accompanied by eosinophilia, high serum IgE levels, and airway hyper-reactivity.[11] While this disease is defined by the hallmark T_H2-mediated inflammation, high levels of IL-17, as well as T_H17 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]. T_H17 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 pro-inflammatory 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 T_H2 polarized.[27] We recently reported that *Ndfip1*−/− mice naturally develop T_H2-mediated inflammation in the skin, gastrointestinal (GI) tract, and lungs, and die prematurely.[27, 30] These mice have increased percentages of activated CD4 T cells in their spleens and lymph nodes (LN) that are T_H2-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 T_H17 cells in lung inflammation and in the recruitment of neutrophils into the airways, we sought to determine whether Ndifp1−/− regulates T_H17 differentiation.

In this report we show that mice lacking Ndfip1 have increased numbers of T_H17 cells. This finding suggested a scenario in which Ndfip1 acts to prevent T_H17 differentiation, much like it does for T_H2 differentiation. On the contrary, *in vitro*, *Ndfip1*−/− T cells were defective in becoming T_H17 cells. This is because increased IL-4 production by *Ndfip1*−/− T cells inhibit T_H17 differentiation. Thus, Ndfip1 promotes T_H17 differentiation by inhibiting production of IL-4. In addition, our data show that while IL-4 directly inhibits T_H17 differentiation *in vitro*, the T_H2 inflammatory environment in *Ndfip1*−/− mice promotes the differentiation of T_H17 *in vivo* by increasing the numbers of IL-6-producing eosinophils. Together, these data suggest that T_H2 responses can promote T_H17 responses *in vivo*. This might help to explain why T_H17 cells develop in the setting of inflammatory diseases such as asthma.

METHODS

Mice

Ndfip1−/− mice have been previously described.[27] They have been backcrossed to the C57BL6 background for more than 9 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*−/− mice were bred from heterozygous parents and their WT littermates were used as controls. The *IL-4*−/− mice[31] were purchased from the Jackson Laboratories and bred with *Ndfip1*−/− mice in our mouse facility. Mice 4–8 weeks old were used for experiments.

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Analysis of lung bronchial alveolar lavage (BAL) fluids and tissue homogenates

Lung tissue homogenates were prepared by perfusing the lungs and removing either a piece or a whole lung, which was then digested with DNase (20μg/ml), collagenase 1 (0.9 mg/ml) and collagenase 1A (0.8 mg/ml) (Sigma) in 20–25 ml of DMEM, shaking for 1 hour at room temperature. The cell suspension was filtered through a 100μm, then 40μm mesh and 10% FCS was added. The cells were then washed and either cultured with PMA, ionomycin, and Golgi-stop (BD Bioseciences) for intracellular cytokine staining, or directly prepared for flow cytometry. Neutrophils in the BAL fluids were collected as previously described[32] and analyzed by flow cytometry using antibodies against CD11b (M1/70) and Ly6G (1A8) from BD Biosciences.

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 4-0 silk suture. BAL fluid was collected from the whole lung using 4 instillation/withdrawal passes of 0.8 ml PBS containing 100 nM Diethylenetriaminepentaacetic acid (DTPA; Sigma), as previously described.[32] BAL fluid (0.17 ml) was centrifuged and placed on glass cytospin 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 antibodies

Unstimulated cells were harvested from lung tissue homogenates (described above) and spleens and then stained with LIVE/DEAD Fixable Dead Cell Blue stain as per manufacturer's instructions (Invitrogen). Cells were then pre-incubated with Fc Block (anti-CD16/32, 2.4G2, BD Biosciences) prior to surface staining. Cells were stained for surface markers for 30 minutes at 4°C, washed and then fixed with the Foxp3 Fixation/ Permeabilization Kit from eBioscience. Samples were analyzed the next day on an LSRFortessa (BD Biosciences). The antibodies 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-FcεRIα (MAR-1) from eBioscience; and anti-SiglecF (E502440) from BD Biosciences.

T_H17 differentiation

Naïve $CD4^+$ T cells were sorted by gating out the $CD25^+$ cells and selecting the $CD44^{low}$ $CD62L^{high}$ population of $CD4^+$ T cells. Cells were cultured in 48 well plates using 5×10^5 cells per well, and activated with plate-bound anti-CD3 (5μg/ml) and anti-CD28 (5μg/ml) (BD Pharmingen). T_H17 differentiating conditions included TGF- β (5ng/ml) (PreproTech) and IL-6 (20ng/ml) (R and D systems), or TGF- β (0.5ng/ml), IL-6 (20ng/ml), IL-1 (20ng/ml) (PreproTech), and IL-23 (50ng/ml) (R and D Systems). Cells were cultured for 5 days and analyzed for IL-17 and IL-4 production by flow cytometry by intracellular cytokine staining. Supernatants were collected at 20–24 hours to measure IL-2 production by ELISA. In some of the experiments, IL-4 (Biolegend) and IL-2 (BD Biosciences) blocking antibodies were added at the initiation of the cultures (20μg/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 4×10^6 splenocytes per well in a round-bottom 96 well plate. Soluble anti-CD3 (5μg/ml) was used to activate T cells. Supernatants were collected at 24 hours after stimulation and placed at

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−80°C until used. For IL-6 production from spleen cultures, splenocytes were stimulated with PMA (1μM) and ionomycin (30μg/ml) for 4.5–5 hours 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 manufacturers protocol, the limit of sensitivity for the ELISA measuring IL-6 was 4pg/ml, however, our extrapolated results showed linear detection of positive controls as low as 2pg/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.

Intracellular cytokine staining

For intracellular cytokine staining, the cells from lung tissue homogenates or T_H17 differentiation cultures were re-stimulated with PMA (1μM) and ionomycin (30μg/ml), in the presence of Golgi-stop for 4.5 hours. Cells were fixed either using the BD Biosciences Kit for intracellular cytokine staining or the Foxp3 Fixation/Permeabilization Kit from eBioscience. For T_H17 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 T helper subsets from lung and spleen samples, cells were stained with the LIVE/DEAD Fixable Cell stain and blocked with Fc Block as described above. The cells were stained for intracellular cytokines using the following antibodies: 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 antibody treatment

Ndfip1−/− mice were treated with IL-6 blocking antibody (R&D Systems), using 1mg in 250μl of PBS, or with 1mg/250μl isotype control antibodies, per injection. Intraperitoneal injections were performed weekly, at 4 and 5 weeks of age, and mice were analyzed at 6 weeks 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 standard deviation of the mean.

RESULTS

Ndfip1−/− mice have an increase in lung neutrophils and T_H17 cells

Previous work from our group has shown that *Ndfip1*−/− mice develop T_H2-mediated pulmonary inflammation characterized by goblet cell hyperplasia and increased numbers of perivascular lymphocytes and eosinophils. Neutrophils, leukocytes that are associated with T_H17 rather than T_H2-mediated inflammation, were also evident within the perivascular space. To quantify the neutrophils from *Ndfip1*−/− and *Ndfip1*+/+ littermates, we analyzed cells from bronchial alveolar lavage (BAL) by both morphological and flow cytometric approaches. Examination of cytospin preparations revealed high numbers of neutrophils among cells isolated from BAL of *Ndfip1*−/− mice, but not *Ndfip1*+/+ littermate controls (Figure 1A and Supplementary Figure 1A). This result was confirmed using flow cytometry of cells isolated from BAL. Antibodies against Ly6G and CD11b, markers used to identify neutrophils (Figure 1B), revealed a significant increase in the number of neutrophils in the airspace of lungs from *Ndfip1*−/− animals. This increase in neutrophils in the lungs of

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Ndfip1−/− mice suggested that T_H17 cells might be present amongst the T_H2 cells that have been reported previously.

T_H17 cells are known to recruit neutrophils into the lung during inflammatory conditions.[33] To test whether *Ndfip1*−/− mice contained increased numbers of T_H17 cells, we isolated cells from the lungs of *Ndfip1*−/− and *Ndfip1*+/+ littermates and used flow cytometry to determine the percentage of $CD4^+$ T cells that expressed intracellular IL-17. Analysis of lung tissue homogenates from 6 week old *Ndfip1*−/− mice or *Ndfip1*+/+ littermates showed a significant increase in the percentage of T_H17 cells in the lungs of *Ndfip1*−/− mice (Figure 1C). As shown in figure 1D, the percentage of cells in the lung tissue homogenates that were $CD4^+$ and IL-17^+ was increased approximately 7 fold in *Ndfip1*−/− mice compared to *Ndfip1*+/+ littermate controls.

We next sought to analyze the percentages of T_H2 cytokine producing cells in the lung and spleen of *Ndfip1*−/− and *Ndfip1*+/+ mice, as well as with other cytokine producing CD4+ subsets. The most frequent cytokine producing subsets in both lung and spleens of *Ndfip1*−/− animals were those producing IL-4 or IL-5 (Supplementary Figure 1B). The percentages of these T_H2 cytokine-producing cells in *Ndfip1*−/− mice were, on average, 12 fold higher than those in *Ndfip1*+/+ littermates. Other cytokine producing subsets were also increased, but to a lesser extent.

Together, these data show that *Ndfip1*−/− mice have an ongoing T_H17 immune response in addition to their previously described T_H2 mediated inflammation.

Ndfip1−/− T cells have a defect in T_H17 differentiation *in vitro*

Ndfip1−/− T cells are more likely to become T_H2 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 T_H2 differentiation. Given the increased percentages of T_H17 cells in the lungs of Ndfip1-deficient mice, we hypothesized that Ndfip1 might also inhibit T_H17 differentiation. To test this, we analyzed T_H17 differentiation of *Ndfip1*+/+ and *Ndfip1*−/− T cells *in vitro*. Naïve, sorted CD4 T cells from *Ndfip1*−/− mice or *Ndfip1*+/+ littermate controls were activated with anti-CD3 and anti-CD28 in the presence of TGF-β and IL-6, conditions shown previously to induce T_H17 differentiation.[4,5] Consistent with these reports, approximately 11% of the *Ndfip1*+/+ T cells became IL-17 producing cells under these conditions. Contrary to our expectations, *Ndfip1*−/− CD4 T cells showed a reduced capacity to differentiate into T_H17 cells (Figure 2A and B), as fewer than 4% of the cells were IL-17 producers. Thus, after 5 days in culture, *Ndfip1*−/− 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 T_H17 differentiation using 0.5ug/mL of TGF-β together with IL-6, IL-1, and IL-23 (data not shown). We therefore tested the ability of naïve *Ndfip1*−/− CD4 T cells to differentiate into T_H17 cells under these conditions. After five days of culture, naïve *Ndfip1*−/− $CD4^+$ T cells again showed a reduced capacity for IL-17 production compared to $CD4^+$ T cells isolated from *Ndfip1*+/+ littermate controls (Figure 2C and D). Approximately 40% of *Ndfip1*+/+ T cells differentiated into T_H17 cells, while fewer than 10% of the Ndfip1−/− T cells became IL-17 producers. The combined data from 5–6 separate experiments is shown in Figure 2 B and D. Taken together these data show that *Ndfip1*−/− T cells are defective at differentiating into T_H17 cells *in vitro*.

IL-4 and IL-2 are cytokines that have been previously shown to inhibit T_H17 differentiation.[8–10] Ndfip1−/− T cells can produce increased levels of IL-2 and IL-4 under

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certain conditions.[27, 34] To test whether *Ndfip1*−/− T cells produce higher levels of IL-2 than their *Ndfip1*+/+ counterparts, we measured IL-2 produced by *Ndfip1*−/− or *Ndfip1*+/+ T cells after activation with anti-CD3 and anti-CD28 in the presence or absence of T_H17 polarizing conditions (Supplementary Figure 2A). When cultured in the presence of anti-CD3 and anti-CD28, *Ndfip1*−/− T cells produced a similar amount of IL-2 compared to *Ndfip1*+/+ T cells. Furthermore, under T_H17 polarizing conditions, IL-2 production was also similar between the two genotypes. These data suggest that differences in IL-2 production are unlikely to account for the defect in T_H17 differentiation in *Ndfip1*−/− T cells. Thus, we next considered a possible role for IL-4.

A previous report from our lab has shown that *Ndfip1*−/− CD4 T cells are biased towards the T_H2 subtype and make more IL-4 than *Ndfip1*+/+ T cells when cultured under T_H2 differentiating conditions.[27] Importantly, when these cells were cultured under neutral conditions, in the absence of polarizing cytokines, higher percentages of *Ndfip1*−/− CD4 T cells produced IL-4 after 5 days in culture than their WT counterparts (Supplementary Figure 2B). Given the increased capacity of *Ndfip1*−/− T cells to become IL-4 producing cells in the absence of T_H2 polarizing conditions, we sought to test whether these cells make IL-4 when activated under T_H17 polarizing conditions. Based on our previous results (Figure 2) we tested IL-4 production under conditions that produce the highest percentages of T_H17 cells, namely with TGF-β, IL-6, IL-1 and IL-23. Under these conditions, fewer than 1% of WT T cells produced IL-4. In WT cells, these percentages were similar to WT cells cultured under neutral conditions (Supplementary Figure 2B). *Ndfip1*−/− T cells, on the other hand, continued to produce IL-4 even under T_H17 polarizing conditions. However, the percentages of IL-4 producing T cells were lower in *Ndfip1*−/− T cells cultured under T_H17 conditions than under neutral conditions. Thus, TGF-β likely prevents some of the IL-4 production in these cells. Importantly, *Ndfip1*−/− T cells that produced IL-4 were not also producing IL-17 (Figure 3). These data suggest that *Ndfip1*−/− T cells can become T_H2 cytokine producing cells even under T_H17 polarizing conditions. T_H17 differentiation is unlikely to occur in these IL-4 producing cells. Furthermore, IL-4 production by these cells could prevent T_H17 differentiation by other cells in the cultures.

Blocking IL-4 restores T_H17 differentiation of *Ndfip1*−/− T cells

Given the capacity of IL-4 and IL-2 to inhibit T_H17 differentiation, we next tested whether IL-4 production or a combination of both IL-4 and IL-2 production by *Ndfip1*−/− T cells could prevent T_H17 differentiation *in vitro*. To test this, we cultured naïve *Ndfip1*+/+ and *Ndfip1*−/− CD4+ T cells under T_H17 polarizing conditions *in vitro* in the presence or absence of IL-4 and IL-2 blocking antibodies. We then measured the percentages of these $CD4^+$ T cells that differentiated into T_H17 cells. As shown above, when activated in the presence of TGF-β, IL6, IL-1, and IL-23, *Ndfip1*−/− CD4 T cells were defective in differentiating into T_H17 cells when compared to *Ndfip1*+/+ CD4 T cells. Importantly, in the presence of IL-4 blocking antibodies, the ability of *Ndfip1*−/− T cells to differentiate into T_H17 cells was restored (Figure 3A and B). Both the percentage of cells making IL-17 and the amount of intracellular IL-17 being made by the T_H17 cells reached levels similar to those of Ndfip1+/+ CD4 T cells. Furthermore, T cells from *Ndfip1*−/− *IL-4*−/− mice do not have a defect in T_H17 differentiation (Supplementary Figure 2C). These data indicate that IL-4 produced by *Ndfip1*−/− CD4 T cells prevents their differentiation into T_H17 cells.

It has been shown previously that blocking IL-2 can enhance T_H17 differentiation.[8,35] Consistent with these observations, we saw an increase in the percentage of *Ndfip1*+/+ cells that produced IL-17 in the presence of IL-2 blocking antibodies. *Ndfip1*−/− CD4 T cells also showed an increased percentage of T_H17 producing cells in the presence of the IL-2 blocking antibody. *Ndfip1*+/+ cells showed an average of a 2 fold increase, compared to a 6 fold increase for *Ndfip1*−/− cells. However, in the presence of IL-2 blocking antibodies, the

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levels of IL-4 produced by *Ndfip1*−/− T cells, and to some extent *Ndfip1*+/+ T cells, also decreased significantly (Figure 3A). This suggests that in these cultures blocking IL-2 is promoting T_H17 differentiation of *Ndfip1*−/− T cells by reducing IL-4 production. Furthermore, when used together, anti-IL4 and anti-IL2 blocking antibodies restored T_H17 differentiation to levels seen in *Ndfip1*+/+ cultures (Figure 3 A and B). Taken together these data indicate that IL-4 produced by *Ndfip1*−/− T cells prevents their differentiation into T_H17 cells. Furthermore, these results show that Ndfip1 facilitates T_H17 differentiation by inhibiting the production of IL-4.

Ndfip1−/− mice have elevated levels of IL-6

Our results show that *Ndfip1*−/− mice have an increase in T_H17 cells in the lung. Surprisingly, however, *Ndfip1*−/− CD4 T cells are less likely to differentiate into T_H17 cells *in vitro*. This suggests that conditions exist in *Ndfip1*−/− mice that support T_H17 differentiation. One possible factor required for T_H17 differentiation *in vivo* is IL-6. Thus, we analyzed the levels of IL-6 in the serum of *Ndfip1*+/+ and *Ndfip1*−/− mice. Analysis of IL-6 levels in the serum of 6 week old mice by ELISA showed higher amounts of IL-6 in *Ndfip1*−/− mice than *Ndfip1*+/+ littermate controls (Figure 4A). Thus, increased IL-6 *in vivo* could promote the T_H17 differentiation of CD4+ T cells observed in *Ndfip1*−/− mice. To test whether the increased level of IL-6 seen in *Ndfip1*−/− mice could account for the increased percentages of T_H17 cells in the lungs, we treated *Ndfip1*−/− mice with an anti-IL-6 blocking antibody. Starting at 4 weeks of age, IL-6 blocking antibody was administered for two weeks. Following this treatment, we analyzed BAL fluid for the numbers of neutrophils and lung tissue homogenates for the presence of T_H17 cells. *Ndfip1*−/− mice treated with isotype control showed similar numbers of neutrophils in their BAL fluid compared to untreated *Ndfip1*−/− mice (Figure 4B versus Supplementary Figure 1A), however treatment with IL-6 blocking antibodies led to a significant reduction in neutrophil numbers (Figure 4C). Eosinophil numbers were also reduced (Figure 4C). While isotype control-treated controls showed similar percentages of T_H17 cells to untreated mice, *Ndfip1*−/− mice that received IL-6 blocking antibody had significantly fewer T_H17 cells than isotype control-treated animals (Figure 4D). While the percentages of T_H17 cells were reduced, the percentages of other cytokine producing CD4+ T cells were less affected (Supplementary Figure 3). Importantly, the reduced percentages of T_H17 cells correlated with decreased lung inflammation (Figure 4E). These results indicate that mice treated with anti-IL-6 antibodies have lower levels of T_H17 cells in the lungs, supporting our hypothesis that the high levels of IL-6 facilitate T_H17 differentiation in *Ndfip1*−/− mice.

Together our data show that *Ndfip1*−/− CD4 T cells are partially defective in T_H17 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 T_H17 differentiation *in vivo*, and explains why we observe increased percentages of the T_H17 effector cell subtype in *Ndfip1*−/− mice compared to 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 T_H17 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 (Figure 5A and 5B and data not shown). While we could detect IL-6 production in CD4+ cells, the majority (about 86%) of the splenocytes

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making IL-6 were CD4 negative cells (Figure 5B). Amongst 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+ (Figure 5C) population 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 (Figure 5D and 5E). Moreover, more of the eosinophils in *Ndfip1*−/− mice are activated as indicated by FcεRIα expression and are making IL-6 (Figure 5F and 5G). Based on these data, eosinophils are the source of IL-6 that promotes T_H17 differentiation *in vivo* in mice lacking Ndfip1.

The T_H2 inflammatory environment in Ndfip1-deficient mice promotes T_H17 differentiation

Our *in vitro* data shows that IL-4 can directly decrease T_H17 differentiation, while our *in vivo* data suggests that T_H2-mediated inflammation may promote T_H17 differentiation indirectly by increasing the levels of IL-6. It is possible that even in the presence of IL-4-producing T_H2 cells, the inflammatory conditions in *Ndfip1*−/− mice promote the differentiation of T_H17 cells. To test this, we crossed *Ndfip1*−/− mice to *IL-4*−/− animals, and analyzed the percentages of T_H17 cells in the lungs, IgE levels in the serum (Supplementary Figure 4A) and IL-6 production from splenocytes in the resulting *Ndfip1*−/−, *IL-4*−/− and *Ndfip1*−/−*IL-4*−/− progeny.

As illustrated in figure 6, *Ndfip1*−/− *IL-4*−/− double knockout (DKO) mice showed a lower level of IL-6 in the serum compared to *Ndfip1*−/− *IL-4*+/+ littermate controls (Figure 6A) as well a decreased IL-6 production by *ex-vivo* splenocytes (Figure 6B), indicating that the T_H2 response in *Ndfip1*−/− mice is contributing to the high levels of IL-6 in these mice. Supporting this, eosinophils in the spleens of *Ndfip1*−/− *IL-4*−/− DKO mice showed reduced intracellular IL-6 (Figure 6C). Furthermore, *Ndfip1*−/− *IL-4*−/− DKO mice contained fewer neutrophils in the BAL fluid (Supplementary Figure 4B) and, in addition to the decrease in T_H2 cytokine producing cells (Supplementary Figure 4C), also showed lower percentages of T_H17 cells in the lungs compared to *Ndfip1*−/− *IL-4*+/+ controls (Figure 6D). Additionally, there is much less inflammation in the lungs of *Ndfip1*−/− *IL-4*−/− DKO mice compared to *Ndfip1*−/− mice (unpublished results Natalia Ramos and Allison Beal). These results indicate that the T_H2 inflammatory environment seen in *Ndfip1*−/− mice contributes to the differentiation of T_H17 cells. 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 T_H2-mediated inflammation.

DISCUSSION

A previously published report from our lab shows that mice deficient in Ndfip1 develop T_H2-mediated inflammation.[27] This phenotype can be explained by the role of Ndfip1 in the regulation of Itch-mediated ubiquitilation and degradation of JunB, a transcription factor that regulates the expression of the T_H2 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 *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 T_H2 response, *Ndfip1*−/− mice have elevated numbers of neutrophils and increased percentages of T_H17 cells in the lungs.

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The increase in lung T_H17 cells in these mice led us to hypothesize that Ndfip1 might prevent T_H17 differentiation, and that its absence would cause an increase in T_H17 cells *in vitro* and *in vivo*. Thus, we tested the role of this adaptor protein in T_H17 differentiation *in vitro*. Unexpectedly, our results showed that *Ndfip1*−/− CD4 T cells are defective in differentiating into the T_H17 subset *in vitro*. *Ndfip1*−/− CD4 T cells are more likely to become T_H2 cells compared to WT counterparts, in the presence of T_H2 driving conditions. Our results further show that even under neutral or T_H17-driving conditions, *Ndfip1*−/− CD4 T cells make IL-4 after activation. IL-4 has been previously shown to inhibit T_H17 differentiation.[9,10,38] Consistent with these results, the IL-4 produced by *Ndfip1*−/− CD4 T cells significantly inhibited their T_H17 differentiation *in vitro*. This is not necessarily due to a direct inhibition of T_H17 differentiation by JunB, as these results indicated that the defect in T_H17 differentiation seen in *Ndfip1*−/− cells is dependent on signaling through the IL-4 receptor. Given the role of Ndfip1 in the negative regulation of T_H2 cytokine expression, our results show that this adaptor protein acts to promote T_H17 differentiation by preventing IL-4 production.

It has been reported that TGF-β plays an important role in the inhibition of T_H2 cytokine expression during T_H17 differentiation as well as during iTreg cell differentiation.[39] We showed recently that TGF-β dampens IL-4 production by Ndfip1-dependent as well as Ndfip1-independent mechanisms.[34] We now show that this is true during T_H17 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 T_H17 subtype. Thus, CD4 T cells have at least two mechanisms that prevent IL-4 production during T_H17 differentiation, one of which depends on Ndfip1.

Our *in vitro* experiments show that *Ndfip1*−/− CD4 T cells are defective in differentiating into T_H17 cells; however, *in vivo*, *Ndfip1*−/− mice show increased numbers in lung T_H17 cells. Given that IL-6 plays a major role in the differentiation of T_H17 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 T_H17 cells in *Ndfip1*−/− mice indicating that this cytokine promotes T_H17 differentiation in our system. Therefore, although *Ndfip1*−/− CD4 T cells are less likely to undergo T_H17 differentiation *in vitro*, the presence of IL-6 promotes T_H17 differentiation *in vivo* leading to an increase in lung T_H17 cells and neutrophilia as compared to healthy *Ndfip1*+/+ mice.

The inflammatory environment within Ndfip1-deficient mice contains factors that promote and inhibit T_H17 differentiation. Our results indicate that high levels of IL-6 in *Ndfip1*−/− mice are conducive to T_H17 differentiation. On the other hand, *Ndfip1*−/− mice have a highly T_H2 polarized cytokine environment, where IL-4 could prevent T_H17 differentiation. In order to test the effect of the observed T_H2 environment on T_H17 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 to mice lacking only Ndfip1, indicating that the T_H2 inflammation in *Ndfip1*−/− mice might lead to higher IL-6 expression. Furthermore, *Ndfip1*−/− *IL-4*−/− mice contained fewer lung T_H17 cells compared to an *Ndfip1*−/−*IL-4*+/+ littermate controls. These results suggest that the T_H2 cytokine production in *Ndfip1*−/− mice leads to recruitment and activation of IL-6 producing eosinophils, which promotes T_H17 differentiation. While IL-4 can directly decrease the percentage of CD4 T cells differentiating into the T_H17 subtype, a T_H2 response can lead to significant inflammation and promotes the production of IL-6 by eosinophils, which increases T_H17 differentiation. Therefore, a balance between the direct inhibition of IL-4 and that of inflammation and IL-6 can determine the outcome of an

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accompanying T_H17 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*−/− mice can tilt the balance towards T_H17 differentiation (Figure 7). Furthermore, our results help to resolve the apparent discrepancy between *in vitro* data showing that IL-4 can directly impair T_H17 differentiation[9,10,38] and *ex vivo* data showing elevated levels of T_H17 cells in asthmatic patients despite a predominant T_H2 response[12–14]. Based on the results presented here, we propose that the missing link connecting the T_H2 and T_H17 responses *in vivo* is eosinophil production of IL-6.

Here we show that *Ndfip1*−/− mice have an ongoing T_H17 response in the lung in addition to their previously described T_H2-mediated inflammation. This is also seen in atopic asthma, since high levels of IL-17, as well as an increase in T_H17 cells, have been observed in the lungs of asthmatic patients.[12–14] Using mouse models of asthma, it has been shown that T_H17 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 T_H17 response can promote a T_H2 response and increase lung eosinophilia,[20–22] which is mediated by an increase in the expression of eotaxin.[40] Here we show that a T_H2 response can further promote T_H17 differentiation through the production of the T_H17-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 T_H2 and T_H17 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 T_H1-mediated autoimmune disease such as rheumatoid arthritis or CD,[41] but also expressed during T_H2-mediated diseases such as asthma.[42] Although there are cases where an immune response is directly guided towards a T_H17 response, it is possible that inflammation and its consequent tissue damage generally promote T_H17 differentiation, explaining why T_H17 cells are found along with both T_H1 or T_H2 responses. The results presented here support the idea that inflammation, even if it is highly T_H2-polarized, can lead to the differentiation of T_H17 cells through the induction of the proinflammatory cytokine IL-6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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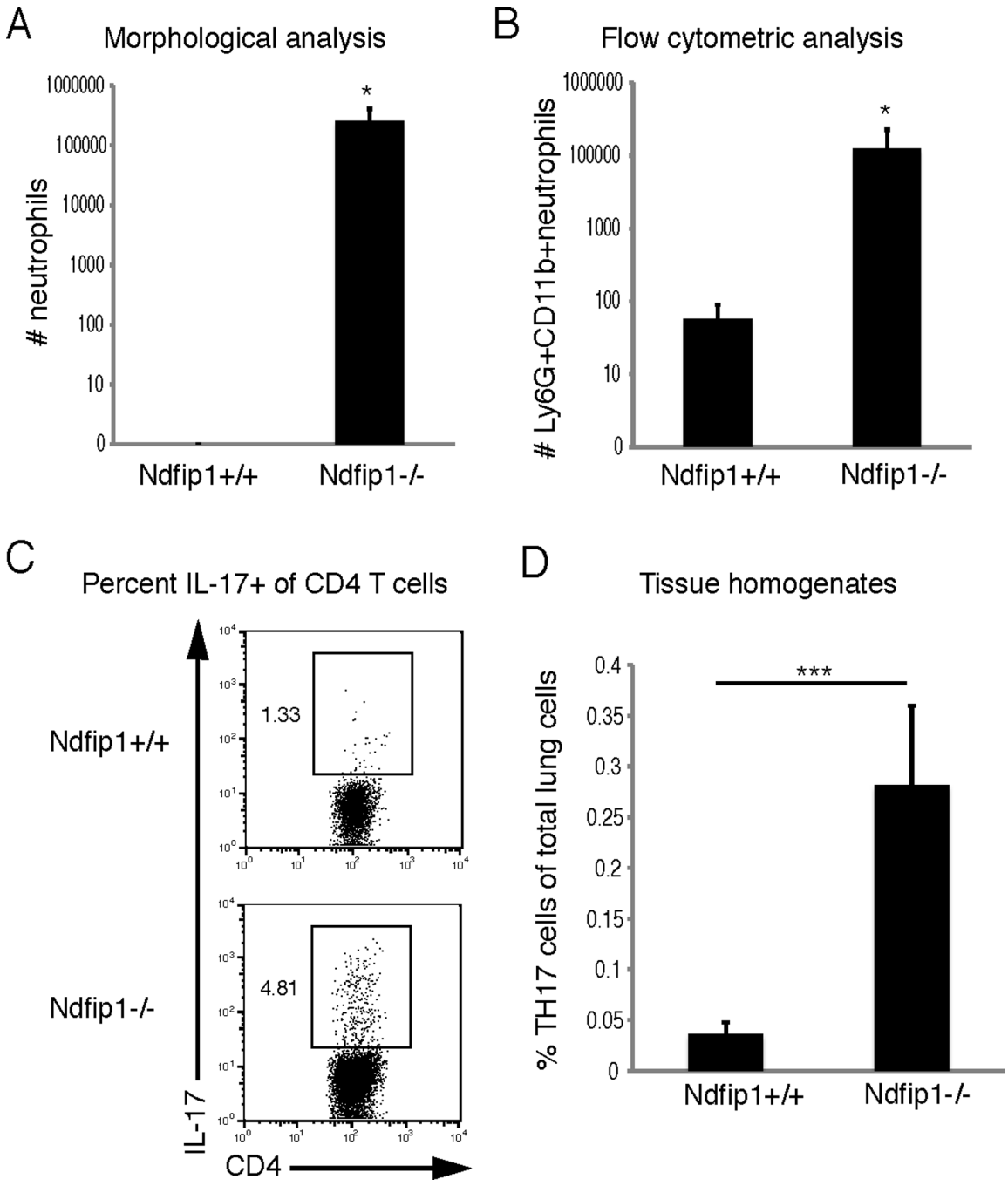

Figure 1. *Ndfip1*−/− mice have increased lung neutrophils and T_H17 cells

(A) Number of neutrophils in the BAL fluids form *Ndfip1*+/+ or *Ndfip1*−/− mice as determined by cytospin. This graph includes the average and standard deviation from 3 different mice of each genotype. (B) Number of neutrophils ($Ly6G^+$ $CD11b^+$) from the BAL fluids of these mice, analyzed by flow cytometry, using antibodies against Ly6G and CD11b, including data from 5 different mice of each genotype. (C) The percentage of CD4 T cells that are IL-17^+ in lung tissue homogenates from *Ndfip1*−/− vs. *Ndfip1*+/+ mice. (D) The percentage of lung cells that are $CD4^+$ IL-17^+ (T_H17) in lung tissue homogenates from *Ndfip1*+/+ or *Ndfip1*−/− mice, including the average and standard deviation from 7 different

mice of each genotype. * refers to a significant difference between *Ndfip1*+/+ and Ndfip1−/−, $p<0.01$; *** refers to $p<0.0001$.

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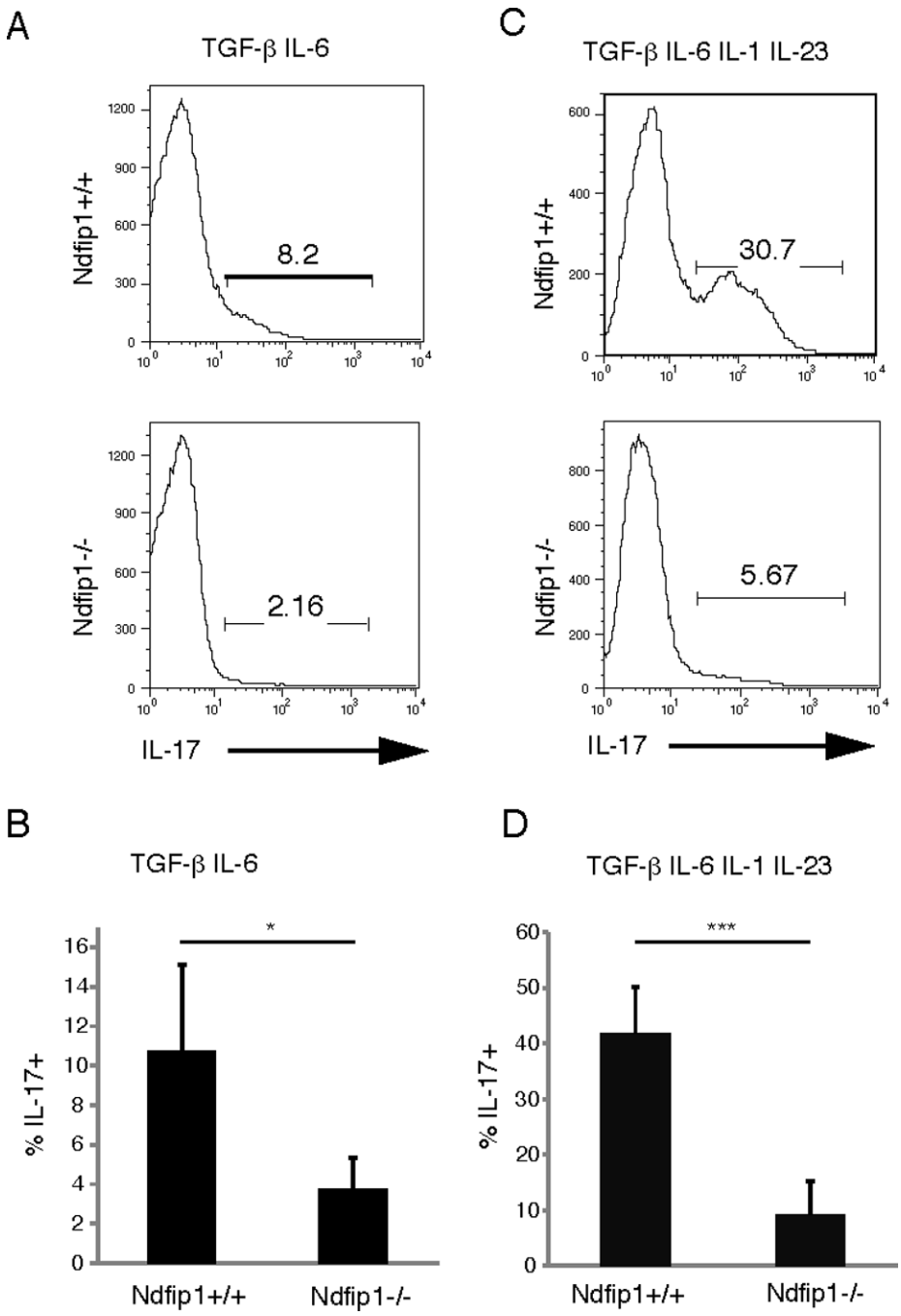

Figure 2. *Ndfip1*−/− CD4 T cells are defective in differentiating into T_H17 cells

(A) Naïve sorted CD4 T cells were activated with plate-bound anti-CD3 and anti-CD28 in the presence of TGF-β and IL-6. The percentage of IL-17$^+$ cells was analyzed by flow cytometry after 5 days of culture. (B) Same as A, shown as the combined data from 5 different experiments. (C) Naïve sorted CD4 T cells were activated with CD3 and CD28 in the presence of TGF-β, IL-6, IL-1, and IL-23. The percentage of IL-17$^+$ cells was analyzed by flow cytometry after 5 days of culture. (D) Same as B, shown as the combined data from 6 different experiments. * refers to $p<0.01$; *** refers to $p<0.001$.

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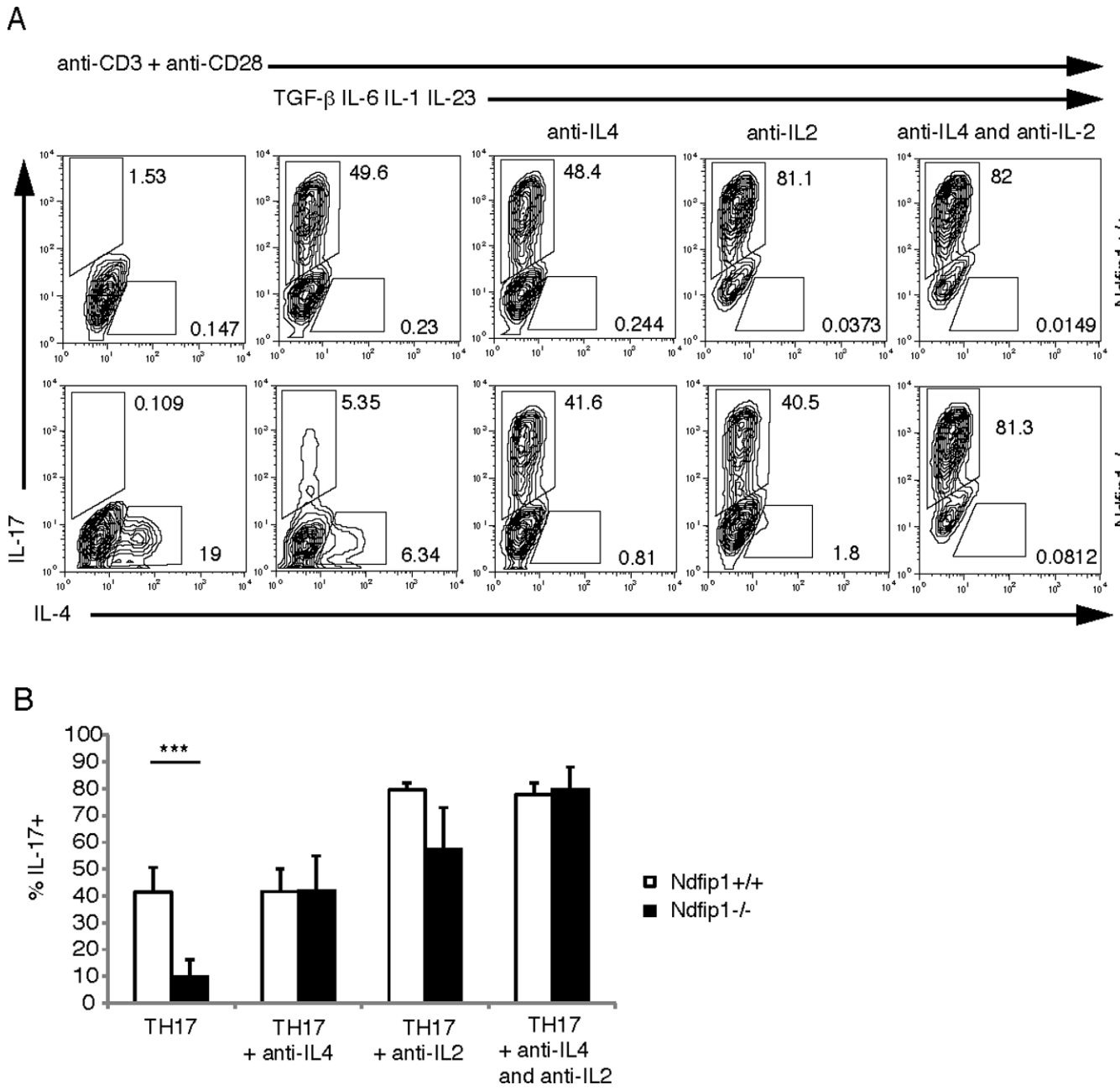

Figure 3. Blocking of IL-4 restores the defect in T_H17 differentiation

(A) Sorted naïve CD4 T cells were activated with plate-bound anti-CD3 and anti-CD28 in the presence of TGF-β, IL-6, IL-1, and IL-23 and blocking antibodies against IL-4 and/or IL-2 as specified. The percentage of IL-17 and IL-4 producers was analyzed using flow cytometry on day 5 of culture. This data is representative of 5 different experiments. (B) Combined data from 5 experiments. Open bars represent *Ndfip1*+/+ and black bars represent *Ndfip1*−/− T cells. *** p< 0.005.

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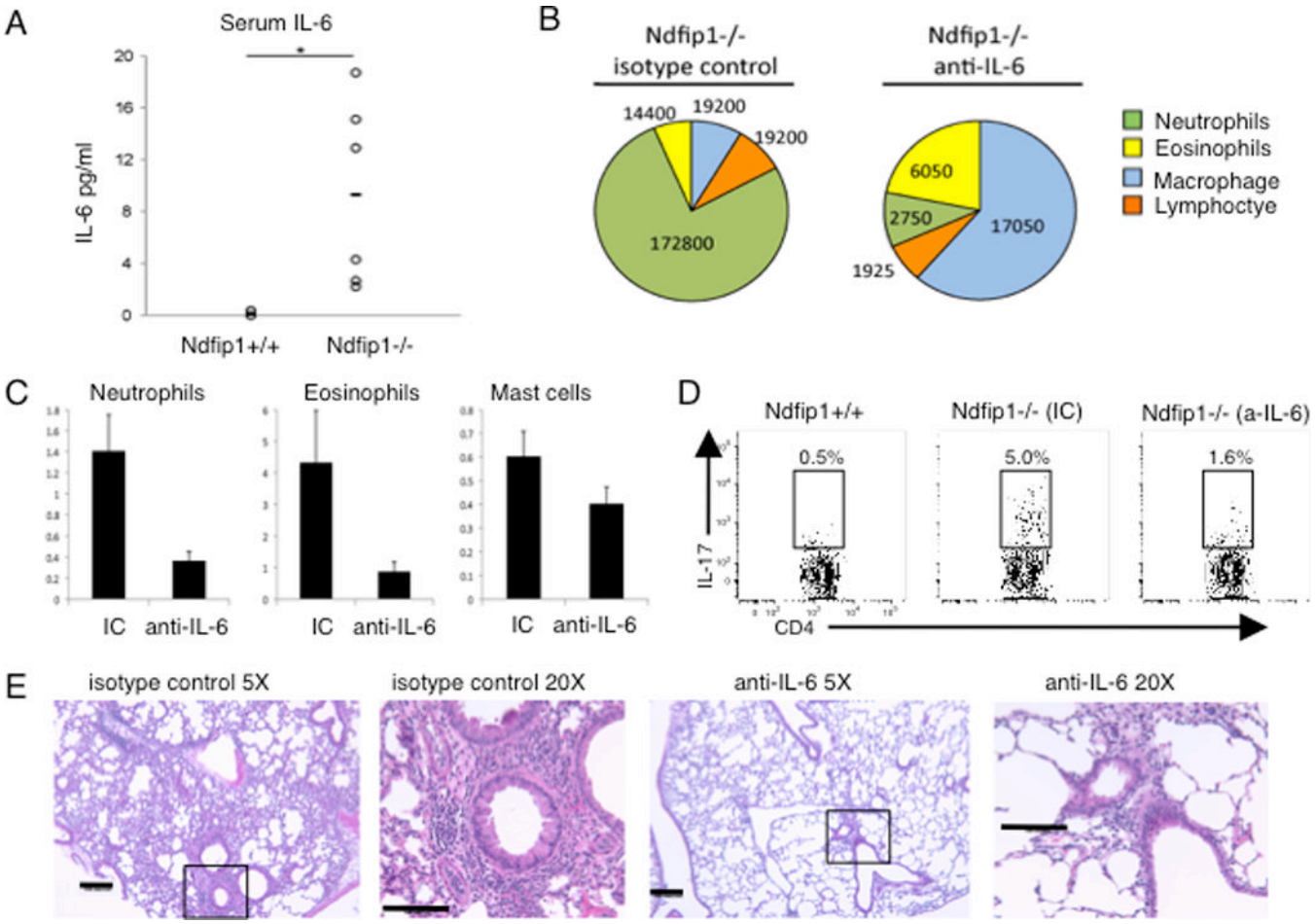

Figure 4. ***Ndfip1*−/− mice have elevated levels of IL-6 that promotes T_H17 differentiation**

(A) IL-6 levels in the serum from *Ndfip1*−/− or *Ndfip1*+/+ littermate controls measured by ELISA. Each circle represents a different mouse and there are 6 mice of each phenotype included. *p<0.05. (B–D) *Ndfip1*−/− mice were treated with anti-IL6 or isotype control (IC) on weeks 4 and 5 and analyzed on week six of life. (B) BAL fluid from *Ndfip1*$^{-/-}$ mice treated either with anti-IL-6 or IC was collected and the number of the indicated cells was determined by cytospin. A representative pie chart is shown for each treatment group, n=2 (IC) and n=3 (anti-IL-6). (C) The number of neutrophils (Ly6G$^+$ CD11b$^+$), eosinophils (SiglecF$^+$, Ly6G$^-$) and Mast cells (FcεRIα$^+$ SiglecF$^-$) in the lung tissue homogenate was determined by flow cytometry. The homogenate was from half of the lung tissue. Graphs show the mean + s. d. (D) The percentage of lung cells from tissue homogenates that were CD4$^+$ and IL-17$^+$ were analyzed by flow cytometry. The plots shown are representative of 2–3 mice of the indicated genotypes and treatments. (E) Lungs from *Ndfip1*−/− mice treated with isotype control (IC) or anti-IL-6 were perfused, inflated with fixative and sectioned. H and E stained sections were analyzed at 5X and 20X. Bar shows 100 microns.

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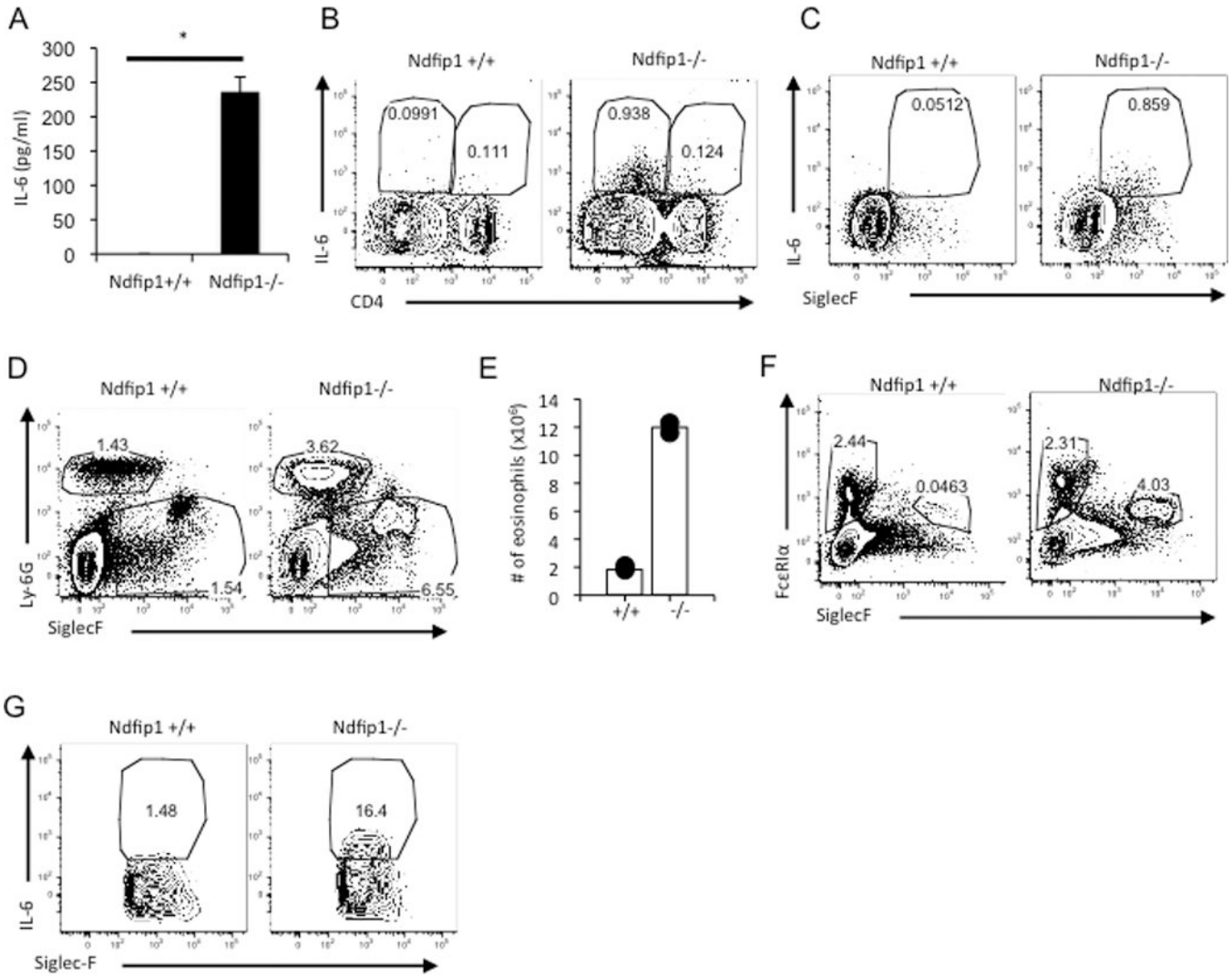

Figure 5. Eosinophils are significant producers of IL-6 in *Ndfip1*−/− mice

(A) The amount of IL-6 in the supernatants of splenocytes restimulated for 4.5–5 hours with PMA and ionomycin was measured by ELISA. Bar graph represents the mean + s.d. of triplicate samples from one mouse for each genotype. The results are representative of 3–4 mice per genotype. *p<0.00005. (B and C) Splenocytes were restimulated with PMA and ionomycin and then stained and analyzed by flow cytometry as described in materials and methods for surface markers and intracellular IL-6. (B) Representative flow plots gated on live cells are shown, n=3 mice per genotype. (C) Representative flow plots gated on live $CD4^-$ cells are shown, n=3 mice per genotype. (D) Representative facs plots of flow cytometric analysis from the spleens of indicated mice. Plots are gated on live cells and show the $Ly6G^{high}$ neutrophil population and $Ly6G^{low/neg}$ $SiglecF^+$ eosinophil population, n=2 mice per genotype. (E) The numbers of eosinophils ($Ly6G^{low/neg}$ $SiglecF^+$) from the mice in D are shown. (F) Splenocytes were analyzed for the percentage of $Fc\varepsilon RI\alpha^+$ $SiglecF^-$ mast cells and $Fc\varepsilon RI\alpha^+$ $SiglecF^+$ activated eosinophils. Representative plots are shown. n=2–3 mice per genotype. (G) Cells were stimulated as in B and C. IL-6 production by the eosinophil population is shown in the plots (Gated on $CD4^-$ $Ly6G^{low/neg}$ $SiglecF^+$ cells), n=3 mice per genotype.

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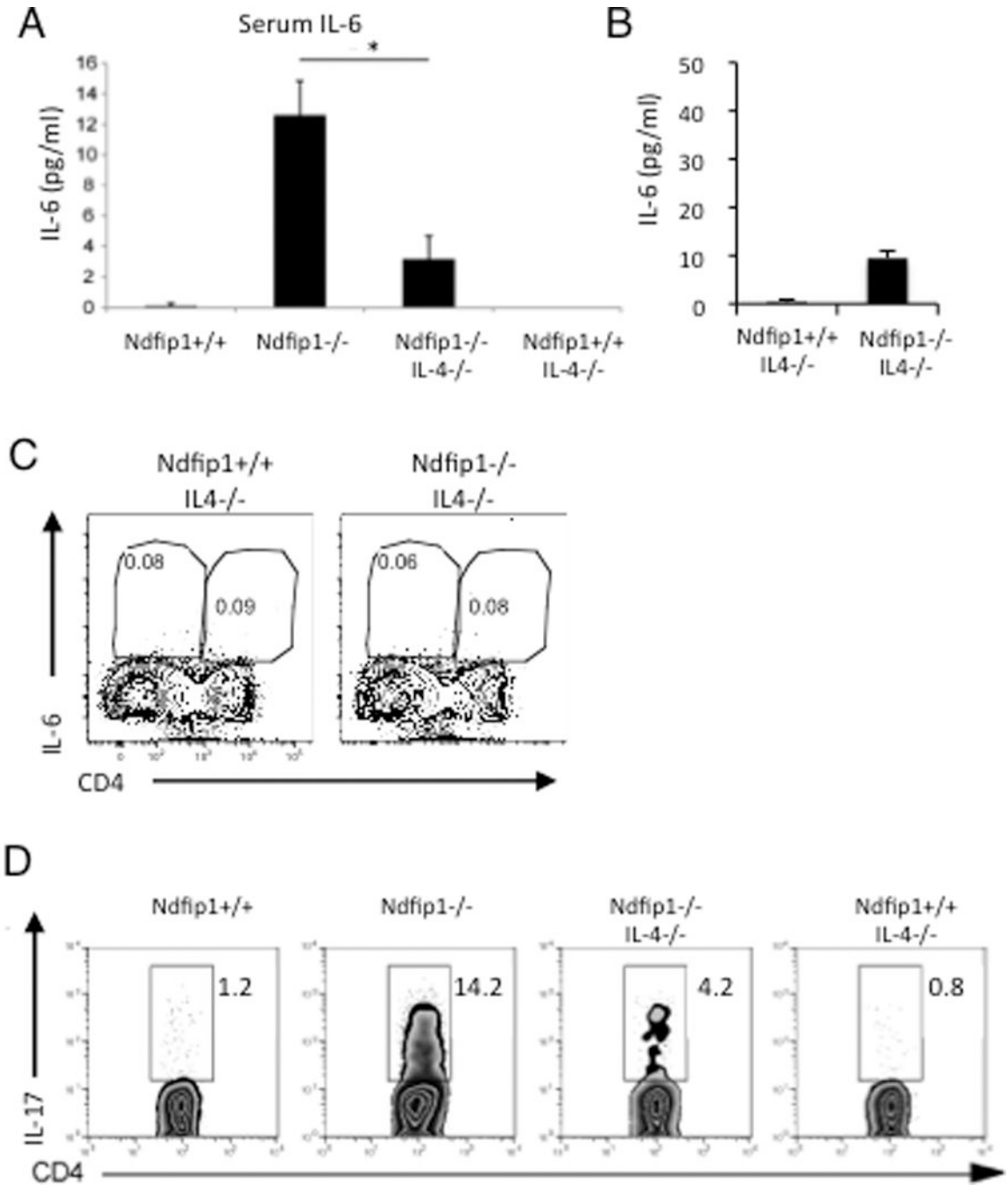

Figure 6. The T_H2 environment in *Ndfip1*−/− mice contributes to IL-6 expression and T_H17 differentiation

(A) IL-6 from the serum of the indicated mouse phenotypes analyzed through ELISA. *p<0.05. (B) The amount of IL-6 in the supernatants of splenocytes restimulated with PMA and ionomycin was measured by ELISA. Bar graph represents the mean + s.d. of triplicate samples from one mouse for each of the indicated genotype. The results are representative of 3 mice per genotype. **p<0.001. (C) Splenocytes were restimulated as indicated in the materials and methods and then analyzed by flow cytometry as described in materials and methods. (B) Representative flow plots gated on live cells are shown, n=3 mice per genotype. (D) Percentage of $CD4^+$ T cells that express IL-17, from lungs tissue

homogenates, analyzed through flow cytometry. Data are representative of three mice of the *Ndfip1*$^{+/+}$, *Ndfip1*$^{-/-}$ *IL-4*$^{-/-}$, and *Ndfip1*$^{+/+}$ *IL-4*$^{-/-}$ genotypes, and 4 *Ndfip1*$^{-/-}$ mice.

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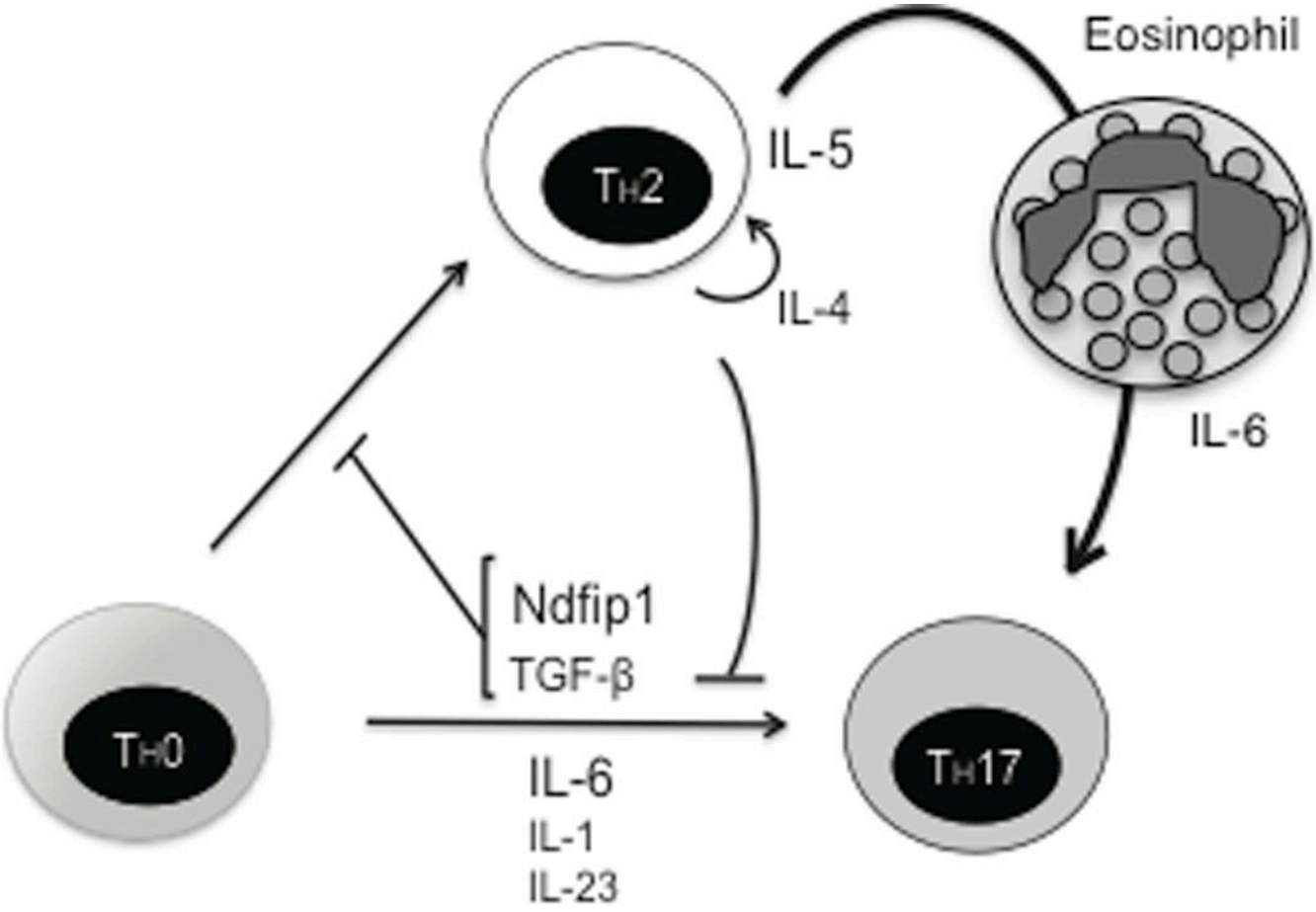

Figure 7. Model representing the role of Ndfip1 in T_H17 differentiation

Ndfip1, along with TGF-β, promotes T_H17 differentiation by inhibiting IL-4 expression. While IL-4 can directly inhibit T_H17 differentiation, a T_H2 response can lead to significant inflammation and IL-6 production predominantly by eosinophils to create an environment that supports the differentiation of T_H17 cells.