The EMBO Journal (2011) 30, 2071–2082 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
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THE EMBO JOURNAL

NFIL3/E4BP4 controls type 2 T helper cell cytokine expression

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Type 2 T helper (T_H2) cells are critical for the development of allergic immune responses; however, the molecular mechanism controlling their effector function is still largely unclear. Here, we report that the transcription factor NFIL3/E4BP4 regulates cytokine production and effector function by T_H2 cells. NFIL3 is highly expressed in T_H2 cells but much less in T_H1 cells. Production of interleukin (IL)-13 and IL-5 is significantly increased in *Nfil3*$^{-/-}$ T_H2 cells and is decreased by expression of NFIL3 in wild-type T_H2 cells. NFIL3 directly binds to and negatively regulates the *Il13* gene. In contrast, IL-4 production is decreased in *Nfil3*$^{-/-}$ T_H2 cells. Increased IL-13 and IL-5 together with decreased IL-4 production by antigen-stimulated splenocytes from the immunized *Nfil3*$^{-/-}$ mice was also observed. The ability of NFIL3 to alter T_H2 cytokine production is a T-cell intrinsic effect. Taken together, these data indicate that NFIL3 is a key regulator of T_H2 responses.

The EMBO Journal (2011) **30,** 2071–2082. doi:10.1038/emboj.2011.111; Published online 15 April 2011
Subject Categories: immunology
Keywords: helper T-cell differentiation; IL-13; IL-4; transcriptional regulation

Introduction

Immune responses to certain parasites or those underlying atopy are primarily driven by type 2 helper T (T_H2) cells through the production of specific cytokines including interleukin (IL)-4, IL-5, and IL-13. These cytokines have separate but overlapping functions. IL-4 has a role in the regulation of immunoglobulin (Ig) class switching to IgE in B cells, in the activation and proliferation of B cells, and also in promoting T_H2 responses. Furthermore, IL-4 upregulates MHC class II on B cells and IgE receptors on inflammatory cells, resulting in the enhancement of antigen presenting ability to T cells and IgE-mediated secretion of inflammatory mediators, respectively (Nelms *et al*, 1999). IL-13 induces IgE class switching in human B cells and allergic inflammation (Wills-Karp *et al*, 1998). IL-5 has a central role in eosinophilia by chemoattracting eosinophils and regulating the activation, expansion, and differentiation of eosinophils. IL-5 is produced not only by T_H2 cells but also by eosinophils themselves, which serves to autoregulate and augment their accumulation in the airways. Other T_H2 cytokines including IL-9 and IL-25 (IL-17E) are also thought to be involved in the pathogenesis of atopic immune responses (Barrett and Austen, 2009; Hamid and Tulic, 2009).

The production of T_H2 cytokines is controlled by several transcription factors including STAT6, GATA-3, NFAT, NF-κB, c-Maf, and AP-1 (Li-Weber and Krammer, 2003; Gilmour and Lavender, 2008). STAT6 directly mediates IL-4 signalling leading to the transcriptional activation of target genes (Nelms *et al*, 1999). Expression of GATA-3 is very low in naive CD4$^+$ T cells but is upregulated during T_H2 differentiation (Ho *et al*, 2009). Studies utilizing mice lacking genes encoding these factors have demonstrated their importance in T_H2 cytokine production (Cousins *et al*, 2008). Recently, Dec2 was also reported as critical for the commitment and promotion of T_H2 differentiation (Liu *et al*, 2009; Yang *et al*, 2009). These transcription factors are believed to act as positive regulators for T_H2 differentiation and T_H2 cytokine production. The transcriptional repressor Mina has been shown to repress *Il4* gene expression by directly binding to its promoter, thereby, controlling T_H2 bias (Okamoto *et al*, 2009). Under T_H1 conditions, IRF1 and IRF2 have been shown to repress *Il4* promoter activity (Elser *et al*, 2002). However, the mechanism of negative regulation by which T_H2 differentiation and thus T_H2-driven pathologies are prevented is still largely unknown.

To elucidate the regulation of helper T-cell differentiation, many studies have focused on the identification of genes specifically expressed or repressed during the differentiation process. Microarray studies have shown that *Nfil3* (nuclear factor, IL-3 regulated, also called *E4bp4*) expression increases during T_H2 differentiation (Chen *et al*, 2003; Lund *et al*, 2003, 2005; Lu *et al*, 2004), suggesting that NFIL3 may be involved in the regulation of T_H2 differentiation and effector function. NFIL3 is a basic leucine zipper transcription factor that was identified as a transcriptional activator for the human IL-3 promoter and a transcriptional repressor for the adenovirus E4 promoter (Cowell *et al*, 1992; Zhang *et al*, 1995). NFIL3 has structural similarity in its basic region and extended region to PAR (proline and acidic residue-rich) family proteins, and NFIL3 and PAR family protein bind to similar DNA sequences (Cowell, 2002). To date, most studies of NFIL3 have focused on its role in the regulation of circadian rhythm due to its oscillatory pattern of expression in both the suprachiasmatic nucleus and peripheral tissues as well as its association with the regulation of *Per2* gene expression (Mitsui *et al*, 2001; Cowell, 2002; Ueda *et al*, 2005; Akashi *et al*, 2006; Ohno *et al*, 2007). *Nfil3* expression is very low in many cell types and is induced in response to cytokines and hormones (Ikushima *et al*, 1997; Altura *et al*, 1998; Lang *et al*, 2002; Chen *et al*, 2003; Ozkurt and Tetradis, 2003; Sartipy and Loskutoff, 2003; Ramsborg and Papoutsakis, 2007). *Nfil3* expression is minimal in immune cells including B cells and T cells, but is strongly induced by IL-4 stimulation in these cells (Chu and Paul, 1998; Schroder *et al*, 2002; Chen *et al*,

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Received: 12 August 2010; accepted: 17 March 2011; published online: 15 April 2011

2003; Kashiwada *et al*, 2010). Recently, we demonstrated that *Nfil3*$^{-/-}$ mice showed impaired IgE class switching in B cells, suggesting a role for NFIL3 in IL-4-induced IgE class switching (Kashiwada *et al*, 2010). *Nfil3* is highly expressed in natural killer (NK) cell and NKT cells (Gascoyne *et al*, 2009; Kamizono *et al*, 2009). Interestingly, *Nfil3*$^{-/-}$ mice lack NK cells, suggesting that NFIL3 is critical for NK cell development (Gascoyne *et al*, 2009; Kamizono *et al*, 2009; Kashiwada *et al*, 2010). These observations suggest that NFIL3 has several important roles in the immune system.

To explore the role of NFIL3 in T_H2 differentiation and T_H2 cytokine production, we examined T_H2 cytokine production in *Nfil3*$^{-/-}$ T_H2 cells. We found that *Nfil3*$^{-/-}$ T_H2 cells produced significantly more IL-13 and IL-5, but less IL-4, compared with wild-type (WT) T_H2 cells. In addition, we found that NFIL3 directly binds and negatively regulates *Il13* gene expression. In contrast, NFIL3 indirectly regulates *Il4* gene expression. Immunization of *Nfil3*$^{-/-}$ mice demonstrated that NFIL3 modulated T_H2 responses *in vivo*. These findings demonstrate that NFIL3 is a critical regulator of T_H2 cytokine production by CD4$^+$ T cells.

Results

Reduced IL-4-producing T_H2 cells in the absence of NFIL3 in vitro

Microarray studies have demonstrated that several sets of genes are differentially expressed during T_H1 and T_H2 cell differentiation. Expression of *Nfil3* gene is induced in both T_H1 and T_H2 cultures although its expression level in T_H2-skewed cells is significantly higher than that in T_H1-skewed cells (Chen *et al*, 2003; Lund *et al*, 2003, 2005; Lu *et al*, 2004). This preferential expression of *Nfil3* mRNA in T_H2 cells suggests a role for NFIL3 in T_H2 differentiation and/or T_H2 effector function. In order to further evaluate this, we examined the expression of *Nfil3* mRNA and NFIL3 protein in T_H1- and T_H2-skewed cells. Naive CD4$^+$ T cells from the spleen of WT and *Nfil3*$^{-/-}$ mice were cultured for 7 days under T_H1 or T_H2 polarizing conditions, and the levels of *Nfil3* mRNA and NFIL3 protein were determined by real-time RT–PCR and western blotting, respectively. As expected from the microarray data, both mRNA and protein for NFIL3 were highly expressed in T_H2-skewed cells (Figure 1A). Apparent but lower expression of *Nfil3* mRNA was also detected in T_H9 cells compared with T_H2 cells, which also require IL-4 for their differentiation (Dardalhon *et al*, 2008; Veldhoen *et al*, 2008), and *Nfil3* expression was much lower in other helper T-cell lineages (Supplementary Figure S1A). To assess the role of NFIL3 in T_H1/T_H2 cell differentiation, we examined T_H1/T_H2 differentiation of naive CD4$^+$ T cells from *Nfil3*$^{-/-}$ mice by monitoring the production of IFN-γ and IL-4. Under T_H1-skewing conditions, *Nfil3*$^{-/-}$ T cells expressed levels of *Ifng* mRNA comparable to WT T cells whereas *Il4* mRNA expression in T_H2-skewed *Nfil3*$^{-/-}$ T cells was significantly reduced (Figure 1B). Consistent with these observations, levels of the expression of transcription factors critical for

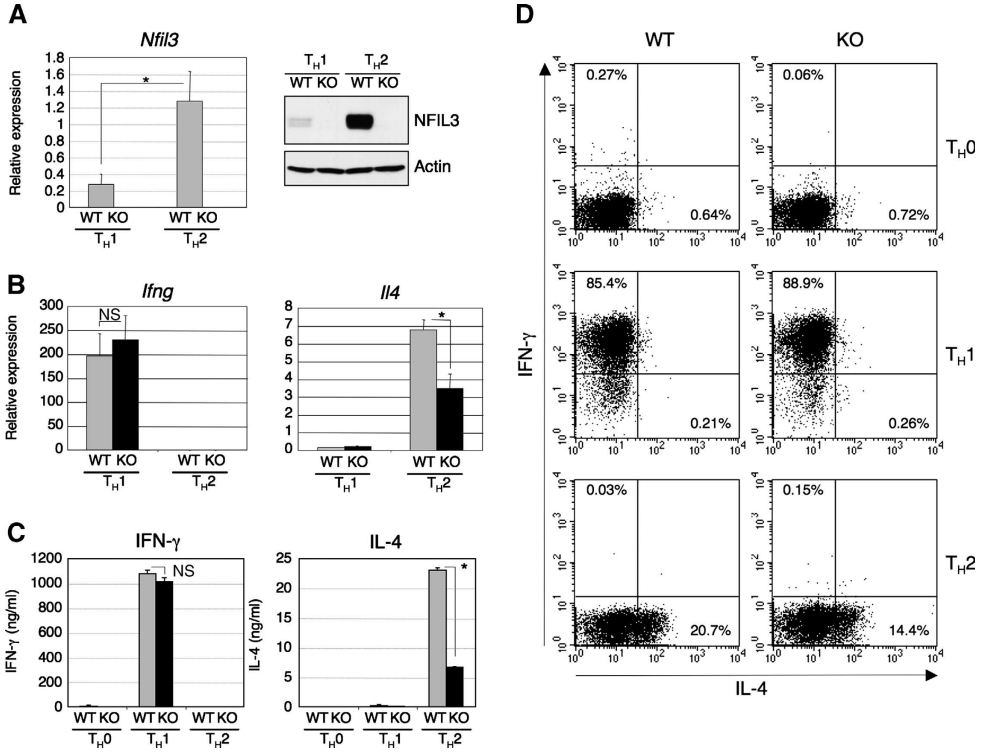

Figure 1 Altered IL-4-producing T_H2 differentiation in the absence of NFIL3. (**A**) Expression of *Nfil3* mRNA and NFIL3 protein in T_H1 and T_H2 cells differentiated *in vitro*. Expression of *Nfil3* mRNA was determined by quantitative real-time RT–PCR and data shown are the mean and s.d. from four experiments (left, $^*P<0.01$). Expression of NFIL3 protein was determined by western blot analysis and data are representative of four experiments with similar results (right). (**B**) Quantitative real-time RT–PCR analysis of *Ifng* and *Il4* mRNA in T_H1 and T_H2 cells differentiated *in vitro*. Data show the mean and s.d. from four experiments (n.s., not significant; $^*P<0.001$). (**C**) Secretion of IFN-γ and IL-4 by T_H1 and T_H2 cells restimulated with anti-CD3/CD28 for 24 h. Cytokine concentration was determined by ELISA and data show the mean and s.d. from four experiments (n.s., not significant; $^*P<0.0001$). (**D**) Intracellular staining for IFN-γ and IL-4 of T_H0, T_H1, and T_H2 cells differentiated *in vitro*. Restimulated cells were stained and analysed by flow cytometry. Data are representative of four experiments with similar results.

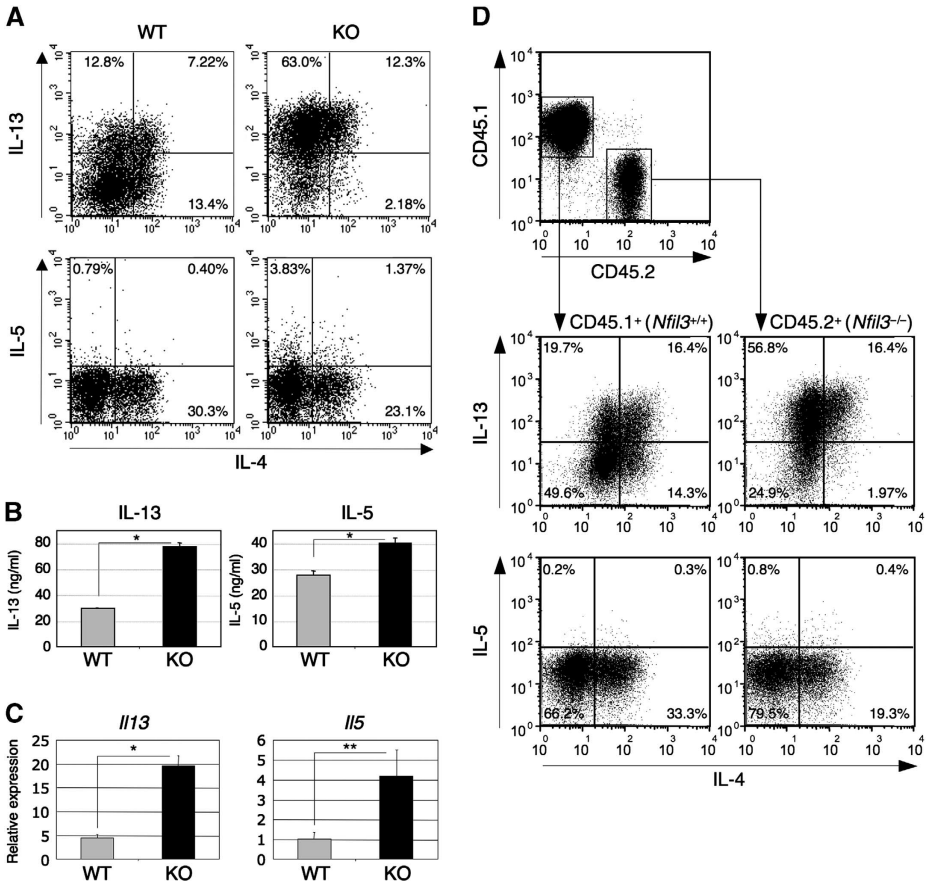

Figure 2 Altered T_H2 cytokine production in *Nfil3*$^{-/-}$ T_H2 cells. (**A**) Increased IL-13 and IL-5 production in *Nfil3*$^{-/-}$ T_H2 cells. Cytokine production of T_H2 cells restimulated with PMA/ionomycin was determined by intracellular staining and analysed by flow cytometry. Data are representative of four experiments with similar results. (**B**) Increased secretion of IL-13 and IL-5 by *Nfil3*$^{-/-}$ T_H2 cells restimulated with anti-CD3/CD28 for 24 h. Cytokine concentration was determined by ELISA and data show the mean and s.d. from four experiments ($^*P<0.001$). (**C**) Increased expression of *Il13* and *Il5* mRNA in *Nfil3*$^{-/-}$ T_H2 cells. Expression of mRNA was determined by quantitative real-time RT–PCR. Data show the mean and s.d. from four experiments ($^*P<0.0001$, $^{**}P<0.005$). (**D**) Altered cytokine production in *Nfil3*$^{-/-}$ T_H2 cells is cell intrinsic. Naive CD4$^+$ T cells from WT mice (CD45.1$^+$) and *Nfil3*$^{-/-}$ mice (CD45.2$^+$) were co-cultured under T_H2 conditions for 7 days and cytokine production by cells restimulated with PMA/ionomycin was determined by intracellular staining and analysed by flow cytometry. Data are representative of three experiments with similar results.

T_H1 differentiation were similar between *Nfil3*$^{-/-}$ and WT T cells (Supplementary Figure S2). IL-4 secretion by T_H2-skewed *Nfil3*$^{-/-}$ T cells was also significantly reduced compared with WT T cells although the secretion of IFN-γ by T_H1-skewed cells from WT and *Nfil3*$^{-/-}$ mice was comparable (Figure 1C). Intracellular cytokine staining also confirmed that T_H1 differentiation is not affected by the loss of expression of NFIL3, whereas under T_H2-skewing conditions the number of IL-4-producing *Nfil3*$^{-/-}$ T cells was reduced compared with that of WT T cells (Figure 1D). These results indicate that NFIL3 has a role in the regulation of IL-4 production during T_H2 differentiation *in vitro*.

T-cell intrinsic NFIL3 regulates T_H2 cytokine production in vitro

In the absence of NFIL3 expression, IL-4 production was significantly reduced in T_H2-skewed cells. The genes encoding the T_H2 cytokines, IL-4, IL-13, and IL-5 are located in the same chromosomal region called the T_H2 cytokine locus and their expression is coordinately regulated (Wilson *et al*, 2009). Given the decreased levels of IL-4 expression in the absence of NFIL3, we determined whether the production of IL-13 and IL-5 was similarly affected. Interestingly, the number of IL-13 and IL-5-producing *Nfil3*$^{-/-}$ cells was significantly increased compared with that of WT cells (Figure 2A). Additionally, the mean fluorescence intensity (MFI) of IL-13$^+$ *Nfil3*$^{-/-}$ cells was significantly higher than that of WT cells (MFI: 129.2 ± 5.7 for WT cells and 224.8 ± 20.1 for *Nfil3*$^{-/-}$ cells). The secretion of these cytokines by *Nfil3*$^{-/-}$ T cells was also increased (Figure 2B), as was the expression of both *Il13* and *Il5* mRNA (Figure 2C). Interestingly, expression of *Il13* in T_H1 and T_H9 cells was also significantly increased in the absence of *Nfil3* expression although *Il13* expression in these cells was very low compared with that of T_H2 cells. This suggests that NFIL3 regulates *Il13* expression in these helper cell subsets (Supplementary Figure S1B). Additionally, under T_H2 conditions *Nfil3*$^{-/-}$ T cells had increased IL-9 production and *Il9* mRNA compared with WT T cells, although these levels were extremely low (Supplementary Figure S3A and B). However, when cultured under T_H9 conditions, no differences in the production of IL-9 and *Il9* mRNA expression were observed between WT and *Nfil3*$^{-/-}$ cells. Moreover, while IL-10 and IL-3 production was significantly decreased in *Nfil3*$^{-/-}$ T_H2 cells, no difference in IL-10 production was observed

between WT and *Nfil3*$^{-/-}$ T_H1 cells (Supplementary Figure S3C–H).

Because perturbed IL-4 production might affect IL-13 and IL-5 production in the absence of NFIL3, we determined whether the altered T_H2 cytokine production in *Nfil3*$^{-/-}$ cells was T-cell intrinsic. Naive CD4$^+$ T cells from WT mice (CD45.1) and *Nfil3*$^{-/-}$ mice (CD45.2) were co-cultured under T_H2 condition for 7 days and were examined for T_H2 cytokine production. The number of IL-13 and IL-5-producing cells from CD45.2$^+$ *Nfil3*$^{-/-}$ mice was significantly higher when compared with the cells from CD45.1$^+$ WT mice (Figure 2D). Similarly, the number of IL-4-producing cells was lower from CD45.2$^+$ *Nfil3*$^{-/-}$ mice compared with the cells from CD45.1$^+$ WT mice. These data suggest that the level of NFIL3 within the T cell regulates T_H2 cytokine production. Taken together, these results suggest that NFIL3 specifically regulates cytokine production by T_H2 cells *in vitro*.

Normal IL-4 signalling and proliferation of Nfil3$^{-/-}$ *T cells*

In the presence of exogenous IL-4 under T_H2-skewing condition, *Nfil3*$^{-/-}$ T cells produced fewer cells that were capable of expressing IL-4. This may be due to altered IL-4 signalling in *Nfil3*$^{-/-}$ T cells, and thus we examined IL-4 signalling in *Nfil3*$^{-/-}$ T cells. Splenic CD4$^+$ T cells were stimulated with IL-4, and the activation of STAT6 was examined by flow cytometry. STAT6 activation by IL-4 stimulation occurred in *Nfil3*$^{-/-}$ T cells at a level comparable to WT cells, suggesting IL-4 signalling in CD4$^+$ T cells is not affected in the absence of NFIL3 expression (Supplementary Figure S4A). We also examined IL-4Rα expression in cells cultured under T_H0 and T_H2 conditions. Expression of *Il4ra* gene was similar between *Nfil3*$^{-/-}$ cells and WT cells subjected to either condition (Supplementary Figure S4B). Thus, *Nfil3* deficiency did not alter IL-4 signalling.

IL-4 production of T_H2 cells is dependent on cellular proliferation (Gett and Hodgkin, 1998). Thus, the impaired production of IL-4 by *Nfil3*$^{-/-}$ T cells could be secondary to defective proliferation. Therefore, we next examined the proliferative response of *Nfil3*$^{-/-}$ CD4$^+$ T cells. In response to CD3/CD28 stimulation, thymidine incorporation of *Nfil3*$^{-/-}$ CD4$^+$ T cells was comparable to those of WT CD4$^+$ T cells (Supplementary Figure S4C). The addition of IL-4 to this culture showed similar enhancement of proliferation of *Nfil3*$^{-/-}$ and WT T cells. These results showing normal proliferation suggest the altered T_H2 cytokine production of *Nfil3*$^{-/-}$ CD4$^+$ T cells is not due to a proliferative defect.

We also asked whether IL-4 negatively regulate *Il13* and *Il5* expression by effector T_H2 cells by upregulating the expression of *Nfil3*. Although the addition of neutralizing anti-IL-4 antibody in the T_H2 cell culture reduced *Nfil3* expression, expression of *Il13* and *Il5* genes was not affected (Supplementary Figure S5). This suggests that *Il13* and *Il5* expression is not altered by IL-4 stimulation of differentiated T_H2 cells.

NFIL3 is required for control of Il13 and Il5 at an early stage of T_H2 differentiation

We next assessed whether the abnormalities displayed by T_H2 effector cells extended to the earlier stages in T_H2 differentiation by examining the expression of cytokine genes and transcription factors essential for T_H2 differentiation. Naive CD4$^+$ T cells from *Nfil3*$^{-/-}$ and WT mice were cultured under T_H2 condition for 1–4 days, and gene expression was analysed by real-time RT–PCR. *Nfil3* expression increased with time during T_H2 differentiation as did the expression of the *Il13* and *Il5* genes (Figure 3A). Notably, expression of these cytokine genes was significantly higher in *Nfil3*$^{-/-}$ cells compared with WT cells at all time points, suggesting that NFIL3 negatively regulates these genes from the very early stages of T_H2 differentiation (Figure 3A). Interestingly, early expression of the *Il4* gene was higher in *Nfil3*$^{-/-}$ T cells than that of WT T cells. Consistent with this observation, *Nfil3*$^{-/-}$ cells cultured under neutral conditions also expressed more *Il4* mRNA than WT cells (Supplementary Figure S6). Gene expression of the T_H2 transcription factors *Gata3*, *Junb*, *Bhlhe41* (which encodes Dec2), and *Stat6*, was comparable between WT and *Nfil3*$^{-/-}$ T cells (Figure 3A). GATA-3 is the master transcription factor for T_H2 differentiation. Therefore, we examined whether GATA-3 is required for the expression of *Nfil3* gene in response to IL-4. Efficient knockdown of GATA-3 expression did not alter *Nfil3* expression in response to IL-4, indicating that GATA-3 is not required for the expression of IL-4-induced *Nfil3* gene (Supplementary Figure S7). Taken together, these results suggest that NFIL3 is involved in the regulation of cytokine production but not in the regulation of transcription factors induced during the early stages of T_H2 differentiation.

Next, we asked whether the restoration of NFIL3 expression in *Nfil3*$^{-/-}$ T cells during T_H2 differentiation is sufficient to induce normal T_H2 cytokine production. We expressed NFIL3 in *Nfil3*$^{-/-}$ naive CD4$^+$ T cells cultured under T_H2 condition using retroviral transduction. Six days after infection, transduced cells were analysed for T_H2 cytokine production by flow cytometry. Transduction of NFIL3 into *Nfil3*$^{-/-}$ CD4$^+$ T cells resulted in increased IL-4 production and decreased IL-13 and IL-5 production in comparison to *Nfil3*$^{-/-}$ CD4$^+$ T cells transduced by empty vector (Figure 3B). These results indicate that the induced NFIL3 expression seen at early stages of T_H2 differentiation is required for the normal cytokine production during T_H2 differentiation.

We also expressed NFIL3 during the later stage of T_H2 differentiation. Similar to the transduction into the early stage of T_H2 differentiation, transduction of NFIL3 resulted in decreased IL-13 production, but the effect of NFIL3 transduction on IL-5 production was less clear (Supplementary Figure S8A). Surprisingly, IL-4 production was also decreased by NFIL3 transduction in contrast to NFIL3 transduction at the early stage. Furthermore, we examined the effect of knockdown of NFIL3 expression in polarized T_H2 cells. Consistent with NFIL3 transduction, knockdown of NFIL3 expression resulted in increased IL-13 and IL-4 production (Supplementary Figure S8B). These results suggest that altered expression of NFIL3 in polarized T_H2 cells may affect IL-4 production in a different manner to that in the early stage of T_H2 differentiation.

NFIL3 regulates T_H2 response in vivo

In order to determine whether NFIL3 regulates T_H2 responses *in vivo*, mice were immunized with ovalbumin (OVA) adsorbed to alum, and cytokine production by splenic T cells after restimulation with OVA was examined by ELISA. In response to OVA restimulation, IL-13 and IL-5 production

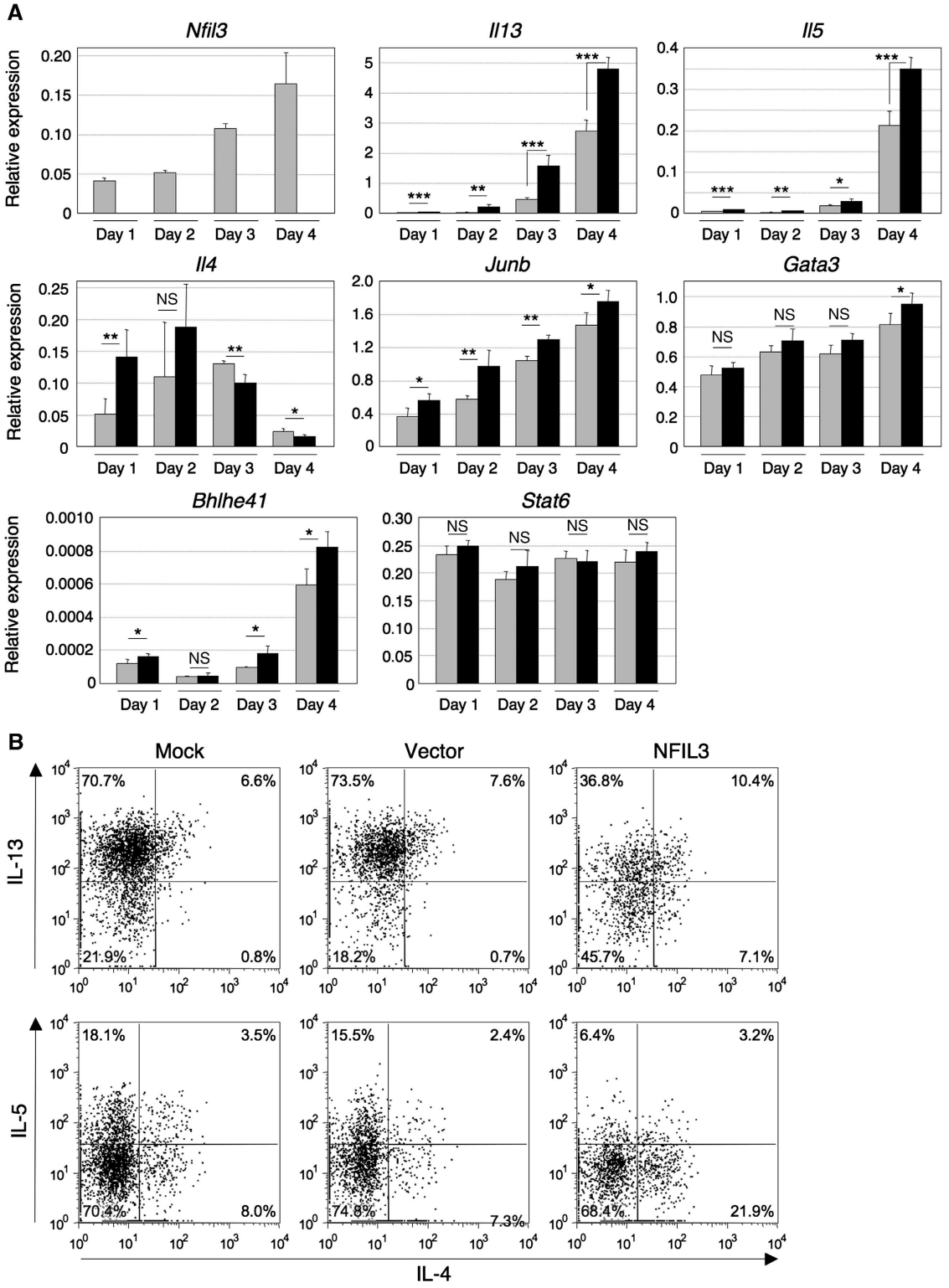

Figure 3 Role of NFIL3 expression at an early stage of T_H2 differentiation. (**A**) Required expression of *Nfil3* at an early stage of T_H2 differentiation for the normal expression of *Il13* and *Il5* genes. Naive $CD4^+$ T cells from WT (grey bar) and *Nfil3*$^{-/-}$ (closed bar) mice were cultured under T_H2 conditions up to 4 days and RNA was prepared for quantitative RT–PCR. Relative expression of each gene was normalized by the expression of *Hprt1* mRNA. Data show the mean and s.d. from four experiments (NS, not significant; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$) (**B**) Introduction of NFIL3 into *Nfil3*$^{-/-}$ T cells at an early time point of T_H2 differentiation restores the impairment of T_H2 cytokine production. $CD4^+$ T cells under T_H2 conditions for 30 h were infected with retroviruses carrying NFIL3 or vector (pMiT). Infected cells were cultured for an additional 6 days under T_H2 conditions and then restimulated with PMA/ionomycin in the presence of Brefeldin A to examine cytokine production by intracellular staining. Data are representative of three experiments with similar results.

by *Nfil3*$^{-/-}$ cells was significantly increased compared with WT cells (Figure 4A). In contrast, IL-4 production was decreased in *Nfil3*$^{-/-}$ mice. These observations are consistent with cytokine production observed by T_H2 cells differentiated *in vitro* (Figures 1 and 2). Thus, these results suggest that NFIL3 regulates T_H2 cytokine production *in vivo*.

We also examined OVA-specific proliferation of the cells from OVA-immunized mice. *Nfil3*$^{-/-}$ cells were hyperproliferative in response to OVA at lower concentration in comparison to WT cells (Figure 4B). However, freshly isolated CD4$^+$ T cells from unimmunized *Nfil3*$^{-/-}$ and WT mice showed comparable proliferation in response to CD3 stimulation (Supplementary Figure S4C). These results may implicate the role of *Nfil3* in the antigen-specific cell expansion leading to the efficient desired effector function such as cytokine production.

NFIL3 directly binds to CGRE region and regulates IL-13 gene expression

In T_H2 cells, both *Il13* mRNA expression and IL-13 production were increased in the absence of NFIL3 (Figure 2), suggesting that NFIL3 may directly regulate the *Il13* gene. Therefore, we searched the mammalian *Il13* gene locus for NFIL3-binding sites using the TRANSFAC program (Matys *et al*, 2003). We found an evolutionarily conserved consensus sequence for NFIL3 binding 1.6–1.7 kb upstream of the first exon of the *Il13* gene (Figure 5A). Interestingly, this putative NFIL3-binding sequence is located next to the GATA-3-binding site in the CGRE/HS1/CS1 region, which was identified as a DNase I hypersensitivity site and corresponds to the 5′ border of the histone acetylation region in T_H2 cells (Agarwal and Rao, 1998; Kishikawa *et al*, 2001; Yamashita *et al*, 2002).

We first determined whether NFIL3 binds to this sequence *in vivo*. We performed chromatin immunoprecipitation (ChIP) analysis by amplifying the CGRE region of WT and *Nfil3*$^{-/-}$ T_H2 cells. ChIP analysis of WT but not *Nfil3*$^{-/-}$ T_H2 cells clearly demonstrated a significant enrichment of NFIL3 binding to the CGRE region upstream of *Il13* gene (Figure 5B). No enrichment of NFIL3 binding to β-globin gene was observed as a negative control. Therefore, NFIL3 specifically binds to the CGRE region *in vivo*. Because the NFIL3-binding site and GATA-3-binding site are very close it is possible that binding of NFIL3 to the CGRE could interfere with GATA-3 binding to the CGRE. However, the level of GATA-3 binding to the CGRE was not affected in *Nfil3*$^{-/-}$ T_H2 cells compared with WT cells (Supplementary Figure S9).

Next, we asked whether NFIL3 binding to the CGRE region is functionally relevant for *Il13* gene transcription. D10.G4.1 cells were transfected with a CGRE-containing 1.8 kb promoter-luciferase construct (WT), NFIL3-binding site mutant construct (NFIL3mut) (Ozkurt and Tetradis, 2003), GATA-3-binding site mutant construct (GATA3mut) (Yamashita *et al*, 2002) or CGRE-deletion mutant construct (ΔCGRE), followed by CD3/CD28 stimulation for 48 h, after which cell lysates were subjected to luciferase assay (Figure 5C, left). CD3/CD28 stimulation induced six-fold transcriptional activation of the WT-reporter gene (Figure 5C, right). Notably, mutation of the NFIL3-binding site significantly enhanced transcriptional activity (14-fold). On the other hand, mutation in the GATA-3-binding site and CGRE deletion diminished transcriptional activity (two-fold and four-fold, respectively). These results

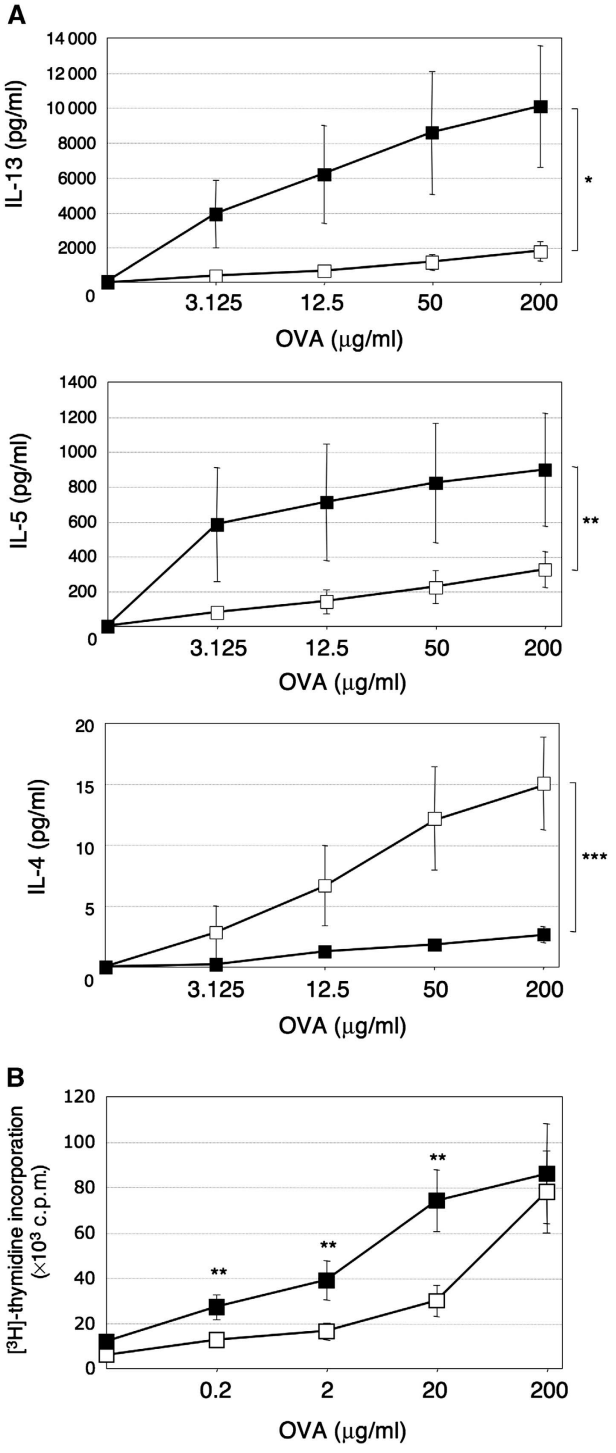

Figure 4 NFIL3 regulates T_H2 response *in vivo*. Mice ($n=4$ for each genotypes, WT mice: open square, *Nfil3*$^{-/-}$ mice: closed square) were immunized with OVA plus alum. After 6 days, splenocytes were prepared and cultured in the presence of OVA for 3 days. (**A**) Increased IL-13 and IL-5 but decreased IL-4 production in response to OVA in *Nfil3*$^{-/-}$ mice. Supernatants were harvested and secreted T_H2 cytokines were examined by ELISA ($*P=0.078$, $**P=0.16$, $***P<0.05$). (**B**) Increased OVA-specific cell proliferation in *Nfil3*$^{-/-}$ mice. Proliferation was measured during the last 16 h by measurement of thymidine incorporation in triplicate. Data show the mean and s.e. from four mice in each group and representative of two independent experiments ($**P<0.05$).

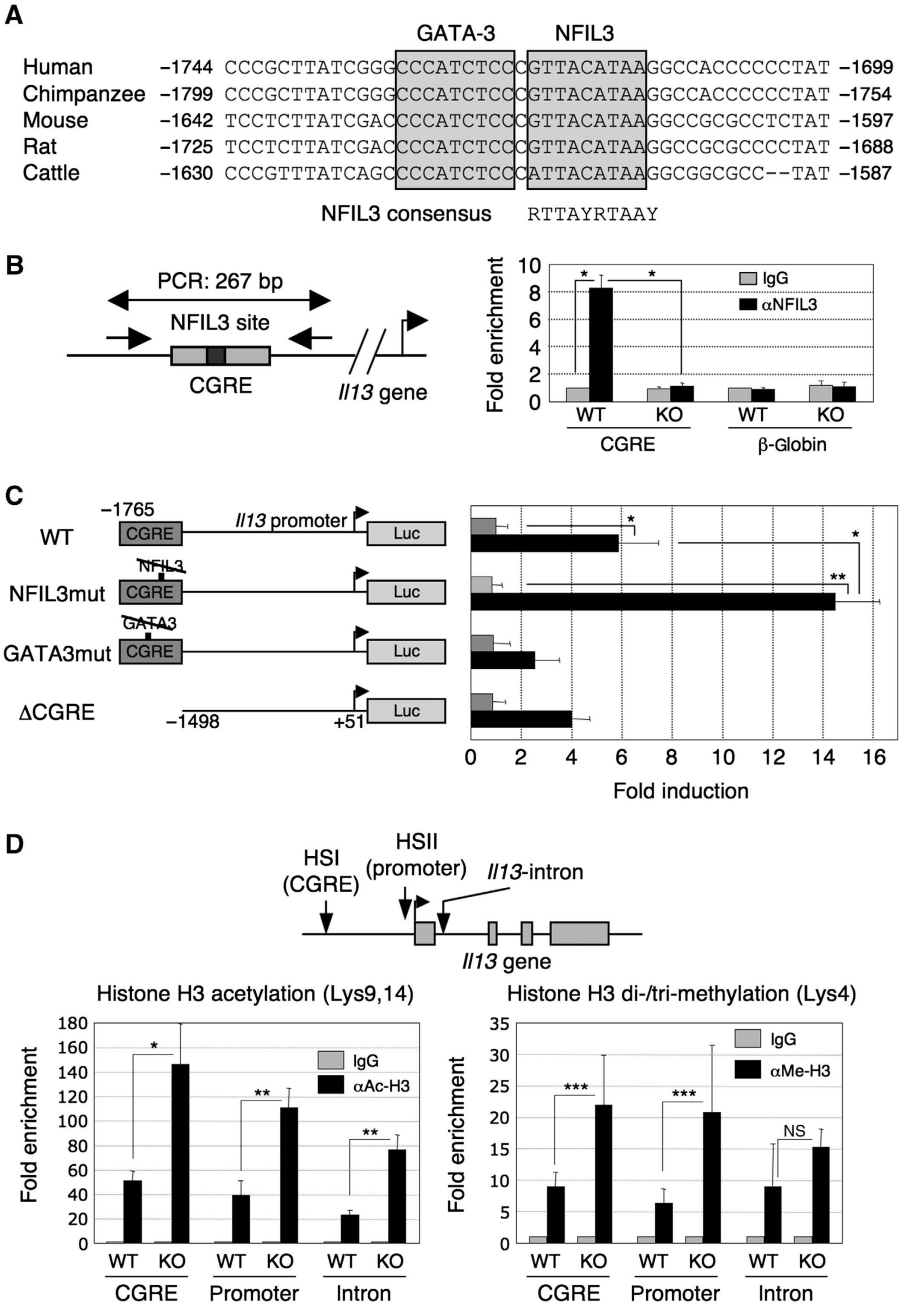

Figure 5 NFIL3 binds to CGRE and suppresses *Il13* gene expression. (**A**) Sequence alignment around the putative NFIL3-binding site in the CGRE region. GATA-3 and NFIL3-binding sequences are boxed and the consensus NFIL3-binding sequence is shown. The numbers of positions relative to the transcriptional start site (human, mouse, and cattle) or the translational start site (chimpanzee and rat) are indicated, respectively. (**B**) NFIL3 binds to CGRE *in vivo*. The region amplified by PCR and the primers are indicated (left). Splenic CD4 T cells from WT and *Nfil3*$^{-/-}$ mice cultured under T_H2 conditions for 7 days were crosslinked and soluble chromatin complexes were immunoprecipitated by anti-NFIL3 antibody or control IgG. The region including CGRE in the co-precipitated DNA was amplified by PCR. The β-globin gene was used as a negative control. The average and s.d. of enrichment from four experiments are indicated (right). *$P<0.0001$. (**C**) Negative regulation of *Il13* gene transcription by NFIL3. The reporter constructs of WT, NFIL3-binding mutant, GATA-3-binding mutant, and CGRE-deletion mutant used are shown (left). The T_H2 cell line, D10.G4.1, was transfected with the indicated reporter constructs and stimulated with anti-CD3/CD28 antibodies for 48 h. Cell lysates were subjected to luciferase assay. Five experiments were performed with similar results. *$P<0.01$, **$P<0.001$. (**D**) Chromatin modification in the *Il13* gene locus. The region amplified by PCR indicated (top). Splenic CD4 T cells from WT and *Nfil3*$^{-/-}$ mice cultured under T_H2 conditions for 7 days were crosslinked and soluble chromatin complexes were immunoprecipitated by anti-acetyl Histone H3, anti-di + tri-methyl Histone H3, or control IgG. The region including the CGRE, promoter region, and first intron in the co-precipitated DNA were amplified by PCR. The average and s.d. of enrichment from four experiments are indicated (*$P<0.005$, **$P<0.001$, ***$P<0.05$; NS, not significant).

suggest that the NFIL3-binding site in the CGRE region functions to regulate *Il13* transcription.

Previous studies have demonstrated that epigenetic changes are observed in the *Il13* gene locus during T_H2 differentiation (Yamashita *et al*, 2002; Baguet and Bix, 2004). Therefore, we examined histone modification at the *Il13* locus in the absence of NFIL3 expression by ChIP analysis. Interestingly, *Nfil3*$^{-/-}$ T_H2 cells showed increased

histone H3 acetylation in the *Il13* gene locus including the CGRE region, promoter region, and the first intron sequence compared with WT T_H2 cells (Figure 5D, left). In addition, *Nfil3*$^{-/-}$ T_H2 cells also showed increased histone H3 methylation at lysine 4 in the *Il13* locus compared with WT T_H2 cells (Figure 5D, right). These changes of chromatin modification are strongly correlated with active transcription of the affected genes (Fischle *et al*, 2003). Thus, NFIL3 may be involved in the regulation of epigenetic changes in the *Il13* locus leading to altered transcription of the *Il13* gene.

NFIL3 regulates the levels of AP-1

As we could not identify a likely NFIL3-binding site within the *Il4* gene locus, we hypothesized that NFIL3 regulation of *Il4* expression might be indirect. In order to assess this possibility, we examined the expression levels of transcription factors involved in the regulation of *Il4* gene expression. Among these, expression of the *Junb*, *Fosl2*, *Bmhle41* genes were slightly reduced in *Nfil3*$^{-/-}$ T_H2 cells (Figure 6A). Both JunB and Fra-2 are components of the AP-1 complex in T_H2 cells (Rooney *et al*, 1995; Li *et al*, 1999). At the protein level, JunB was markedly reduced in *Nfil3*$^{-/-}$ T_H2 cells (Figure 6B). Expression of Fra-2 protein was also slightly reduced. These data suggest that diminished upregulation of JunB and Fra-2 might be the mechanism by which NFIL3 deficiency causes reduced *Il4* expression in T_H2 cells. Next, we tested this hypothesis through assessment of the level of JunB protein bound to the *Il4* promoter by ChIP analysis. As predicted, ChIP analysis demonstrated significantly reduced JunB binding to the *Il4* promoter region in *Nfil3*$^{-/-}$ T_H2 cells compared with WT T_H2 cells following stimulation with PMA/ionomycin (Figure 6C). A small level of JunB binding to the *Il4* promoter in WT but not *Nfil3*$^{-/-}$ T_H1 cells was observed (Supplementary Figure S10) likely because small amounts of JunB protein is also detectable in T_H1 cells (Li *et al*, 1999). We confirmed the lower JunB protein level in both unstimulated and PMA/ionomycin-stimulated *Nfil3*$^{-/-}$ T_H2 cells in comparison to the levels in WT T_H2 cells (Figure 6D). We also asked whether the reduced JunB binding to the *Il4* promoter in the absence of NFIL3 alters histone modification. The levels of both histone H3 acetylation and methylation were similar between WT T_H2 cells and *Nfil3*$^{-/-}$ T_H2 cells, indicating that JunB reduction in the absence of NFIL3 results in downregulation of *Il4* transcription independent of histone modification (Supplementary Figure S11). Last, we examined whether transduction of JunB into *Nfil3*$^{-/-}$ T_H2 cells restores reduced IL-4 production. Interestingly, transduction of JunB into *Nfil3*$^{-/-}$ T_H2 cells could not rescue the impairment of IL-4 production (Supplementary Figure S12). This observation suggests that transduction of JunB alone is not sufficient to rescue IL-4 production.

Discussion

There have been extensive studies over the past two decades focused on elucidating the mechanisms that regulate helper T-cell differentiation and effector function. Specific cytokines direct naive CD4$^+$ T cells to induce the required lineage-specific transcription factors at the appropriate stage of lineage specification in the context of T-cell receptor (TCR) signalling. For T_H2 differentiation, IL-4 is critical for initiating, as well as maintaining, T_H2 phenotypes. Indeed, TCR-stimulated naive CD4$^+$ T cells produce IL-4, which stimulates these T cells by positive autocrine feedback. IL-4 signalling induces STAT6 activation, which is constitutively expressed, leading to GATA-3 expression. GATA-3 has a critical role in the chromatin remodelling of the T_H2 cytokine locus, driving the commitment to T_H2 differentiation (Ansel *et al*, 2006; Lee *et al*, 2006). The collaboration of GATA-3 with other transcription factors including JunB, c-Maf, NFATc, and AP-1 facilitates T_H2 differentiation and T_H2 cytokine production. However, dysregulated or inappropriate production of these T_H2 cytokines leads to pathogenic allergic immune responses. Our data described here suggest that NFIL3 may be a key regulator of normal cytokine production.

NFIL3 expression in T_H2 cells is much higher when compared with T_H1 cells, implicating a role for NFIL3 in T_H2-mediated responses, such as allergic inflammation. We demonstrated that NFIL3 functions as a negative regulator of IL-13 and IL-5 production and as a positive regulator of IL-4 production under T_H2 condition *in vitro* and *in vivo*. A previous report describing a putative NFIL3-binding site in the proximal promoter of *Ifng* gene suggests a potential role for NFIL3 in T_H1 cytokine production (Zhang *et al*, 1995). Despite this implication T_H1 differentiation of *Nfil3*$^{-/-}$ T cells appeared normal as determined by the expression and production of IFN-γ and transcription factors. However, *Il13* expression in *Nfil3*$^{-/-}$ cells was slightly increased compared with WT cells under T_H1 conditions (Supplementary Figure S1B), suggesting that NFIL3 may also contribute to negative regulation of the *Il13* gene in T_H1 cells. Moreover, expression of *Nfil3* in T_H9 cells, which is induced by IL-4 plus TGF-β from naive CD4 T cells, was higher than T_H1 cells, and *Nfil3*$^{-/-}$ T_H9 cells highly expressed *Il13* gene compared with WT cells (Supplementary Figure S1A and B). These observations suggest that NFIL3 regulates expression of the *Il13* gene not only in T_H2 cells but also T_H1 and T_H9 cells.

How does NFIL3 regulate *Il13* gene expression? Our analysis demonstrated that NFIL3 directly bound to the CGRE region and negatively regulated *Il13* gene expression. Enhanced *Il13* expression in *Nfil3*$^{-/-}$ T_H2 cells was observed not only in the differentiated T_H2 cells but also at the very early stages of T_H2 differentiation. Interestingly, the CGRE region corresponds to the 5′ border of the histone acetylation region in T_H2 cells (Yamashita *et al*, 2002). The NFIL3-binding site is very close to the GATA-3-binding site in the CGRE region. However, the level of GATA-3 binding to the CGRE was similar between WT and *Nfil3*$^{-/-}$ T_H2 cells, suggesting that NFIL3 regulated *Il13* gene expression without affecting GATA-3 activity in T_H2 cells. Moreover, epigenetic analysis revealed increased levels of histone acetylation and methylation leading to transcriptional activation in *Nfil3*$^{-/-}$ T_H2 cells compared with WT T_H2 cells. Therefore, NFIL3 may contribute to the chromatin remodelling on the *Il13* gene locus.

Similarly to IL-13 production, we also showed increased IL-5 production in *Nfil3*$^{-/-}$ T_H2 cells and OVA-immunized mice. Although we could not find putative NFIL3-binding sites in the *Il5* gene locus, preliminary experiments showed increased acetylation and methylation of histone H3 in the *Il5* gene locus in *Nfil3*$^{-/-}$ T_H2 cells compared with WT T_H2 cells (data not shown). These chromatin modifications were correlated with the transcription level of the *Il5* gene in T_H2 cells from *Nfil3*$^{-/-}$ and WT mice. Thus, NFIL3 may regulate *Il5* gene expression either indirectly through controlling other

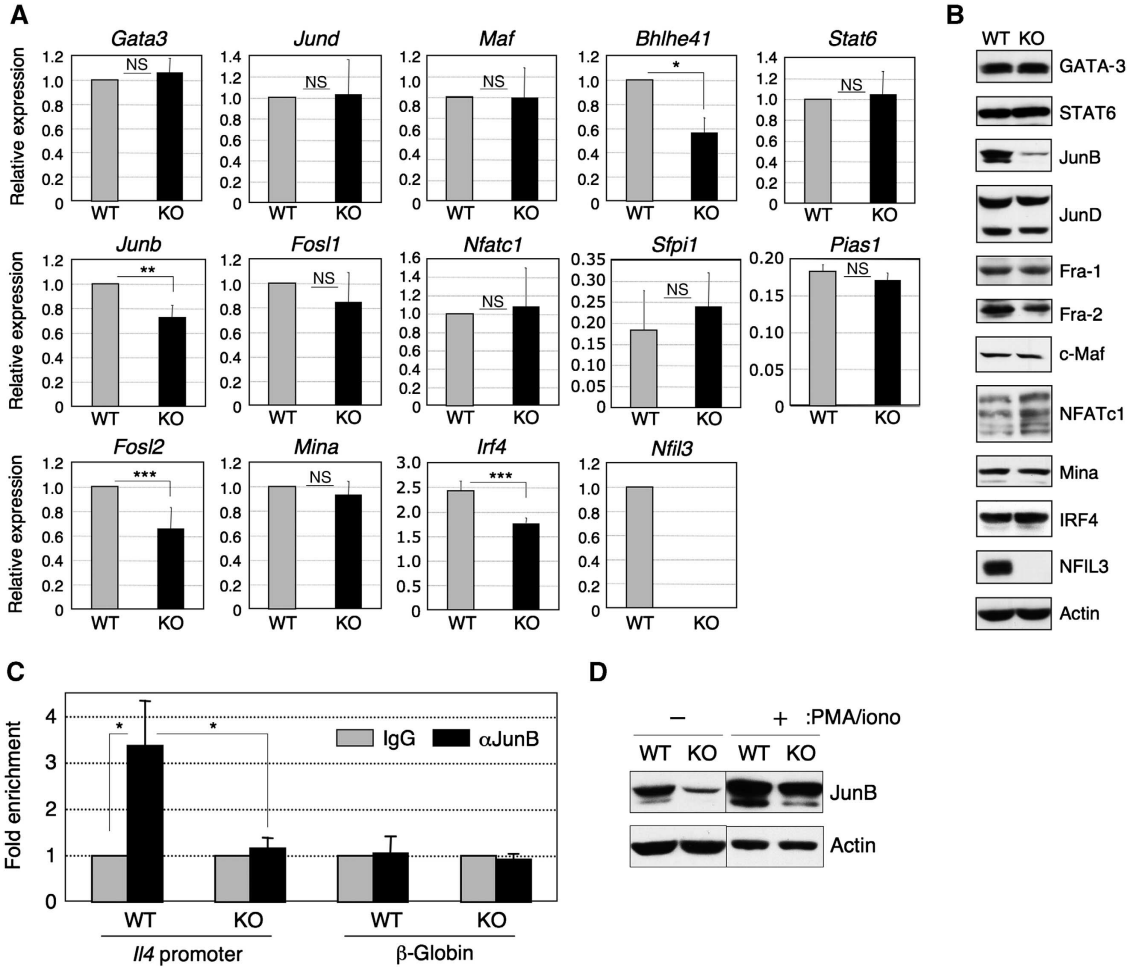

Figure 6 Indirect regulation of *Il4* gene expression by NFIL3. (**A**) Gene expression levels of transcription factors involved in the regulation of *Il4* gene expression. Real-time RT–PCR analysis for the genes listed in *Nfil3*$^{-/-}$ and WT cells cultured under T_H2 condition for 7 days was performed. Relative expression of each gene was normalized to the expression of *Hprt1* mRNA and WT value was set as 1. Data show the mean and s.d. from four experiments (*$P<0.001$, **$P<0.002$, ***$P<0.01$). (**B**) Protein expression levels of transcription factors listed in (**A**). Cell lysates from the cells above were subjected to western blot analysis. At least four experiments were performed with similar results. (**C**) Reduced JunB binding to the *Il4* promoter in *Nfil3*$^{-/-}$ T_H2 cells (left). *Nfil3*$^{-/-}$ and WT T_H2 cells were restimulated with PMA/ionomycin for 4 h and were crosslinked and soluble chromatin complexes were immunoprecipitated by anti-JunB antibody or control IgG. *Il4* promoter region in the co-precipitated DNA were amplified by PCR. The β-globin gene was used as a negative control. The average and s.d. of enrichment from three experiments are indicated (*$P<0.05$). (**D**) Reduced JunB expression in *Nfil3*$^{-/-}$ cells both before and after PMA/ionomycin restimulation. Cells were unstimulated or stimulated with PMA/ionomycin for 4 h and prepared cell lysates for western blot analysis. Data are representative from more than five independent experiments and two independent experiments for the unstimulated and PMA/ionomycin-stimulated samples, respectively. The lanes are from the same blot.

transcription factors involved in the regulation of *Il5* gene transcription, or directly through intrachromosomal interaction between the *Il13* promoter region containing CGRE and the *Il5* gene. Indeed, the *Il5* promoter region has been shown to co-localize with the *Il13* promoter region in T_H2 cells (Spilianakis and Flavell, 2004; Cai *et al*, 2006), and the CGRE functions as an enhancer if fused to the *Il5* promoter (Yamashita *et al*, 2002). By elucidating the mechanism of *Il13* and *Il5* regulation by NFIL3, we could identify putative targets for therapeutic intervention to control allergic immune responses. Although a coordinate regulation of the T_H2 cytokine locus to express *Il4*, *Il5*, and *Il13* genes is well documented, discordant expression of these cytokine genes has also been demonstrated (Kishikawa *et al*, 2001; Wilson *et al*, 2009). Our data indicate that the IL-13$^+$IL-4$^-$ population is dramatically increased in the absence of NFIL3, and that NFIL3 regulates *Il13* and *Il5* gene expression but not *Il4* gene expression at very early stages of T_H2 differentiation, suggesting that NFIL3 may differentially regulate the expression of these cytokines independent of the coordinated T_H2 cytokine locus accessibility. These observations may explain why there is heterogeneity in the expression of other T_H2 cytokine genes in the individual T_H2 cells or clones (Bucy *et al*, 1995; Kelso *et al*, 1999; Kishikawa *et al*, 2001). It has been shown that expression of PU.1, IRF4, and Pias1 contributes to T_H2 heterogeneity (Chang *et al*, 2005; Zhao *et al*, 2007; Ahyi *et al*, 2009). However, we could not find a potential role for NFIL3 in regulating these genes, suggesting that NFIL3 may not be involved in the regulation of these transcriptional regulators. How NFIL3 contributes to T_H2 heterogeneity needs to be elucidated.

In contrast to the differentiated T_H2 cells, early expression of IL-4 by *Nfil3*$^{-/-}$ T cells under T_H2 condition and neutral condition were higher than that of WT T cells. Initial

expression of the *Il4* gene is induced by TCR stimulation, and this induction is independent on GATA-3 (Ansel *et al*, 2006). Under these conditions, expression of *Junb* and *Gata3* in *Nfil3*$^{-/-}$ T cells was similar to that of WT T cells, suggesting that NFIL3 may regulate *Il4* expression in the different mechanisms between the early stage of T_H2 differentiation and T_H2-polarized cells.

We have shown a correlation between reduced *Il4* expression and a significant reduction of JunB protein in *Nfil3*$^{-/-}$ T cells. This marked decrease in JunB protein was found in the context of only a modest decrease in *Junb* expression. These observations have led us to speculate that NFIL3 may regulate both *Junb* expression and perhaps JunB protein stability. Importantly, JunB transduction into *Nfil3*$^{-/-}$ T_H2 cells did not restore reduced IL-4 production (Supplementary Figure S12). Therefore, JunB alone may be insufficient to rescue IL-4 production. Alternately, JunB protein may be unstable in the absence of NFIL3. It has been demonstrated that JunB protein stability was controlled by Itch, an E3 ubiquitin ligase, whose activity is regulated by Ndfip1 (Fang *et al*, 2002; Oliver *et al*, 2006). It is also known that transcriptional activity of JunB is regulated by SUMOylation in T cells (Garaude *et al*, 2008). SUMOylation is one of the post-translational modifications with SUMO protein that, unlike ubiquitin, modifies the target protein's function but dose not cause protein degradation. However, how JunB stability is regulated is still unknown. Therefore, the mechanism by which NFIL3 modifies the JunB protein level needs to be elucidated.

Similar to the increased *Il13* and *Il5* expression, *Il9* expression was also increased in *Nfil3*$^{-/-}$ T_H2 cells. In contrast, reduced *Il10* and *Il3* expression (along with *Il4*) was observed in *Nfil3*$^{-/-}$ T_H2 cells. NFIL3 was previously identified as a transcriptional activator for human *IL3* promoter (Zhang *et al*, 1995). Our data show that *Nfil3*$^{-/-}$ T_H2 cells produced decreased *Il3* expression and IL-3 production, suggesting that NFIL3 directly regulates *Il3* transcription. Currently, the molecular mechanisms by which NFIL3 regulates the expression of *Il9* and *Il10* genes are unclear.

In summary, we demonstrated that NFIL3 has a critical role in the T_H2 cytokine gene expression. Previously, we have also demonstrated that NFIL3 controls IgE class switching. In addition, NFIL3 controls NK cell development. Therefore, NFIL3 is a pleiotropic transcriptional regulator in both acquired immunity and innate immunity.

Materials and methods

Mice and cell line

Generation of *Nfil3*$^{-/-}$ mice was previously described (Kashiwada *et al*, 2010). *Nfil3*$^{-/-}$ mice backcrossed with C57BL/6 for at least 10 generations were used for all experiments. CD45.1$^+$ C57BL/6 mice were obtained from the Jackson Laboratory. All mice were bred and maintained under specific pathogen-free conditions. All experimental mouse protocols were adhered to Institutional Animal Care and Use Committee guidelines and were approved by the IACUC of University of Iowa. The T_H2 cell line, D10.G4.1, was maintained in complete RPMI1640 medium containing sodium pyruvate, non-essential amino acids and IL-2 (5 ng/ml). D10.G4.1 were stimulated with conalbumin every 2 weeks and used 2 weeks after stimulation.

In vitro helper T-cell differentiation

Naive CD4$^+$ T cells (CD4$^+$CD62L$^+$CD25$^-$) purified with CD4$^+$CD62L$^+$ T-Cell Isolation Kit II (Miltenyi) were cultured with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (5 μg/ml) in the presence of the following cytokines and antibodies; IL-12 (20 ng/ml), anti-IL-4 (5 ng/ml), IL-2 (20 ng/ml) for T_H1 differentiation; IL-4 (20 ng/ml), anti-IFN-γ (5 ng/ml), IL-2 (20 ng/ml) for T_H2 differentiation; anti-IFN-γ (5 ng/ml), anti-IL-4 (5 ng/ml), IL-2 (20 ng/ml) for T_H0 condition; IL-4 (20 ng/ml), TGF-β (5 ng/ml), anti-IFN-γ (5 ng/ml), IL-2 (20 ng/ml) for T_H9 differentiation; TGF-β (5 ng/ml), anti-IFN-γ (5 ng/ml), anti-IL-4 (5 ng/ml) for iTreg differentiation; IL-6 (10 ng/ml), TGF-β (5 ng/ml), anti-IFN-γ (5 ng/ml), anti-IL-4 (5 ng/ml) for T_H17 differentiation; 1α, 25-dihydroxyvitamin D_3 (4×10^{-8} M; Sigma), dexamethasone (5×10^{-8} M; Sigma) for Tr1 differentiation. All recombinant cytokines except IL-4 (BD Bioscience) were purchased from R&D Systems. All antibodies for helper T-cell differentiation were purchased from eBioscience.

Western blotting

Preparation of cell lysates and western blot analysis was performed as described previously (Kashiwada *et al*, 2010). Briefly, cell lysates prepared in lysis buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl with protease inhibitor cocktail obtained from Roche) were subjected to SDS–PAGE and transferred onto Immobilon^{TM} PVDF membrane (Millipore). Membranes were probed with the antibodies indicated, and detected with ECL detection system (Amersham). Antibodies used are anti-NFIL3, STAT6, NFATc1, IRF4 (Santa Cruz), GATA-3 (eBioscience), actin (Sigma), JunB (Cell Signaling Technology), JunD (R&D Systems), Mina (Proteintech Group), Fra-1 (Aviva Systems Biology), Fra-2 (Bioworld Technology), and c-Maf (Novus Biologicals).

Real-time RT–PCR

RNA was prepared from unstimulated cells or cells stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h, and cDNA was synthesized using the SuperScript® First-Strand Synthesis System (Invitrogen). PCR analysis of mRNA expression was performed using SYBR® GREEN PCR Master Mix on the 7900HT Fast Real-Time PCR System (Applied Biosystems). The expression levels of each gene were normalized to the expression of *Hprt1*. The primers used are listed in Supplementary Table 1.

Detection of cytokine by ELISA

After a 7-day culture for differentiation, cells (1×10^6 cells) were restimulated with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (5 μg/ml) for 24 h, and then the culture supernatants were harvested. The concentration of cytokines was measured by ELISA. All cytokine antibodies were obtained from eBioscience, and alkaline phosphatase-conjugated avidin was obtained from BD Biosciences.

Intracellular staining

After a 7-day culture for differentiation, cells were restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h in the presence of Brefeldin A (eBioscience). After stimulation, cells were stained with anti-CD4, CD45.1, CD45.2, and anti-Thy1.1 for virus-infected cells, fixed, permeabilized, and then stained with antibodies for the cytokines indicated. For detection of phospho-STAT6, cells were stimulated with IL-4 for 20 min, then fixed, permeabilized, and then stained with anti-phospho-STAT6 antibody (BD Biosciences). The stained cells were analysed with LSR II and CellQuest (BD Biosciences). All antibodies for cytokine detection were obtained from eBioscience except the anti-IL-9 antibody (Biolegend).

Viral infection

Retroviral construct for NFIL3 in pMiT was described previously (Kashiwada *et al*, 2010). The lentiviral construct for JunB was generated by cloning JunB cDNA into pCDH-Thy1.1, which was generated by inserting an IRES-Thy1.1 fragment from pMiT into pCDH-CMV-MCS-EF1-copGFP (System Biosciences). The shRNA constructs for the *Nfil3* gene were generated using pLKO.1-Thy1.1 constructed by replacing Puro^r gene in pLKO.1 (Addgene) with a Thy1.1 fragment. The scramble shRNA construct was obtained from Addgene. For infection at the early stage of T_H2 differentiation, naive CD4$^+$ T cells were cultured under T_H2 conditions overnight, and then activated cells were spin infected with the viruses produced by transient transfection of viral constructs into 293T cells. After infection, cells were grown under T_H2 conditions for another 6 days before being subjected to assays. For infection at the late stage of T_H2 differentiation, *Nfil3*$^{-/-}$ CD4$^+$ T cells cultured for 6 days under T_H2 condition were spin infected with viruses and cultured for 2 days. For *Nfil3* knockdown experiments, WT CD4$^+$

T cells cultured for 6 days under T_H2 condition were spin infected with shRNA viruses, and cultured for 3 days. Infected cells were identified as Thy1.1$^+$ cells by flow cytometry. For *Gata3* knockdown experiments, D10.G4.1 cells were spin infected with lentivirus produced by transient transfection of viral constructs into 293T cells. The shRNA constructs for the *Gata3* gene were kindly provided by Dr Soumen Paul (University of Kansas Medical Center, KS) (Home *et al*, 2009).

Luciferase assay

Luciferase-reporter plasmids were generated with pGL2-basic (Promega) and mutations were introduced using QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the primers as follows: for the NFIL3-binding mutant, 5′-CATCTCCCGTTACATTAGGCC GCGCCTCTAT-3′ and 5′-ATAGAGGCGCGGCCTAATGTAACGGGAGA TG-3′, for the GATA-3-binding mutant, 5′-TCCTCTTATCGACCCA AAATCCCGTTACATAAGG-3′ and 5′-CCTTATGTAACGGGATTTTG GGTCGATAAGAGGA-3′. D10.G4.1 cells were transiently transfected by electroporation with the reporter gene constructs in pGL2 and pRL-TK (Promega) as an internal control. Cells were cultured with or without plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (5 μg/ml). After 48 h of transfection, the Dual-Reporter Luciferase Assay® system (Promega) was used for the preparation of cell lysates and subsequent assays.

Proliferation assay

Naive CD4$^+$ T cells were seeded at a density of 2×10^5 cells per well in 96-well plates in complete RPMI 1640 with varying final concentrations of plate-bound anti-CD3 (0.3, 1, and 3 μg/ml), soluble anti-CD28 (5 μg/ml), mIL-4 (20 ng/ml) as indicated in triplicate. One μCi of [^{3}H]-thymidine (Perkin-Elmer) was added 40 h after stimulation followed by incubation for 8 h before analysis. For OVA-specific proliferation, splenocytes were cultured in the presence of OVA at the indicated concentration for 56 h followed by incubation with 1 μCi of [^{3}H]-thymidine for 16 h before analysis. [^{3}H]-thymidine incorporation was measured with a liquid scintillation counter.

Chromatin immunoprecipitation

ChIP assay was performed as described previously (Kashiwada *et al*, 2010). Briefly, cells were crosslinked with formaldehyde and chromatin was fragmented by sonication. Chromatin was immunoprecipitated with the antibodies indicated or control IgG, and then purified co-precipitated DNA was quantified by real-time PCR as above. Data are normalized to input Ct values and indicated as fold enrichment relative to the values for control IgG. The primers used are listed in Supplementary Table 1. Antibodies used for IP are anti-NFIL3, JunB, GATA-3 (Santa Cruz), anti-acetyl Histone H3 (Millipore), and anti-di + tri-methyl Histone H3 (Abcam).

Statistical analysis

Statistical significance was determined using a two-tailed Student's *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

This study was supported by National Institutes of Health Grant R01 AI54821 (to PBR).

Author contributions: MK and SLC performed the experiments; MK and JDC analysed the data; MK, JDC, and PBR designed the experiments; and MK and PBR wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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