

THE TRANSCRIPTIONAL REGULATOR NFIL3 CONTROLS IgE PRODUCTION

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ABSTRACT

Cytokines are essential modulators of the immune response that underlies the inflammatory component of atopic asthma and other allergic diseases. Interleukin-4 is an important cytokine for the regulation of allergic immune responses. However, the molecular mechanisms that regulate the response of cells to IL-4 are still not completely defined. IL-4 plays an important role in B cell biology. It can regulate B cell differentiation. For example, IL-4 induces immunoglobulin heavy chain class switching to IgE by inducing germline immunoglobulin heavy chain transcription. It also induces expression of CD23 and MHC class II. Further understanding of the mechanisms by which IL-4 mediates these biologic responses may lead to novel mechanisms for therapeutic intervention and control of allergy. To define how different signaling pathways activated by IL-4 regulate gene transcription, we identified many differentially expressed genes by IL-4 stimulation by microarray analysis. NFIL3 (nuclear factor, interleukin 3 regulated) is the most strongly induced transcription factor by IL-4 stimulation in a STAT6-dependent manner. To analyze the role of NFIL3 in the immune system, we have generated NFIL3-deficient mice. NFIL3-deficient mice showed greatly impaired IgE production in response to antigen. NFIL3-deficient B cells fail to produce IgE in response to LPS plus IL-4. These defects may be due to the reduced production of immunoglobulin heavy chain germline epsilon transcripts in the absence of NFIL3. Moreover, NFIL3 KO mice sensitized and challenged with ovalbumin showed reduced airway hyper-responsiveness when compared to wild-type mice. Therefore, we hypothesize that NFIL3 is a critical regulator for IgE production and airway hyper-responsiveness.

The inflammation found in allergic states is extremely complex and involves a variety of inflammatory cells, including B and T lymphocytes, mast cells, eosinophils, macrophages and dendritic cells. In

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response to challenge with allergen, cells in the lung initiate a cascade of events characterized by the production of TH2 cytokines and the recruitment of inflammatory cells, including eosinophils, into the lung (1). This inflammatory response results in bronchial hyper-responsiveness and the clinical manifestations of asthma. Current research in this field has been directed at understanding this inflammatory process in hopes of identifying essential molecules responsible for this immune response. These molecules would be targets for the development of therapeutic interventions. Recent work has demonstrated a variety of molecules involved in this immune response. Although the importance of many of these molecules in the immune response in asthma remains unclear, IL-4 and IL-13 have been demonstrated to be essential for the development of atopic immune responses (2).

Our laboratory has been interested in IL-4 and how it regulates the immune system since this cytokine was cloned over 20 years ago. Our interest has been in understanding the molecular mechanisms by which IL-4 controls lymphocyte function. My initial work began as a postdoctoral fellow in Dr. Fred Alt's laboratory, where we demonstrated that IL-4 regulates immunoglobulin class switching to IgE by modulating the production of germline transcription at the epsilon constant region locus (3–5). We also defined the first promoter regulated by IL-4, the $I\epsilon$ promoter (6). Since starting my own laboratory in 1991, we have continued working on the molecular mechanisms by which IL-4 functions. We have helped identify many of the molecules involved in IL-4 signaling, including STAT6 (7). We have helped define the mechanisms by which the IL-4 receptor transmits its signal to the nucleus (8–10). Recently, we have identified at least two proteins important in inhibiting IL-4 signaling, SOCS-1 and BCL-6 (11–13).

The pathways by which cytokines exert their biologic effects have been intensely researched over the past several years. Much of this work has defined the mechanisms by which binding of cytokines to their specific receptors initiates, downstream signaling. This work has defined several major signaling pathways initiated by this process. For type I and II cytokines, one of these pathways is the JAK-STAT signaling pathway. Our laboratory has been involved in defining the pathway for IL-4, a cytokine involved in the activation, proliferation and differentiation of a variety of hematopoietic cells, including B cells, T cells and mast cells (14). When IL-4 is provided to a cell, it initiates signaling by oligomerizing the heterodimeric IL-4 receptor, which in hematopoietic cells is composed of the ligand-specific IL-4 receptor chain (IL-4R α and the common gamma chain (γ C) (15). This oligomer-

ization initiates signaling by causing the activation of two non-receptor tyrosine kinases, JAK1 and JAK3, which are constitutively associated with receptor chains (JAK1 with IL-4R α and JAK3 with γ C). IL-13 also uses the IL-4R α and JAK1 to initiate signaling. In addition, IL-13 also binds to IL-13-specific receptor chains (15–17). The IL-13R α 1 chain binds Tyk2. The IL-13R α 2 chain does not appear to bind JAK kinases and may function as a decoy receptor. After binding of either IL-4 or IL-13, the activated JAK kinases phosphorylate tyrosines within the cytoplasmic domain of the IL-4R α , which act as docking sites for signaling molecules that contain either PTB or SH2 domains.

One signaling molecule important for IL-4 and IL-13 function is a member of the STAT (Signal Transducer and Activator of Transcription) family of transcription factors, STAT6. STAT6 is recruited to the activated IL-4R α chain in response to either IL-4 or IL-13 and is thereafter tyrosine-phosphorylated by the JAK kinases. Phosphorylated STAT6 dimerizes, translocates to the nucleus, and activates transcription of genes involved in B cell differentiation, including the germline epsilon gene and the low affinity Fc ϵ cell receptor, CD23. STAT6-deficient mice are deficient in class switching to IgE and in the development of atopic immune responses (18–20). However, these mice can develop atopic immune responses in certain experimental systems, suggesting that there may be other signaling pathways that perform functions redundant to that of STAT6.

Although the JAK-STAT signaling pathway is important for IL-4 function, recent data suggest that IL-4 signaling is more complex. The receptors and JAK kinases used by IL-4 are not uniform. The γ C receptor chain and the JAK3 kinase are not expressed in non-hematopoietic cells (21–23). In these cells, IL-4 signals either through homodimerization of the IL-4R α (JAK1) or through heterodimers of IL-4R α and the IL-13R α 1 chain (Tyk2). It is unclear if signaling using these different receptors and JAK kinases is distinct. In addition to this complexity, other kinases may be involved in IL-4 signaling. The Fes tyrosine kinase can be activated in response to IL-4 and appears to be involved in the activation of a subset of IL-4-activated pathways (24).

Asthma and other allergic diseases are increasingly prevalent in both developing and westernized nations (25). Environmental components clearly have a prominent role in asthma frequency (26). However, heritable factors have also been shown to have a major influence on atopic disease development, as illustrated by large-cohort twin studies (27). On a broader scale, human genome-wide linkage or candidate gene association studies have collectively implicated as many as

80 genes as contributing factors for allergy (28–30). With this information comes the challenge of understanding how these factors contribute to atopic disease at the molecular level.

Cytokines and their downstream signaling pathways are critical regulators of the immune response. Human genetic studies have demonstrated that polymorphisms affecting genes encoding cytokines or components of cytokine signaling pathways are strongly associated with allergic disease (31). One of the most replicated findings is the association between atopy and polymorphisms within human chromosome 5q31-33, which contains the genes encoding the cytokines IL-4, IL-5, and IL-13 (32–34). Similarly, compelling genetic evidence indicates that polymorphisms in the IL-4R α chain, and to a lesser extent in the IL-13R α 1 chain, contribute to atopic disease development (35, 36). In addition, polymorphisms in the signaling molecule STAT6 have also been associated with an increased risk of asthma (37–39). Considered together with functional studies establishing that IL-4 and IL-13 are central mediators of allergic inflammation, these data argue that the IL-4 and IL-13 signaling pathways have a critical and perhaps predominant role in atopic disease development.

The immune response underlying the inflammation found in allergic states is extremely complex, involving interplay between cytokines, chemokines and cells within the innate and adoptive immune system. Current models invoke activation of Th2-type memory/effector CD4⁺ T cells by allergen as a major initiator of allergic inflammation (40). The ensuing production of the Th2 cytokines IL-4, IL-5 and IL-13 by activated CD4⁺ T cells promotes the migration and activation of inflammatory cell types such as basophils, eosinophils and mast cells within the affected tissue. These cells in turn release soluble factors that promote and exacerbate the inflammatory state. In addition to cells within the immune system, IL-4 and IL-13 also interact with receptors expressed on non-hematopoietic cells, thus contributing to allergic inflammation by inducing functional responses in these populations.

Besides excessive production of Th2 cytokines, another clinical feature that is strongly associated with human allergic disease is increased serum IgE levels. Indeed, atopic individuals can exhibit increased “basal line” IgE levels and IgE-reactive cells even prior to an allergic reaction. As with allergic inflammation, IL-4 and IL-13 are the principal inducers of class switching to IgE by B cells (41). Conversely, IFN- γ can suppress class switching to IgE (42, 43). These observations, combined with the associations between IL-4, IL-13 and atopy from human genetic studies, argue that a detailed molecular analysis of how these cytokine signaling pathways are regulated will yield valuable

insights regarding the molecular mechanisms underlying atopic disease.

IL-4 SIGNALING AND IgE CLASS SWITCHING

IL-4 regulates several important aspects of B cell biology. One of the important functions is the regulation of immunoglobulin class switching to IgE and IgG1. Elevated levels of IgE are observed in patients with allergic diseases (44). Cross-linking of IgE induces the activation of mast cells and basophils and leads to the release of inflammatory mediators such as histamine and leukotriene (45). Thus, IgE production is very tightly controlled. When immature B cells migrate from bone marrow, they express IgM on their surface. Stimulation of these B cells with cytokines and co-stimulatory factors in germinal centers of peripheral lymphoid organs can cause them to produce other immunoglobulin isotypes through the process of immunoglobulin class switch recombination. Stimulation of B cells with IL-4 and CD40 ligand (or LPS) induces class switching to IgE and IgG1. For IgE class switching, IL-4 and CD40 stimulation induce germline epsilon transcription from I ϵ promoter. This promoter activity is regulated by several transcription factors, including STAT6, NF κ B, Pax-5, AP1, C/EBP, PU.1 and E2A (46). These transcription factors activate promoter activity to induce germline epsilon transcripts in response to IL-4 and CD40 stimulation. Transcriptional repressors such as Bcl-6 and Id2 are implicated in repression of the I ϵ promoter (11, 47). Indeed, Bcl-6-deficient mice show increased IgE production (13). Thus, both positive and negative transcriptional regulators can control the production of IgE through their regulation of this promoter. IL-4 induction of genes, germline epsilon and CD23 are maximally induced at 12–24 hours, and their induction by IL-4 is sensitive to cycloheximide. This leads us to hypothesize that germline epsilon and CD23 genes are actually induced by transcription factors that lie downstream of STAT6.

Germline transcription permits access of several enzymes to the S ϵ region, which is located immediately downstream of I ϵ exon (48, 49). Switch recombination includes the deletion and ligation steps between S μ and S ϵ region mediated by recombinases and cooperating molecules, including activation-induced cytidine deaminase (AID), histone H2AX, Ku 70/80, DNA-PK, 53BP1, XRCC4, DNA ligase IV, ATM and Bach2. However, these molecules involved in switch recombination regulate immunoglobulin class switching to all isotypes. Therefore, a critical step for determining the specificity of class switching to IgE

may be the regulation of germline transcription induced by IL-4 and CD40. Understanding how germline epsilon transcription is regulated by IL-4 leads to the development of therapeutic reagents that control allergy.

NFIL3 (NUCLEAR FACTOR INTERLEUKIN-3 REGULATED)

NFIL3 is a basic leucine zipper (bZIP) type transcription factor and highly related to PAR family transcription factors (50). NFIL3 was initially identified as E4BP4 (E4 binding protein 4) through its ability of binding to and repress adenoviral E4 promoter ATF sites (51). NFIL3 was shown to act as a transcriptional repressor, with a functional repression domain in its C-terminal region. This repressor domain binds to the transcriptional repressor protein Dr1 (52). NFIL3 was also identified as a transcriptional activator of IL-3 promoter in T cells (53). The functional differences in its ability to repress or activate transcription may be dependent on the cell-types, target genes or binding partners. NFIL3 binds to the sequence 5'-(G/A)T(G/T)A(C/T)GTAA(C/T)-3', which is similar to the binding sequences of CREB/ATF, AP-1, C/EBP and PAR family proteins. This binding sequence has been identified in the promoter regions, including IL-3 and IFN- γ genes (53). The NFIL3 gene is localized in chromosome 9q22 in humans and chromosome 13 in mice.

NFIL3 is induced by several cytokines and hormones (54–60). Interestingly, *de novo* synthesis of protein is not required for NFIL3 induction by IL-4, but is required for the induction of the gene by IL-3 (57). IL-4- and IL-10-induced NFIL3 expression are STAT6- and STAT3-dependent, respectively (61, 62). Both IL-4 and IL-10 are multifunctional cytokines produced by a subset of activated T cells and crucial for the development of an allergic reaction. This evidence suggests that NFIL3 is induced via the JAK-STAT pathway at early time point after cytokine stimulation as an “immediate early gene”.

The function of NFIL3 *in vivo* is not fully understood. NFIL3 may have an important role in the regulation of the circadian clock (50, 63–65). NFIL3 drives the circadian expression of Per2 gene by direct binding to the promoter region of Per2 gene in cell line experiments (66). However, direct evidence has yet to support a requirement for NFIL3-mediated regulation of the circadian clock. Another biological role of NFIL3 is in suppression of apoptosis. NFIL3 is expressed in motor neurons, and its overexpression induces neuronal cell growth—hand reduces the number of dying motoneurons (67). In immune cells, overexpression of NFIL3 in IL-3-dependent B-cell lines prevents apo-

ptosis induced by IL-3 depletion, suggesting an anti-apoptotic role of NFIL3 in the cytokine-dependent manner (57). Interestingly, in *C. elegans*, the NFIL3 homologue, CES-2, has a pro-apoptotic function by suppressing the survival gene, CES-1 (68). NFIL3 is also implicated in malignant transformation that involves STAT3 activation in human tumors (69). The finding that NFIL3 regulates cell survival in B cells, suggests that NFIL3 may cooperate with other STAT3-target genes in oncogenesis through the regulation of cell survival. NFIL3 suppresses the transcription of hepatitis virus genes and production of virions (70), suggesting NFIL3 may function as an anti-viral infection factor at the level of transcription of this virus.

IDENTIFICATION OF NFIL3 AS AN IL-4-INDUCIBLE GENE IN B CELLS

Previously, we identified genes that are regulated by IL-4 in a STAT6-dependent manner (62). Splenic naive B cells were isolated from wild-type (WT) and STAT6 KO mice and stimulated with LPS and IL-4 for 24 hours. Total RNA was then isolated and hybridized to Affymetrix U74A GeneChip microarrays in order to obtain gene expression profiles. Analysis of the acquired data identified 114 probe sets that gave statistically significant differences in signal when WT and STAT6 KO samples were compared. These probe sets represent 106 distinct cDNAs, 70 of which are derived from known genes, and 36 of which are expressed sequence tags (ESTs). Differentially expressed genes were grouped into transcription factors/transcription-related proteins, kinases and phosphatases, other enzymes, cytokines/cell surface receptors, immunoglobulin heavy chain genes, and miscellaneous. The expression levels of selected IL-4-induced STAT6-dependent genes as identified by microarray analysis were confirmed by Northern and Western blot analysis (62). This approach identified several genes known to be induced by IL-4, including *Bcl-6*, the STAT6 target genes *Cd23* and *Il4r*, and the gene *E4bp4/Nfil3* (56), which encodes the transcription factor NFIL3. Among these genes, the expression level of *E4bp4/Nfil3* was strikingly different between WT and STAT6 KO samples, suggested that expression of NFIL3 protein is dramatically increased in response to IL-4 stimulation, and that NFIL3 has an important role in responses to IL-4 in the context of an immune response.

NFIL3 RNA AND PROTEIN EXPRESSION ARE RAPIDLY INDUCED BY IL-4 STIMULATION OF B CELLS

To confirm that NFIL3 expression is regulated by IL-4, we used the B cell line, M12, to examine the induction of NFIL3 mRNA and protein in response to stimulation with LPS alone, IL-4 alone or LPS plus IL-4. NFIL3 mRNA expression was not increased above baseline by LPS stimulation at any time point examined. In contrast, NFIL3 mRNA expression was sharply up-regulated after 1 hour of exposure to IL-4 alone or LPS plus IL-4 and reached peak levels at 2 hours after stimulation. In contrast to that described previously regarding induction of NFIL3 mRNA by IL-3 (57), IL-4-induced expression of NFIL3 mRNA was not blocked by cycloheximide, indicating that this effect does not require *de novo* protein synthesis. To assess NFIL3 protein expression, cell lysates were prepared from M12 cells treated with LPS alone, IL-4 alone, or LPS plus IL-4 and subjected to Western blot analysis. In the absence of stimulation, NFIL3 protein was maintained at low levels. Similar to that seen for NFIL3 mRNA, LPS treatment of cells did not affect NFIL3 protein levels, while exposure to IL-4 alone or LPS plus IL-4 elicited increased expression of NFIL3 for up to 24 hours after stimulation. The rapid induction of NFIL3 in response to IL-4 suggests that NFIL3 plays a role in the modulation of gene regulation that occurs downstream of IL-4 signaling.

GENERATION OF NFIL3 KNOCKOUT (KO) MICE

To determine the importance of NFIL3 expression in the immune system, the gene encoding this protein was disrupted by homologous recombination in embryonic stem (ES) cells. The second exon of *Nfil3* contains the entire protein coding region. A targeting vector was constructed so that this exon was replaced with the neomycin resistance gene. Homologous recombination in ES cells between the endogenous gene and the targeting vector was confirmed by PCR analysis and Southern blotting. Successfully targeted ES cells were injected into C57BL/6 blastocysts to generate chimeric mice, which were then bred to obtain germ-line transmission of the disrupted *Nfil3* allele. Heterozygous founder mice were obtained and bred with C57BL/6 mice to produce F1 heterozygotes; these were intercrossed to generate mice homozygous for the disrupted *Nfil3* allele [NFIL3 knockout (KO) mice]. Genotypes were determined by Southern blotting and PCR analysis. NFIL3 KO mice appear grossly normal and are fertile. To determine if the gene targeting resulted in the loss of NFIL3 protein expression in NFIL3 KO mice, splenocytes were stimulated with IL-4 and cell lysates

analyzed by Western blotting. NFIL3 protein was not detected in lysates prepared from NFIL3 KO splenocytes, indicating that the *Nfil3* gene was successfully disrupted and protein expression abolished.

PRODUCTION OF IgE IS IMPAIRED IN NFIL3 KO MICE

To determine if loss of NFIL3 alters immunoglobulin heavy chain class switching, we examined baseline immunoglobulin concentrations in sera from WT and NFIL3 KO mice. Concentrations of IgM, G1, G2a, G2b, G3 and A were similar in sera from WT and NFIL3 KO mice. In contrast, serum concentrations of IgE in NFIL3 KO mice were, on average, decreased more than 2-fold relative to those in WT mice, suggesting that the loss of NFIL3 specifically affects IgE synthesis. To further pursue this possibility, WT NFIL3 KO and mice were immunized and boosted with ovalbumin (OVA) emulsified in Alum. Fourteen days after boosting, serum was collected and immunoglobulin levels examined. Concentrations of total IgM, all IgGs, and IgA were comparable in sera from immunized WT and NFIL3 KO mice. Increased concentrations of IgE were detected in sera from immunized WT mice relative to baseline (un-immunized), indicating that IgE synthesis was induced in response to OVA challenge. In contrast, total IgE levels in sera from immunized NFIL3 KO mice were ~5-fold lower compared to that in sera from WT. Further OVA-specific IgE in sera from NFIL3 KO mice was reduced relative to that seen WT mice. These data suggest that NFIL3 is specifically required for efficient production of IgE.

REDUCED IGE PRODUCTION BY NFIL3 KO B CELLS IN RESPONSE TO IL-4 STIMULATION

To determine if B cells from NFIL3 KO mice are intrinsically altered in their ability to class switch, we assessed production of IgG1 and IgE by cultured B cells. Isolated splenic B cells were stimulated with LPS and IL-4 for 5 days. Cells were then harvested, stained for surface IgE and IgG1 expression, and analyzed by flow cytometry. Only 1.8 % of NFIL3 KO B cells expressed surface IgE following stimulation, while 14% of WT B cells were surface IgE⁺. Interestingly, although the serum levels of IgG1 in NFIL3 KO mice were normal, only 7.5 % of NFIL3 KO B cells were IgG1⁺ in contrast to 13% of WT B cells.

To further quantify the impairment of IgE production by NFIL3 KO B cells, culture supernatants from B cells were collected after 5 days of stimulation with LPS alone or LPS plus IL-4 and the levels of secreted immunoglobulin determined. IgM, IgG2a, G2b and G3 production by

LPS-activated B cells was unaltered in NFIL3 KO B cells. IgG1 produced by LPS/IL-4-stimulated NFIL3 KO B cells was similar to that of WT cells. In sharp contrast, IgE secretion by B cells from NFIL3 KO stimulated with LPS/IL-4 was significantly decreased.

We next examined whether NFIL3 is important for production of the germline epsilon (ϵ) mRNA by B cells in response to IL-4. B cells were stimulated for 5 days with LPS and IL-4 and then RNA was isolated. Northern blot analysis of RNA was performed using a DNA probe encoding a portion of the IgE constant region in order to detect both germline epsilon and post-switch VDJ-C epsilon mRNA. Germline epsilon transcripts were strongly induced in WT B cells but not in NFIL3 KO B cells. After 4 days of stimulation, post-switch transcripts were induced in WT B cells, but expression levels of these transcripts in NFIL3 KO B cells were significantly lower. These data suggest that NFIL3 regulates transcription of germline epsilon transcription and class switching to IgE.

NORMAL STAT6 ACTIVATION BUT IMPAIRED CD23 UPREGULATION IN NFIL3 KO B CELLS

It has been shown that JAK kinase and STAT6 activation induced by IL-4 receptor engagement is critical for upregulation of germline epsilon gene transcription. Therefore, we tested whether the lack of NFIL3 in B cells affects STAT6 activation in response to IL-4 stimulation. WT and NFIL3 splenic B cells were cultured for 1 hour in the absence or presence of LPS plus IL-4. Cell lysates were then prepared and probed for activated STAT6 using anti-phospho-STAT6 antibodies. Similar levels of activated STAT6 were detected in lysates from WT and NFIL3 KO B cells, suggesting that the JAK-STAT6 activation pathway is not impaired by the absence of NFIL3.

The cell surface protein, CD23, plays critical roles in the regulation of IgE levels and the production of proinflammatory cytokines (45). Expression of CD23 is also induced by IL-4 stimulation in a STAT6-dependent manner. Therefore, we determined if NFIL3 deficiency affects the induction of CD23 mRNA production in response to IL-4. Splenic B cells were stimulated with LPS alone, IL-4 alone, or LPS plus IL-4 for 2 days, and cell surface expression of CD23 was determined by flow cytometry. WT B cells responded to IL-4 stimulation by upregulating CD23 expression. However, CD23 upregulation on NFIL3 KO B cells was less efficient. This result indicates that NFIL3 is required for CD23 induction. CD23 upregulation by IL-4 requires *de novo* protein synthesis, while NFIL3 induction does not. These results

suggest that IL-4-induced NFIL3 expression may cooperate with other transcription factors to induce CD23 mRNA transcription. Overall, these data demonstrate that NFIL3 is important for the regulation of CD23 expression by B cells.

ALTERED AIRWAY HYPER-RESPONSIVENESS IN NFIL3 KO MICE

As supported by studies involving humans and mice, IL-4 has a central role in the development of allergic airway inflammation and airway hyper-responsiveness. We therefore asked whether these processes would be affected by the absence of NFIL3 expression as assessed in a mouse model.

Mice were sensitized and challenged with OVA using a previously described model (71). Consistent with the data described above, serum IgE levels in OVA-sensitized NFIL3 KO mice were significantly decreased compared to those in WT mice. In contrast, IgG1 levels were comparable in serum from OVA-sensitized WT and NFIL3 KO mice. After exposure to aerosolized OVA, leukocyte populations present in the airways of mice were recovered by bronchoalveolar lavage (BAL). Recovered cells were counted and leukocyte subsets enumerated based on morphology (as visualized by differential staining and microscopy).

The major population of cells recovered by BAL of WT or NFIL3 KO mice were eosinophils, indicating that exposure of OVA-sensitized mice to aerosolized OVA elicited the expected immune response. The total number of cells present in BAL fluid from NFIL3 KO mice was greater than that in BAL fluid from WT mice; this correlated with greater numbers of eosinophils being recovered from NFIL3 KO mice. Based on percentage, the relative proportions of cell types present in BAL fluid from WT or NFIL3 KO mice were similar, with the majority being eosinophils (79.5% in WT versus 85.75% in NFIL3 KO). Taken together, these data suggest that the lack of NFIL3 causes increased influx of leukocytes into the airway in response to aerosolized antigen.

To examine the extent of airway hyper-responsiveness elicited by aerosolized OVA challenge, methacholine-induced airway obstruction was assessed two days after the last exposure to OVA. Analysis of unsensitized mice showed that the basal responses of WT and NFIL3 KO mice to methacholine were similar. As expected, OVA-sensitized WT mice showed increased airway obstruction in response to methacholine challenge. In striking contrast, the responses of OVA-sensitized NFIL3 KO mice to methacholine were similar to those of unim-

munized WT or NFIL3 KO mice. These data suggest that NFIL3 plays a role in controlling the development of airway hyper-responsiveness in a mouse asthma model.

DISCUSSION

Our data demonstrate that expression of NFIL3 RNA and protein is dramatically upregulated in B cells in response to IL-4 signaling and STAT6 activation. We have successfully generated NFIL3 KO mice and our analysis of these mice allows for several preliminary conclusions.

1) NFIL3 is an important regulator of B cell responses to IL-4

Our data show that basal and antigen-specific IgE production in NFIL3 KO mice is impaired. Our data also demonstrate that cultured NFIL3 KO B cells generate less IgE in response to stimulation with LPS and IL-4; this correlates strongly with reduced expression of germline epsilon and class-switched epsilon mRNA transcripts and decreased upregulation of surface CD23 expression in response to IL-4. Thus, our data raise the possibility that IL-4-activated STAT6 immediately induces NFIL3, and that the induced NFIL3 is involved in the transcriptional regulation of the ϵ and CD23 promoters. This may be in cooperation with other transcription factors including STAT6, NF κ B, Pax5, AP1, C/EBP and Bcl-6.

2) NFIL3 influences the extent of allergic airway inflammation and airway hyper-responsiveness

Our preliminary data suggest that the absence of NFIL3 alters the responses of mice to immunization by antigen (OVA). The decreased airway response in these mice, along with the increased number of cells, suggests that NFIL3 regulates other aspects of the immune response in addition to IgE production. B cell-deficient mice systemically sensitized with OVA (i.p.) can develop normal levels of airway hyper-responsiveness (72). In addition, IgE-deficient mice sensitized with *Aspergillus fumigatus* intranasally showed significant airway hyper-responsiveness comparable to control mice, suggesting that airway responsiveness can be elicited via IgE-independent mechanisms (73). These data suggest that IgE is not required for the development of airway hyper-responsiveness in mouse asthma models. Thus, the altered airway hyper-responsiveness we observe in NFIL3 KO mice is not likely due to decreased levels of IgE. We hope that defining the molecular mechanisms by which NFIL3 regulates IgE and airway

hyper-responsiveness will lead to novel therapeutic approaches for controlling allergic diseases.

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DISCUSSION

Czeisler, Boston: On a historical note I would just point out that a former member of the Society, George Thorne, actually wrote a fellowship paper in the *New England Journal* many years ago in which he used eosinophil counts in college rowers who were on the Harvard crew team to demonstrate circadian variations and to track them. So there is some connection with the society going back. Now in terms of the *DEC2* gene you just mentioned, there was just a publication in *Science* a couple of months ago showing that a mutation that produced a particular variant in the *DAK2* gene was related to spontaneous sleep duration, that could be transmitted to different, not just where it was found in a particular human family but also it was put into mice and they slept a shorter amount per day. So, that may also relate to sleep duration. Steve Shay in our group is looking at the relationship in nocturnal asthma, where it's principally a sleep-related phenomenon or a circadian variation. So, I think the links go back many years, and you may perhaps uncover some of the relationships between why there is this prominent circadian variation in many of these conditions.

Rothman, Iowa City: Actually it's that same group that published the *Science* paper that has this other paper coming out on this and since I rowed for MIT, I am very interested that Harvard rowers actually have circadian changes too.

Martin, Chevy Chase: Paul that was a wonderful presentation. Since asthma has early life origins *in utero* or the first months of life, is *NFIL3* under some type of epigenetic control or are there other types of epigenomic components to its expression?

Rothman, Iowa City: That's a great question. We haven't so far found any epigenetic control in the mice. You can induce it in many, many tissues with IL-4, but possibly in humans it would be a great candidate gene to look at for control by environment.

Martin, Chevy Chase: So your knockouts, your *NFIL3*-knockouts, are in pathogen-free housing, right?

Rothman, Iowa City: Yes.

Martin, Chevy Chase: What happens if they go into a pathogen-rich environment? Do they develop parasites?

Rothman, Iowa City: It's a great question. We haven't done it, nor have we seen if they actually don't sleep at night. So, we have a lot yet to do on the mice.