Genome-Wide Methylation Study Identifies an IL-13–induced Epigenetic Signature in Asthmatic Airways

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Abstract

Rationale: Epigenetic changes to airway cells have been proposed as important modulators of the effects of environmental exposures on airway diseases, yet no study to date has shown epigenetic responses to exposures in the airway that correlate with disease state. The type 2 cytokine IL-13 is a key mediator of allergic airway diseases, such as asthma, and is up-regulated in response to many asthma-promoting exposures.

Objectives: To directly study the epigenetic response of airway epithelial cells (AECs) to IL-13 and test whether IL-13–induced epigenetic changes differ between individuals with and without asthma.

Methods: Genome-wide DNA methylation and gene expression patterns were studied in 58 IL-13–treated and untreated primary AEC cultures and validated in freshly isolated cells of subjects with and without asthma using the Illumina Human

Methylation 450K and HumanHT-12 BeadChips. IL-13–mediated comethylation modules were identified and correlated with clinical phenotypes using weighted gene coexpression network analysis.

Measurements and Main Results: IL-13 altered global DNA methylation patterns in cultured AECs and were significantly enriched near genes associated with asthma. Importantly, a significant proportion of this IL-13 epigenetic signature was validated in freshly isolated AECs from subjects with asthma and clustered into two distinct modules, with module 1 correlated with asthma severity and lung function and module 2 with eosinophilia.

Conclusions: These results suggest that a single exposure of IL-13 may selectively induce long-lasting DNA methylation changes in asthmatic airways that alter specific AEC pathways and contribute to asthma phenotypes.

Keywords: epigenetic signature; asthma; airway epithelial cells; IL-13

Pathogenic changes in complex airway diseases result from combinations of factors that drive specific arms of disease progression across multiple tissues (1). It has long been suggested that epigenetic changes to airway cells modulate the effects of environmental exposures on

airway diseases (reviewed in References 2–5), yet no study to date has directly shown epigenetic responses to relevant exposures in the lung that correlate with disease state. IL-13 is a type 2 cytokine that is a key mediator of airway inflammation and remodeling in many inflammatory

diseases of the lung (6, 7), such as asthma (8–10). Although other T-helper cell type 2 (Th2) cytokines (IL-4, -5, -9) are associated with symptoms of asthma, only IL-13 has been shown to be necessary and sufficient to induce all the features of asthma in mouse models (10). This finding is

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At a Glance Commentary

Scientific Knowledge on the

Subject: Epigenetic changes to airway cells have been proposed as important modulators of the effects of environmental exposures on airway diseases, yet no study to date has shown epigenetic responses to exposures in the airway that correlate with disease state.

What This Study Adds to the

Field: We demonstrate that a single exposure to IL-13, a cytokine critically involved in asthma pathogenesis, creates an epigenetic signature in airway epithelial cell cultures that persists in freshly isolated airway epithelial cells from individuals with asthma. This signature is highly correlated with nearby gene expression and enriched near genes associated with asthma. This signature is mainly comprised of two modules of comethylation that are correlated with asthma severity and lung function or eosinophilia. Collectively, our data indicate that IL-13-mediated methylation changes contribute to asthma pathogenesis through alterations to specific arms of IL-13-mediated pathways and interaction with asthma-associated genetic variation.

consistently supported in human studies (reviewed in Reference 11). IL-13 signaling mediates the airway response to many environmental exposures, such as air pollution (12), viruses (13), and inhaled allergens (14), among others, which are facilitated across disease states through coordinated effects in airway epithelial, smooth muscle, and local immune cells (reviewed in References 15 and 16).

Yet, despite the established central role that IL-13 plays in airway inflammation and remodeling and the abundance of information about the downstream signaling pathways that mediate its effects (7, 17–23), little is known about the underlying molecular mechanisms that influence airway transcriptional response to IL-13 in general, or about IL-13-mediated changes in DNA methylation in particular. It is currently

unknown whether IL-13 exposure alters DNA methylation patterns in the airway, if potential changes target specific pathways/genes, or if changes are long-lasting. Because of the reversible nature of DNA methylation, many alterations that occur in response to environmental stimulation may be short-lived or lost over time. Therefore, identifying IL-13-mediated methylation changes within a short window after exposure may elucidate new therapeutic targets and advance the understanding of the underlying epigenetic mechanisms that promote IL-13-mediated pathogenesis.

To this end we used cultured, primary human airway epithelial cells (AECs) to identify IL-13-mediated changes in DNA methylation and to characterize this epigenetic signature with respect to the transcript abundance of nearby genes. To assess the biologic relevance of our model, we compared these data with DNA methylation data from freshly isolated AECs to identify IL-13-responsive CpGs that also differ between subjects with and without asthma. We show that epigenetic modifications at thousands of CpG sites occur after only one 24-hour exposure to IL-13 in a cell culture model, and that the IL-13-responsive CpG sites are enriched near genes that have been associated with asthma. Moreover, we show that a significant portion of this IL-13-mediated epigenetic signature is mirrored in freshly isolated AECs of subjects with compared to those without asthma. These epigenetic changes validate our cell culture model data and likely represent changes that are more permanently altered by IL-13 exposure. Importantly, this epigenetic signature clusters in fibrotic and inflammatory pathways, demonstrating the biologic relevance of our cell culture model and implicating epigenetic modifications in the development and persistence of key pathognomonic features of asthma in the airways. Some of the results of these studies have been previously reported in an abstract (24).

Methods

An overview of the study design can be found in Figure E1 in the online supplement.

Sample Composition and Nucleic Acid Extraction

Cultured cells. Human donor lungs that were not suitable for transplantation were obtained from deceased individuals through Gift of Hope. Primary AEC cultures were established from these lungs at the University of Chicago Lung Biospecimen Core. Sample composition information can be found in the online METHODS section.

Cells at passage 0 or 1 were cultured in duplicate in a six-well plate format in BEGM media (Lonza, Basel, Switzerland) to 70% confluency and then treated with either 10 ng/ml IL-13 (Peprotech, Rocky Hills, NJ) or vehicle (5% fetal bovine serum) for 24 or 48 hours, as in previous studies (18, 23, 25). RNA for expression studies and DNA for methylation studies were extracted from vehicle and IL-13-treated cultures after 24-hours using the Qiagen AllPrep kit (Qiagen, Valencia, CA).

Freshly isolated cells. Seventy-five adults with asthma and 41 adults without asthma were recruited for bronchoscopic studies at the University of Chicago. Asthma diagnosis criterion can be found in the online METHODS section.

DNA and RNA for methylation studies was isolated from epithelial cell brushings using the Qiazol lysis reagent (Qiagen). DNA was concentrated using the Millipore Amicon Ultra centrifugal filters-0.5 ml 30K membrane according to manufacturer's instructions (Millipore, Billerica, MA).

Methylation Studies

Methylation was assessed in freshly isolated and cultured AEC samples using the Infinium Human Methylation 450K Bead Chip (Illumina, San Diego, CA; see online METHODS) (26). Methylation data were processed using the minfi package (27) and Infinium type I and type II probe bias was corrected for using the SWAN algorithm (28). We corrected raw probe values for color imbalance and background by controls normalization. At each CpG site the methylation level is reported as a β value, which is the fraction of signal obtained from the methylated beads over the sum of methylated and unmethylated bead signals.

Gene Expression Studies

Microarray. RNA from the cultured cells was hybridized to the Illumina Human HT-12 v4 array in the Functional Genomics

Core at the University of Chicago. Probe level raw intensity values across arrays were normalized using quantile normalization and background corrected normalized expression values were obtained for each probe (see online METHODS). We removed probes that were indistinguishable ($P < 0.01$) from background intensity, contained greater than one Hapmap single-nucleotide polymorphism, or mapped to several locations in the genome (29), and selected the median probe intensity to represent the transcriptional abundance of each gene. Of the 47,231 transcripts assayed on the Illumina HTv12 expression arrays, 13,532 (29%) were detected as expressed in at least one sample.

RNAseq. We obtained RNAseq data from AECs from 82 of the 116 individuals in the methylation studies of freshly isolated AECs. cDNA libraries were constructed using the TruSeq RNA Sample Preparation v2 Guide (Illumina) and run on the Illumina HiSeq2500 platform. RNAseq data was mapped to the transcriptome using BWA (Burrows-Wheeler Aligner) (30). Sequences that overlapped with protein coding regions were determined using BEDTools (31). The median number of mapped reads was 19,210,000, with a range of 10,100,000 to 51,150,000 per individual. The gene counts were adjusted for gene length and variation in sample read depth and exported. Expression estimates were obtained for 26,393 genes, and estimates were filtered to genes that had at least five reads in two individuals reducing the number of genes to 19,296. Subsequent analyses were performed in R (v2.15.2). Data were normalized using upper quartile normalization. Unwanted variation that was not associated with asthma status were identified using the algorithms of RUVSEquation (32). Three RUVs were ultimately included in our model. Principal component analysis was performed on the residuals after regressing out these sources of variation. No additional technical or biologic covariates were identified; however, three outliers were removed. Differential gene expression was then assessed using the negative binomial generalized linear model as implemented in RUVSEquation (32).

Weighted Gene Coexpression Network Analysis Comethylation Network Analysis

Methylation levels for the 2,020 IL-13-responsive CpG sites that were also

differentially methylated by asthma status were clustered according to coordinated methylation changes using the weighted gene coexpression network analysis (WGCNA) program under the default settings (33). The soft thresholding power was determined to be six. Eigengenes of the comethylation modules were generated according to author instructions. WGCNA calculates an eigengene (i.e., the first principal component) for each module (34). This vector represents a weighted average profile of the methylation changes in each module. The eigengenes for each module were correlated with the clinical phenotypes.

Ingenuity Pathway Analysis of Protein-Protein Interaction Networks

Gene lists of interest were interrogated in ingenuity pathway analysis (IPA) and network associations were constructed using the Ingenuity Knowledge Base. We limited network interactions to those known to occur in primary cells or tissue. All other factors were kept at default settings. The network score is based on the network hypergeometric distribution and is calculated with the right-tailed Fisher exact test to identify enrichment of those genes that were associated with IL-13-responsive CpG-gene pairs in the network relative to the IPA database.

Statistical Analyses

Data were analyzed with R software (version 3.1.2). We used a random effects model with individual ID coded as a random effect to identify CpG sites or genes that were differentially methylated or expressed in culture cells and standard linear regression in freshly isolated cells. Because of the low CpG density of the 450K array we focused our analyses on single CpG sites. The Kolmogorov-Smirnov test was used to compare the distributions of Pearson correlation P values. Permutations were performed by randomly selecting 6,522 CpGs (the number of IL-13-responsive CpGs in the cultured cells) from the asthma dataset, calculating their P values for differential methylation by asthma status, and then assessing significance using q value. We recorded the number of times (out of 10,000) that the number of CpGs with a q value less than 0.05 was greater than our observed value of 1,636.

Results

IL-13 Induces Changes in Genome-Wide DNA Methylation Levels

We first determined the extent to which IL-13 treatment alters DNA methylation patterns in primary AEC cultures derived from 57 unrelated white adults (see METHODS). Overall, 6,522 CpG sites (2.0%) were differentially methylated between vehicle (5% fetal bovine serum) and IL-13-treated cells out of 327,174 probes on the Illumina 450k array that passed quality control checks (see METHODS) (Figure 1A). The median absolute change in methylation was 1.1% (range, 0.1–7.1%). Among these IL-13-responsive CpGs, 2,920 (44%) became more methylated and 3,602 (56%) became less methylated after exposure to IL-13 (see Table E2). Most of these sites (5,042/6,522–77%) are located in a gene body or within 1,500 bp of a gene transcription start site (41% and 36%, respectively), similar to the overall distribution of CpGs on the array. These 5,042 IL-13-responsive CpG sites are in or near 3,771 unique genes. Information on their locations relative to genes can be found in Table E3. Although most genes were associated with one IL-13-responsive CpG site, some were associated with multiple sites, such as tenascin B (*TNXB*), which had 12 hypomethylated IL-13-responsive CpG sites in its gene body. Many genes shown to be responsive to IL-13 in this study and others (21, 35) were near differentially methylated CpGs, including chitinase 3-like 1 (*CHI3L1*) (encoding YKL-40), a biomarker for asthma in the blood (36).

IL-13 Induces Changes in Transcript Abundance

We next measured transcript abundance in these same cells. Sixty-three percent (8,524 genes) of the 13,532 genes detected as expressed on the Illumina HT12v4 array were differentially expressed between vehicle and IL-13-treated cells (Figure 1B). Transcript levels at these differentially expressed genes increased for 4,445 (52%) and decreased for 4,079 (48%) (see Table E4), and included up-regulation of chemokine (C-C Motif) ligand 26 (*CCL26*, encoding eotaxin 3), a cytokine known to be differentially expressed in response to IL-13 treatment (37), and the genes

encoding periostin and serpin B2, both of which are considered to be markers of the Th2-high asthma phenotype (17, 38).

IL-13-mediated CpG Site Methylation Levels Are Correlated with Nearby Gene Expression

To assess the relationship between IL-13-induced changes in DNA methylation and IL-13-responsive genes, we first identified all CpG sites in or near genes detected as expressed in our study. Of the 8,524 IL-13-responsive transcripts, 7,553 were within 1,500 kb of a CpG site assayed on the methylation array. A total of 1,590 (21%) of these genes were in or near at least one IL-13-responsive CpG site. We refer to these as IL-13-responsive CpG-gene pairs.

We then calculated Pearson correlation P values between methylation levels at all CpG sites within or near a gene and the transcript abundance of its nearest gene. The distribution of P values for IL-13-responsive CpG-gene pairs was significantly different from the distribution of correlation P values for all CpG-gene pairs (Figure 1C, *dashed* vs. *solid* line, respectively; $P < 2.2 \times 10^{-16}$), reflecting the many more low correlation P values between methylation response and transcriptional response to IL-13 among these pairs. Similarly, small P value enrichments were observed in separate analyses of CpG sites located in promoters or in gene bodies (see Figure E2).

IL-13-responsive CpG-gene pairs included genes, such as neutrophil cytosolic factor 2 (*NCF2*), a component of the NADPH oxidase complex, which has been associated with pulmonary fibrosis (39) and metalloproteinase 14 (*MMP14*), a molecule critical to IL-13-mediated fibrosis (21). IL-13-responsive CpG-gene pairs are enriched in many proinflammatory and fibrotic networks (see Figure E3) consistent with AEC IL-13 responsiveness. Overall, our data show that a large portion (21%) of the IL-13-mediated transcriptional response is associated with nearby methylation changes and that these associations are enriched for correlations with small P values. Our data collectively demonstrate a strong epigenetic component to IL-13 treatment of AECs and likely IL-13-mediated disease.

Genes Near IL-13-Responsive CpG Sites Are Enriched for Asthma Genes

Because of the central role that IL-13 has in asthma pathogenesis, we directly assessed the relationship between IL-13-mediated epigenetic changes and asthma. We first asked whether the IL-13-responsive CpG-gene pairs are enriched for genes with previously reported genetic or epidemiologic associations with asthma, as defined in the HuGE Navigator database (40). Indeed, the IL-13-responsive CpG-gene pairs were significantly enriched for genes associated with asthma

(Figure 2A). No such enrichment was present when all IL-13-responsive transcripts were evaluated ($P = 0.76$) (Figure 2A). There was evidence for enrichment of asthma genes among all genes with a nearby IL-13-responsive CpG site ($P = 0.031$) (Figure 2A), and for the subset of IL-13-responsive CpG-gene pairs (correlation $P < 0.2$; $P = 0.023$) (Figure 2A). The distributions of P values for the correlations between DNA methylation and gene expressions levels among the IL-13-responsive CpG-gene pairs was significantly different from the other subsets ($P < 2.2 \times 10^{-16}$) (Figure 2B, *dashed* line vs. others), with an abundance of highly correlated CpG-asthma gene pairs among the former. An example of an IL-13-responsive CpG-gene pair is shown in Figure 2C. Neutrophil cytosolic factor 2 is a component of the NADPH oxidase complex. This complex produces reactive oxygen species that aid in cell growth, adhesion, and antimicrobial defense. Overproduction of reactive oxygen species by NADPH oxidase is commonly associated with respiratory inflammatory diseases including asthma (reviewed in Reference 41). Collectively, these data show that IL-13-induced epigenetic modifications in AECs are enriched near and correlated with the expression of genes that have been associated with asthma, suggesting that such IL-13-mediated epigenetic changes near these genes may

Figure 1. Methylation and gene expression levels differ between vehicle and IL-13-treated primary airway epithelial cells. (A) Volcano plot showing methylation differences between vehicle and IL-13-treated cells. The negative $\log_{10}$ -transformed P values are shown on the y -axis, and the mean difference in methylation β values are shown on the x -axis. CpGs that are differentially methylated at a q value < 0.05 are shown in gray. (B) Volcano plot showing gene expression differences between vehicle and IL-13-treated cells. Transcripts that are differentially expressed at a q value < 0.05 are shown in gray. (C) Density plot of CpG-gene pair Pearson correlation P values for the IL-13-responsive CpGs that are also associated with a differentially expressed gene (*dashed* line), compared with the remaining CpG-gene pairs (*solid* line). Distributions are significantly different from each other ($P < 2.2 \times 10^{-16}$).

underlie some proportion of the risk for asthma.

IL-13 Epigenetic Signature Is Mirrored *In Vivo*

The data from our cell culture model represent the immediate epigenetic responses to IL-13. We reasoned, however, that a portion of this epigenetic signature should be recapitulated in AECs from individuals with and without asthma because of the persistently elevated levels of IL-13 in the asthmatic airway (9, 10). To determine whether IL-13-induced methylation patterns in our cell culture model mirror methylation patterns *in vivo*, we studied freshly isolated AECs from 59 adults with asthma and 27 adults without asthma and analyzed the 6,522 IL-13-responsive CpG sites for differential methylation in asthmatic and nonasthmatic airway cells (Figure 3A).

The *P* value distribution revealed a clear enrichment for low *P* values in the freshly isolated AECs (Figure 3A). Of the 6,522 IL-13-responsive CpGs in our cell culture model, 2,020 (31%) were also differentially methylated in freshly isolated AECs from adults with asthma versus without asthma (Figure 3B).

Moreover, the direction of effect was the same in 74% (1,497/2,020) of these CpG sites that were either more methylated after IL-13 treatment and more methylated in asthmatic airways or less methylated after IL-13 treatment and less methylated in asthmatic airways (Figure 3B, *black dots*). Among this subset of overlapping CpGs, the median absolute change in methylation was 1.4% (range, 0.13–6.6%) between IL-13-treated and -untreated samples and 1.8% (range, 0.10–24.3%) between asthmatic and nonasthmatic samples. The number of overlapping CpGs between the *in vitro* and *in vivo* studies are significantly higher than expected by chance ($P < 10^{-5}$; see METHODS), and suggest that differences in DNA methylation patterns observed between individuals with and without asthma at the IL-13-responsive CpG sites were caused at least in part by chronic IL-13 exposure in asthmatic airways. Finally, methylation levels at these CpG sites were also enriched for correlation with gene expression in the primary cells, as evidenced by the abundance of CpG–gene pairs with low Spearman correlation *P* values in the freshly isolated cells (Figure 3D).

WGCNA Reveals Two Distinct Comethylation Networks among the IL-13 Epigenetic Signature in AECs That Are Associated with Clinical Asthma Phenotypes

To better define the relationship between the IL-13-induced epigenetic signature in asthmatic airway cells and clinical phenotypes, we used WGCNA to cluster CpG sites with correlated methylation patterns into modules and then examined correlations of the modules with clinical outcomes. Of the 2,020 overlapping CpG sites, 1,746 (86%) clustered into two comethylation modules: one module (module 1) was comprised of 722 CpG sites (477 unique genes), and the other (module 2) was comprised of 1,024 CpG sites (642 unique genes). The remaining 274 CpG sites (211 unique genes) were not significantly associated with a comethylation pattern (see Table E5). We next tested for associations between each of these modules and clinical phenotypes in the subjects with asthma (see Table E1; see METHODS). Each module was significantly correlated with distinct clinical phenotypes with module 1 associated with measures of asthma severity, percent predicted FEV₁, inhaled

Figure 2. IL-13-responsive transcripts near IL-13-responsive CpG sites are enriched for genes that have been implicated in asthma. (A) Bar graphs showing the proportion of asthma genes (*y*-axis) present in various subsets of genes (*x*-axis). *Black bar*, all genes expressed on the HT12v4 array with nearby CpGs on the 450k methylation array. *First gray bar*, subset of all genes that are IL-13-responsive. *Second gray bar*, subset of all genes near an IL-13-responsive CpG. *Third gray bar*, subset of IL-13-responsive genes and IL-13-responsive CpGs that are correlated (Pearson, $P < 0.2$). (B) Density plot of Pearson correlation *P* values for the IL-13-responsive CpG–asthma gene pairs (*dashed line*), IL-13-responsive CpG–gene pairs (*solid gray line*), or all CpG–gene pairs (*solid black line*). (C) Representative example of a CpG–gene correlation. *Gray dots* are IL-13-treated samples, and *black dots* correspond to vehicle-treated samples. Neutrophil cytosolic factor 2 (NCF2) has been implicated in asthma and is involved in the oxidative stress pathway.

Figure 3. Thirty-four percent ($n = 2,020$) of IL-13-responsive CpGs in cell culture are also differentially methylated between freshly isolated airway epithelial cells (AECs) from individuals with and without asthma. (A) P value distribution from analysis of methylation levels for IL-13-responsive CpGs in AECs from individuals with compared to individuals without asthma. (B) Mean percent methylation differences between IL-13-treated and untreated AEC cultures (vehicle–IL-13; y -axis) versus mean percent methylation differences between freshly isolated AECs from individuals with and without asthma (asthmatic–nonasthmatic). Black dots are CpG sites showing the same direction of effect in both cultured/treated and freshly isolated epithelial cells; gray dots are CpG sites that show opposite direction of effect. The number of genes in each quadrant is shown in the four corners. (C) Boxplot showing the distribution of permutation P values ($n = 10,000$) for the 1,636 overlapping CpGs that go in the same direction (gray dots in B). The whiskers extend to the 5th and 95th percentile; circles indicate values outside of this range. The X indicates the observed value. (D) Density plot of Spearman correlation P values for methylation levels and nearby gene expression in freshly isolated AECs from individuals with and without asthma for CpGs that go in the same direction between studies (solid line) and CpGs that are opposite in direction (dashed line).

corticosteroid usage, body mass index, and atopy, whereas module 2 was associated with eosinophil counts in blood and in bronchoalveolar lavage (Table 1). Neither module was significantly associated with sex, fractional exhaled nitric oxide levels, smoking status, oral corticosteroid use, total IgE levels, or the Th2 phenotype. These results suggest that IL-13 exposure elicits coordinated changes in methylation patterns that are correlated with distinct asthma phenotypes.

Comethylation Modules Are Enriched for Genes in Distinct Networks Implicated in IL-13-mediated Pathophysiology

To identify specific pathways and molecules that contribute to the observed associations between the comethylation modules and clinical phenotypes, we focused on the differentially methylated CpG sites that are near or within a gene (477 sites in module 1 and 642 sites in module 2). We generated protein–protein interaction networks on these gene sets using IPA. There were four significant networks

(score >30) in module 1 and five significant networks in module 2. Module 1 contained one network centered around ERK1/2, a molecule central for IL-13-mediated fibrosis in AECs (Figure 4A) (22); two networks with genes involved in cell proliferation, motility, and growth (see Figure E4); and one centered around the proinflammatory molecule nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which is involved in IL-13-mediated inflammation, fibrosis, and remodeling (see Figure E4) (19). This indicates that associations between module 1 with percent predicted FEV₁ may be caused by IL-13-mediated ERK1/2 remodeling (among other remodeling pathways; see Figure E4).

Network analysis of module 2 genes revealed networks centered on IFN- γ (Figure 4B) and NF- κ B (Figure 4C), suggesting that the association of this module with eosinophilia is likely caused by IFN- γ - and NF- κ B-mediated pathways. IFN- γ inhibits many Th2/IL-13-mediated effects in AECs in a mouse model, including AEC-mediated eosinophilia (42). The NF- κ B network contains a hub centered around NOD-like receptors, reflecting potential involvement of these methylation changes in AEC-mediated innate immune responses (43). Additional hubs ELF3 (44) and YKL-40 (*CHI3L1*) (36) implicate this module in bronchial epithelial repair processes and/or cell survival through NF- κ B-mediated pathways (see Figure E5). Collectively, these networks highlight pathways and molecules through which coordinated IL-13-mediated changes in methylation promote clinical phenotypes associated with each comethylation module.

Discussion

The importance of epigenetic modifications in human disease is evidenced by many recent studies showing differences in global CpG site methylation patterns in tissues from individuals with and without specific diseases (reviewed in References 45–47), including airway diseases (48–50). However, these studies are generally limited in two respects. First, studying tissues or cells from individuals with established disease makes it impossible to distinguish between changes that are

Table 1. Correlation of Comethylation Modules with Clinical Phenotypes in Individuals with Asthma

Clinical Phenotypes	Comethylation Module Correlation <i>P</i> Value	
	Module 1	Module 2
Asthma severity*	2.37×10^{-5}	0.060
Th2 phenotype [†]	0.79	0.54
Steroid resistance*	3.91×10^{-5}	0.11
Inhaled corticosteroid use	1.04×10^{-5}	0.044
Oral steroid use	0.050	0.12
FEV ₁ , % predicted	7.20×10^{-5}	0.020
F _{ENO}	0.26	0.24
Total serum IgE	0.006	0.12
Blood eosinophil count	0.26	1.04×10^{-5}
Bronchoalveolar lavage eosinophil count	0.39	1.26×10^{-6}
Atopy [§]	0.0020	0.025
Body mass index	3.21×10^{-6}	0.15
Sex	0.87	0.81
Current smoker	0.45	0.57

Definition of abbreviation: F_{ENO} = fractional exhaled nitric oxide; Th2 = T-helper cell type 2. The Bonferroni-adjusted threshold of significance is 0.0036.

*Severity was defined using stepwise classification of asthma per NHLBI guidelines (<http://www.nhlbi.nih.gov/health-pro/guidelines/current/asthma-guidelines>).

[†]Th2 classifications were determined following published guidelines (55).

*Defined as an asthma severity (STEP classification of asthma) score >4 or 6.

[§]One or more positive skin prick test.

related to disease onset or susceptibility from those that result from severity, progression, or response to disease, because associative changes may not be permanent or may change through an individual's lifetime. Second, many DNA methylation studies have not included measures of gene expression in the same cells or tissues. Correlations between methylation and nearby gene expression not only serve to validate epigenetic variation as important biologic markers, but support functional roles for a subset of the correlated CpG-gene pairs (reviewed in Reference 51).

Our study was designed to address these two limitations. By using an *in vitro* model of methylation and gene expression response to IL-13, we first identified methylation and transcriptional responses to an environmental stimulus that induces the key features of asthma in AECs (reviewed in Reference 52). Our study revealed that IL-13-mediated methylation changes are associated with a significant proportion of the AEC transcriptional response to IL-13. In particular, most IL-13-responsive CpGs (77%) in cultured AECs were in or near genes and associated with approximately 21% of the IL-13-mediated

transcriptional responses. To our knowledge this is the first demonstration that a cytokine can coordinately alter genome-wide DNA methylation profiles, many of which are near genes that are transcriptionally responsive to IL-13. Importantly, these IL-13-responsive CpG-gene pairs include many of the key molecules that promote IL-13-mediated inflammation and fibrosis in AECs, such as the PPAR γ cofactor RXRA (53, 54), and are enriched for genes associated with asthma, suggesting these epigenetic changes contribute to risk for asthma.

We also showed *in vivo* differences in methylation patterns between asthmatic and nonasthmatic AECs that mirror, to a large extent, the patterns observed in our cell culture model. Indeed, 31% of IL-13-induced CpG site methylation changes were at sites that were also differentially methylated between freshly isolated AECs from individuals with and without asthma, with 73% of these overlapping sites occurring in the same direction, that is, increased methylation after IL-13 treatment and increased methylation in freshly isolated airway cells of individuals with asthma (compared with subjects without asthma)

or vice versa. This concordance between our cell culture model and freshly isolated cells was unlikely to be caused by chance ($P < 10^{-5}$), thereby validating the biologic relevance of our cell culture model and identifying CpG site methylation patterns in asthmatic airways that are likely caused by *in vivo* exposure to IL-13. Moreover, these CpG sites cluster into two distinct comethylation modules that are correlated with clinical phenotypes: one with severity and lung function and one with eosinophilia. These particular epigenetic changes are also enriched in, and may specifically modulate, the proinflammatory and profibrotic pathways that contribute to the pathogenic effects of this cytokine, suggesting that IL-13 exposure may result in persistent changes in DNA methylation that reprograms the cells' inflammatory, fibrotic, and innate responses to external factors.

We note limitations to our study. The usage of a submersion cell culture model consists primarily of epithelial basal cells, and therefore observed methylation and gene expression changes likely do not encompass IL-13-mediated changes in goblet or ciliated epithelial cell types. Additionally, we chose to use one IL-13 concentration and exposure time, therefore interpretation of our data is limited to immediate IL-13-mediated changes. We cannot assess the permanency of IL-13-mediated changes, nor can we address the effects of varying concentrations of IL-13 on these changes in our cell culture model.

An understanding of how response phenotypes contribute to susceptibility to common, complex diseases is the first step in designing personalized therapies. Although most studies to date have focused on the role of genetic variation in conferring susceptibility, associated variants for most common diseases continue to account for relatively small proportions of phenotypic variation in risk. We identify here an IL-13-mediated epigenetic signature that is highly correlated with nearby gene expression, persists in freshly isolated AECs of individuals with asthma, is correlated with clinical asthma phenotypes, and is enriched for genes associated with asthma. The latter observation suggests that nearby genetic variation may influence DNA

Figure 4. Ingenuity pathway analysis protein-protein interaction networks derived from two modules grouped by comethylation using weighted gene coexpression network analysis shows that IL-13-responsive CpG sites that are significantly differentially methylated by asthma status are enriched in proinflammatory and fibrotic pathways. Three significant networks are shown. (A) Network 3 in module 1 is centered on ERK1/2 signaling, a known contributor to IL-13-mediated fibrotic phenotype (IPA network score, 35). The most significant module 2 networks are centered on IFN- γ (IPA network score, 35) and NF- κ B (IPA network score, 42), important modulators of inflammation and eosinophilia. Genes that were associated with a differentially methylated CpG are colored gray in all networks. Molecule shapes: oval = transcriptional regulator; diamond = enzyme; dashed line rectangle = channel; up triangle = phosphatase; down triangle = kinase; trapezoid = transporter; circle = other.

methylation levels, gene expression, or both, and potentially provides a mechanistic explanation for many reported associations.

Regardless, our study shows that variation in DNA methylation may serve as a read out of an individual's responses to exposures, in this case IL-13. More broadly,

epigenetic variation may contribute to asthma inception or to severity and disease course on exposure to IL-13, for example in response to inhaled allergens

or viruses. Regardless, epigenetic signatures in airway cells, and possibly in other relevant cell types, provide a rich source of variation that potentially accounts for a significant proportion of interindividual differences in asthma susceptibility or severity. Elucidating epigenetic variation associated with pathogenic pathways could lead to new insights into disease mechanisms and development of

personalized therapy for modulating inflammatory responses in asthma and possibly in other inflammatory airway diseases. ■

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