Epigenetic mechanisms and the development of asthma

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Asthma is a complex heritable disease affecting more than 8% of the US population, approximately 7 million children and approximately 18.7 million adults.1,2 This disease has been increasing in prevalence, incidence, and severity,3 although recent evidence suggests that the prevalence of asthma and allergies might have reached a plateau in developed countries.4 Asthma accounts for more than $10 billion of direct health care costs in the United States.5 In 2008, persons with asthma missed 10.5 million school days and 14.2 million work days because of their disease.6 Sex and ethnic differences exist for women and African American asthmatic patients, with both having a significantly higher rate of outpatient asthma visits, emergency department evaluations, and hospitalizations than non-Hispanic male subjects.7 Consistent with these data is an increased mortality rate in women and African American asthmatic patients that is 45% and 200% higher, respectively, than that seen in non-Hispanic white male patients. What is most disturbing is that ongoing increases in disease prevalence, incidence, and severity are occurring despite the intense national and international investigation into the pathobiology, genetics, and treatment of asthma. Consequently, it is essential to consider alternative explanations for the growing health problems associated with the development and persistence of asthma.

Several separate lines of evidence support a role for epigenetics in asthma (Fig 1). First, asthma, like epigenetic mechanisms, is inheritable. Although asthma is a strongly familial condition (36% to 79% heritability) with a non-Mendelian pattern of inheritance and polymorphisms in more than 100 genes,5,8 these associations have infrequently been replicated, and genetics has explained only a small portion of the cause of this disease.6 Second, asthma, like epigenetic mechanisms, shows a parent-of-origin transmission of inheritance, with an affected mother significantly more likely to transmit the disease than an affected father.9 These parent-of-origin effects can result from immune interactions between the fetus and the mother.10 Alternatively, the maternal effect might be the result of epigenetically regulated genomic imprinting.11 Several known genes show parent-of-origin effects on allergic disease; these genes include the FCERIB locus,12 and the Spink5 gene.13

Third, asthma, like epigenetic mechanisms,14,15 is affected by in utero exposures.16,17 Prenatal exposure to maternal and grand-maternal cigarette smoke18-20 and traffic-related air

Key words: Asthma, atopy, epigenetics, gene expression, DNA methylation, histone marks, noncoding RNAs
pollution are among the in utero exposures that contribute to the development of this disease. On the other hand, higher maternal fruit and vegetable intake and oily fish consumption during gestation have been associated with a lower risk of asthma.

Fourth, asthma, like epigenetics, is influenced by the general environment. Environmental factors are known to play important roles in the pathogenesis of asthma, both in terms of main effects and those exerted indirectly through complex interactions with gene variants. The dramatic increase in the prevalence, incidence, and severity of asthma over the last 20 years provides strong evidence that exposures, including diet, play an important role in the development of this disease; these changes have occurred too rapidly to be accounted for by changes in primary DNA sequences alone. Although allergens are classically associated with asthma, many other exposures, including smoking behavior, agents in the workplace, indoor and outdoor air pollution, viruses, domestic and occupational exposure to endotoxin, and immunization against certain infectious diseases, are associated with the development and progression of this disease, and several of these agents have been shown to alter epigenetic marks.

Finally, asthma is an immune-mediated disease characterized mainly by skewing toward a TH2 phenotype, although other T-cell subtypes might be involved. Epigenetic mechanisms regulate the expression of transcription factors that are involved in T-cell differentiation (T_H1, T_H2, and regulatory T [Treg] cells).

**EPGENETIC MECHANISMS**

Epigenetics is traditionally defined as the study of heritable changes in gene expression caused by molecules that bind to DNA rather than changes in the underlying DNA sequence (Table I). Recent evidence suggests that the epigenome is dynamic and changes in response to the environment, diet, and aging. In addition to a set of inherited epigenetic marks, there are likely nonheritable epigenetic marks that are more dynamic and change in response to environmental stimuli. Three main classes of epigenetic marks are DNA methylation, modifications of histone tails, and noncoding RNAs (Fig 2).

Methylation of cytosine residues in CpG dinucleotides (5-methylcytosine) within the context of CpG islands is the simplest form of epigenetic regulation in eukaryotes, with hypermethylation of CpG islands in gene promoters leading to gene silencing and hypomethylation leading to active transcription. CpG island methylation has long been studied in patients with cancer, with findings that hypermethylation of tumor

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**GLOSSARY**

3': A single strand of DNA is oriented based on the direction of the phosphodiester bonds that join each nucleotide. A 5'-3' phosphodiester bond involves a bond between the fifth carbon of one pentose ring and the third carbon of the next pentose ring. DNA strands have opposite polarity, with one strand running in a 5' to 3' direction and the other in a 3' to 5' direction.

CHROMATIN: Chromatin is a substance within a chromosome consisting of DNA and protein. The major proteins in chromatin are histones. Changes in chromatin structure are associated with DNA replication and gene expression.

CpG DINUCLEOTIDES: Shorthand for cytosine-phosphate-guanine or a cytosine base located adjacent to a guanine base in a linear sequence (as opposed to cytosine forming chemical bonds to guanine on the opposite DNA strand).

CYTOTOXIC T LYMPHOCYTE–ASSOCIATED ANTIGEN 4 (CTLA-4): A transmembrane protein containing immune tyrosine inhibitory motifs. CTLA-4 is expressed on peripheral T cells after activation and plays an important role in terminating T-cell responses in vivo.

GENOMIC IMPRINTING: Differential expression of genes depending on whether they are inherited from the maternal or paternal parent.

HERITABILITY: The proportion of observed variation in a particular trait that can be attributed to inherited genetic factors in contrast to environmental factors.

INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS): An enzyme that converts L-arginine into nitric oxide. iNOS is found in macrophages, fibroblasts, neutrophils, and smooth muscle cells. Many proinflammatory cytokines increase the expression of iNOS.

MICROARRAY: A technology used to study the expression, methylation, noncoding RNAs, and chromatin marks of many genes at once. It involves placing thousands of gene sequences in known locations on a glass slide called a gene chip. A sample containing DNA or RNA is placed in contact with the gene chip. Complementary base pairing between the sample and the gene sequences on the chip produces light that is measured. Areas on the chip producing light identify genes that are expressed in the sample.

MORTALITY RATE: The ratio of deaths to the number of subjects in a population usually expressed as the number of deaths per thousand subjects per year.

MyD88: MyD88 is key downstream adapter for most Toll-like receptors. MyD88 deficiency is an autosomal recessive immune deficiency involving life-threatening, often recurrent pyogenic bacterial and mycobacterial infections, including invasive pneumococcal disease.

NUCLEOSOME: A subunit of chromatin containing DNA and histone complex.

OILY FISH: Oily fish include salmon, trout, mackerel, sardines, pilchards, herring, kipper, eel, whitebait, and fresh tuna. Omega-3 fatty acids are found in these fish.

PM2.5: Particles, such as those found in smoke and haze, that are 2.5 μm in diameter or less. These particles can be directly emitted from sources such as forest fires, or they can form when gases emitted from power plants, industries, and automobiles react in the air.

POLYCYCLIC AROMATIC HYDROCARBON (PAH): PAH compounds are a generally hazardous class of organic compounds found in petroleum and emissions from fossil fuel use and conversion processes. PAHs are comprised of 2 or more benzene rings arranged in various configurations.

RETROTRANSPOSON: An intermediate RNA transcript that has a copy of the DNA of the transposable element made by using a reverse transcriptase. This DNA is then inserted into the genome at a new location.

Spink5: A gene that encodes proteins important for epidermal integrity. Mutations in Spink5 are associated with Netherton syndrome. Spink5 mutations have also been found in children with atopic dermatitis and food allergy.

UBIQUITYLATION: Ubiquitin is a 76-amino-acid peptide. Ubiquitylation involves ubiquitin conjugation to a lysine residue of a target protein or another ubiquitin molecule, thereby forming a branching structure. This conjugation serves to modulate protein signaling.

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suppressor genes and hypomethylation of oncogenes contribute to the process of carcinogenesis.\textsuperscript{11,47} More recent studies have demonstrated that methylation of less CpG-dense regions near CpG islands (CpG island shores) controls expression of tissue-specific genes, as well as genes relevant to carcinogenesis and lineage-specific cell differentiation,\textsuperscript{48,49} suggesting that DNA

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**TABLE I. Key components of the epigenetic regulation of gene expression**

<table>
<thead>
<tr>
<th>Epigenetics</th>
<th>DNA methylation</th>
<th>Histone modifications</th>
<th>Noncoding RNA</th>
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<tr>
<td>DNA methylation</td>
<td>DNA methylation is the extent of methylation of cytosines in approximately 30 million CpG sites in the human genome. Sixty percent to 90% of CpGs across the human genome are methylated, whereas regulatory regions containing more dense areas of CpG motifs (CpG islands; stretches of DNA &gt;200 bp in length with &gt;50% GC content and observed/expected CpG &gt;0.644) are generally unmethylated. DNA methylation also correlates with spatial organization of the chromatin (proximity of chromosomal loci).\textsuperscript{45}</td>
<td>In eukaryotes DNA is packaged and ordered into nucleosomes, the basic structural unit of chromatin, by wrapping around the octamer, which consists of 2 copies each of histone proteins (H2A, H2B, H3, and H4). Histone modifications include methylation, acetylation, phosphorylation, and other modifications of specific amino acids in nucleosomal histones. Generally, histone marks are described as “permissive” (active promoters), “repressive” (inactive promoters), or “poised” (accessible promoters).</td>
<td>The term noncoding RNA is commonly used for RNA that does not encode a protein. Noncoding RNAs include both small and large classes of RNA molecules that control gene expression through a variety of mechanisms.</td>
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**FIG 1.** An overview of epigenetic regulation of gene expression in asthmatic patients. Environmental exposures and dietary factors to which a subject is exposed \textit{in utero} and postnatally influence epigenetic marks, which in turn regulate genes expression. Underlying genetic variation can regulate gene expression by affecting epigenetic marks or through other mechanisms (eg, alteration of transcription factor binding sites). Alterations in epigenetic marks have consequences on expression of key immune genes that regulate T\textsubscript{h} subtype cell skewing, which in turn leads to the development of disease. It is likely that distinct epigenomic profiles are associated with the development of allergic and nonallergic forms of asthma. ncRNA, Noncoding RNA; ROR\textsubscript{yt}, retinoic acid–related orphan receptor; STAT, signal transducer and activator of transcription.
methylation outside of CpG islands is an important mechanism that controls gene transcription. Additionally, recent evidence suggests that DNA methylation is more prevalent within gene bodies than in promoters. Intragenic DNA methylation functions at least in part by regulating transcription from alternative promoters, but it is likely that other mechanisms are also involved. The DNA “methylole” of the H1 human embryonic stem cell line uniquely revealed that nearly one quarter of all methylation is in non-CpG context, suggesting that embryonic stem cells might use different methylation mechanisms to control gene expression. 5-Methylcytosine can be oxidized to 5-hydroxymethylcytosine by the recently discovered TET family of enzymes. Although the role of 5-hydroxymethylcytosine in epigenetic regulation of gene expression is not fully elucidated, it has been suggested that 5-hydroxymethylcytosine is a mark of demethylation and that it potentially plays a role in the regulation of specific promoters and enhancers.

Methylation, acetylation, phosphorylation, and ubiquitylation of histone tails occur at specific sites and residues and control gene expression by regulating DNA accessibility to RNA polymerase II and transcription factors. H3K4 trimethylation (H3K4me3), for example, is strongly associated with transcriptional activation, whereas H3K27 trimethylation (H3K27me3) is frequently associated with gene silencing. Similarly, histone tail acetylation leads to active gene transcription, whereas deacetylation is a repressive mark and leads to gene silencing. Histone acetyltransferases are enzymes that acetylate histone tails, whereas histone deacetylases remove acetyl groups from histone tails. Analogous to DNA methylation, deregulation of these histone modifications has been linked to misregulation of gene expression in patients with cancer.

MicroRNAs (miRNAs) are the most studied class of noncoding RNAs and control gene expression by binding to the 3’ untranslated regions of mRNA, which leads to either mRNA degradation or inhibition of protein translation. Almost 2000 mature miRNAs have been identified in the human genome (http://www.mirbase.org/), but it is expected that more miRNAs will be identified in the near future. Alterations of expression of miRNAs contribute to the pathogenesis of most malignancies, with miRNAs acting as both oncogenes and tumor suppressor genes, but miRNAs also have well-established roles and are therapeutic targets in cardiovascular disease and liver injury. More recently, noncoding RNAs, such as PIWI-interacting RNAs, small nucleolar RNAs, transcribed ultraconserved regions, and large
intergenic noncoding RNAs are emerging as a key component of deregulated transcription not only in tumorigenesis but also in patients with many nonmalignant diseases.\textsuperscript{61}

An emerging paradigm for epigenetic regulation of gene expression is the relationship between DNA methylation and histone modification. One example of these interactions is binding of DNA methyltransferase (DNMT) 3L, a regulatory factor related in sequence to mammalian \textit{de novo} methyltransferases DNMT3A and DNMT3B, to the N-terminus of histone H3 tail.\textsuperscript{62,63} DNMT3L recognizes unmethylated H3 tails at lysine 4 and induces \textit{de novo} DNA methylation by using recruitment or activation of DNMT3A2; these findings establish the N-terminus of the histone H3 tail with an unmethylated lysine 4 as a \textit{chromatin} determinant for DNA methylation. Similarly, DNMTs preferentially target \textit{nucleosome}-bound DNA.\textsuperscript{64} The relationship of histones and DNA methylation is bidirectional; in addition to histones playing a role in the establishment of DNA methylation patterns, DNA methylation is important for maintaining patterns of histone modification through cell division.\textsuperscript{65} Cross-talk between DNA methylation and miRNAs has also been identified.\textsuperscript{66,67}

In addition to cross-talk between different epigenetic marks, it is becoming evident that underlying genetic variation and epigenetic marks work together. The best example is allele-specific gene expression, in which differences can arise because of sequence variation that might be marked by differences in DNA methylation.\textsuperscript{68-70} histone modifications, or chromatin structure.

Epigenetic marks (DNA methylation and histone marks) are a key component of cell-specific gene expression and, as such, are erased during germ cell development (meiosis) and re-established after fertilization. This process is referred to as epigenetic reprogramming\textsuperscript{71} and constitutes of comprehensive erasure and re-establishment of DNA methylation and extensive remodeling of histone modifications in 2 steps. Epigenetic reprogramming is a key feature of inheritance of epigenetic marks. Genes that are expressed from only 1 parental allele, known as imprinted genes, are protected during the second reprogramming step by mechanisms that are being unraveled.\textsuperscript{72}

**EPIGENOMIC STUDY DESIGN**

The first step in epigenomic analysis is experimental design, including the choice of tissues/cells to be profiled and study design. Challenges in selection of the material to be used include limited availability of lung tissue for asthma studies and cell heterogeneity in available samples (eg, DNA from whole blood). One way to address the first challenge will be to analyze paired lung-blood samples to identify epigenetic marks that carry over from the lung to the peripheral blood and test whether surrogate tissues (eg, PBMCs, nasal epithelia, and sputum) adequately reflect activity in the lung. The second challenge can be addressed by collecting white blood cell count data and including the constituent cell counts in the analysis. If this information is not available, established epigenetic profiles for constituent cells (eg, data generated by the Roadmap Epigenomic project, http://www.roadmapepigenomics.org/) can be used to estimate the relative abundance of different cell types.\textsuperscript{73}

Study design and power calculations based on previously collected data are important in designing studies that are able to identify significant epigenetic changes after adjustment for genome-wide comparisons. The most powerful study design for epigenomic analysis uses monozygotic twins who are essentially identical genetically so that all differences in phenotype can be attributed to environmental factors, with a paired-sample design allowing for better statistical power.\textsuperscript{74} Another design with reasonably high power includes siblings (not necessarily twins) discordant for disease phenotypes with parental DNA also available for estimates of heritability. A case-control design with a large enough number of subjects included in the analysis is often used because of availability of samples. The final considerations in the study design are clinical and immune phenotypes of interest. Before sample selection from available specimens for epigenomic profiling, clinical/immune variables must be analyzed to identify those who have reliable measurements, normal distribution, and a strong clinical or biological rationale to be included in statistical models. Once epigenomic profiles are collected and data are normalized, principal components analysis can be used to prioritize variables based on the amount of variance in the dataset for which they account.

**EPIGENOMIC PROFILING**

Epigenetic marks can be studied by using focused and genome-wide approaches (Table II).\textsuperscript{75} Generally, studies begin with genome-wide approaches to identify targets, followed by focused approaches to internally (confirmation in the same cohort) or externally (independent cohort) validate the initial findings. \textit{Microarray} have been the tool of choice for profiling epigenetic marks on a genomic scale, with several platforms and protocols available for DNA methylation (Table II).\textsuperscript{76} The most commonly used array platforms for DNA methylation are the Illumina 450k BeadChip (Illumina, San Diego, Calif), the Comprehensive Analysis of Relative DNA Methylation (CHARM) platform,\textsuperscript{77} and the Methylated DNA immunoprecipitation (MeDIP) arrays (Agilent Technologies, Santa Clara, Calif, and Roche NimbleGen, Madison, Wis). Array platforms have also been used to examine histone modifications by using chromatin immunoprecipitation (ChIP) followed by hybridization on microarrays (ChIP-chip),\textsuperscript{76} as well as for miRNAs.\textsuperscript{78}

However, the most substantial advance in the area of technologies for the assessment of epigenetic marks on the genome scale in recent years has been the introduction of next-generation sequencing technologies.\textsuperscript{79} Application of next-generation sequencing to epigenomic research has been recently reviewed.\textsuperscript{80} These technologies have been widely used for the study of histone marks (ChIP-seq) and miRNAs (miRNA-seq) because they provide superb-quality data compared with array platforms. They have also been used to identify open chromatin areas of the genome (FAIRE-seq)\textsuperscript{81} and spatial chromatin organization (3C-seq).\textsuperscript{82} The majority of methylation profiling is still done on array platforms because bisulfite-converted DNA sequencing (BS-seq) on the genomic scale is expensive. However, a number of techniques that examine only regions of the genome enriched for methylation marks have been developed and are being increasingly used.\textsuperscript{83} Recent advances in the development of techniques for epigenomic profiling include attempts to define genome-wide patterns of DNA hydroxymethylation\textsuperscript{84,85} and to study DNA methylation and histone modifications in one experiment.\textsuperscript{86,87}

Pyrosequencing\textsuperscript{88} and Epityper assays on the Sequenom MassARRAY platform (Sequenom, San Diego, Calif) are commonly used techniques for interrogation of a small number of CpG sites, whereas quantitative PCR methods are typically
of integrative epigenomic analyses have not reached maturity but gene regulation genome wide. Analytic strategies for these types of epigenetic mechanisms are independently complex, but when combined, the complexity of these interactions presents unique analytic challenges. We are just beginning to understand how different epigenetic mechanisms affect the expression of cytokines and binding of transcription factors that control the lineages of TH1, TH2, and Treg cells. In the context of TH1/TH2 differentiation, the most extensively studied are the TH1 cytokine IFN-γ and the TH2 cytokines IL-4 and IL-13. It has been shown that de novo DNMT3A methylates CpG-53 in the Ifng promoter35 and cord blood CD4+ cells enhance the development of the TH1 (but not TH2) lineage through progressive demethylation of the Ifng promoter.36 Methylation of the Ifng promoter was reduced in CD8+ cells from atopic children in the age range during which hyperproduction of IFN-γ occurs, suggesting that DNA methylation at this locus might be a contributing factor in the development of atopy in children. Differentiation of human CD4+ cells into the TH2 subtype is accompanied by the appearance of DNase I hypersensitive sites and CpG demethylation around these DNase I hypersensitive sites within IL-4 and IL-13 promoters.37,38 Extensive studies of the TH2 cytokine locus control region (LCR)40 have shown that RAD50-hypersensitive site 7 within the TH2 cytokine LCR underlies methylation status (methyl-QTL) or methylation marks that control expression changes (methyl-expression QTL). Similarly, coexpression network analysis strategies that have been applied to expression analysis can be applied to epigenomic analysis.32

EPIGENETIC MARKS AND THE IMMUNE SYSTEM

A substantial body of evidence suggests that epigenetic mechanisms affect the expression of cytokines and binding of transcription factors that control the lineage of TH1, TH2, and Treg cells. In the context of TH1/TH2 differentiation, the most extensively studied are the TH1 cytokine IFN-γ and the TH2 cytokines IL-4 and IL-13. It has been shown that de novo DNMT3A methylates CpG-53 in the Ifng promoter35 and cord blood CD4+ cells enhance the development of the TH1 (but not TH2) lineage through progressive demethylation of the Ifng promoter.36 Methylation of the Ifng promoter was reduced in CD8+ cells from atopic children in the age range during which hyperproduction of IFN-γ occurs, suggesting that DNA methylation at this locus might be a contributing factor in the development of atopy in children. Differentiation of human CD4+ cells into the TH2 subtype is accompanied by the appearance of DNase I hypersensitive sites and CpG demethylation around these DNase I hypersensitive sites within IL-4 and IL-13 promoters.37,38 Extensive studies of the TH2 cytokine locus control region (LCR)40 have shown that RAD50-hypersensitive site 7 within the TH2 cytokine LCR underlies methylation status (methyl-QTL) or methylation marks that control expression changes (methyl-expression QTL). Similarly, coexpression network analysis strategies that have been applied to expression analysis can be applied to epigenomic analysis.32

EPGENOMIC DATA ANALYSIS

The first step in analysis of collected epigenomic data is to identify statistically significant differences between disease states. Statistical methods used for microarray analysis have been generally applicable to epigenomic profiles collected on arrays or sequence data after alignments and tag counts have been performed.80 Strategies for analyzing tiling arrays have also been used in epigenomic analyses (eg, the CHARM platform or ChIP-chip).90 One of the problems with this type of analysis is that it is only associative and does not demonstrate causality. Methods used in epidemiology and genetical genomics are beginning to be applied to epigenomes to identify causal relationships.91

The second and most complex step in the analysis of epigenomic data is understanding how different epigenetic mechanisms together influence gene expression. Each of the 3 epigenetic mechanisms is independently complex, but when combined, the complexity of these interactions presents unique analytic challenges. We are just beginning to understand how one type of epigenetic mark affects gene expression.92 However, the evidence for cross-talk among different types of epigenetic marks is accumulating. The complexity of epigenetic regulation of gene expression is high, even when one is interested in examining only 1 gene or locus, and there are considerable challenges associated with understanding these interactions and the effect on gene regulation genome wide. Analytic strategies for these types of integrative epigenomic analyses have not reached maturity but are starting to be applied to disease datasets.93 Two types of integrative analysis will be important to apply to epigenomic data: mapping strategies and network analysis. Expression quantitative trait locus (QTL) mapping approaches34 can be applied to identify genetic variants that underlie methylation status (methyl-QTL) or

<table>
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<tr>
<th>Type of epigenetic mark</th>
<th>Type of sample preparation approach</th>
<th>Type of profiling approach</th>
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<tr>
<td>DNA methylation</td>
<td>Bisulfite conversion</td>
<td>Microarray (Illumina), high-throughput sequencing (BS-seq and RRBS-seq), EpiTYPER (mass spectrometry, focused), or pyrosequencing (focused)</td>
</tr>
<tr>
<td>Methylated DNA immunoprecipitation (MeDIP)</td>
<td>Microarray or high-throughput sequencing (MeDIP-seq)</td>
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<tr>
<td>Methyl-binding domain (MBD) precipitation</td>
<td>Microarray or high-throughput sequencing (MBD-seq)</td>
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<tr>
<td>Restriction digest with methylation-sensitive restriction enzymes (eg, Mv/Bc, MspI, HpaII, and MspII)</td>
<td>Microarray (CHARM, HELP) or high-throughput sequencing (MRE-seq)</td>
<td></td>
</tr>
<tr>
<td>Histone modifications</td>
<td>ChIP</td>
<td>Microarray (ChIP-chip), high-throughput sequencing (ChIP-seq), or quantitative PCR (focused)</td>
</tr>
<tr>
<td>DNA methylation associated with chromatin modifications</td>
<td>ChIP followed by bisulfite conversion</td>
<td>High-throughput sequencing (ChIP-BS-seq and Bis-ChIP-seq)</td>
</tr>
<tr>
<td>Noncoding RNAs</td>
<td>Size selection of appropriate RNA molecules</td>
<td>Microarray, high-throughput sequencing (miRNA-seq and RNA-seq) or quantitative RT-PCR (focused)</td>
</tr>
<tr>
<td>Chromatin accessibility</td>
<td>DNAse 1 cleavage</td>
<td>High-throughput sequencing (DNase sensitivity-seq)</td>
</tr>
<tr>
<td>Chromatin accessibility, chromatin spatial organization</td>
<td>Formaldehyde cross-linking</td>
<td>High-throughput sequencing (FAIRE-seq)</td>
</tr>
<tr>
<td>Chromosome conformation capture</td>
<td>High-throughput sequencing (3C-seq, 4C-seq, and 5C-seq)</td>
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methylation and histone modifications are highly dynamic and represent important determinants of T\textsubscript{H}1 and T\textsubscript{H}2 cell lineages.

Although miRNAs were discovered relatively recently, there is already a substantial body of evidence for the role of miRNAs in the development and function of the immune system. A number of differentially expressed miRNAs have been identified in response to innate and adaptive immune stimuli, with many commonalities in miRNA expression (miR-21, miR-103, miR-155, and miR-204). miR-155 is the most often identified differentially regulated noncoding RNA in studies involving the immune system. A recent study revealed that miR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte–associated antigen 4.

Epigenetic mechanisms controlling T cell development are also beginning to be explored. T cell clones are a unique T-cell lineage with an important role in immunologic tolerance. The development of which is primarily regulated by the transcription factor forkhead box protein 3 (FOXP3). Evidence for the role of DNA methylation and histone modifications is summarized in 2 recent reviews. There is also clear evidence that miRNAs are involved in Treg cell development and function.

**ROLE OF THE ENVIRONMENT AND IN UTERO EXPOSURES IN MODULATING THE EPIGENOME**

Unlike a patient’s genetic make-up, epigenetic marks can be influenced much more easily by exposures, diet, and aging. Randy Jirtle’s seminal experiments showed that a maternal diet supplemented with methyl donors (folic acid, vitamin B12, choline, and betaine) shifts coat color distribution of progeny toward the brown pseudoguaiti phenotype and that this shift in coat color resulted from an increase in DNA methylation in a retrotransposon adjacent to the agouti gene. These studies also revealed that mice with yellow coat color are obese and develop cancer, suggesting for the first time that changes in DNA methylation caused by diet in utero might be linked to disease development. Other studies have shown that pesticides and fungicides can alter the methylome, resulting in changes in male fertility, and that aging is also associated with changes in DNA methylation and gene expression. The concepts associated with environmental epigenetics were reviewed recently elsewhere.

More recent evidence suggests that environmental exposures relevant to the development of asthma, such as air pollution and cigarette smoke, also affect the epigenome. Decreased DNA methylation in peripheral blood (as measured by LINE-1 repeats) was found to be associated with exposure to particulate matter of 2.5 \( \mu \)m in diameter or less (PM\textsubscript{2.5}) among 718 elderly subjects in the Boston area, and although this correlated with time-dependent variables, such as day of the week and season, there was no association with air pollution–related health effects. Another study demonstrated that hypomethylation of the inducible nitric oxide synthase (iNOS [Nos2]) promoter in buccal cells was associated with elevated nitric oxide levels and PM\textsubscript{2.5} exposure among 940 participants in the Children’s Health Study.

Several epidemiologic studies have examined the relationship between exposure to cigarette smoke and epigenetic marks. Among 384 children, a global reduction in DNA methylation, as measured by the extent of methylation of Alu repeats, and differential methylation of 8 specific CpG motifs was found to be associated with in utero exposure. Fifteen specific genomic loci were significantly associated with current smoking, 2 with cumulative smoke exposure, and 3 with time since quitting cigarettes in 1085 subjects enrolled in the International COPD Genetics Network and validated in the Boston Early-Onset COPD study (n = 369). Cigarette smoke exposure has also been shown to have a significant influence on the expression of miRNAs. Comparing current with never smokers, 28 miRNAs were differentially expressed and mostly downregulated in the human bronchial airway epithelia of smokers. miR-218 was found to be one of the strongly associated miRNAs with cigarette smoke exposure, and it was further shown that a change in mi-R-218 expression in primary bronchial epithelial cells and the H1299 cell line resulted in a corresponding anticorrelated change in the expression of predicted mRNA targets for miR-218.

Other studies have examined the influence of cigarette smoke exposure on epigenetic marks in vitro or in animal models. Normal human airway epithelial cells and immortalized bronchial epithelial cells exposed to cigarette smoke condensate identified time- and dose-dependent changes in histone modifications (decrease in H4K16Ac and H4K20Me3 and increase in H3K27Me3) accompanied by decreased DNMT1 and increased DNMT3b expression; these changes are characteristic of lung cancer progression. Two other studies also demonstrated changes in miRNA expression in lungs of mice and rats exposed to cigarette smoke, with substantial overlap between mice and rats and some overlap of rodent miRNA expression changes in the lung with those observed in human airway epithelium.

In addition to influencing epigenetic marks as a result of direct exposure, in utero exposure to components of air pollution or cigarette smoke results in changes in global and site-specific DNA methylation. Maternal exposure to benzo(a)pyrene, a representative airborne polycyclic aromatic hydrocarbon, was associated with hypermethylation of IFN-\( \gamma \) in cord blood DNA from 53 participants in the Columbia Center for Children’s Environmental Health cohort. In another study global hypomethylation has been associated with maternal smoking and cotinine levels in umbilical cord blood from 30 newborns. In a birth cohort of 90 women born from 1959 to 1963 in New York City, prenatal tobacco exposure, measured at the time of pregnancy and not retrospectively reported, was associated with a decrease in Sat2 methylation but not LINE-1 or Alu methylation. Examination of 2 differentially methylated regions (DMRs) regulating 2 imprinted loci (H19 and Igf2) in infants born to 418 pregnant women demonstrated that infants born to smokers had higher methylation at the Igf2 DMR than those born to never smokers or those who quit during pregnancy (no differences were seen in the H19 DMR). Similarly, DNA methylation in Axl, a receptor tyrosine kinase relevant in cancer and immune function, was 2.3% higher in peripheral blood of children exposed to maternal smoking in utero. Finally, one study has demonstrated association of maternal cigarette smoking during pregnancy with downregulation of several miRNAs in the placenta; expression of one of the miRNAs (miR-146a) was downregulated in a dose-dependent manner in immortalized placental cell lines exposed to nicotine and benzo(a)pyrene.

**ASTHMA EPIGENETICS: ANIMAL STUDIES**

Given the evidence for the strong influence of environmental exposures on epigenetic marks and the role of epigenetic...
regulation in T-cell differentiation, it is becoming clear that epigenetic changes might be one of the factors to explain the increasing prevalence of asthma. Our group hypothesized that these dietary influences are, at least in part, mediated by the epigenome. To test this hypothesis, we conducted a study in which pregnant female mice were fed either a low- or high-methylation diet and progeny were sensitized and challenged with ovalbumin.\(^1\) We observed an increase in airway inflammation, serum IgE levels, and airway hyperresponsiveness (AHR) in pups of mothers who were fed a high-methylation diet compared with those of mothers on a low-methylation diet. Importantly, we reversed the immune phenotype by treatment with a demethylating agent (5-aza-deoxycytidine). Epidemiologic evidence for association of folic acid with the development of asthma in children has been mixed,\(^12\) but it might be that folate, together with other methyl donors in the diet, plays a role in this disease.

Importantly, a direct link between epigenetic control of the TH2 cytokine locus and development of allergic airway diseases was further demonstrated in mice with deficiency in the TH2 LCR.\(^12\) A more recent study also identified a DNase I hypersensitive site 2 element in the second intron of the Ifng gene as the strongest of all known Ifng enhancers and showed that this enhancer is strictly controlled by GATA-3 binding.\(^12\) Moreover, Tanaka et al\(^12\) propose a new model in which independent enhancers and decreased expression of Runx3, a gene known to regulate allergic airway disease in mice. Importantly, we reversed the immune phenotype by treatment with a demethylating agent (5-aza-deoxycytidine). Epidemiologic evidence for association of folic acid with the development of asthma in children has been mixed,\(^12\) but it might be that folate, together with other methyl donors in the diet, plays a role in this disease.

A number of other animal studies have since examined DNA methylation in the context of allergic airway disease. Fedulov et al\(^12\) demonstrated DNA methylation changes in splenic CD11c\(^+\) dendritic cells from neonate mice born to allergic mothers (mothers sensitized and challenged with ovalbumin). Brand et al\(^12\) observed increased methylation of the Ifng promoter (and increased IFN-\(\gamma\) cytokine production) in CD4\(^+\) T lymphocytes after ovalbumin sensitization challenge and demonstrated that methylation of the Ifng promoter is required for development of allergic airway disease by using 5-aza-deoxycytidine (demethylating agent) and adoptive transfer experiments transferring CD4\(^+\) T cells from sensitized/challenged to naive animals and the reverse. Although both demethylation and adoptive transfer experiments clearly demonstrate the importance of methylation marks in CD4\(^+\) cells in the development of allergic airway disease, loci other than Ifng might be important in this process and should be examined. Finally, DNMT3A, but not DNMT3B, deficiency in CD4\(^+\) lymphocytes (conditional mutant mice) was shown to result in increased expression of IL-13 (and other TH2 cytokines), decreased DNA methylation and changes in H3K27 acetylation/methylation in the IL-13 promoter, increased airway inflammation, and AHR in the ovalbumin model of allergic airway disease.\(^12\) This study clearly demonstrates the role of DNA methylation in controlling the expression of TH2 cytokines and the development of allergic airway disease in mice.

Several recent studies have also begun to shed light on the role several miRNAs play in the development of allergic airway disease in animal models.\(^13\) Selective miR-126 blockade resulted in a diminished TH2 response, inflammation, and AHR in the house dust mite model; these effects were shown to be mediated by activation of the MyD88 innate immune signaling pathway. By using the same house dust mite model, this group also demonstrated that inhibition of miR-145 inhibited eosinophilic inflammation, mucus hypersecretion, TH2 cytokine production, and AHR and that the anti-inflammatory effects of miR-145 antagonism were comparable with those of glucocorticoid treatment.\(^13\)

Two studies identified a controversial role for the let-7 family of miRs in the ovalbumin model of allergic airway disease.\(^12,13\) The first study showed that multiple members of the highly conserved let-7 miRNA family are the most increased lung miRNAs in response to allergen.\(^12\) The authors confirmed that IL-13 is regulated by let-7a in vitro and demonstrated that inhibition of let-7 miRNAs in vivo using a locked nucleic acid profoundly inhibited allergic inflammation and AHR, suggesting a proinflammatory role for let-7. The second independent study demonstrated that let-7 miRNAs regulate IL-13 production in A549 cells and primary cultured T cells and that intranasal administration of mature let-7 mimic to the lungs of mice with allergic inflammation resulted in decreased IL-13 levels, AHR, and mucus metaplasia, implying an anti-inflammatory role for let-7.\(^13\) More studies are needed to understand the discrepancy in these findings, but this illustrates the complexity of miRNA regulation of gene expression.

Finally, 3 recent studies have demonstrated how miRNAs play a crucial role in the regulation of IFN-\(\gamma\) and therefore T-cell polarization. Targeted ablation of miR-21 led to reduced lung eosinophilia after ovalbumin sensitization and challenge, with a broadly reprogrammed immunoinactivation transcriptome and significantly increased levels of the TH1 cytokine IFN-\(\gamma\). Consistent with the miR-21 binding site in IL-12p35, dendritic cells from miR-21–deficient mice produced more IL-12 after LPS stimulation, and OVA-challenged CD4\(^+\) T cells from the same mice produced more IFN-\(\gamma\) and less IL-4. Two studies showed that miR-29 suppresses IFN-\(\gamma\) production.\(^13,14\) Steiner et al\(^13\) performed gene expression profiling of cells that do not produce miRNAs (DGCR8-deficient cells)\(^13\) transfected with a synthetic miR-29 and wild-type cells with antisense inhibitors of miR-29, respectively. In this elegant experiment they found reduced expression of 2 transcription factors that regulate IFN-\(\gamma\) production (Tbx21/T-bet and Eomes) under gain-of-function conditions and increased expression of these 2 transcription factors under loss-of-function conditions. They further proved the role of miR-29 regulation of the expression of these transcription factors in CD4\(^+\) lymphocytes in vitro and in both CD4\(^+\) and CD8\(^+\) T cells in an in vivo viral infection model. Ma et al\(^15\) demonstrated an inverse correlation between IFN-\(\gamma\) production and levels of miR-29 in natural killer cells and T cells from mice infected with Listeria monocytogenes or Mycobacterium bovis. Mice lacking miR-29 infected with M bovis showed less inflammation, lower bacterial burden, and increased numbers of IFN-\(\gamma\)-producing CD4\(^+\) T cells in their lungs compared with control mice.

**ASTHMA EPIGENETICS: HUMAN STUDIES**

Although animal studies have begun to decipher the role of epigenetic regulation of gene expression associated with the development of allergic airway disease in the lung, several recent publications in human cohorts have examined DNA methylation in cells outside of the lung: peripheral blood cells,\(^13\) buccal cells,\(^13,14\) and nasal cells.\(^14\) These early studies have only...
demonstrated the statistical association of DNA methylation and specific exposure or asthma phenotype but have not elucidated the role of DNA methylation in the control of gene expression in human asthma. Breton et al demonstrated that DNA methylation in promoters of 2 arginase genes (Arg1 and Arg2) is associated with exhaled nitric oxide levels in children with asthma from the Children’s Health Study and indicates a role for epigenetic regulation of nitric oxide production. In a pilot study in the Columbia Center for Children’s Environmental Health cohort, Kurikose et al found that iNOS methylation was not significantly associated with fraction of exhaled nitric oxide (FeNO) but was associated inversely with bronchial nitric oxide flux. This latter study emphasizes the importance of careful selection of clinical parameters used in the association study. A more recent study of DNA methylation in nasal cells from 35 asthmatic children 8 to 11 years old identified inverse association of FeNO levels and promoter methylation of both Il6 and iNOS. Finally, data from 2 independent pregnancy cohorts in Spain (discovery and validation) showed that DNA hypomethylation in Alox12 in peripheral blood of children was associated with a higher risk of persistent wheezing at age 4 years. In aggregate, these studies suggest that DNA methylation in easily obtained samples (buccal, nasal, or peripheral blood cells) might be a useful biomarker for airway inflammation in pediatric research.

A recent study has also examined DNA methylation in Foxp3 and Treg cell function in peripheral blood from children with and without asthma and with high and low exposures to air pollution. Treg cell suppression was impaired, and Treg cell chemotaxis was reduced as a result of exposure to air pollution. Changes in DNA methylation have also been associated with the development of asthma among older smokers in the Lovelace Smokers Cohort. Comparison of 184 smokers with asthma with 511 control subjects with a similar smoking history (patients with chronic obstructive pulmonary disease were excluded) identified an association of DNA methylation in the protocadherin-20 gene in sputum DNA with asthma, as well as a significant synergistic interaction between methylation of protocadherin-20 and paired box protein transcription factor 5α on the odds of having asthma.

A set of earlier studies suggested that acetylation of histones can also play a role in asthma. Increased acetylation of H4 has been demonstrated in asthmatic patients and is associated with an increase in the expression of several inflammatory genes in the lung. It has also been shown that increased acetylation of histones results in decreased histone deacetylase activity, which might be responsible for enhanced expression of inflammatory genes. In addition, glucocorticoids appear to suppress inflammation by altering acetylation of histones that regulate inflammatory and anti-inflammatory genes; these studies are described in detail in a review and suggest that targeting histone acetylation (and possibly other epigenetic marks) might lead to novel anti-inflammatory therapies, especially in corticosteroid-resistant cases of asthma. A more recent study found that TGF-β2 suppresses expression of a disintegrin and metalloprotease 33 (ADAM33), one of the most replicated asthma susceptibility genes, in fibroblasts from healthy subject or asthmatic patients and that this occurs by altering chromatin structure (deacetylation of histone H3, demethylation of lysine 4 on H3, and hypermethylation of lysine 9 on H3) and not by gene silencing through DNA methylation, as in epithelial cells.

The role of miRNAs in asthma and atopy in human subjects is also emerging. Although no detectable differences in the expression of miRNAs from airway biopsy specimens were observed between patients with mild asthma and healthy subjects in an early study, only patients with mild asthma were included in this study, and the number of miRNAs examined was limited. However, this study demonstrated cell type--specific expression of miRNAs in cells isolated from airways and lung tissue, suggesting a possible role for miRNAs in asthmatic patients. A more recent study has indeed identified miRNAs that play a role in specific cells in asthmatic patients. In a study of 8 control subjects, 4 patients with mild asthma, and 12 patients with severe asthma, widespread changes in mRNA and noncoding RNA expression in circulating CD8+ but not CD4+ T cells were associated with severe asthma. miRNA expression profiles showed selective downregulation of miR-28-5p in CD8+ lymphocytes and reduction of miR-146a and miR-146b in both CD4+ and CD8+ T cells. It is likely that some of the other miRNAs identified in animal models play a yet uncovered role in the development of asthma in human subjects.

**CHALLENGES IN UNDERSTANDING THE ASTHMA EPIGENOME**

Some of the key questions in regard to future studies in asthma epigenetics revolve around understanding how the epigenome contributes to inheritance of asthma, developmental vulnerability of the epigenome, effect of the environment/diet/aging, and influence of asthma (and other diseases) on the epigenome. Although sorting out these factors will be challenging, it is absolutely essential that the proper tissue be chosen to study the effects of the epigenome on asthma. The more pure and relevant the cell population is to the disease state, the more likely the epigenetic marks will regulate the expression of key genes involved in the pathogenesis of asthma. In the absence of airway biopsy specimens in pediatric asthma, nasal epithelial cells or sputum might be the closest surrogate for disease-relevant cells. Specific cell populations, such as CD4+ and CD8+ T lymphocytes isolated from peripheral blood, might also be informative in identifying immune genes with expression that is dysregulated by epigenetic marks in the disease state. Despite these concerns, epigenetic marks in peripheral blood might provide biomarkers to identify those at risk, responses to different forms of environmental stress, or the likelihood of responding to specific therapeutic agents.

Analogous to asthma genetics studies, the choice of the study population will be crucial to the success of future epigenetic studies. The ancestry of study subjects will likely need to be taken into account given the early evidence for the role of genetic variation and DNA methylation at asthma-associated loci, such as Ormd3. Moreover, a recent study suggests that DNA methylation is highly divergent between populations of European and African descent and that this divergence might be due to a combination of differences in allelic frequencies and complex epistasis or gene-environment interactions. Based on this, population stratification might be a confounder in population-based genome-wide DNA methylation studies and might have to be accounted for by using principal components from the methylation profile, genome-wide association studies, or ancestry-specific marker panels. Given the strong influence of the environment on epigenetic marks, environmental and dietary exposures, as well as medication use, must be measured/recorded in the study not only for exposures of interest but also for any confounding
exposures that need to be adjusted for in the analysis. Despite the differences between disease phenotypes in human cohorts and animal models of allergic airway disease, animal models with a fixed genetic background and controlled exposures are likely to remain a crucial component of future studies in the field.

One of the major hurdles to overcome in future asthma epigenetics research will be the validation component. Necessary components of the validation process include internal validation of epigenetic marks in the same samples by using a different technique, association of epigenetic marks with changes in gene expression in the study population, and external validation of epigenetic marks in an independent cohort (Fig 3). Some of the difficulties encountered in the validation process are platform differences in technologies; differences in DNA methylation measurements are encountered based on the approach used to capture methylated marks (restriction digest, immunoprecipitation, and bisulfite conversion) and probes used to measure the extent of methylation (single CpG site vs a region covered by overlapping probes). Another major challenge in validations studies is interpretation of epigenetic marks in the context of changes in gene expression. Both cis and trans effects of methylation marks are likely to be important in gene regulation, and this process is very complex. Depending on the site of methylation (promoter vs intron), epigenetic marks might play different roles in control of gene expression in cis. Mapping studies of methylation marks on gene expression (methyl-expression QTL) will be essential in the identification of cis and trans effects. The final major challenge will be identification of cohorts with comparable genetic background and environmental exposures to use as an independent validation step. It is likely that cohorts with similar exposures and phenotypes will be of most utility for broad validation of a large number of epigenetic marks and identification of specific phenotype- and exposure-driven epigenetic changes, whereas more divergent cohorts might still be useful in validation of a small number of epigenetic marks associated with disease, regardless of other factors.

FIG 3. An overview of the validation process for discovery of epigenetic marks associated with the development of asthma. Internal validation refers to confirmation in the same cohort by using a different technique and association of epigenetic marks with changes in gene expression, whereas external validation refers to validation of epigenetic marks in an independent cohort. Genome-wide analysis of gene expression is preferable to focused approaches because of complexities in the relationship of epigenetic marks and gene expression alterations.

POTENTIAL EFFECT OF EPIGENETICS RESEARCH ON ASTHMA

Although we know that inheritance, parent of origin, environment, in utero exposures, and TH2 immunity play important roles in the cause of asthma, there is no well-developed unifying mechanism accounting for these causative events/triggers. Although the hygiene hypothesis is appealing conceptually and ties a number of these basic causative events together, there are several competing hypotheses (eg, T-cell skewing, infection, diet, and obesity), and none of them fully account for the complex interaction between host and environmental determinants of asthma. For example, the hygiene hypothesis suggests that a decrease of exposure to microbes would, through enhanced atopic immune responses, increase the incidence of allergies and allergic asthma. However, the prevalence of atopy and asthma are not concordant, allergic mechanisms account for at most 50% of asthma cases, very high asthma rates are present in some countries in which hygienic conditions are less than ideal, and although the prevalence and incidence of asthma continue to increase in inner cities in the United States, housing conditions in these communities are becoming more hygienic.

Although epigenetic mechanisms provide a unique cause of asthma, these basic transcriptional controls potentially serve to explain some of the prevailing hypotheses underlying the development of asthma. For example, the hygiene hypothesis is dependent on activation of innate immune genes, including genes activated by the Toll-like receptors; importantly, epigenetic mechanisms control the activation of these innate immune genes and, consequently, the extent of the inflammatory response. Moreover, a recent study demonstrated that microbes can also operate by means of epigenetic mechanisms. In this animal study prenatal administration of the farm-derived gram-negative bacterium \textit{Acinetobacter lwoffii} F78A prevented the development of an asthmatic phenotype in progeny, and this effect was IFN-γ dependent. Prenatal microbial exposure was also associated with a significant protection against loss of H4 acetylation in the \textit{Ilfng} promoter, which was closely associated with IFN-γ expression in CD4+ lymphocytes, as well as a decrease in H4 acetylation at the \textit{Il4} promoter. Pharmacologic inhibition of H4 acetylation in offspring abolished the asthma-protective phenotype. Therefore although epigenetic mechanisms have the potential of changing our basic concepts about asthma, these mechanisms might not only account for the causative events/triggers related to asthma but also help explain some of the prevailing hypotheses attributed to this disease.

Furthermore, identification of key epigenetic marks has the potential to transform asthma therapy from palliative to preventive and might alter our recommendations for pregnancy throughout the world. Currently, other than avoidance of cigarette smoke, we are simply unable to prevent asthma. Most patients with asthma rely on chronic medications to reduce the severity of their symptoms. Understanding the importance of epigenetic mechanisms in the development of asthma and the periods of vulnerability in establishing epigenetic marks has the potential to prevent the development of this disease not only in our offspring but also in their children. Identification of critical epigenetic marks associated with the development of asthma and influenced by specific environmental factors at certain time points, in utero or postnatally, would allow us to advise our patients on intake of dietary supplements and limiting harmful exposures during the critical windows when these dietary and environmental factors have the strongest influence on the development of disease. Understanding the complex interactions between in utero exposures and epigenetic vulnerability will provide insight into future interventions for subjects at risk for allergic asthma and might lead to the prevention of this disease altogether.

However, asthma is a complex disease, and although epigenetic mechanisms might contribute to the cause and pathogenesis of
this disease, there are multiple pieces to the asthma puzzle. The challenge will be to understand how genetic variation, the transcriptome, epigenetic marks, the environment, and the immune system interface with each other to result in the development of allergic and nonallergic forms of asthma.

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