

Elucidation of asthma phenotypes in atopic teenagers through parallel immunophenotypic and clinical profiling

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Background: Current treatment strategies for asthma in teenagers derive primarily from information on chronic disease in adults. More detailed understanding of risk factors related to teenage asthma might aid in the development of improved preventive and treatment strategies for this age group.

Objective: We sought to identify biomarkers associated with asthma phenotypes in teenagers, particularly atopic asthma, and to identify markers that aid in discriminating between atopic subjects at high versus low risk of asthma.

Methods: We studied 1380 unselected 14-year-olds and collected data on clinical history, allergic sensitization, and respiratory and immunoinflammatory function. The latter comprised measurements of circulating inflammatory markers and *in vitro* innate and adaptive immune functions, including house dust mite T-cell responses. We integrated the data into regression models to identify variables most strongly associated with asthma risk and severity among atopic subjects.

Results: Eight hundred twenty-seven subjects were atopic, 140 subjects were asthmatic, and 81% of asthmatic subjects were also atopic. We identified asthma risk variables related to atopy intensity, including specific IgE and eosinophil levels, plus an additional series external to the T_H2 cascade but that modified risk only in atopic subjects, including IFN- γ , IL-10, and IL-12 responses and neutrophil numbers in blood. Moreover, bronchial hyperresponsiveness was associated strongly with atopic but not nonatopic asthma, and the bronchial hyperresponsiveness risk profile was itself dominated by atopy-associated variables.

Conclusions: Asthma in teenagers is predominantly driven by atopy acting in concert with a second tier of T_H2-independent immunoinflammatory mechanisms, which contribute to pathogenesis only against the background of pre-existing inhalant allergy. (J Allergy Clin Immunol 2009;124:463-70.)

Key words: Asthma, atopy, bronchial hyperresponsiveness, IgE, eosinophils, neutrophils, teenagers, cytokines, immunoepidemiology

Persistent asthma in teenagers is a major cause of morbidity in this age group and in many cases persists into adulthood. The available drug treatments provide symptomatic relief but do not modify the overall course of the disease. One factor contributing to this less-than-ideal picture is that current treatment strategies derive mainly from the study of older patient groups with more advanced disease.

One approach to this problem that is gaining momentum involves prospectively tracking birth cohorts over extended periods through different stages of asthma development.¹⁻⁵ An important common aim in these studies is to identify variations in the spectrum of asthma phenotypes in relation to age and disease stage because target organ tissues and the inflammatory effector mechanisms affecting them adapt to the effects of chronic stimulation. Under such circumstances, the importance of individual risk factors is likely to vary significantly with disease progression, and failure to account for this variation might be a significant impediment to the development of effective asthma treatments.⁶

In the present study we have taken a targeted approach to this issue in relation to asthma in 14-year-olds by using an unselected study population to ensure that the phenotypes studied are representative of the burden of disease in the overall community. This approach extends an earlier pilot study of 11-year-olds⁷ that suggested novel relationships between immunophenotypes and wheezing phenotypes at this age. In the present study we have adopted an in-depth approach to phenotyping study subjects, encompassing detailed clinical, physiologic, and immunoinflammatory profiling using a large sample size to maximize statistical power to detect disease-associated risk variables.

An initial audit of the study population identified most asthmatic subjects as atopic, and we therefore focused our analyses principally on this subgroup. However, only 13.7% of the atopic subjects express asthma, and in the analyses below we sought to identify the key covariates that distinguish asymptomatic atopic subjects from their counterparts with symptomatic atopic asthma. Previous cohort studies in Perth have found house dust mite (HDM) to be the dominant local allergen,^{4,7} and this finding was reiterated in the current larger study. In light of this, we focused our analyses within the 14-year-old cohort on HDM-sensitized subjects as representative of atopic subjects.

METHODS

Patients and sample collection

Subjects were from an unselected longitudinal birth cohort broadly representative of the West Australian population, as detailed previously.⁸

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Supported by the National Health and Medical Research Council of Australia, the Stanley Trust (UK), and the Raine Medical Research Foundation of the University of Western Australia. Reagents for antibody and eosinophil protein X arrays were provided gratis by Phadia AB.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication February 9, 2009; revised May 21, 2009; accepted for publication June 23, 2009.

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0091-6749/\$36.00

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doi:10.1016/j.jaci.2009.06.019

Abbreviations used

BHR: Bronchial hyperresponsiveness
 EPX: Eosinophil protein X
 HDM: House dust mite
 OR: Odds ratio
 PGF_{2a}: Prostaglandin F_{2a}
 TLR: Toll-like receptor

One thousand three hundred eighty subjects volunteered at a mean age of 14.1 years (range, 13.01-15.03 years) for clinical assessment and provision of blood and urine samples. All aspects of the study were approved by our institutional human ethics committee, and parents provided written consent, with assent provided by the teenagers. Analyses presented below were entirely cross-sectional and did not use information collected earlier.

Clinical phenotyping

Current asthma was defined as wheeze or cough without a cold in the last 12 months, use of any asthma medication in the last 12 months, and a doctor's diagnosis of asthma ever. Current allergic rhinoconjunctivitis was assessed on the basis of parental response to a standardized questionnaire regarding the child's symptoms (runny, blocked, or itchy nose in the presence of runny or itchy eyes) over the preceding 12 months. Respiratory assessments followed standard American Thoracic Society guidelines (see the Methods section in the Online Repository at www.jacionline.org for more details). Baseline spirometry was performed, with the subject performing maximal forced expiratory maneuvers while seated; FEV₁/forced vital capacity is reported. Bronchial hyperresponsiveness (BHR) was assessed by means of administration of increasing doses of methacholine, with FEV₁ measured at 30 and 90 seconds after each dose. Testing continued until either FEV₁ decreased by 20% or more or the maximum cumulative dose had been administered (8 mg/mL). Dose-response curves were constructed, and 2 outcome variables were calculated: PC₂₀, which was defined as the cumulative concentration resulting in a 20% decrease in FEV₁, and the dose-response slope, which was defined as the 2-point slope regardless of the decrease in FEV₁. Results of the methacholine challenge were used in 2 ways: (1) as a binary variable where children with a PC₂₀ value of less than 8 mg/mL were classified as BHR positive and (2) as dose-response slope used as a continuous variable.

An asthma severity scale was developed from asthma management guidelines in use in Australia (www.nationalasthma.org.au). Three grades of asthma severity were defined as mild, moderate, or severe on the basis of symptoms in the past 12 months, as follows: wheeze frequency (mild, 1-2/y; moderate, 3-12/y; severe, >12/y), weekly average nocturnal awakenings caused by asthma (mild, nil; moderate, 1; severe, >1), and severe episodes limiting speech (mild, nil; moderate, nil; severe ≥1). Children were classified into the most severe category for which they qualified: if a child had 1 severe asthma episode limiting speech within the past 12 months, he or she was classified as having severe asthma regardless of the number of wheezing episodes during the year.

Atopy status

Atopic family history was obtained by means of a parental questionnaire. Antibodies were measured with ImmunoCAP (Phadia AB, Uppsala, Sweden), comprising total IgE and specific IgE and IgG4 levels to HDM and a broad panel of additional allergens (see the Methods section of this article's Online Repository). Atopy was defined as any specific IgE titer of 0.35 kU/L or greater, a total IgE titer of 300 kU/L or greater, or both.

In vitro culture of PBMC and cytokine measurements

See the Methods section of this article's Online Repository for more detail. PBMCs were cryopreserved and subsequently cultured as described

previously by our laboratory,⁷ with some modifications. PBMCs were cultured for 48 hours in medium alone with HDM or rye grass pollen allergen or with the T-cell mitogen PHA. For measurement of innate immune responses, PBMCs were cultured for 24 hours with the Toll-like receptor (TLR) 3 stimulant poly I:C or the TLR4 stimulant LPS (with or without IFN- γ). Cytokine proteins were measured by using time-resolved fluorometry (IL-5, IL-10, IL-12, IL-13, IFN- γ , and TNF- α), or mRNAs were measured by using quantitative RT-PCR (IL-4 and IL-9). Cytokine production at greater than the unstimulated control (Δ) value was calculated and used in subsequent analyses.

Measurement of inflammatory markers

Levels of the mast cell and eosinophil products prostaglandin F_{2a} (PGF_{2a}) and eosinophil protein X (EPX) were measured from urine and normalized against creatinine values by using an ELISA (Cayman Chemical, Ann Arbor, Mich; Phadia AB, Uppsala, Sweden). Soluble CD14 levels were measured in plasma by using an ELISA (BioScientific, Gynema, Australia).

Statistical methods

Univariate analyses of group differences in biomarker expression used the Mann-Whitney *U* test. After adjustment of measurements below the assay detection limits (see the Methods section of this article's Online Repository), continuous variables (excluding body mass index and respiratory measures) were log₁₀ transformed for use in multivariate analyses. Stepwise logistic regression was used to identify variables significantly associated with asthma and BHR risk, whereas ordinal regression was used to identify variables associated with asthma severity. Cases missing data for variables were excluded from relevant regression analyses. The cutoff level of significance used for regression analyses was a *P* value of less than .05. The selection of variables in the overall test matrix (listed in Tables E1 and E2 in this article's Online Repository at www.jacionline.org) was based on *a priori* knowledge from the literature of their contributions to asthma- or atopy-related processes, and they are therefore included as potential confounding factors in relevant regression models. The percentage of asthma cases attributable to atopy variables (attributable risk) were obtained by using Aflogit in STATA version 10.0 (StataCorp, College Station, Tex). All other analyses were implemented in SPSS 13 for the Mac OS system (SPSS, Inc, Chicago, Ill). Although we looked at several combinations of cytokine levels and disease outcomes, the results of all are presented, and therefore no formal adjustment to the *P* values was carried out.⁹

RESULTS**Asthma- and atopy-related phenotypes: Population characteristics**

The frequencies of relevant phenotypes are shown in Fig 1. The prevalence of current asthma in the cohort was 10.1% (*n* = 140); 81% (*n* = 113) of the asthmatic subjects were atopic, and 49% (*n* = 56) of atopic asthmatic subjects showed BHR. Atopy was significantly associated with risk for asthma (odds ratio [OR], 3.11; *P* < .0005; attributable risk, 0.521 ± 0.080 [0.335-0.655]) and BHR (OR, 2.87; *P* < .0005; attributable risk, 0.463 ± 0.064 [0.323-0.574]), with the strongest association in both cases being with sensitization to HDM (data not shown), which is the most frequent cause of inhalant allergy in this population (*n* = 571). Risk for both asthma and BHR increased progressively with total IgE titers in the overall atopic population and with specific IgE titers in the HDM-sensitized atopic subjects (Fig 2). This relationship was again clearest and strongest in the HDM-sensitized subgroup, particularly for more severe asthma with BHR (Fig 2, B). We accordingly focused in more detail on this subgroup as exemplary of the

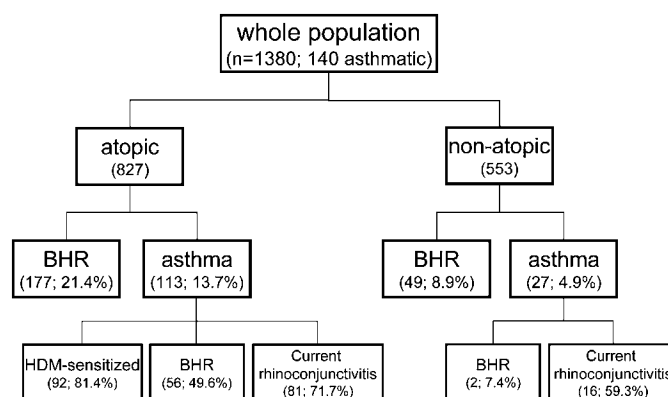


FIG 1. Wheezing and related phenotypes in the study cohort: spectrum of current asthma, rhinoconjunctivitis, and BHR in the unselected population stratified by atopy.

overall atopic population, including specific testing of HDM-specific T_H memory responses (see below).

Univariate analyses: Immunophenotypes

Fig 3, A, shows the spectrum of immunophenotypes within the overall population stratified by HDM sensitization, including both allergen-specific and more general inflammation-associated biomarkers (see Table E1 in this article's Online Repository for detail of all variables examined). The high fidelity of the *in vitro* cellular immunity methodology is demonstrated by the increased expression of atopy-associated T_H2 cytokines (IL-4, IL-5, IL-9, and IL-13) in HDM-stimulated PBMC cultures from the HDM-sensitized subjects. Of additional interest, their responses included hyperproduction of the T_H1 cytokine IFN- γ and the regulatory cytokine IL-10. The sensitized subgroup also demonstrated increases in antibody levels, circulating eosinophil counts, and levels of the inflammatory metabolites EPX and PGF $_2$ a in urine.

Data from the HDM-sensitized subgroup were further stratified for comparisons between atopic subjects with or without current asthma, and the biomarkers differentially expressed are shown in Fig 3, B (see Table E2 in this article's Online Repository for full detail). The majority of the atopy-associated inflammatory biomarkers from Fig 3, A, notably IL-5, IL-9, IL-13, and allergen-specific antibodies, are expressed at significantly higher levels in the asthmatic subjects. Additionally, parallel increases are evident in numbers and activation status (EPX) of IL-5-dependent eosinophils, together with an apparent reciprocal relationship between eosinophil and neutrophil numbers in association with the atopic asthma phenotype. Increased expression of the non- T_H2 cytokines IL-10 and IFN- γ is also more prominent among the asthmatic subset, suggesting that immunophenotypic variations external to the atopy cascade itself might also contribute to the expression of symptomatic atopy.

Multivariate analyses of asthma risk and severity

Multivariate regression analyses were performed on data from the HDM-sensitized atopic subjects, integrating the biomarkers in Fig 3 with variables derived from the clinical assessment of subjects, including respiratory function measures (the full list of variables included is shown in Table E2 in this article's Online Repository). Spearman correlation coefficients were calculated to demonstrate the associations between the various sets of

variables (see Tables E3-E6 in this article's Online Repository at www.jacionline.org), and although associations were observed between some atopy-associated variables, these were generally weak (Spearman ρ rarely exceeding 0.400), so that colinearity was unlikely to be an issue in the ensuing multivariate analyses.

Table I lists the significant variables identified in the logistic regression model for asthma risk, and consistent with other studies, atopic family history contributed the highest OR for asthma. Additional significant T_H2 -associated variables contributing to asthma risk were levels of IgE antibody and the activation product EPX derived from IL-5-dependent eosinophils. In addition to the core T_H2 -associated variables, the model contains a second tier of significant variables that are T_H2 independent. These comprise PHA-induced IFN- γ (associated with increased risk) and LPS-induced IL-12 (associated with decreased risk).

Table II summarizes ordinal regression analyses aimed at identifying risk variables associated with asthma severity among HDM-sensitized atopic subjects. The list of significant variables identified parallels those in Table I but with some significant variations. Notably, the T_H2 -associated variables associated with disease severity include current rhinoconjunctivitis, peripheral blood eosinophil numbers (as opposed to EPX levels), and mold-specific as opposed to mite-specific IgE. The ostensibly atopy-independent series includes polyclonal IL-10 (associated with increased severity) and circulating neutrophil numbers (associated with diminished risk for high severity asthma).

Association of BHR with atopy

BHR to methacholine was consistently associated with the risk for and severity of asthma (Fig 1 and Tables I and II). We compared biomarker expression between HDM-sensitized atopic subjects with and without BHR, and continuous variables showing significant differences are listed in Table III (the complete list is shown in Table E7 in this article's Online Repository at www.jacionline.org). BHR-positive atopic subjects were characterized by very high-level production of the proinflammatory T_H2 cytokines IL-5 and IL-13, with correspondingly high levels of circulating eosinophils and IgE antibodies (Fig 2, A). Follow-up multivariate analyses examined BHR as a binary outcome by using a modified version of the logistic regression model used in Table I (lung function and BHR dose slope were excluded).

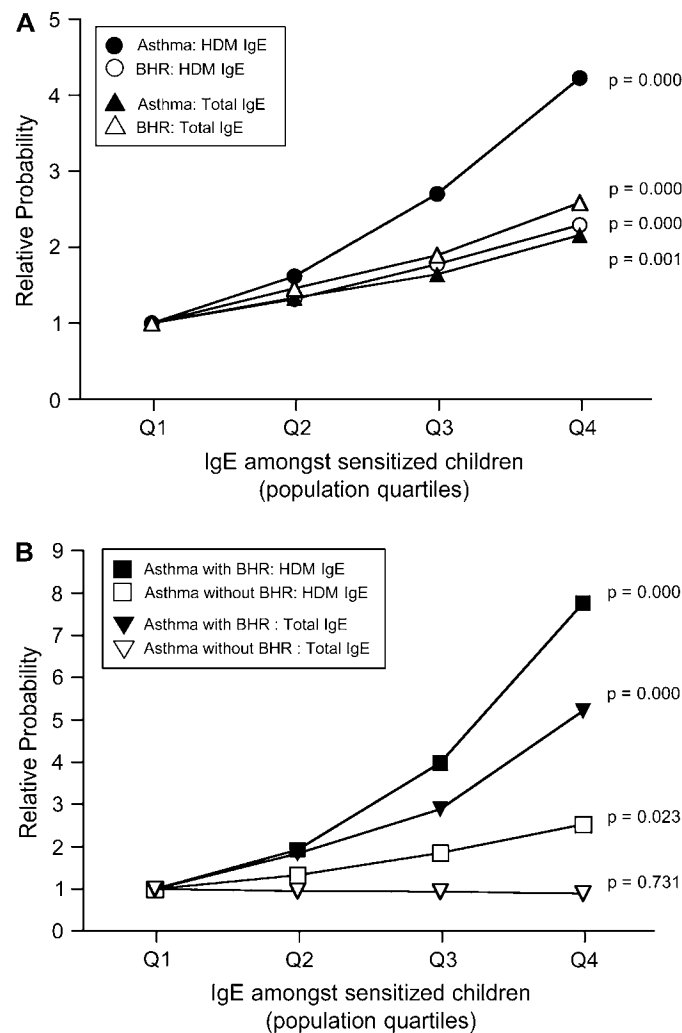


FIG 2. Degree of sensitization as a risk determinant for asthma and BHR among atopic teenagers. HDM-sensitized subjects (HDM IgE level ≥ 0.35 kU/L) or subjects with any allergy (any specific IgE level ≥ 0.35 , total IgE level ≥ 300 kU/L, or both) were analyzed separately. HDM-sensitized subjects ($n = 540$) were separated into quartiles based on HDM-IgE levels, and the any allergy group ($n = 827$) were quantified by total IgE level; average probability of asthma, BHR, or both was calculated for each quartile by using univariate logistic regression (P values shown) and normalized against probability for quartile 1. **A**, Asthma (with or without BHR) versus BHR (with or without asthma). **B**, Asthma with BHR versus asthma without BHR.

The results of these analyses, summarized in Table IV, further confirmed the strong association between BHR expression and atopy-associated markers. Male sex came up as protective in the model because there was a higher prevalence of BHR among HDM-sensitized female subjects (69/211) than male subjects (71/289; $P = .045$, χ^2 test).

Association of T_H2 cascade-independent biomarkers with asthma

Subsequent analyses focused on the atopy cascade-independent variables identified above as modulators of asthma risk in teenagers by using additional logistic regression analyses to test the robustness of the associations. Analyses relating to neutrophils and eosinophils were prompted by the observations (Table II) that these cell types exerted reciprocal effects in relation to the risk for severe asthma and by the knowledge that these cells derive from common bone marrow precursors. When entered

together into a regression model with asthma risk as a binary outcome, the resultant interaction term was highly significant (OR, 52.93 [3.94-709.55]; $P = .003$), reinforcing the view that the balance between these 2 cell types in relevant inflammatory responses might contribute to disease pathogenesis. Fig 4, A, shows a clear reciprocal linear relationship between circulating numbers of these cells and asthma risk, which moreover is restricted to the atopic subjects. A similar approach examining the effects of IL-10 and IFN- γ demonstrated a clear linear trend toward increase in the risk for asthma with increasing T-cell production of these cytokines, and these effects were again restricted to the atopic subjects (Fig 4, B). Similar stratification strategies applied to the IL-12 data did not detect comparable linear trends (data not shown), suggesting a more complex underlying relationship between this cytokine response and asthma susceptibility. Follow-up regression modeling of IL-12 and asthma risk among nonatopic subjects did not detect risk-modifying effects in this group (data not shown).

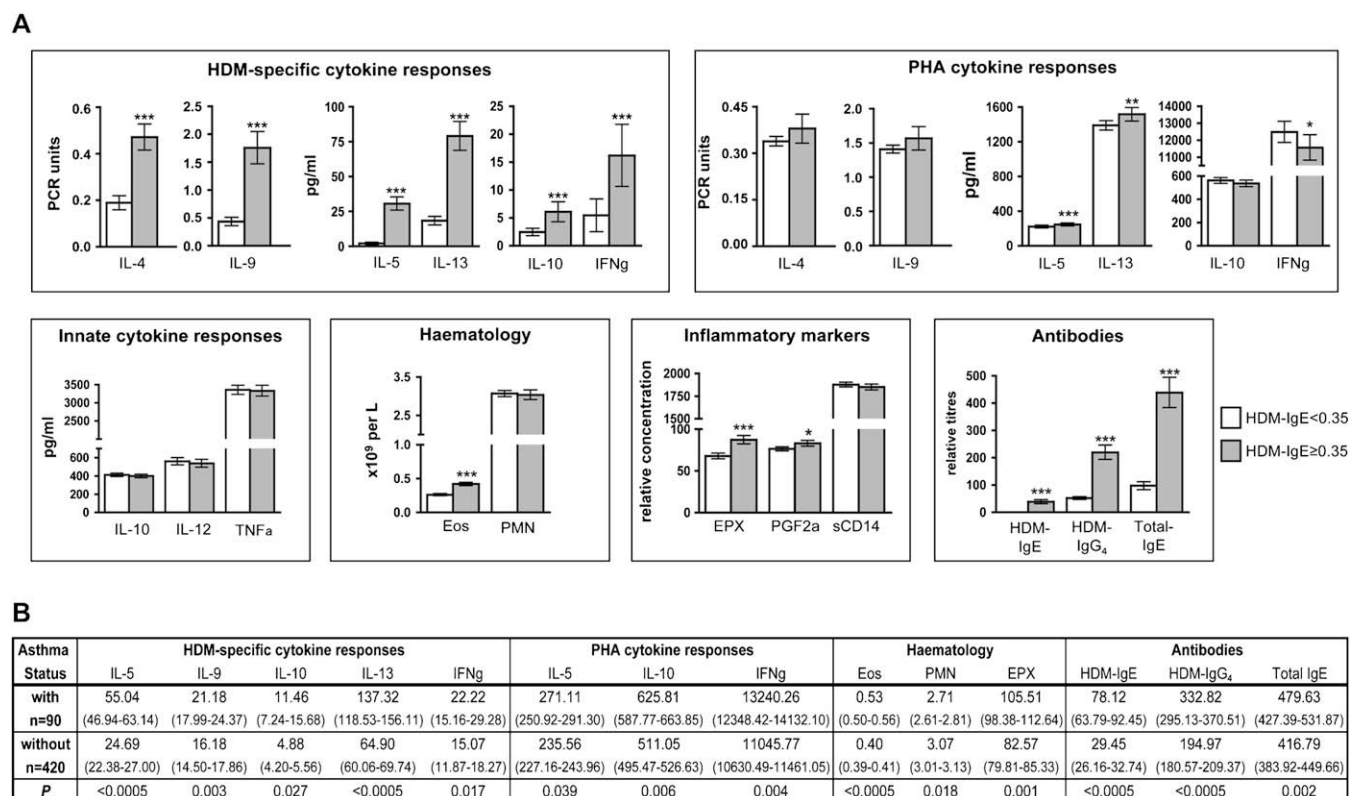


FIG 3. Immunophenotypic profiling of the study cohort. **A**, Biomarker expression in the whole population ($n = 1380$) stratified by HDM sensitization (nonsensitized, $n = 840$; sensitized, $n = 540$). Means and SEMs are shown. $*P < .05$, $**P < .01$, and $***P < .0005$, Mann-Whitney U test. **B**, Biomarker expression in HDM-sensitized atopic subjects stratified by current asthma status. Means, 95% CIs, and P values (Mann-Whitney U test) are shown.

Asthma and BHR risk among nonatopic subjects

As noted above, the focus of these analyses was on atopic subjects, who account for the bulk of asthma-related disease in this age group, but for purposes of comparison, we have included data relating to the smaller subpopulations of nonatopic subjects in the cohort manifesting asthma ($n = 27$; univariate analyses are shown in Table E8 in this article's Online Repository at www.jacionline.org) or BHR ($n = 50$; univariate analyses are shown in Table E9 in this article's Online Repository at www.jacionline.org). These data were reanalyzed by using multivariate regression (see Tables E10 and E11 in this article's Online Repository at www.jacionline.org), and significant variables identified in the asthma model were rhinoconjunctivitis (OR, 6.98; $P = .000$), FEV₁/forced vital capacity (OR, 0.94; $P = .035$) For BHR, significant variables were PGF_{2a} (OR, 5.57; $P = .041$), neutrophil counts (OR, 11.82; $P = .016$), and LPS-induced IFN- γ levels (OR, 0.81; $P = .015$).

DISCUSSION

The general association between asthma and atopy has been recognized for many years; however, the magnitude of the contribution of atopy to asthma pathogenesis^{10,11} and the proposed mechanistic basis for the association have been questioned.¹² There is increasing evidence that the relative importance of different asthma pathogenic factors might vary significantly with disease stage, and in this regard the present study

has targeted teenagers in whom the association between atopy and asthma appears strongest.¹³ Eighty-one percent of current asthma in these teenagers occurs among the atopic subjects, which is indeed higher than in the same children at age 6 years (55.5%)¹⁴ and considerably in excess of what has been reported cross-sectionally in adults. However, despite this strong association, asthma occurs in only a minor proportion of the overall atopic teenage population, which is inconsistent with a simple unidimensional causal role for atopy in the disease process. Hence the key issue in relation to understanding the basis for asthma risk in this age group is the nature of the underlying phenotypic differences distinguishing atopic subjects who are symptomatic from those who are asymptomatic; defining these differences represented the primary aim of this study.

Such differences are clearly demonstrated by our univariate analyses, which show that expression of various phenotypic markers related to the T_H2 cascade was more intense in the asthmatic subgroup of atopic subjects, particularly allergen-specific antibody levels in serum, allergen-induced T-cell cytokine production, and measures of the activity of the IL-5–eosinophil axis (Fig 3). A comparable pattern of T_H2-related hyperexpression is evident in atopic subjects with BHR, which is a common feature of the atopic (but not nonatopic) asthmatic phenotype (Fig 1) and which represents an independent risk factor for asthma severity (Table II). Therefore one determinant of risk for expression of wheeze-associated symptomatology among these teenage atopic subjects is intensity of sensitization. The results of follow-up multivariate analyses are consistent with an important role for

TABLE I. Logistic regression analysis of asthma risk in atopic 14-year-olds

Significant variables	Asthma risk		
	OR	95% CI	P value
Atopic family history	10.154	2.723-37.685	.001
HDM IgE	2.061	1.420-2.990	.000
EPX	6.997	2.128-23.005	.001
LPS-induced IL-12	0.646	0.453-0.920	.015
PHA-induced IFN- γ	3.464	1.413-8.492	.007
BHR dose slope	3.004	1.685-5.358	.000

The sample of HDM-sensitized atopic subjects comprised 324 nonasthmatic subjects and 72 asthmatic subjects.

atopy in the expression or maintenance of BHR because the significant variables (excepting sex) appearing within the relevant regression model (Table IV) are all T_H2 associated. However, parallel regression modeling of asthma risk reveals a more complex picture. As shown in Tables I and II, the overall risk profile in these atopic asthmatic subjects includes an additional layer of immunophenotypic biomarkers that are not part of the T_H2 -dependent atopic cascade: capacity of the T-cell system to produce IFN- γ and IL-10, responsiveness of the innate immune system to LPS, and an apparent protective signal associated with neutrophil numbers. The analyses in Fig 4 confirm that the effects of these risk-modifying variables are discernible only against the background of pre-existing atopy.

Although the precise mechanism or mechanisms by which these T_H2 -independent factors affect asthma risk remain to be determined, there is existing evidence in the literature of a role for IFN- γ in asthma pathogenesis. Although T_H1 and T_H2 cytokines are mutually antagonistic during initiation of immune responses, once T_H memory is established, this cross-regulation wanes; responses in atopic children typically manifest the T_H0 cytokine phenotype, which is characterized by high-level production of the T_H1 cytokine IFN- γ , along with T_H2 effector cytokines.^{15,16} In our recent study in 11-year-olds,⁷ the risk for BHR was more pronounced in atopic subjects at the high end of the IFN- γ response spectrum, suggesting that this cytokine can potentially add to inflammatory damage caused by T_H2 cytokines. This idea is consistent with the demonstration of increased IFN- γ levels in blood and bronchoalveolar lavage fluid during experimental asthma exacerbations.^{17,18} Our present results reiterate the clinical implications of IFN- γ hyperexpression in atopic subjects and extend them to include risk for severe asthma, emphasizing the potential underlying dangers of immunotherapeutic approaches to atopy control that are based on immune deviation alone.

Although this scenario has biologic plausibility with respect to the role of IFN- γ , no direct parallels can be drawn for the other atopy-independent covariates identified above. It is more likely that these contribute to asthma through pathways related to susceptibility to other environmental factors implicated in disease, in particular respiratory tract infection. A broad range of recent studies in children have demonstrated that the highest ORs for initial asthma development^{1,3,4,14,19} and for asthma exacerbations^{20,21} ensue from interactions between atopy and viral infections. Thus immunophenotypes influencing host antiviral immunity could markedly modify asthma risk. Prime candidates in this context are IL-10 and IL-12. IL-12 plays a central role in the initiation and T_H1 polarization of host antiviral defense, and

TABLE II. Ordinal regression analysis of asthma severity in atopic 14-year-olds

Significant variables	Asthma severity		
	OR	95% CI	P value
Atopic family history	11.870	2.863-49.156	.001
Current rhinoconjunctivitis	3.222	1.522-6.821	.002
Mold IgE	1.659	1.132-2.430	.009
Eosinophils	9.516	1.840-49.205	.007
Neutrophils	0.049	0.005-0.504	.011
LPS-induced IL-12	0.531	0.351-0.803	.003
PHA-induced IL-10	4.604	1.085-19.511	.038
BHR dose slope	3.212	1.533-6.740	.002

The sample of HDM-sensitized atopic subjects comprised 324 nonasthmatic subjects and 25 subjects with mild, 27 subjects with moderate, and 20 subjects with severe asthma. Couch grass-specific IgE produced a negative OR (0.355), but this was considered spurious on the grounds of implausibility and the lack of confirmation through similar effects of IgE of any other specificities.

its production during the crucial early stages of immune responses is antagonized by IL-10.²² Immunophenotypes associated with high-level production of either IL-12 or IL-10 could therefore be expected to show inverse relationships with asthma risk, as observed here, and precedents for such findings are available from earlier smaller studies.^{23,24} It is also noteworthy that the LPS used to trigger IL-12 binds cells through TLR4, which is also the main receptor for respiratory syncytial virus, and triggering this receptor initiates innate defense mechanisms against this pathogen.²⁵ Additionally, LPS responsiveness has been linked with induction of intercellular adhesion molecule 1, the receptor for rhinovirus, on airway epithelial cells.²⁶

Neutrophils are also significant in these models, either as an independent variable or potentially through interactions with eosinophils. There is no immediate explanation for how their effects here are mediated, particularly in light of other literature arguing for a role for neutrophilic inflammation in some wheezing phenotypes,²⁷ but several related possibilities merit investigation. For example, neutrophil recruitment has been identified as a significant component of the initial cellular infiltrate triggered during the early phase of viral replication in infected airway epithelial cells.^{28,29} Their function in this context is uncertain but might involve surveillance for opportunistic bacterial pathogens (compare with Bisgaard et al³⁰) or contribution to local immunoregulation through their capacity to produce high levels of nitric oxide, which is a potent modulator of T_H2 cell activation.³¹

We have also included preliminary findings related to the minority nonatopic subset of asthmatic subjects and those with BHR, and the differences in risk variables relative to atopic subjects with the same clinical phenotypes are noteworthy, particularly with respect to PGF_{2a} and neutrophils, which appear associated with risk for BHR in nonatopic subjects. However, we emphasize that these data should be interpreted with caution because of the low sample size. In contrast, the study provides stronger support for a central role for atopy in asthma pathogenesis in this cohort. Atopic effector mechanisms appear to contribute independently to disease risk among teenagers, and additionally, they might unmask susceptibility to other proinflammatory immunophenotypic cofactors that are insufficient to initiate asthma themselves but that can enhance expression of established disease by adding to the inflammatory milieu of the atopic airway. The findings demonstrating a

TABLE III. Biomarker expression in atopic subjects stratified by BHR status

BHR status	HDM-specific cytokines (pg/mL)		Hematology	Antibodies		
	IL-5	IL-13	Eosinophils ($\times 10^{-9}$ cells/L blood)	HDM IgE (kU/L)	HDM IgG4 (μ g/L)	Total IgE (kU/L)
Without (n = 359)	26.40 (2.70)	69.56 (5.76)	0.40 (0.02)	28.34 (3.51)	200.60 (13.23)	384.14 (34.16)
With (n = 139)	42.29 (5.84)	106.07 (12.87)	0.50 (0.03)	62.59 (10.17)	247.89 (23.41)	585.28 (59.69)
P value	.012	.043	<.0005	<.0005	.045	<.0005

Means (SEMs) are shown. *P* values are determined by using the Mann-Whitney *U* test. Cat IgE and mold IgE levels were also increased in the BHR-positive group (*P* < .0005).

TABLE IV. Logistic regression analysis of BHR risk in atopic 14-year-olds

Variables	BHR risk		
	OR	95% CI	P value
Sex, male	0.611	0.386-0.967	.035
Atopic family history	1.904	1.028-3.526	.041
Cat IgE	1.404	1.081-1.823	.011
Total IgE	1.638	0.974-2.753	.063
Eosinophils	2.494	1.048-5.936	.039

The sample of HDM-sensitized atopic subjects comprised 117 BHR-positive and 287 BHR-negative subjects.

attenuation of wheezing symptomatology in pollen-sensitized schoolchildren³² and in HDM-sensitized young adults³³ through currently available immunotherapeutic treatments. We argue that further improvement of these and related therapies targeting T_H2 -associated pathways has significant potential for reducing the community burden of asthma in atopic teenagers, who represent the subpopulation at highest risk of this disease among their age group.

We thank Jenny Tizard for her skilled technical assistance and the families and members of the Raine Study team who took part in the study.

Key messages

- Risk for asthma and BHR among teenagers is maximal among atopic subjects and increases with intensity of sensitization.
- Atopy contributes directly to asthma risk and in addition creates susceptibility to atopy-independent proinflammatory mechanisms, which can then act as cofactors in disease pathogenesis.

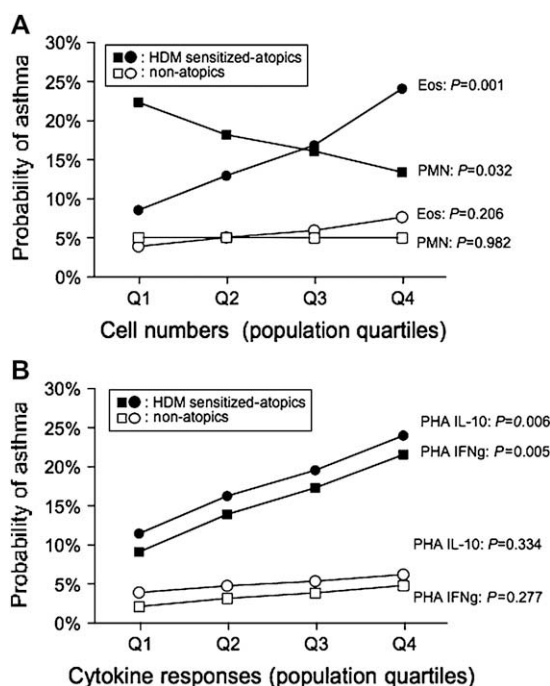


FIG 4. Asthma risk and quantitative expression of biomarkers. Subjects in the nonatopic and HDM-sensitized groups were separated into quartiles based on eosinophil (*Eos*) and neutrophil (*PMN*) numbers in blood (**A**) or IL-10 and IFN- γ PHA response capacity (**B**). Average probability of asthma was calculated for each quartile by using individual univariate logistic regression models (*P* are values shown). Probabilities for IFN- γ responses were graphed offset by -1.5%.

quantitative relationship between the degree of atopic sensitization and the level of risk for severe asthma also argue for more detailed intervention studies targeting inhalant allergy as an asthma preventive strategy in this age group. In this context initial proof-of-concept has already been demonstrated for

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METHODS

Allergen test panel

Allergen specificities tested with the Phadia ImmunoCAP were as follows: HDM (*Dermatophagoides pteronyssinus*), rye grass pollen (*Lolium perenne*), cat, couch grass (*Cynodon dactylon*), mold mix (*Penicillium notatum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Candida albicans*, *Alternaria alternata*, and *Helminthosporium halodes*), and peanut and food mix (egg white, milk, fish, wheat, peanut, and soybean).

In vitro culture of PBMCs

Cryobanked PBMCs were cultured for 48 hours in AIM-V (Life Technologies, Carlsbad, Calif) medium with 4×10^{-5} mol/L 2-mercaptoethanol alone or with 10 μ g/mL HDM extract (prepared in house), 30 μ g/mL rye grass pollen extract (prepared in house), or 1 μ g/mL PHA (Murex Diagnostics Ltd, Dartford, United Kingdom). PBMCs were cultured for 24 hours in RPMI with 10% FCS alone or with 50 μ g/mL poly I:C (Sigma-Aldrich, Castle Hill, Australia), 1.0 ng/mL LPS (from *Escherichia coli* serotype R515; Alexis Biochemicals, Lausanne, Switzerland), or 10 ng/mL IFN- γ (PharMingen, San Diego, Calif) with 1.0 ng/mL LPS added after 3 hours. Cells were cultured in round-bottom 96-well plates (Nunc, Roskilde, Denmark) at a concentration of 1.2×10^6 cells/mL; 1.2×10^6 cells were used for 48-hour cultures, whereas 0.6×10^6 cells were used for 24-hour cultures. Cultures were performed in batches by the same team using carefully standardized reagents throughout, with progressive cross-validation of growth media and allergen batches throughout the approximately 2-year period over which experiments were performed to avoid reagent-associated variations in *in vitro* reactivity. The culture and analytic teams were blind to the IgE level and clinical status of the cohort members.

At the end of the study, we compared the expression of HDM-induced cytokines by HDM-sensitized subjects whose blood was collected in different seasons and found that there was no seasonal difference in expression of IL-4, IL-5, IL-10, IL-13, or IFN- γ (as determined by using the Kruskal-Wallis test). The only significant seasonal variation we saw was slightly higher expression of IL-9 in autumn than in the other 3 seasons. When we performed equivalent comparisons for all rye-sensitized subjects, we saw no significant differences in rye-induced cytokine expression between seasons.

Cytokine and mRNA measurements

Coating and biotin-labeled detection antibodies were purchased from BD PharMingen: IL-5 coating, catalog number 554393; IL-5 detection, catalog number 554491; IL-10 coating, catalog number 554495; IL-10 detection, catalog number 554499; IL-12 coating, catalog number 555065; IL-12 detection, catalog number 554660; IL-13 coating, catalog number 554570; IL-13 detection, catalog number 555054; IFN- γ coating, catalog number 554393; IFN- γ detection, catalog number 554550; TNF- α coating, catalog number 551220; and TNF- α detection, catalog number 554511. Recombinant standards were purchased from PharMingen (IL-5, catalog no. 554606; IL-10, catalog no. 554611; IL-12, catalog no. 554613; and IFN- γ , catalog no. 554616) or R&D Systems, Inc (Minneapolis, Minn; IL-13, catalog no. 213-IL-005; TNF- α , catalog no. 210-TA-010). Limits of detection were 30 pg/mL for IL-12 and TNF- α and 10 pg/mL for the remaining assays.

mRNA levels for *IL4*, *IL9*, and the reference gene *UBE2D2*^{E1} were measured from the 48-hour cultures by using quantitative RT-PCR on the ABI Prism 7900HT (Applied Biosystems, Austin Tex) with QuantiTect SYBR Green (Qiagen, Doncaster, Australia). RNA was isolated from cell pellets by using the RNeasy-96 kit (Ambion/Applied Biosystems, Austin, Tex) and reverse transcribed with the Omniscript RT kit (Qiagen) by using oligo(dT) (Promega, Madison Wis) and RNasin (Promega), with cDNA diluted 1:5 before PCR. cDNA-specific PCR assays were developed in house, and relative cDNA copy numbers were measured from plasmid standard curves, with copy numbers for *IL4* and *IL9* normalized against those for *UBE2D2* for each cDNA sample. Amplification of unique products was verified by means of dissociation curve analysis, and standard cycling conditions were used (95°C for 15 minutes [95°C for 15 seconds, 60°C for 1 minute] $\times$ 40), with the exception that data were collected at 80.5°C for *IL4*. Primers used were as follows: *UBE2D2* mRNA (accession no. U39317), forward 5'ATGGCAGCATTTGTCTTGATATTCTAC3' and reverse 5'TGGATTGGGATCACACAACAGA3'; IL-4 mRNA (accession no.

MGC79402), forward 5'AACAGCCTCACAGAGCAGAAGACT3' and reverse 5'CAGCGAGTGTCTTCTCATGGT3'; and IL-9 mRNA (accession no. X81851), forward 5'GGTGAAAAAATCAGTTGAAGTACTAAAGAA3' and reverse 5'TCAGAAATGTCAGCGCGTTG3'.

Standardized quality assurance standards (containing known amounts of all cytokines measured) were included in every set of assays to control for drift in sensitivities of the different cytokine assays over the life of the project.

Log transformation of cytokine data

To allow log₁₀ transformation, cytokine Δ values of 0 or less were converted to 0.01 for protein and 0.000001 for mRNA. Undetected cases for hematology variables were ascribed the value 3×10^{-4} . Antibody values of less than the limits of detection were ascribed a value equivalent to half the limit of detection for each assay.

Additional study cohort details

Population. The West Australian Pregnancy Cohort is a longitudinal birth cohort; mothers were enrolled from antenatal clinics from the main tertiary maternity hospital in Perth, Western Australia. Mothers in the West Australian Pregnancy Cohort were not selected based on any other criteria than having enrolled for antenatal care at the hospital.^{E2} Two thousand eight hundred sixty-eight children were examined at birth, with extensive antenatal data collected. Follow-ups occurred at 1, 2, 3, 6, 8, 10, and 14 years of age, with physical examinations at 1 and 6, 8, 10, and 14 years of age.

At the age of 14 years, follow-up data included questionnaires with social, demographic, respiratory history, environmental, and smoking statuses obtained for 1795 subjects. There were no significant differences between those who participated in the 14-year follow-up and nonparticipants with regard to birthweight, gestational age, and sex. The only significant difference seen was fewer children with young mothers (maternal age <20 years at the time of birth) participating at the 14-year follow-up (7.1 participating vs 13.3 not participating, $P < .001$).

Respiratory assessment. All spirometric measurements were performed in the morning (10–12 PM) and conducted according to American Thoracic Society guidelines with the subjects seated.^{E3} Subjects were eligible to participate in challenge testing if they obtained reproducible spirometric results, had an FEV₁ of at least 80% of predicted value, had no respiratory illness in the last 14 days, and had withheld from their asthma medications for the standard period.^{E3} Methacholine challenges were performed with a Koko digidoser (nSpire Health, Inc, Longmont, Colo) by using a modified dosimeter technique.^{E4} Subjects were given an initial saline dose followed by doubling doses of methacholine (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, and 8 mg/mL) delivered through DeVilbiss 646 nebulizers (DeVilbiss Healthcare, Somerset, Pa). Testing was stopped once a patient's FEV₁ had decreased by 20% or greater or the highest dose was administered. Participation in respiratory assessment is detailed in Table E12 of this article's Online Repository at www.jacionline.org.

BHR was defined as a binary outcome, with subjects who had a PC₂₀ of less than 8 mg/mL classified as positive. The dose-response slope was calculated as the 2-point slope of the curve regardless of the decrease in FEV₁.

Atopic family history assessment. Family history was obtained by means of parent-completed questionnaires. Questions were asked about first-degree relatives (biologic mother, biologic father, or sibling), seeking information about asthma, eczema, allergic rhinoconjunctivitis, and other allergies. Parents were asked to provide information about whether the allergies had been confirmed and by whom. Information was recorded about maternal and paternal allergic history separately. A child was considered to have a positive family history if 1 or more first-degree relatives had confirmed asthma, eczema, or allergic rhinoconjunctivitis.

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TABLE E1. Comparison of biomarkers in the whole population stratified by HDM sensitization (defined as HDM-specific IgE level ≥ 0.35 kU/L serum)

Variable	No.	Not sensitized to HDM				Sensitized to HDM				Mann-Whitney test
		No.	Mean	SE	Mean rank	No.	Mean	SE	Mean rank	P value
Clinical										
Body mass index	1363	829	21.058	0.137	670.17	534	21.414	0.184	700.37	.167
Current rhinoconjunctivitis: positive	527	227	NA			300	NA			.000*
Current rhinoconjunctivitis: negative	808	587	NA			221	NA			
Sex: male	713	402	NA			311	NA			.000*
Sex: female	666	437	NA			229	NA			
Atopic family history: positive	945	565	NA			380	NA			.324*
Atopic family history: negative	292	184	NA			108	NA			
Antibodies										
HDM IgE	1379	839	0.050	0.002	420.00	540	38.839	3.715	1109.50	.000
HDM IgG4	1379	839	52.235	2.558	546.17	540	219.891	13.369	913.47	.000
Rye IgE	1379	839	4.268	0.827	535.69	540	21.841	2.536	929.75	.000
Rye IgG4	1379	839	26.562	2.991	608.95	540	97.742	9.143	815.93	.000
Cat IgE	1379	839	0.252	0.086	532.37	540	1.595	0.248	934.91	.000
Cat IgG4	1379	839	241.664	15.621	638.05	540	354.308	33.454	770.71	.000
Couch IgE	1379	839	2.195	0.424	545.70	540	12.594	1.573	914.21	.000
Couch IgG4	1379	839	18.345	2.279	612.15	540	111.421	16.294	810.96	.000
Mold IgE	1379	839	0.502	0.069	609.38	540	2.147	0.272	815.27	.000
Mold IgG4	1379	839	143.640	10.863	670.74	540	175.481	19.448	719.92	.024
Total IgE	1379	839	97.782	7.497	506.50	540	438.893	28.208	975.11	.000
Lung function										
BHR dose slope	1272	777	1.161	0.013	562.39	495	1.388	0.021	752.84	.000
FEV ₁ /FVC ratio	1339	815	91.040	0.248	673.08	524	91.020	0.298	665.20	.716
Inflammatory markers										
Soluble CD14	1379	839	1877.735	12.844	701.85	540	1851.979	16.187	671.59	.168
PGF _{2a}	1292	782	76.417	1.249	621.87	510	83.156	1.754	684.26	.003
EPX	1292	782	68.007	1.719	578.80	510	87.393	2.588	750.31	.000
Hematology										
Eosinophils	1376	837	0.264	0.008	580.54	539	0.420	0.013	856.15	.000
Neutrophils	1376	837	3.071	0.041	698.04	539	3.004	0.054	673.69	.267
Innate cytokine responses										
LPS IL-10	1368	829	1046.212	23.309	685.83	539	1006.971	24.588	682.46	.877
LPS IFN-γ	1368	829	194.463	17.598	666.68	539	196.572	13.008	711.91	.038
LPS TNF-α	1368	829	984.879	23.589	676.11	539	1033.694	31.327	697.40	.330
LPS + IFN-γ IL-10	1367	829	412.590	9.403	686.10	538	399.036	10.242	680.76	.807
LPS + IFN-γ IL-12	1358	827	560.762	19.903	685.75	531	538.997	21.802	669.76	.463
LPS + IFN-γ TNF-α	1367	829	3360.372	64.635	685.89	538	3334.158	77.046	681.08	.826
Poly I:C IL-10	1367	829	1756.224	44.366	688.29	538	1673.431	47.629	677.39	.618
Poly I:C IFN-γ	1367	829	1805.027	64.624	679.62	538	1901.509	90.670	690.75	.611
Poly I:C TNF-α	1367	829	717.825	18.502	681.92	538	721.812	22.683	687.20	.809
HDM-specific cytokine responses										
IL-4	1341	814	0.002	0.000	584.53	527	0.005	0.000	804.55	.000
IL-5	1374	835	2.057	0.467	596.44	539	30.610	2.411	828.56	.000
IL-9	1341	814	0.044	0.004	557.23	527	0.176	0.015	846.72	.000
IL-10	1374	835	2.488	0.339	667.38	539	6.095	0.912	718.68	.000
IL-13	1374	835	18.346	1.563	574.15	539	79.107	5.233	863.09	.000
IFN-γ	1374	835	5.459	1.506	660.59	539	16.203	2.827	729.19	.000
Rye-specific cytokine responses										
IL-4	1328	804	0.0003	0.00004	607.90	524	0.001	0.000	751.34	.000
IL-5	1374	835	3.303	1.103	675.11	539	4.196	0.822	706.69	.001
IL-9	1328	804	0.019	0.003	587.54	524	0.036	0.004	782.58	.000
IL-10	1374	835	27.305	1.751	684.37	539	27.768	2.063	692.34	.684
IL-13	1374	835	13.196	1.353	645.89	539	22.948	2.376	751.96	.000

(Continued)

TABLE E1. (Continued)

Variable	No.	Not sensitized to HDM				Sensitized to HDM				Mann-Whitney test P value
		No.	Mean	SE	Mean rank	No.	Mean	SE	Mean rank	
IFN- γ	1374	835	20.987	6.708	684.79	539	14.395	4.073	691.70	.576
PHA cytokine responses										
IL-4	1342	814	0.003	0.000	670.53	528	0.004	0.000	673.00	.909
IL-5	1374	835	221.379	7.815	647.81	539	245.662	8.130	748.99	.000
IL-9	1343	815	0.141	0.006	667.60	528	0.157	0.009	678.79	.605
IL-10	1374	835	562.226	13.098	693.20	539	536.654	14.560	678.68	.508
IL-13	1374	835	1388.127	28.213	660.37	539	1516.146	39.736	729.53	.002
IFN- γ	1374	835	12489.475	317.997	705.53	539	11567.016	381.128	659.56	.036

Data shown are as follows: (1) body mass index (ratio of weight [kg] divided by height² [m]); (2) antibodies (IgE as kU/L serum and IgG4 as μ g/L serum); (3) lung function (FEV₁/FVC ratio as L/L $\times$ 100; BHR dose slope as log₁₀[L/mg/mL]); (4) inflammatory markers (soluble CD14 as ng/mL serum; PGF_{2a} as ng/mmol creatinine in urine; EPX as μ g/mmol creatinine in urine); (5) hematology (as cell numbers $\times$ 10⁹/L blood); and (6) data shown for IL-4 and IL-9 are PCR units normalized to housekeeping gene, and for all other cytokines, units are pg/mL culture supernatant.

NA, Not applicable; FVC, forced vital capacity.

*P value from χ^2 test.

TABLE E2. Comparison of biomarker expression within HDM-sensitized atopic subjects stratified by current asthma

Variable	No.	Without asthma				With asthma				Mann-Whitney test
		No.	Mean	SE	Mean rank	No.	Mean	SE	Mean rank	P value
Clinical										
BMI	517	425	21.510	0.206	261.43	92	21.213	0.436	247.70	.427
Current rhinoconjunctivitis: positive	299	231	NA			68	NA			.000*
Current rhinoconjunctivitis: negative	219	197	NA			22	NA			
Sex: male	300	248	NA			52	NA			.839*
Sex: female	222	182	NA			40	NA			
Atopic family history: positive	378	295	NA			83	NA			.000*
Atopic family history: negative	107	101	NA			6	NA			
Antibodies										
HDM IgE	522	430	29.450	3.293	244.51	92	78.125	14.330	340.90	.000
HDM IgG4	522	430	194.968	14.404	249.56	92	332.820	37.691	317.32	.000
Rye IgE	522	430	23.494	3.003	263.17	92	12.415	2.613	253.72	.585
Rye IgG4	522	430	89.884	8.343	258.09	92	128.496	34.437	277.42	.244
Cat IgE	522	430	1.063	0.147	251.18	92	3.669	1.147	309.74	.001
Cat IgG4	522	430	343.278	40.170	253.49	92	411.736	56.612	298.92	.009
Couch IgE	522	430	13.773	1.856	265.04	92	5.554	1.279	244.96	.245
Couch IgG4	522	430	96.267	13.957	257.99	92	178.974	68.948	277.92	.229
Mold IgE	522	430	2.015	0.303	255.39	92	2.775	0.699	290.05	.033
Mold IgG4	522	430	176.389	23.028	263.10	92	159.297	33.563	254.02	.598
Total IgE	522	430	416.788	32.868	252.06	92	479.631	52.241	305.63	.002
Lung function										
BHR dose slope	482	402	1.332	0.022	226.42	80	1.674	0.062	317.29	.000
FEV ₁ /FVC ratio	510	420	91.361	0.321	261.55	90	89.457	0.818	227.29	.045
Inflammatory markers										
Soluble CD14	522	430	1847.750	18.668	258.41	92	1869.808	34.472	275.95	.311
PGF _{2a}	492	403	83.883	1.998	247.97	89	81.326	4.226	239.85	.626
EPX	492	403	82.571	2.764	236.66	89	105.510	7.133	291.04	.001
Hematology										
Eosinophils	521	429	0.395	0.014	247.33	92	0.528	0.032	324.76	.000
Neutrophils	521	429	3.068	0.063	268.20	92	2.714	0.097	227.41	.018
Innate cytokine responses										
LPS IL-10	521	429	1006.858	26.492	263.17	92	993.535	64.428	250.87	.477
LPS IFN-γ	521	429	194.078	14.557	258.98	92	193.032	30.817	270.40	.509
LPS TNF-α	521	429	1053.616	34.860	266.73	92	941.042	79.292	234.28	.061
LPS + IFN-γ IL-10	520	428	398.708	11.021	262.99	92	395.472	28.768	248.90	.414
LPS + IFN-γ IL-12	513	423	547.720	25.404	259.86	90	451.114	39.751	243.54	.343
LPS + IFN-γ TNF-α	520	428	3374.014	87.231	263.69	92	3200.371	185.169	245.67	.297
Poly I:C IL-10	520	428	1680.331	52.616	262.45	92	1634.328	121.873	251.42	.523
Poly I:C IFN-γ	520	428	1850.362	98.650	258.49	92	1920.841	196.057	269.85	.511
Poly I:C TNF-α	520	428	741.191	26.255	265.89	92	631.314	46.302	235.41	.078
HDM-specific cytokine responses										
IL-4	510	420	0.004	0.000	250.15	90	0.006	0.001	280.45	.071
IL-5	521	429	24.693	2.307	249.08	92	55.041	8.103	316.57	.000
IL-9	510	420	0.162	0.017	246.56	90	0.212	0.032	297.22	.003
IL-10	521	429	4.879	0.679	256.86	92	11.459	4.224	280.32	.027
IL-13	521	429	64.896	4.838	246.68	92	137.324	18.795	327.79	.000
IFN-γ	521	429	15.070	3.204	256.20	92	22.219	7.058	283.40	.017
Rye-specific cytokine responses										
IL-4	506	416	0.001	0.0003	250.09	90	0.001	0.0002	269.24	.179
IL-5	521	429	4.397	0.995	261.76	92	2.666	1.029	257.47	.614
IL-9	506	416	0.038	0.005	248.95	90	0.028	0.004	274.52	.127
IL-10	521	429	28.367	2.395	261.71	92	25.098	4.336	257.67	.794
IL-13	521	429	22.101	2.799	254.38	92	27.597	4.648	291.86	.011
IFN-γ	521	429	11.263	2.989	259.35	92	28.047	19.297	268.68	.344

(Continued)

TABLE E2. (Continued)

TABLE 22 (Continued)										
Variable	No.	Without asthma				With asthma				Mann-Whitney test
		No.	Mean	SE	Mean rank	No.	Mean	SE	Mean rank	P value
PHA cytokine responses										
IL-4	510	421	0.004	0.000	255.86	89	0.004	0.001	253.79	.904
IL-5	521	429	235.560	8.403	254.68	92	271.111	20.195	290.46	.039
IL-9	510	421	0.154	0.010	254.58	89	0.161	0.021	259.83	.760
IL-10	521	429	511.046	15.578	252.62	92	625.806	38.036	300.09	.006
IL-13	521	429	1473.657	42.165	255.85	92	1691.010	118.227	285.01	.092
IFN- γ	521	429	11045.769	415.275	252.26	92	13240.257	891.837	301.74	.004

Data shown are as follows: (1) body mass index (ratio of weight [kg] divided by height² [m]); (2) antibodies (IgE as kU/L serum; IgG4 as μ g/L serum); (3) lung function (FEV₁/FVC ratio as L/L $\times$ 100; BHR dose slope as log₁₀[L/mg/mL]); (4) Inflammatory markers (soluble CD14 as ng/mL serum; PGF₂a as ng/mmol creatinine in urine; EPX as μ g/mmol creatinine in urine); (5) hematology (as cell numbers $\times$ 10⁹/L blood); and (6) data shown for IL-4 and IL-9 are PCR units normalized to a housekeeping gene, and for all other cytokines, units are pg/mL culture supernatant.

BMI, Body mass index; *NA*, not applicable; *FVC*, forced vital capacity.

**P* value from χ^2 test.

TABLE E3. Spearman correlation matrix using data relating to HDM-sensitized atopic subjects in Table E2

Variable	BMI	HDM IgE	HDM IgG4	Rye IgE	Rye IgG4	Cat IgE	Cat IgG4	Couch IgE	Couch IgG4	Mold IgE	Mold IgG4
BMI	1.000	−0.044	−0.029	−0.042	−0.071	0.022	−0.057	−0.050	−0.070	−0.065	0.009
HDM IgE	−0.044	1.000	0.566†	0.025	0.001	0.370†	0.103*	−0.005	0.019	−0.035	0.064
HDM IgG4	−0.029	0.566†	1.000	0.030	0.318†	0.230†	0.518†	0.018	0.407†	0.054	0.266†
Rye IgE	−0.042	0.025	0.030	1.000	0.605†	0.383†	0.104*	0.916†	0.440†	0.423†	0.118†
Rye IgG4	−0.071	0.001	0.318†	0.605†	1.000	0.250†	0.232†	0.591†	0.792†	0.316†	0.259†
Cat IgE	0.022	0.370†	0.230†	0.383†	0.250†	1.000	0.197†	0.338†	0.203†	0.309†	0.134†
Cat IgG4	−0.057	0.103*	0.518†	0.104*	0.232†	0.197†	1.000	0.076	0.288†	0.077	0.176†
Couch IgE	−0.050	−0.005	0.018	0.916†	0.591†	0.338†	0.076	1.000	0.474†	0.420†	0.108*
Couch IgG4	−0.070	0.019	0.407†	0.440†	0.792†	0.203†	0.288†	0.474†	1.000	0.270†	0.271†
Mold IgE	−0.065	−0.035	0.054	0.423†	0.316†	0.309†	0.077	0.420†	0.270†	1.000	0.209†
Mold IgG4	0.009	0.064	0.266†	0.118†	0.259†	0.134†	0.176†	0.108*	0.271†	0.209†	1.000
Total IgE	−0.114†	0.389†	0.242†	0.473†	0.326†	0.457†	0.140†	0.455†	0.281†	0.434†	0.119†
Soluble CD14	0.040	0.000	0.013	−0.004	0.050	0.006	0.075	0.022	0.097*	0.014	−0.022
PGF2a	−0.130†	0.061	0.026	−0.018	−0.033	0.030	−0.021	0.008	−0.014	0.045	0.045
EPX	−0.096*	0.172†	0.100*	−0.014	−0.026	0.131†	0.062	−0.033	0.013	0.096*	−0.024
Eosinophils	−0.086*	0.385†	0.242†	0.009	0.017	0.190†	0.068	−0.019	0.008	0.117†	0.036
Neutrophils	0.258†	−0.054	−0.017	−0.157†	−0.128†	−0.100*	−0.049	−0.142†	−0.101*	−0.071	0.019
LPS IL-10	−0.018	0.024	0.003	−0.014	−0.035	0.011	−0.028	0.006	−0.038	−0.066	0.040
LPS IFN- γ	0.022	0.043	0.048	−0.048	−0.014	−0.024	0.017	−0.070	−0.010	0.040	−0.084
LPS TNF- α	0.020	0.045	0.024	0.005	0.005	−0.025	−0.053	0.018	0.004	−0.069	−0.067
LPS + IFN- γ IL-10	0.017	0.034	−0.008	−0.007	−0.033	0.017	−0.063	0.004	−0.056	−0.039	0.072
LPS + IFN- γ IL-12	−0.080	0.047	0.066	−0.046	0.018	0.010	0.018	−0.029	0.040	−0.053	−0.061
LPS + IFN- γ TNF- α	−0.019	0.073	0.054	−0.028	−0.034	0.008	0.034	−0.015	−0.031	−0.073	0.015
Poly I:C IL-10	−0.009	0.051	0.032	0.000	−0.039	0.052	−0.060	0.011	−0.007	−0.066	0.079
Poly I:C IFN- γ	−0.007	0.047	0.004	−0.040	−0.023	−0.035	−0.079	−0.044	−0.045	0.000	−0.047
Poly I:C TNF- α	−0.004	0.068	0.037	−0.004	−0.011	0.003	−0.056	0.006	0.004	−0.072	−0.001
HDM IL-4	0.033	0.405†	0.180†	−0.064	−0.118†	0.121†	−0.071	−0.079	−0.077	−0.137†	−0.025
HDM IL-5	−0.054	0.597†	0.348†	−0.105*	−0.115†	0.140†	0.036	−0.129†	−0.073	−0.139†	−0.004
HDM IL-9	−0.012	0.514†	0.244†	−0.148†	−0.183†	0.113†	−0.032	−0.156†	−0.131†	−0.174†	−0.036
HDM IL-10	−0.024	0.241†	0.188†	0.008	−0.007	0.102*	0.067	−0.010	−0.027	−0.024	0.016
HDM IL-13	−0.019	0.569†	0.303†	−0.106*	−0.141†	0.146†	0.023	−0.123†	−0.112†	−0.084	−0.034
HDM IFN- γ	0.028	0.199†	0.121†	0.049	−0.007	0.083	0.043	0.053	−0.008	0.033	0.050
PHA IL-4	0.057	0.020	0.070	0.059	0.012	−0.027	−0.004	0.038	−0.002	0.023	0.027
PHA IL-5	−0.112†	0.088*	0.076	0.033	0.025	0.089*	0.076	0.035	−0.011	0.204†	0.009
PHA IL-9	0.039	0.016	0.069	0.027	0.025	−0.037	0.095*	0.037	0.005	−0.016	0.055
PHA IL-10	0.039	0.007	0.016	−0.035	−0.026	−0.011	0.097*	−0.027	−0.077	0.050	−0.014
PHA IL-13	−0.032	0.040	0.043	0.033	0.010	0.044	0.085*	0.046	−0.022	0.160†	0.025
PHA IFN- γ	0.043	0.075	0.093*	−0.013	0.019	0.024	0.063	−0.044	0.056	0.022	0.011
BHR dose slope	−0.044	0.281†	0.137†	−0.014	−0.006	0.186†	−0.030	0.005	−0.041	0.098*	0.034
FEV1/FVC ratio	−0.138†	−0.072	−0.070	−0.070	−0.079	−0.111*	0.043	−0.090*	−0.085	−0.056	0.009

Data shown are Spearman correlation coefficients.

BMI, Body mass index; FVC, forced vital capacity.

*Correlation is significant at the .05 level (2-tailed).

†Correlation is significant at the .01 level (2-tailed).

TABLE E4. Spearman correlation matrix using data relating to HDM-sensitized atopic subjects in Table E2

Variable	Total IgE	Soluble CD14	PGF _{2a}	EPX	Eosinophils	Neutrophils	LPS IL-10	LPS IFN- γ	LPS TNF- α	LPS- γ IL-10	LPS- γ IL-12
BMI	-0.114†	0.040	-0.130†	-0.096*	-0.086*	0.258†	-0.018	0.022	0.020	0.017	-0.080
HDM IgE	0.389†	0.000	0.061	0.172†	0.385†	-0.054	0.024	0.043	0.045	0.034	0.047
HDM IgG4	0.242†	0.013	0.026	0.100*	0.242†	-0.017	0.003	0.048	0.024	-0.008	0.066
Rye IgE	0.473†	-0.004	-0.018	-0.014	0.009	-0.157†	-0.014	-0.048	0.005	-0.007	-0.046
Rye IgG4	0.326†	0.050	-0.033	-0.026	0.017	-0.128†	-0.035	-0.014	0.005	-0.033	0.018
Cat IgE	0.457†	0.006	0.030	0.131†	0.190†	-0.100*	0.011	-0.024	-0.025	0.017	0.010
Cat IgG4	0.140†	0.075	-0.021	0.062	0.068	-0.049	-0.028	0.017	-0.053	-0.063	0.018
Couch IgE	0.455†	0.022	0.008	-0.033	-0.019	-0.142†	0.006	-0.070	0.018	0.004	-0.029
Couch IgG4	0.281†	0.097*	-0.014	0.013	0.008	-0.101*	-0.038	-0.010	0.004	-0.056	0.040
Mold IgE	0.434†	0.014	0.045	0.096*	0.117†	-0.071	-0.066	0.040	-0.069	-0.039	-0.053
Mold IgG4	0.119†	-0.022	0.045	-0.024	0.036	0.019	0.040	-0.084	-0.067	0.072	-0.061
Total IgE	1.000	0.070	0.097*	0.175†	0.395†	-0.123†	-0.035	0.098*	0.019	-0.010	0.031
Soluble CD14	0.070	1.000	-0.057	0.005	0.018	0.070	0.057	-0.014	0.049	0.015	0.034
PGF _{2a}	0.097*	-0.057	1.000	0.154†	0.122†	0.006	0.005	-0.020	0.026	0.010	0.015
EPX	0.175†	0.005	0.154†	1.000	0.432†	-0.035	-0.019	0.031	-0.019	-0.035	0.023
Eosinophils	0.395†	0.018	0.122†	0.432†	1.000	0.098*	0.010	0.103*	0.057	0.007	0.016
Neutrophils	-0.123†	0.070	0.006	-0.035	0.098*	1.000	0.181†	0.008	0.144†	0.198†	-0.022
LPS IL-10	-0.035	0.057	0.005	-0.019	0.010	0.181†	1.000	-0.124†	0.284†	0.800†	0.012
LPS IFN- γ	0.098*	-0.014	-0.020	0.031	0.103*	0.008	-0.124†	1.000	0.316†	-0.105*	0.266†
LPS TNF- α	0.019	0.049	0.026	-0.019	0.057	0.144†	0.284†	0.316†	1.000	0.298†	0.312†
LPS + IFN- γ IL-10	-0.010	0.015	0.010	-0.035	0.007	0.198†	0.800†	-0.105*	0.298†	1.000	-0.084
LPS + IFN- γ IL-12	0.031	0.034	0.015	0.023	0.016	-0.022	0.012	0.266†	0.312†	-0.084	1.000
LPS + IFN- γ TNF- α	0.002	0.057	0.051	-0.007	0.078	0.184†	0.416†	0.128†	0.769†	0.330†	0.373†
Poly I:C IL-10	-0.020	0.026	0.049	-0.054	0.009	0.142†	0.778†	-0.199†	0.150†	0.660†	0.006
Poly I:C IFN- γ	0.026	-0.036	0.047	0.003	0.070	0.048	0.076	0.533†	0.304†	0.021	0.278†
Poly I:C TNF- α	0.019	0.040	0.040	-0.029	0.022	0.116†	0.314†	0.100*	0.763†	0.256†	0.332†
HDM IL-4	0.039	-0.117†	0.022	0.106*	0.171†	0.088*	0.019	0.050	0.023	0.042	-0.060
HDM IL-5	0.152†	-0.010	-0.040	0.152†	0.345†	-0.018	0.091*	0.130†	0.090*	0.081	0.010
HDM IL-9	0.072	-0.114†	0.023	0.131†	0.283†	0.019	0.038	0.102*	0.012	0.083	-0.073
HDM IL-10	0.118†	-0.081	0.046	0.060	0.182†	-0.049	0.163†	0.073	0.049	0.169†	-0.030
HDM IL-13	0.149†	-0.082	0.017	0.158†	0.352†	0.005	0.079	0.178†	0.105*	0.107*	-0.030
HDM IFN- γ	0.163†	-0.071	0.022	0.028	0.183†	-0.004	-0.092*	0.235†	0.032	-0.034	-0.024
PHA IL-4	0.048	-0.153†	-0.004	0.017	-0.075	0.025	-0.100*	0.069	0.050	-0.022	-0.059
PHA IL-5	0.262†	0.037	0.044	0.153†	0.271†	-0.022	0.050	0.153†	0.047	0.064	0.013
PHA IL-9	0.067	0.007	0.018	-0.014	-0.007	0.046	0.126†	0.081	0.160†	0.150†	0.020
PHA IL-10	0.008	0.042	0.023	-0.063	0.002	0.056	0.186†	0.155†	0.052	0.162†	0.005
PHA IL-13	0.148†	0.001	0.048	0.104*	0.138†	0.030	0.136†	0.031	0.072	0.159†	-0.054
PHA IFN- γ	0.019	-0.026	-0.005	-0.029	0.048	0.038	-0.017	0.301†	0.110*	-0.034	0.074
BHR dose slope	0.231†	0.020	0.040	0.111*	0.266†	-0.010	0.070	0.039	0.018	0.122†	-0.004
FEV ₁ /FVC ratio	-0.103*	-0.018	0.007	-0.031	-0.036	0.057	-0.044	0.000	-0.085	-0.079	-0.026

Data shown are Spearman correlation coefficients.

BMI, Body mass index; FVC, forced vital capacity.

*Correlation is significant at the .05 level (2-tailed).

†Correlation is significant at the .01 level (2-tailed).

TABLE E5. Spearman correlation matrix using data relating to HDM-sensitized atopic subjects in Table E2

Variable	LPS- γ TNF- α	Poly I:C IL-10	Poly I:C IFN- γ	Poly I:C TNF- α	HDM IL-4	HDM IL-5	HDM IL-9	HDM IL-10	HDM IL-13	HDM IFN- γ
BMI	-0.019	-0.009	-0.007	-0.004	0.033	-0.054	-0.012	-0.024	-0.019	0.028
HDM IgE	0.073	0.051	0.047	0.068	0.405†	0.597†	0.514†	0.241†	0.569†	0.199†
HDM IgG4	0.054	0.032	0.004	0.037	0.180†	0.348†	0.244†	0.188†	0.303†	0.121†
Rye IgE	-0.028	0.000	-0.040	-0.004	-0.064	-0.105*	-0.148†	0.008	-0.106*	0.049
Rye IgG4	-0.034	-0.039	-0.023	-0.011	-0.118†	-0.115†	-0.183†	-0.007	-0.141†	-0.007
Cat IgE	0.008	0.052	-0.035	0.003	0.121†	0.140†	0.113†	0.102*	0.146†	0.083
Cat IgG4	0.034	-0.060	-0.079	-0.056	-0.071	0.036	-0.032	0.067	0.023	0.043
Couch IgE	-0.015	0.011	-0.044	0.006	-0.079	-0.129†	-0.156†	-0.010	-0.123†	0.053
Couch IgG4	-0.031	-0.007	-0.045	0.004	-0.077	-0.073	-0.131†	-0.027	-0.112†	-0.008
Mold IgE	-0.073	-0.066	0.000	-0.072	-0.137†	-0.139†	-0.174†	-0.024	-0.084	0.033
Mold IgG4	0.015	0.079	-0.047	-0.001	-0.025	-0.004	-0.036	0.016	-0.034	0.050
Total IgE	0.002	-0.020	0.026	0.019	0.039	0.152†	0.072	0.118†	0.149†	0.163†
Soluble CD14	0.057	0.026	-0.036	0.040	-0.117†	-0.010	-0.114†	-0.081	-0.082	-0.071
PGF _{2a}	0.051	0.049	0.047	0.040	0.022	-0.040	0.023	0.046	0.017	0.022
EPX	-0.007	-0.054	0.003	-0.029	0.106*	0.152†	0.131†	0.060	0.158†	0.028
Eosinophils	0.078	0.009	0.070	0.022	0.171†	0.345†	0.283†	0.182†	0.352†	0.183†
Neutrophils	0.184†	0.142†	0.048	0.116†	0.088*	-0.018	0.019	-0.049	0.005	-0.004
LPS IL-10	0.416†	0.778†	0.076	0.314†	0.019	0.091*	0.038	0.163†	0.079	-0.092*
LPS IFN- γ	0.128†	-0.199†	0.533†	0.100*	0.050	0.130†	0.102*	0.073	0.178†	0.235†
LPS TNF- α	0.769†	0.150†	0.304†	0.763†	0.023	0.090*	0.012	0.049	0.105*	0.032
LPS + IFN- γ IL-10	0.330†	0.660†	0.021	0.256†	0.042	0.081	0.083	0.169†	0.107*	-0.034
LPS + IFN- γ IL-12	0.373†	0.006	0.278†	0.332†	-0.060	0.010	-0.073	-0.030	-0.030	-0.024
LPS + IFN- γ TNF- α	1.000	0.343†	0.314†	0.789†	-0.032	0.109*	-0.011	0.033	0.121†	-0.018
Poly I:C IL-10	0.343†	1.000	-0.003	0.232†	0.121†	0.133†	0.129†	0.207†	0.149†	-0.054
Poly I:C IFN- γ	0.314†	-0.003	1.000	0.346†	0.063	0.115†	0.080	0.036	0.119†	0.110*
Poly I:C TNF- α	0.789†	0.232†	0.346†	1.000	-0.009	0.075	-0.027	0.012	0.055	-0.053
HDM IL-4	-0.032	0.121†	0.063	-0.009	1.000	0.386†	0.687†	0.235†	0.529†	0.155†
HDM IL-5	0.109*	0.133†	0.115†	0.075	0.386†	1.000	0.646†	0.376†	0.760†	0.329†
HDM IL-9	-0.011	0.129†	0.080	-0.027	0.687†	0.646†	1.000	0.387†	0.836†	0.388†
HDM IL-10	0.033	0.207†	0.036	0.012	0.235†	0.376†	0.387†	1.000	0.455†	0.353†
HDM IL-13	0.121†	0.149†	0.119†	0.055	0.529†	0.760†	0.836†	0.455†	1.000	0.464†
HDM IFN- γ	-0.018	-0.054	0.110*	-0.053	0.155†	0.329†	0.388†	0.353†	0.464†	1.000
PHA IL-4	-0.067	-0.095*	-0.006	0.008	0.297†	-0.002	0.124†	0.044	0.057	0.068
PHA IL-5	0.071	0.084	0.040	0.038	0.136†	0.290†	0.206†	0.208†	0.278†	0.052
PHA IL-9	0.136†	0.188†	0.125†	0.109*	0.266†	0.096*	0.233†	0.161†	0.136†	0.081
PHA IL-10	0.113†	0.226†	0.169†	-0.020	0.126†	0.109*	0.155†	0.242†	0.195†	0.085*
PHA IL-13	0.174†	0.160†	0.013	0.103*	0.120†	0.126†	0.154†	0.216†	0.257†	0.072
PHA IFN- γ	0.104*	0.027	0.220†	0.010	0.023	0.138†	0.048	0.112†	0.128†	0.143†
BHR dose slope	-0.039	0.039	0.017	-0.054	0.113*	0.216†	0.173†	0.108*	0.202†	0.110*
FEV ₁ /FVC ratio	-0.006	-0.039	0.020	-0.015	0.019	-0.044	0.002	0.030	-0.039	-0.023

Data shown are Spearman correlation coefficients.

BMI, Body mass index; FVC, forced vital capacity.

*Correlation is significant at the .05 level (2-tailed).

†Correlation is significant at the .01 level (2-tailed).

TABLE E6. Spearman correlation matrix using data relating to HDM-sensitized atopic subjects in Table E2

Variable	PHA IL-4	PHA IL-5	PHA IL-9	PHA IL-10	PHA IL-13	PHA IFN- γ	BHR dose slope	FEV ₁ /FVC ratio
BMI	0.057	-0.112†	0.039	0.039	-0.032	0.043	-0.044	-0.138†
HDM IgE	0.020	0.088*	0.016	0.007	0.040	0.075	0.281†	-0.072
HDM IgG4	0.070	0.076	0.069	0.016	0.043	0.093*	0.137†	-0.070
Rye IgE	0.059	0.033	0.027	-0.035	0.033	-0.013	-0.014	-0.070
Rye IgG4	0.012	0.025	0.025	-0.026	0.010	0.019	-0.006	-0.079
Cat IgE	-0.027	0.089*	-0.037	-0.011	0.044	0.024	0.186†	-0.111*
Cat IgG4	-0.004	0.076	0.095*	0.097*	0.085*	0.063	-0.030	0.043
Couch IgE	0.038	0.035	0.037	-0.027	0.046	-0.044	0.005	-0.090*
Couch IgG4	-0.002	-0.011	0.005	-0.077	-0.022	0.056	-0.041	-0.085
Mold IgE	0.023	0.204†	-0.016	0.050	0.160†	0.022	0.098*	-0.056
Mold IgG4	0.027	0.009	0.055	-0.014	0.025	0.011	0.034	0.009
Total IgE	0.048	0.262†	0.067	0.008	0.148†	0.019	0.231†	-0.103*
Soluble CD14	-0.153†	0.037	0.007	0.042	0.001	-0.026	0.020	-0.018
PGF _{2a}	-0.004	0.044	0.018	0.023	0.048	-0.005	0.040	0.007
EPX	0.017	0.153†	-0.014	-0.063	0.104*	-0.029	0.111*	-0.031
Eosinophils	-0.075	0.271†	-0.007	0.002	0.138†	0.048	0.266†	-0.036
Neutrophils	0.025	-0.022	0.046	0.056	0.030	0.038	-0.010	0.057
LPS IL-10	-0.100*	0.050	0.126†	0.186†	0.136†	-0.017	0.070	-0.044
LPS IFN- γ	0.069	0.153†	0.081	0.155†	0.031	0.301†	0.039	0.000
LPS TNF- α	0.050	0.047	0.160†	0.052	0.072	0.110*	0.018	-0.085
LPS + IFN- γ IL-10	-0.022	0.064	0.150†	0.162†	0.159†	-0.034	0.122†	-0.079
LPS + IFN- γ IL-12	-0.059	0.013	0.020	0.005	-0.054	0.074	-0.004	-0.026
LPS + IFN- γ TNF- α	-0.067	0.071	0.136†	0.113†	0.174†	0.104*	-0.039	-0.006
Poly I:C IL-10	-0.095*	0.084	0.188†	0.226†	0.160†	0.027	0.039	-0.039
Poly I:C IFN- γ	-0.006	0.040	0.125†	0.169†	0.013	0.220†	0.017	0.020
Poly I:C TNF- α	0.008	0.038	0.109*	-0.020	0.103*	0.010	-0.054	-0.015
HDM IL-4	0.297†	0.136†	0.266†	0.126†	0.120†	0.023	0.113*	0.019
HDM IL-5	-0.002	0.290†	0.096*	0.109*	0.126†	0.138†	0.216†	-0.044
HDM IL-9	0.124†	0.206†	0.233†	0.155†	0.154†	0.048	0.173†	0.002
HDM IL-10	0.044	0.208†	0.161†	0.242†	0.216†	0.112†	0.108*	0.030
HDM IL-13	0.057	0.278†	0.136†	0.195†	0.257†	0.128†	0.202†	-0.039
HDM IFN- γ	0.068	0.052	0.081	0.085*	0.072	0.143†	0.110*	-0.023
PHA IL-4	1.000	0.163†	0.535†	-0.014	0.188†	-0.069	0.001	0.048
PHA IL-5	0.163†	1.000	0.398†	0.361†	0.646†	0.101*	0.125†	0.019
PHA IL-9	0.535†	0.398†	1.000	0.428†	0.330†	0.204†	0.003	0.025
PHA IL-10	-0.014	0.361†	0.428†	1.000	0.289†	0.339†	0.028	0.011
PHA IL-13	0.188†	0.646†	0.330†	0.289†	1.000	0.047	0.000	0.024
PHA IFN- γ	-0.069	0.101*	0.204†	0.339†	0.047	1.000	-0.044	-0.033
BHR dose slope	0.001	0.125†	0.003	0.028	0.000	-0.044	1.000	-0.212†
FEV ₁ /FVC ratio	0.048	0.019	0.025	0.011	0.024	-0.033	-0.212†	1.000

Data shown are Spearman correlation coefficients.

BMI, Body mass index; FVC, forced vital capacity.

*Correlation is significant at the .05 level (2-tailed).

†Correlation is significant at the .01 level (2-tailed).

TABLE E7. Comparison of biomarker expressions within HDM-sensitized atopic subjects stratified by BHR status

Variable	No.	BHR negative				BHR positive				Mann-Whitney test
		No.	Mean	SE	Mean rank	No.	Mean	SE	Mean rank	P value
Clinical										
Body mass index	500	360	21.251	0.216	247.10	140	21.656	0.382	259.24	.399
Current rhinoconjunctivitis: positive	277	195				82				.425*
Current rhinoconjunctivitis: negative	209	154				55				
Sex: male	289	218				71				.045*
Sex: female	211	142				69				
Atopic family history: positive	358	246				112				.019*
Atopic family history: negative	99	80				19				
Antibodies										
HDM IgE	500	360	28.336	3.510	233.03	140	62.593	10.169	295.42	.000
HDM IgG4	500	360	200.595	13.234	242.43	140	247.889	23.411	271.25	.045
Rye IgE	500	360	22.823	3.263	246.53	140	20.514	4.754	260.70	.324
Rye IgG4	500	360	102.366	12.029	248.09	140	84.664	13.659	256.69	.531
Cat IgE	500	360	0.949	0.156	234.72	140	2.816	0.705	291.08	.000
Cat IgG4	500	360	342.858	38.870	252.00	140	388.912	78.247	246.64	.710
Couch IgE	500	360	12.630	1.969	244.48	140	13.709	3.241	265.98	.134
Couch IgG4	500	360	118.295	21.214	249.90	140	101.531	30.550	252.05	.876
Mold IgE	500	360	1.857	0.322	239.22	140	2.983	0.585	279.51	.003
Mold IgG4	500	360	181.802	26.292	248.44	140	159.191	27.422	255.79	.607
Total IgE	500	360	384.137	34.156	230.60	140	585.284	59.688	301.66	.000
Inflammatory markers										
Soluble CD14	500	360	1841.702	20.182	246.47	140	1863.280	31.150	260.86	.317
PGF _{2a}	474	341	82.997	2.152	237.01	133	82.439	3.340	238.77	.900
EPX	474	341	83.818	2.870	230.93	133	94.710	5.385	254.34	.095
Hematology										
Eosinophils	499	360	0.396	0.015	234.74	139	0.500	0.028	289.58	.000
Neutrophils	499	360	2.950	0.057	248.42	139	3.132	0.136	254.09	.694
Innate cytokine responses										
LPS IL-10	499	359	975.779	28.317	248.69	140	1026.300	53.162	253.37	.745
LPS IFN-γ	499	359	180.764	13.852	242.42	140	244.533	31.366	269.44	.060
LPS TNF-α	499	359	980.325	34.288	241.91	140	1199.234	76.112	270.74	.045
LPS + IFN-γ IL-10	498	359	382.182	11.913	243.84	139	421.099	21.810	264.12	.158
LPS + IFN-γ IL-12	492	354	532.709	26.954	244.52	138	560.869	43.135	251.58	.621
LPS + IFN-γ TNF-α	498	359	3278.576	89.989	246.39	139	3493.560	168.738	257.53	.439
Poly I:C IL-10	498	359	1620.905	55.912	250.27	139	1619.897	96.136	247.52	.848
Poly I:C IFN-γ	498	359	1875.601	110.836	245.46	139	1962.973	182.332	259.93	.314
Poly I:C TNF-α	498	359	708.871	25.321	250.04	139	748.931	51.516	248.11	.893
HDM-specific cytokine responses										
IL-4	487	350	0.005	0.0004	240.75	137	0.005	0.001	252.31	.406
IL-5	499	359	26.397	2.700	241.30	140	42.287	5.841	272.30	.012
IL-9	487	350	0.163	0.019	237.48	137	0.209	0.029	260.65	.102
IL-10	499	359	5.860	1.209	247.36	140	7.118	1.498	256.78	.294
IL-13	499	359	69.562	5.757	242.04	140	106.073	12.872	270.40	.043
IFN-γ	499	359	15.592	3.540	245.98	140	20.576	5.957	260.30	.136
Rye-specific cytokine responses										
IL-4	484	349	0.0009	0.00012	237.82	135	0.0010	0.0001	254.61	.159
IL-5	499	359	4.738	1.168	249.00	140	3.821	0.999	252.57	.627
IL-9	484	349	0.033	0.004	238.85	135	0.040	0.008	251.95	.349
IL-10	499	359	27.732	2.458	250.22	140	30.059	4.605	249.43	.951
IL-13	499	359	21.830	3.101	242.08	140	29.255	4.407	270.31	.022
IFN-γ	499	359	12.413	3.523	249.48	140	21.410	12.746	251.34	.826
PHA cytokine responses										
IL-4	488	352	0.004	0.000	246.34	136	0.004	0.000	239.73	.641
IL-5	499	359	237.174	9.623	244.12	140	268.319	17.239	265.09	.144
IL-9	488	352	0.155	0.010	244.76	136	0.162	0.020	243.82	.947

(Continued)

TABLE E7. (Continued)

Variable	No.	BHR negative				BHR positive				Mann-Whitney test P value
		No.	Mean	SE	Mean rank	No.	Mean	SE	Mean rank	
IL-10	499	359	520.359	17.302	245.68	140	559.108	29.833	261.08	.284
IL-13	499	359	1527.611	48.615	253.24	140	1496.944	82.718	241.69	.422
IFN- γ	499	359	11597.210	466.961	250.34	140	11514.580	763.263	249.12	.932

Data shown are as follows: (1) body mass index (ratio of weight [kg] divided by height² [m]); (2) antibodies (IgE as kU/L serum; IgG4 as μ g/L serum); (3) lung function (FEV₁/FVC ratio as L/L $\times$ 100; BHR dose slope as log₁₀[L/mg/mL]); (4) inflammatory markers (soluble CD14 as ng/mL serum; PGF₂a as ng/mmol creatinine in urine; EPX as μ g/mmol creatinine in urine); (5) hematology (as cell numbers $\times$ 10⁹/L blood); and (6) data shown for IL-4 and IL-9 are PCR units normalized to a housekeeping gene, and for all other cytokines, units are pg/mL culture supernatant.

FVC, Forced vital capacity.

*P value from χ^2 test.

TABLE E8. Comparison of biomarker expressions within nonatopic subjects stratified by current asthma

Variable	No.	Nonatopic without asthma				Nonatopic with asthma				Mann-Whitney test
		No.	Mean	SE	Mean rank	No.	Mean	SE	Mean rank	P value
Clinical										
Body mass index	528	501	21.143	0.176	266.19	27	21.030	1.048	233.19	.274
Current rhinoconjunctivitis: positive	114	98				16				.000*
Current rhinoconjunctivitis: negative	423	412				11				
Sex: male	239	225				14				.431*
Sex: female	298	285				13				
Lung function										
BHR dose slope	499	474	1.140	0.016	248.80	25	1.141	0.057	272.84	.416
FEV ₁ /FVC ratio	517	490	91.059	0.318	261.64	27	89.175	1.219	211.13	.087
Inflammatory markers										
Soluble CD14	537	510	1885.403	16.108	269.29	27	1861.284	58.534	263.59	.853
PGF _{2a}	500	474	74.244	1.573	250.07	26	77.202	6.967	258.31	.777
EPX	500	474	64.368	2.101	247.72	26	80.262	9.462	301.12	.067
Hematology										
Eosinophils	535	508	0.230	0.009	265.85	27	0.259	0.030	308.37	.148
Neutrophils	535	508	3.118	0.052	267.73	27	3.030	0.157	273.07	.861
Innate cytokine responses										
LPS IL-10	528	501	1052.002	29.688	264.87	27	1037.996	135.862	257.70	.812
LPS IFN-γ	528	501	160.115	11.698	263.39	27	357.969	171.035	285.19	.469
LPS TNF-α	528	501	953.906	29.142	267.03	27	768.650	106.090	217.56	.101
LPS + IFN-γ IL-10	528	501	412.447	11.822	264.90	27	410.627	54.524	257.11	.796
LPS + IFN-γ IL-12	530	503	554.369	25.325	266.96	27	463.910	87.456	238.26	.343
LPS + IFN-γ TNF-α	528	501	3324.845	81.388	267.51	27	2635.119	238.554	208.59	.051
Poly I:C IL-10	528	501	1783.571	57.745	264.80	27	1787.016	254.221	258.85	.843
Poly I:C IFN-γ	528	501	1700.732	72.727	264.06	27	1818.354	425.627	272.63	.776
Poly I:C TNF-α	528	501	706.948	22.176	267.29	27	511.379	52.836	212.78	.071
PHA cytokine responses										
IL-4	524	498	0.004	0.000	262.42	26	0.003	0.001	264.04	.958
IL-5	533	506	200.979	9.863	269.07	27	172.310	39.045	228.30	.180
IL-9	525	499	0.142	0.007	264.05	26	0.118	0.025	242.88	.488
IL-10	533	506	551.881	16.327	265.67	27	605.166	72.218	291.85	.389
IL-13	533	506	1337.592	35.286	269.17	27	1088.179	97.542	226.26	.158
IFN-γ	533	506	12738.999	424.784	265.12	27	15358.634	2030.591	302.19	.223

Data shown are as follows: (1) body mass index (ratio of weight [kg] divided by height² [m]); (2) antibodies (IgE as kU/L serum; IgG4 as μ g/L serum); (3) lung function (FEV₁/FVC ratio as L/L $\times$ 100; BHR dose slope as log₁₀[L/mg/mL]); (4) inflammatory markers (soluble CD14 as ng/mL serum; PGF_{2a} as ng/mmol creatinine in urine; EPX as μ g/mmol creatinine in urine); (5) hematology (as cell numbers $\times$ 10⁹/L blood); and (6) data shown for IL-4 and IL-9 are PCR units normalized to a housekeeping gene, and for all other cytokines, units are pg/mL culture supernatant.

FVC, Forced vital capacity.

*P value from χ^2 test.

TABLE E9. Comparison of biomarker expressions within nonatopic subjects stratified by BHR status

Variable	No.	Nonatopic BHR negative				Nonatopic BHR positive				Mann-Whitney test
		No.	Mean	SE	Mean rank	No.	Mean	SE	Mean rank	P value
Clinical										
Body mass index	518	464	21.150	0.190	257.84	54	21.227	0.475	273.76	.459
Current rhinoconjunctivitis: positive	105	95				10				.658*
Current rhinoconjunctivitis: negative	399	355				44				
Sex: male	233	219				14				.003*
Sex: female	285	245				40				
Inflammatory markers										
Soluble CD14	518	464	1884.947	16.965	259.62	54	1877.304	55.616	258.46	.957
PGF ₂ a	488	438	72.970	1.507	241.46	50	85.037	7.011	271.10	.159
EPX	488	438	62.883	1.862	243.60	50	72.708	9.352	252.40	.676
Hematology										
Eosinophils	516	462	0.223	0.009	254.38	54	0.272	0.029	293.73	.056
Neutrophils	516	462	3.088	0.055	253.20	54	3.398	0.150	303.82	.018
Innate cytokine responses										
LPS IL-10	512	458	1039.980	30.891	250.10	54	1243.819	84.635	310.74	.004
LPS IFN-γ	512	458	156.418	9.683	261.49	54	112.200	26.563	214.19	.026
LPS TNF-α	512	458	963.967	33.149	256.46	54	891.567	68.447	256.83	.986
LPS + IFN-γ IL-10	512	458	408.046	12.538	249.04	54	510.822	35.038	319.73	.001
LPS + IFN-γ IL-12	513	459	552.188	26.436	258.99	54	486.080	64.696	240.07	.375
LPS + IFN-γ TNF-α	512	458	3357.974	89.039	257.54	54	3179.144	219.808	247.70	.644
Poly I:C IL-10	512	458	1743.779	57.773	250.51	54	2129.322	174.709	307.31	.008
Poly I:C IFN-γ	512	458	1704.110	76.204	257.62	54	1478.907	164.715	247.02	.619
Poly I:C TNF-α	512	458	711.397	24.654	257.13	54	681.478	64.588	251.17	.779
PHA cytokine responses										
IL-4	504	451	0.004	0.0002	253.89	53	0.003	0.001	240.69	.532
IL-5	515	461	198.894	10.558	256.72	54	201.880	22.198	268.91	.569
IL-9	505	452	0.136	0.007	255.14	53	0.132	0.025	234.75	.336
IL-10	515	461	546.021	16.686	256.02	54	608.320	57.670	274.94	.376
IL-13	515	461	1323.480	36.831	255.24	54	1427.002	108.217	281.56	.219
IFN-γ	515	461	12774.210	457.703	256.89	54	12747.110	1112.879	267.50	.620

Data shown are as follows: (1) body mass index (ratio of weight [kg] divided by height² [m]); (2) antibodies (IgE as kU/L serum; IgG4 as μ g/L serum); (3) lung function (FEV₁/FVC ratio as L/L $\times$ 100; BHR dose slope as log₁₀[L/mg/mL]); (4) inflammatory markers (soluble CD14 as ng/mL serum; PGF_{2a} as ng/mmol creatinine in urine; EPX as μ g/mmol creatinine in urine); (5) hematology (as cell numbers $\times 10^9$ /L blood); and (6) Data shown for IL-4 and IL-9 are PCR units normalized to a housekeeping gene, and for all other cytokines, units are pg/mL culture supernatant.

FVC, Forced vital capacity.

*P value from χ^2 test.

TABLE E10. Logistic regression analysis of asthma risk in nonatopic 14-year-olds

Significant variable	Asthma risk		
	OR	95% CI	P value
Current rhinoconjunctivitis	6.980	2.864-17.009	.000
FEV ₁ /FVC ratio	0.941	0.890-0.996	.035

The sample of nonatopic subjects comprised 425 nonasthmatic and 23 asthmatic subjects. Models are as per [Table I](#) with exclusion of allergen response-associated variables. FVC, Forced vital capacity.

TABLE E11. Logistic regression analysis of BHR risk in nonatopic 14-year-olds

Significant variable	BHR risk		
	OR	95% CI	P value
PGF ₂ a	5.577	1.070-29.073	.041
Neutrophils	11.820	1.577-88.576	.016
LPS-induced IFN- γ	0.805	0.676-0.959	.015

The sample of nonatopic subjects comprised 401 BHR-negative and 50 BHR-positive subjects. The Model is as per [Table IV](#) with exclusion of allergen response-associated variables.

TABLE E12. Participation of subjects in 14-year respiratory assessment

Test	No. participating	Proportion of those eligible at 14 y (n = 1855)	Proportion of original cohort (n = 2868)
Questionnaire (primary caregiver)	1795	96.8%	62.6%
Physical examination	1704	91.9%	59.4%
Respiratory assessment	1587	85.6%	55.3%
Spirometry	1479	84.0%	54.4%
MCh (attempted)	1510	81.4%	52.6%
MCh (successful)	1479	79.7%	51.6%
Blood sample	1380	74.4%	48.2%

MCh, Methacholine.