

An immunoepidemiological approach to asthma: identification of in-vitro T-cell response patterns associated with different wheezing phenotypes in children

Lancet 2005; 365: 142–49

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Summary

Background Increasing evidence suggests that patterns of T-cell immunity to inhalant allergens in genetically diverse human populations are more heterogeneous than previously assumed, and that covert differences in expression patterns might underlie variations in airway disease phenotypes. We tested this proposition in a community sample of children

Methods We analysed data from 172 individuals who had been recruited antenatally to a longitudinal birth cohort study. Of the 194 birth cohort participants, data from the 147 probands (age range 8·6–13·5 years) who consented to blood collection were included along with data from 25 consenting siblings (mean age 11 years [range 7·4–17·4]). We ascertained clinical phenotypes related to asthma and allergy. We measured T-cell responses to allergens and mitogens, together with blood eosinophils and IgE/IgG antibodies, and assessed associations between these indices and clinical phenotypes.

Findings Atopy was associated with allergen-specific T-helper (Th)2 responses dominated by interleukin 4, interleukin 5, interleukin 9, interleukin 13, whereas interleukin 10, tumour necrosis factor α , and interferon γ responses were common to both atopics and non-atopics. The wheal size from skin prick with allergen was positively associated with in-vitro interleukin 5 and interferon γ responses, and negatively associated with interleukin 10. Asthma, especially in atopics, was strongly associated with eosinophilia/interleukin 5, and bronchial hyper-responsiveness (BHR) was associated with eosinophilia plus polyclonal interferon γ production. BHR in non-atopics was associated with elevated allergen-specific and polyclonal interleukin 10 production.

Interpretation Parallel immunological and clinical profiling of children identified distinctive immune response patterns related to asthma and wheeze compared with BHR, in atopics non-atopics. Immunological hyper-responsiveness, including within the Th1 cytokine compartment, is identified as a hallmark of BHR.

Relevance to practice These findings highlight the heterogeneity of immune response patterns in asthmatic children, including those with seemingly homogeneous Th2-driven atopic asthma. Further elucidation of the covert relationships between wheezing phenotypes and underlying immunophenotypes in this age group will potentially lead to more effective treatments for what is an unexpectedly heterogeneous collection of disease subtypes.

Introduction

Current concepts of the mechanisms underlying atopy have evolved from studies in mice which defined the Th1/Th2 paradigm.¹ Support for the general applicability of this model to asthma in human beings comes from findings of activated T-helper (Th)2 cells in airway biopsies from atopic asthmatics,² and allergen-specific Th2-memory cells in their blood.³ However, it is becoming increasingly clear that human allergen-specific Th-memory involves a broader range of immune response phenotypes than that in mice. Notable examples include production of interleukin 10, which is a Th2 marker in mice¹ but is secreted by human Th1 and Th2 clones⁴ and the production in non-atopics of Th2-dependent IgG4 in the absence of IgE.⁵

Attempts to establish a direct causal relation between Th2-polarised immunity to allergens and airway disease in human beings have also been complicated by the

inconsistent epidemiological association between Th2-dependent skin prick reactivity to inhalants and symptomatology. By contrast with inbred mice in which sensitisation and subsequent exposure to inhalants is a highly efficient mechanism for induction of bronchial hyper-responsiveness (BHR), only a few sensitised people develop wheeze,⁶ suggesting that additional cofactors are needed for disease expression.

Genetic variations in mechanisms involved in regulation of tissue responses to inflammation⁷ and bronchoconstrictors⁸ might be important in this context. However, an additional possibility merits consideration; notably that variations in clinical phenotypes in atopics might be the result of covert differences in underlying immune response profiles. The population segment in whom these responses are least understood are children, spanning the age ranges of highest asthma prevalence. The dearth of information on relations between asthma

symptoms and specific immune effector mechanisms in children greatly restricts the capacity for rational drug trial design (and drug target identification) relevant to paediatric asthma. To elucidate these relations, we have undertaken a broad-ranging study of allergen-specific T-cell immunity in 172 children from a cohort being studied prospectively for development of asthma and atopy. We sought to define: (1) in-vitro cytokine response patterns associated with skin prick test reactivity; (2) allergen-specific cytokine response profiles that distinguish symptomatic from asymptomatic atopics; (3) relations between immune parameters and wheezing symptoms within the overall population; and (4) immunological determinants of wheezing phenotypes in non-atopics.

Methods

Patients

Study subjects were participants in the assessment of a longitudinal birth cohort study. Antenatal recruitment of this cohort has been described previously.⁹ Of the 194 birth cohort participants, data from the 147 probands (age range 8.6–13.5 years) who consented to blood collection could be included in the present paper along with data from 25 consenting siblings (mean age 11 years [range 7.4–17.4]). The study was approved by our institutional ethics committee. Written consent was obtained from parents, and verbal assent from children.

Clinical phenotyping

Wheeze and asthma were defined as affirmative answers to the questions “has your child wheezed in the past year?” and “does your child currently have asthma diagnosed by a doctor?”, respectively. We have shown previously¹⁰ that physician diagnosed asthma is associated with abnormalities in airways function in this population.

Skin-prick test reactivity was assessed against: cow's milk, egg white, rye, mixed grass, *Dermatophagoides pteronyssinus* (house dust mite), cat and dog dander (ie, flakes of skin), and the fungi *Alternaria alternans* and *Aspergillus fumigatus* (Hollister-Stier, Elkhart, IN, USA). The positive control (histamine sulphate 10 mg/mL) was read after 10 min, all other tests were read after 15 min. A positive skin-prick test was defined as a wheal 3 mm or larger. Atopy was defined as one or more positive reactions to the skin-prick test.

We assessed BHR using the Yan technique.¹¹ Increasing doses of inhaled histamine were administered from a hand-held dosimeter until either FEV₁ (forced expiratory volume in 1 sec) was reduced by 20% or the maximum cumulative dose had been given (7.8 µmol/L). Increased BHR was defined as 20% or greater fall in FEV₁ after inhalation of up to 7.8 µmol/L of histamine.¹²

Cellular studies

In-vitro studies on peripheral blood mononuclear cells (PBMC) responses were done on cryobanked samples, by

methods detailed previously^{13,14} and previous studies have failed to detect any differential loss of PBMC responses due to the cryobanking process. The PBMC were cultured for 48 h in AIM-V medium with 4×10^{-5} M 2-ME alone or with 10 µg/mL house dust mite extract or purified Der P1 dust mite allergen at 30 µg/mL (gift from Wayne Thomas, Telethon Institute for Child Health Research, Perth, Australia), or 1 µg/mL phytohaemagglutinin A (PHA) (Murex, Dartford, UK). Cell pellets were resuspended in RNALater (Ambion, Austin, Texas) and stored at -20°C.

In-vitro analyses

IgE and IgG assays were undertaken as previously described.^{15,16} Interleukin 5, interleukin 10, interleukin 13, tumour necrosis factor (TNF) α, and interferon γ protein in supernatants was assayed using ELISA or Time Resolved Fluorescence (TRF).¹⁴ Quantification of interleukin 4 and interleukin 9-specific mRNA was done by Taqman PCR.

Statistical methods

Initial univariate analyses compared immunological response indices (continuous variables) between atopics and non-atopics (using skin-prick test as a binary variable) with the Mann Whitney U test. Before multivariate analyses, cytokine responses and total IgE were log-transformed to approximate a normal distribution; values below the limits of detection

Interleukin 5

Principal growth factor for eosinophils; produced mainly by CD4+ Th2 cells.

Interleukin 10

Major anti-inflammatory cytokine; produced by both Th1 and Th2 cells and by cells of innate immune system.

Interleukin 13

Synergies with interleukin 4 in promoting IgE production; also promotes mucus production in airways.

Tumour necrosis factor (TNF) α

Potent pro-inflammatory cytokine produced mainly by cells of innate immune system and by Th1 cells.

Interferon γ

Potent pro-inflammatory cytokine produced by Th1 cells; antagonises Th2 differentiation in early stages of Th2 clonal expansion; major role in elimination of virus-infected cells; produced by CD4+ Th1 cells and CD8+ T cells, and sometimes by cells of innate immune system.

Interleukin 4

Principal growth factor for Th2 cells and B cells; promotes IgE production; produced mainly by CD4+ Th2 cells.

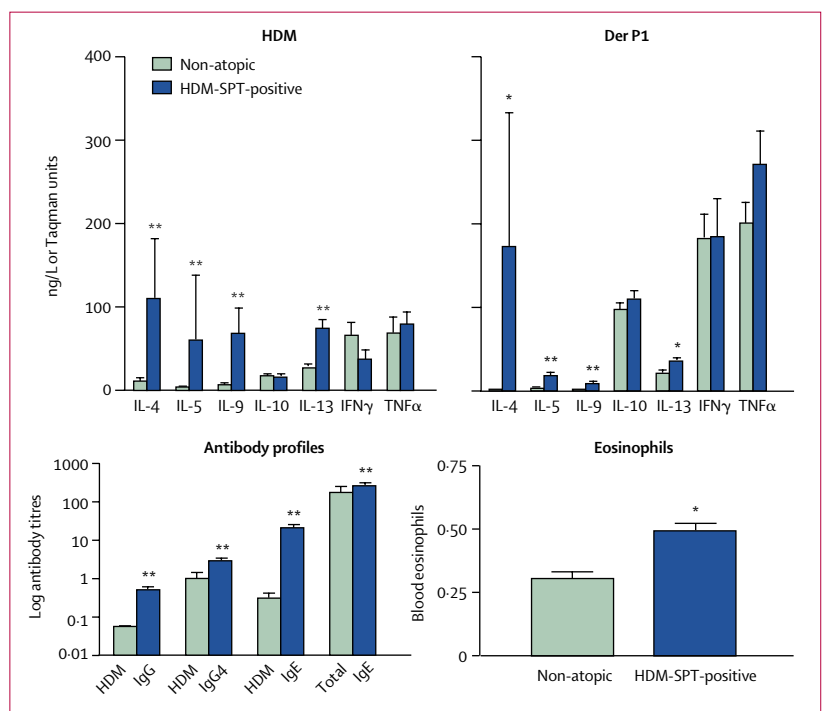


Figure: Immunological profiles of atopic and non-atopic children

SPT=skin-prick test. HDM=house dust mite. IL=interleukin. IFN=interferon. Light bars: non-atopic children (n=77; SPT-negative to all allergens tested); dark bars: HDM-SPT-positive children (n=73). *p<0.01. **p<0.001.

	Interleukin 4 (ng/L)			Interleukin 5 (ng/L)			Interleukin 9 (ng/L)			Interleukin 10 (ng/L)			Interleukin 13 (ng/L)			Interferon γ (ng/L)			TNF α (ng/L)			HDM IgE*	Total IgE†	Eos
	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA			
Spearman's rho	0.344	0.285	..	0.567	0.490	0.315	0.487	0.381	..	0.336	0.094	0.144	0.460	0.295	0.294	0.280	0.011	-0.002	0.250	0.168	0.297	0.491	0.388	0.260
p	0.007	0.052	..	<0.0001	<0.0001	0.015	0.000	0.002	..	0.002	0.476	0.234	0.000	0.005	0.037	0.011	0.101	0.899	0.031	0.150	0.011	<0.0001	0.003	0.027

SPT=skin-prick test. HDM=house dust mite. Data are mean (SE) unless otherwise indicated. Eos=eosinophils, number per mL blood $\times 10^{-12}$. *Rast units/mL serum. †All/mL serum.

Table 1: Determinants of SPT wheal size in HDM-SPT-positive individuals: Spearman correlation

(including zero values) were initially ascribed a value equivalent to 0.5 \times limit of detection.¹⁷ Multiple stepwise linear regression was used to compare relations between skin prick wheal size and immune indices, adjusting for confounding factors identified by Spearman correlation. Stepwise logistic regression was used to examine associations between asthma-related outcomes (as binary variables) and immunological indices, adjusting for potential confounding factors identified in univariate analyses or by reference to the scientific reports. The cut-off for significance was $p < 0.05$ for all values.

Role of the funding source

The funding source had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

95 (55.2%) of the 172 children were atopic as defined by skin-prick test. The dominant allergen in the population was house dust mite (73 [42.4%] positive), followed by rye (55 [32.0%]). 26(15.1%) had asthma at the time of analysis, and of these, 22 (85%) were atopic, 30 (17.4%) had wheeze and 25 (83%) of these were atopic, and BHR was present in 62 (35.9%) of whom 45 (73%) were atopic.

Before undertaking the main study, we assessed the potential effects of bacterial lipopolysaccharide (LPS) contamination in the allergen extracts in preliminary experiments (for details see: <http://image.thelancet.com/extras/03art12146webtable.pdf> and <http://image.thelancet.com/extras/03art12146webfigure.pdf>). These studies indicated that in the AIM-V culture system PBMC were unresponsive to LPS below 100 $\mu\text{g/L}$, by contrast with a sensitivity threshold of 0.1–1.0 $\mu\text{g/L}$ in cultures containing serum-supplemented medium, indicating the absence in AIM-V of carriers such as sCD14 and LPS-binding-protein. Additional experiments with the LPS inhibitor Polymyxin B indicated no LPS-attributable effects on Th1 or Th2 cytokine production by the house dust mite preparation, and moderate stimulatory effects on Th1 responses induced by Der P1.

The figure shows the relation between house dust mite sensitisation and correlates of immune function. The

recall responses of PBMC to house-dust mite and Der P1 in sensitised children contained a prominent Th2 component (interleukin 4, interleukin 5, interleukin 9, interleukin 13); by contrast, interleukin 10, interferon γ , and TNF α responses were common to sensitised and unsensitised children. Polyclonal PHA-induced interleukin 5 and interleukin 13 were also increased in atopics compared with non-atopics (data not shown). This Th2 bias was mirrored in the humoral compartment of atopics by high levels of production of IgG4 and IgE, and accompanying eosinophilia.

The analyses in table 1 focus on associations between in-vitro allergen-specific responses and the magnitude of skin-prick test responses in sensitised children. With the Spearman Correlation, positive associations were noted between wheal size and all allergen-specific cytokine responses tested, including the Th1 cytokine interferon γ , and also with humoral immune parameters. Multiple stepwise linear regression (table 2) identified mite allergen-induced interleukin 5 ($p < 0.0001$) and interferon γ ($p = 0.003$) as the most potent factors associated with wheal size, while interleukin 10 responses were inversely correlated.

Table 3 shows associations between immunological indices and asthma phenotypes. In univariate analyses, current asthma was strongly associated with elevated house dust mite-specific interleukin 5, interleukin 9, and interleukin 13 responses as well as house dust mite-specific IgE and eosinophil counts, and was weakly associated with total IgE. Comparable associations were seen for current wheeze (data not shown). BHR was also associated with allergen-specific interleukin 5 and specific IgE, and additionally with eosinophilia and raised total IgE. A unique additional feature of BHR was the association with polyclonal cytokine responses (interleukin 5, interleukin 13,

Immune response index	Coefficient (SE)	t	p
Log interleukin 5 DerP1	0.484 (0.265)	4.112	<0.0001
Log interferon γ HDM	0.173 (0.363)	3.181	0.003
Log interleukin 10 DerP1	-0.293 (0.309)	-2.593	0.013

SPT=skin-prick test. HDM=house dust mite. The model used included all indices from Spearman correlation that had $p < 0.05$.

Table 2: Determinants of SPT wheal size in HDM-SPT-positive individuals: multiple stepwise linear regression

	Interleukin 4 (ng/L)			Interleukin 5 (ng/L)			Interleukin 9 (ng/L)			Interleukin 10 (ng/L)			Interleukin 13 (ng/L)			Interferon γ (ng/L)			TNF α (ng/L)			HDM (IgE)*	Total (IgE)†	Eos
	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA			
Current asthma																								
No asthma (n=147)	54 (35)	82 (79)	..	22 (4)	8 (121)	409 (31)	1983 (391)	242 (72)	..	18 (2)	99 (978)	186 (14)	41 (5)	24 (3)	1849 (106)	54 (11)	188 (31)	9134 (767)	76 (13)	230 (23)	1048 (88)	7 (2)	148 (21)	0.344 (0.022)
Asthma (n=25)	46 (27)	11 (6)	..	65 (16)	20 (5)	537 (107)	11734 (8513)	1234 (709)	..	20 (6)	116 (13)	171 (27)	95 (18)	44 (9)	2264 (414)	68 (24)	144 (31)	9059 (2284)	67 (25)	235 (54)	795 (123)	18 (6)	623 (314)	0.611 (0.070)
p	0.063	0.173	..	0.0001	0.007	0.447	0.022	0.027	..	0.926	0.110	0.533	0.000	0.003	0.316	0.339	0.488	0.486	0.651	0.877	0.413	0.004	0.059	<0.0001
BHR																								
No BHR (n=107)	24 (7)	3 (2)	..	20 (5)	7 (2)	378 (37)	3537 (1920)	379 (156)	..	18 (3)	96 (8)	168 (143)	43 (7)	27 (3)	1745 (118)	55 (13)	172 (32)	7788 (852)	73 (16)	189 (24)	857 (97)	3 (1)	125 (24)	0.293 (0.021)
BHR (n=60)	115 (89)	211 (203)	..	46 (9)	14 (3)	533 (56)	2997 (933)	440 (213)	..	18 (3)	112 (13)	213 (25)	62 (9)	26 (5)	2257 (226)	45 (11)	195 (49)	11051 (1366)	67 (13)	287 (38)	1245 (131)	18 (5)	411 (143)	0.528 (0.044)
p	0.0207	0.139	..	0.001	0.015	0.0020	0.103	0.558	..	0.388	0.127	0.169	0.059	0.762	0.016	0.674	0.844	0.015	0.250	0.011	0.004	<0.0001	<0.0001	0.003

Data are mean (SE) unless otherwise indicated. HDM=house dust mite. Eos=eosinophils, number per mL blood $\times 10^{12}$. *Rast units/mL serum. †All/mL serum.

Table 3: Asthma-associated outcomes: univariate analysis

interferon γ , and TNF α), which was not seen in those with asthma and wheeze.

Table 4 narrows the focus to house dust mite-sensitive atopics, and seeks to identify immune response indices that differ significantly between children with asthma and those without. Initial univariate analyses identified allergen-specific interleukin 5 and interleukin 13 responses and degree of eosinophilia as factors distinguishing atopics with current asthma from those without, and identical findings were obtained for current wheeze (data not shown). By contrast with findings for asthma and wheeze, univariate analyses identified the characteristics distinguishing the immune response profile of BHR-positive atopics from those without BHR as eosinophilia and associated interleukin 5 production in conjunction with IgE production.

Table 5 focuses on expression of symptoms in non-atopics. The numbers of non-atopic subjects with current asthma or wheeze were insufficient for meaningful analyses. However BHR was present in 16 (22%) of non-atopics, and was positively associated with production of

interleukin 10 in responses to house dust mite and Der P1 and the polyclonal mitogen PHA. Of note, mean production levels of both allergen-specific and polyclonal interleukin 10 in this subgroup of non-atopic/BHR-positive individuals was higher than in their atopic counterparts (table 4). Additionally, polyclonal TNF α and interferon γ were also associated with BHR in this group (table 5).

Table 6 summarises results of logistic regression modelling of associations between wheezing outcomes and these immunological indices; details of potential confounding factors (such as atopic family history) included in the model are shown in the table legend. Within the overall population and among the atopics, the factor most consistently associated with asthma, wheeze, and BHR was eosinophilia. Of further note is the additional association between polyclonal interferon γ and BHR within the overall population, and interleukin 10 responses and BHR in non-atopics.

Repetition of the univariate and multivariate analyses on tables 3–6 with the 147 probands alone confirmed all

	Interleukin 4 (pg/mL)			Interleukin 5 (pg/mL)			Interleukin 9 (pg/mL)			Interleukin 10 (pg/mL)			Interleukin 13 (pg/mL)			Interferon γ (pg/mL)			TNF α (pg/mL)			HDM (IgE)*	Total (IgE)†	Eos
	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA			
Current asthma																								
No asthma (n=56)	123 (89)	211 (203)	..	52 (10)	14 (3)	476 (53)	4019 (914)	529 (177)	..	15 (4)	105 (12)	176 (22)	65 (11)	31 (6)	2018 (156)	33 (13)	201 (63)	8966 (1297)	64 (21)	259 (44)	1243 (178)	21 (6)	245 (38)	0.428 (0.038)
Asthma (n=17)	68 (44)	18 (10)	..	93 (22)	29 (6)	629 (150)	18407 (13880)	1976 (1136)	..	23 (9)	122 (7)	191 (37)	112 (24)	49 (12)	2525 (613)	57 (21)	118 (23)	12307 (3233)	87 (36)	305 (319)	995 (167)	30 (9)	393 (132)	0.689 (0.087)
p	0.441	0.244	..	0.047	0.022	0.737	0.350	0.200	..	0.377	0.180	0.820	0.035	0.030	0.980	0.070	0.587	0.640	0.131	0.456	0.990	0.264	0.695	0.006
BHR																								
No BHR (n=39)	43 (18)	8 (4)	..	47 (12)	15 (3)	464 (72)	8756 (5421)	931 (423)	..	22 (6)	121 (14)	184 (29)	72 (16)	40 (8)	1971 (195)	31 (10)	160 (68)	8779 (1666)	77 (30)	232 (50)	1120 (233)	10 (3)	187 (46)	0.318 (0.028)
BHR (n=35)	197 (157)	378 (364)	..	76 (14)	21 (4)	572 (81)	4766 (1573)	748 (376)	..	12 (3)	96 (13)	174 (25)	80 (14)	30 (7)	2361 (323)	30 (11)	197 (71)	10720 (1933)	56 (19)	319 (59)	1268 (168)	38 (9)	386 (68)	0.650 (0.057)
p	0.265	0.082	..	0.035	0.066	0.326	0.520	0.866	..	0.763	0.372	0.841	0.262	0.417	0.472	0.703	0.673	0.414	0.860	0.276	0.151	<0.0005	0.006	<0.0005

Data are mean (SE) unless otherwise indicated. HDM=house dust mite. Eos=eosinophils, number per mL blood $\times 10^{12}$. *Rast units/mL serum. †All/mL serum.

Table 4: Determinants of symptomatology in HDM-sensitive atopics: univariate analyses

	Interleukin 4 (ng/L)			Interleukin 5 (ng/L)			Interleukin 9 (ng/L)			Interleukin 10 (ng/L)			Interleukin 13 (ng/L)			Interferon γ (ng/L)			TNF α (ng/L)			Eos
	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	HDM	Der P1	PHA	
BHR- (n=56)	16 (6)	0 (0)	..	5 (2)	3 (1)	364 (50)	952 (272)	66 (17)	..	16 (3)	88 (11)	170 (17)	30 (7)	21 (3)	1716 (172)	78 (22)	186 (38)	8060 (1142)	75 (22)	183 (31)	802 (97)	0-229 (0-028)
BHR+ (n=16)	13 (7)	0 (0)	..	2 (1)	1 (1)	490 (108)	480 (121)	25 (7)	..	40 (8)	156 (33)	300 (70)	26 (6)	9 (2)	2401 (449)	80 (32)	141 (84)	12821 (2367)	109 (30)	298 (66)	1630 (305)	0-358 (0-053)
p	0.922	n/a	..	0.786	0.284	0.286	0.691	0.546	..	0.006	0.008	0.017	0.450	0.190	0.120	0.336	0.071	0.017	0.052	0.063	0.004	0.491

Data are mean (SE) unless otherwise indicated. HDM=house dust mite. Eos=eosinophils, number per mL blood $\times 10^{-12}$.

Table 5: BHR in non-atopics: univariate analyses

the substantive associations noted in the overall population, indicating that inclusion of the siblings did not distort the findings in this study.

Discussion

The results of this study show that atopy to inhalant allergens is associated with a mixed Th1/Th2 immune response profile in children. Moreover, the contribution of individual Th1-associated and Th2-associated effector mechanisms to this mixed response profile is highly heterogeneous, and variations in response patterns seem to be associated with variations in clinical phenotypes. The importance of inflammation in the pathogenesis of asthma, and the central role of Th2 cells in driving this process in both atopic and non-atopic asthma, is supported by evidence from immunohistopathological studies on airway lesional sites.² These findings have collectively stimulated broad interest in development of Th2-antagonistic drugs for asthma, including recombinant interleukin 12 to block the overall Th2 pathway,¹⁸ and inhibitors targeted at individual effector molecules, notably interleukin 4,¹⁹ interleukin 5,²⁰ and IgE.²¹ This process is still at an early stage,²² and clinical efficacy to date in trials in adults seems modest, despite significant effects on specific drug targets. This evidence suggests that hyperactivity of Th2 mechanisms might not be sufficient to explain maintenance of ongoing asthma in persistent disease, and that additional covert pro-inflammatory cofactors might be involved. Furthermore, the relative importance of individual inflammatory mechanisms probably varies at different disease stages. In particular, epidemiological evidence shows that the association between asthma and allergy is strongest in mid-late childhood,²³ suggesting that young asthmatics may be a relevant population for testing recently developed Th2 antagonists. However, a substantial limiting factor in the design of such early intervention trials is the paucity of information on the relative importance of individual allergy-associated effector mechanisms in young asthmatics, and a primary aim of the present study was to obtain new information relevant to this question. This study was restricted to immune variables that are measurable in peripheral blood and, as such, might not have detected modifications to response patterns that occur in inflamed tissues. Additionally, the precise cellular

sources of the cytokines measured remains to be established.

The data in the figure provide a comprehensive systemic immune response profile of the study group, stratified by house dust mite allergy. Our results attest to the fidelity of the batch analysis methodology used in the immunological component of the study. In particular, the distinguishing feature of house dust mite-specific T-cell immunity in children who were positive for the skin-prick test is Th2 cytokine production, as predicted by the results in adults, and is most evident with the mixed house dust mite allergen. A qualitatively similar but attenuated Th2 response pattern was noted with the major house dust mite allergen Der P1, which may be the result of negative feedback from Th1 cytokines induced by low level LPS contamination in the Der P1 (<http://image.thelancet.com/extras/03art12146webtable.pdf>).

The prominence of Th2 cytokines in the allergen-specific responses of these atopic children is consistent with the pattern described in atopic adults.³ However, it is noteworthy that responses of both atopics and non-atopics include production of the Th1 cytokines interferon γ and TNF α , and also interleukin 10, since this suggests that the overall response of the atopic children is a mixed Th1/Th2 or Th0-like pattern, as reported elsewhere.^{24,25} The size of the house dust mite-induced skin wheal in those positive for skin-prick test was positively correlated with a wide range of Th1 and Th2 related parameters, and further analysis by stepwise linear regression modelling confirmed the potential importance of the interferon γ component of the response. The negative association between wheal diameter and interleukin 10 responses to mite is consistent with our previous findings relating to house dust mite-induced wheal size in 6-year olds,²⁴ and also with data on the protective effects of parasite-induced interleukin 10 on dermal responses to allergen.²⁶

Current asthma and/or wheeze in the overall population and in atopics was associated in univariate analyses with allergen-specific cytokine responses and eosinophilia. IgE levels were associated with asthma risk within the overall population, but did not discriminate between atopics with and without disease. 85% of the asthmatics in this study were atopic, and these observations suggest that although IgE may be

	Asthma	Wheeze	BHR*
Whole population† (n=172)	Eosinophils (8.39 [2.08–33.84]; p=0.003) log IL-5/HDM (OR 1.57 [7.13–2.17]; p=0.000)	SPT-positive to grass (2.49 [1.06–5.85]; p=0.036)	Eosinophils (29.09 [7.01–120.69]; p=0.000) IFN γ /PHA (1.55 [1.06–2.27]; p=0.023)
HDM-SPT-positive‡§ (n=73)	Eosinophils (12.19 [2.12–70.11]; p=0.005)	Eosinophils (16.46 [2.41–112.64]; p=0.004) log IL-13/DerP1 (2.62 [1.33–5.14]; p=0.005)	Eosinophils (273.48 [16.14–4634.88]; p=0.000)
Non-atopics¶ (n=77)	nd	nd	log IL-10/PHA (8.47 [1.93–37.20]; p=0.005) log IL-10/HDM (2.59 [1.29–5.22]; p=0.008)

Data are odds ratios (95% CI) unless otherwise indicated. Summaries of data used in these analyses, including subject numbers in subgroups, shown in tables 3 and 4. HDM=house dust mite. SPT=skin-prick test. *Combined outcome of asthma plus BHR (n=17 within the whole population) was strongly associated with eosinophilia (OR 149.38 [6.30–3450.53]; p=0.002). †Stepwise model encompassed eosinophils plus log transformed cytokine responses and IgE; additional potential confounding factors included in the model were IgG and IgG4 responses, sex, atopic family history, and SPT response status to a panel of dietary and inhalant allergens. Results shown are final model with all factors included at p<0.05. ‡Identical model, after selection on atopy status. §Findings were comparable if atopics were selected on the basis of "any positive SPT". ¶Model encompassed eosinophils plus log transformed cytokine responses; potential confounding factors included were gender and atopic family history. Deletion of the cytokine responses to DerP1 did not significantly affect these findings. ||Insufficient numbers for meaningful analyses (n=4 for asthma and 5 for wheeze).

Table 6: Logistic regression modelling of relation between asthma-associated outcomes and immune response variables in children

permissive for asthma, additional cytokine-associated stimuli including eosinophilia are needed for disease expression. The situation with respect to BHR seems more complex. In the overall population, eosinophilia again appears as a risk factor in association with IgE, as observed for asthma. However, by contrast with asthma, raised cytokine production, as indicated by heightened responsiveness to the polyclonal mitogen PHA, represents the defining feature of BHR-positive individuals, suggesting that bronchial and immunological hyper-responsiveness could be linked. When responses in atopics and non-atopics were analysed independently, the immunologically hyper-responsive phenotype as illustrated by high level PHA-induced interleukin 10, TNF α , and IFN γ , appears restricted to BHR-positive non-atopics, whereas BHR among atopics associated exclusively with eosinophilia, interleukin 5, and IgE.

The results of logistic regression modelling of these relations provides broad support for the findings above. Of particular note is the consistency of the relation between BHR and polyclonal responses involving interleukin 10 and interferon γ . This observation requires verification in a larger sample of non-atopics, because it has important theoretical implications. The role of interleukin 10 in asthma pathogenesis is controversial. This interleukin is thought to have a major role in down-regulation of airway inflammation,²⁷ possibly via mechanisms analogous to those we outlined in table 1, and the elevated interleukin 10 secretion in this subgroup could represent an exaggerated attempt to control the inflammation. Interleukin 10 production has been reported both as reduced²⁸ and elevated²⁹ in asthmatics, and these differences might represent variation in clinical phenotypes of study participants.²⁹ Interleukin 10 responsiveness to rhinovirus is also

reportedly raised in asthmatics,³⁰ which could restrict the effectiveness of inflammatory mechanisms necessary for viral clearance, thus potentiating the asthmatogenic effects of infection. Notably, the association between elevated interleukin 10 and BHR is restricted to non-atopics, and this link could provide a plausible mechanism for induction of viral-induced BHR within this subgroup. However, additional mechanisms might be involved, since interleukin 10 augments airway smooth muscle contractility,³¹ suggesting that enhanced local production could contribute directly to BHR expression.

An additional finding from this study is the positive association between Th1 cytokine production and end-organ responsiveness, quantified in this study as skin-prick test wheal size and sensitivity to inhaled histamine. These findings seem at first counterintuitive in view of the fact that Th1 cytokines are believed to provide feedback inhibition of Th2-induced allergic inflammation. However, this simplistic interpretation of Th1/Th2 effector interactions is increasingly challenged by data in human beings and animals. In particular, in mice, the Th2-inhibitory effects of interferon γ are seen predominantly during the early phase of Th-memory development, and the capacity of committed Th-cells to repolarise either Th1 or Th2 cytokine production is progressively lost with long-term stimulation.³² A comparable situation is likely to operate in humans since attenuated production of interferon γ is associated with increased risk for atopic sensitisation initially in infancy,³³ whereas individuals sensitised by later childhood show mixed Th1/Th2 response profiles,^{34,35} often with evidence of hyperproduction of interferon γ .^{25,35} This finding suggests that once allergen-specific Th-memory becomes stabilised, allergen-induced interactions can occur between Th1 and Th2 pathways

that are not necessarily antagonistic. One example might be atopic dermatitis, in which early immigration of Th2 cells into dermal challenge sites is supplanted within 12–24 h by a much larger influx of Th1 cells, suggesting that Th1 cytokines might contribute to pathogenesis.³⁶

Accumulating evidence also points to a possible Th1 component, particularly interferon γ , in BHR. Although evidence from some animal models suggests that Th1 responses potentially antagonise Th2-mediated airways inflammation and BHR,³⁷ others show the converse—ie, that interferon γ -producing Th1 cells can synergise with Th2 cells in BHR induction.³⁸ In human beings, elevated levels of interferon γ have been reported during asthma exacerbations in blood³⁹ and also in BAL fluid;⁴⁰ similar elevations have been reported for TNF α .⁴⁰ Furthermore, increased numbers of circulating interferon γ -secreting cells have been associated with BHR in children,⁴¹ and are also a prominent feature of infiltrates in airway biopsies in fatal asthma.⁴²

Thus, despite many scientific reports on the potential protective effects of Th1 immunity in relation to induction of atopic asthma, an equally plausible case can be made for a role for Th1 cytokines in driving disease pathogenesis, a view which is reinforced by our results. These findings have implications in relation to drug development, and provide one possible explanation for the equivocal results of trials employing Th2 antagonists in asthmatics.

Although there has been speculation on the potential role of Th1 cytokines in asthma pathogenesis,⁴³ previous studies have failed to detect relations comparable to those detailed here. A key feature that distinguishes this in-vitro study from others on asthma-related effector mechanisms is the large sample size, which would serve to increase capacity to detect subtle associations. However, further subdivision of our study population into individual clinical phenotypes of interest (eg, BHR-positive non-atopics) ultimately produces modest subgroup sizes. Accordingly, we acknowledge that even larger samples are needed to accurately define associations between asthma phenotypes and related immunophenotypes, and this study provides a blueprint for such investigations. Additionally, we have argued previously⁴⁴ that the link between Th2-driven inflammation and asthma in atopics is likely to weaken beyond childhood, as chronically damaged and remodelled airways undergo progressive phenotypic changes, resulting in hyper-responsiveness to a widening range of (non-allergenic) environmental irritants. Our results, especially the consistent association between eosinophilia and asthma phenotypes in children, suggest that Th2 antagonists such as anti-interleukin 5, which have been tested in adult asthmatics with equivocal results,^{20,22} should be reconsidered for use in younger age groups.

Contributors

The cellular and molecular biological components of the study were undertaken by T Heaton, J Rowe, D Suriyaarachchi, M Serralha, B J Holt,

E Hollams, and S Yerkovich. Antibody studies were undertaken under the direction of R C Aalberse. S Turner was responsible for the clinical studies in collaboration with J Goldblatt and P Le Souef. Data analyses were done by K Holt under the direction of N de Klerk. Interpretation of the data was done by P G Holt, T Heaton, J Rowe, S Turner, and P D Sly. P G Holt was responsible for writing the report.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This work was supported by the National Health and Medical Research Council of Australia. We thank Jenny Tizard for skilled technical assistance, Steven Stapel, and Henk de Vrieze for undertaking the antibody assays, and Sally Young and Lou Landau for their contribution to earlier phases of the cohort study.

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