

Adult Serum Cytokine Concentrations and the Persistence of Asthma

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Key Words

Asthma · Cytokines · Interleukin 10 concentrations

Abstract

Background: Cytokines play a pivotal role in regulating the development and persistence of the inflammatory process in asthma. Our aim was to investigate whether asthma persistence or remission is associated with a specific cytokine profile. **Methods:** The Tasmanian Longitudinal Health Study followed participants from 7 to 44 years of age. Serum concentrations of interleukin (IL)-4, IL-5, IL-6, IL-8, IL-10 and tumor necrosis factor- α (TNF- α) were measured at age 44 years. Participants were categorized into five phenotypes (early-onset noncurrent asthma, early-onset current asthma, late-onset noncurrent asthma and late-onset current asthma). Those who had never had asthma formed the reference group. Multivariable linear regression was used to compare serum cytokine concentrations between each phenotype and the reference group. **Results:** IL-10 concentrations were significantly lower in serum from the early-onset current asthma group than in the reference group (ratio of geometric means 0.58; 95% confidence interval 0.33–0.99; $p = 0.048$). IL-6 concentrations for the late-onset remitted group were also significant-

ly lower than in the reference group ($p = 0.009$). The TNF- α concentrations were significantly lower for both early- and late-onset remitted asthma phenotypes when compared with the reference group. No associations were detected between serum concentrations of IL-4, IL-5 or IL-8 and these specific longitudinal asthma phenotypes. **Conclusion:** Our findings suggest a possible role for deficient IL-10 responses in the persistence of early-onset asthma. Lower IL-6 and TNF- α concentrations in serum from those with remitted asthma suggest that these proinflammatory cytokines may be actively suppressed during asthma remission.

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Introduction

Asthma is a chronic inflammatory disorder of the airways and it is the most prevalent allergic disease in childhood [1]. While for many children asthma remits with age, some have asthma that persists into adulthood. Only a few longitudinal studies have examined factors associated with the remission or persistence of childhood asthma into adulthood. A New Zealand study identified airway hyperresponsiveness (AHR) at 9–12 years, skin prick

test (SPT) positivity to house-dust mite at 13 years and female gender and smoking at 21 years to be associated with asthma persistence at 26 years [2]. The Tasmanian Longitudinal Health Survey (TAHS) reported that childhood allergic rhinitis was associated with asthma persistence into adulthood [3].

Airway inflammation associated with allergic asthma is characterized by skewing of the immune response from type 1 T helper cell (Th1)-predominant to Th2-predominant response, driven by distinct cytokines such as interleukin (IL)-4 and IL-5 [4]. These cytokines play a vital role in the initiation and persistence of airway inflammation, and are hence fundamental to disease pathogenesis [4]. There is some evidence of an association between changes in Th1 or Th2 cytokine profiles in early life and a natural history of asthma over time. Elevated IL-4 production by peripheral blood cells/cord blood/whole blood cultures [5–7] and decreased interferon-gamma (IFN- γ) [5, 8, 9] have been associated with an increased risk for the development of asthma during the first years of life. One longitudinal cohort study of asthma spanning childhood to middle age reported a normalization of IFN- γ production to be associated with resolution of asthma by middle age and defective IFN- γ production to be associated with a persistence of asthma [10], but reported no association between Th2 cytokines and the persistence or resolution of asthma (possibly related to inadequate power, as the study included only 89 participants).

Given the importance of cytokines to asthma pathogenesis, we hypothesized that their concentration in peripheral blood might be associated with asthma remission or persistence into adulthood. Previous studies investigating this were limited by small numbers of participants who were not sampled from the general population, which led to limited statistical power and lack of generalizability. Moreover, in previous studies, information concerning asthma progression or remission over time has not been ascertained. In our study, we aimed to examine the association between several serum cytokines that are known to be important in airway inflammation and asthma persistence or remission.

Methods

Study Design – TAHS

This study included participants from the most recent laboratory phase of the TAHS. The study methodology and some results have been reported elsewhere [11]. In brief, the TAHS began in 1968 when 8,583 Tasmanian children (probands) born in 1961 and attending school in Tasmania were enrolled by their parents

who completed a respiratory health questionnaire for the child, who then underwent clinical examinations and lung function measurements. Subsequent follow-up surveys were completed in 1974, 1981 and 1992 at the ages of 13, 20 and 31 years, respectively. The most recent survey started in 2004 when the probands were 44 years of age. We managed to trace 7,562 (88.1%) of the original 1968 cohort to an address and achieved a response of 5,729 (78.4%) of these to a postal survey [11]. A subgroup of these respondents, selected on the basis of their participation in the previous follow-ups, was invited to participate in a detailed laboratory study which ran from 2005 until 2008. Of the 2,373 individuals invited, 1,389 (58.5%) attended the laboratory, 354 (15%) completed a telephone questionnaire only and 630 (26.5%) withdrew. At the laboratory visit, participants completed a detailed interviewer-administered questionnaire, performed lung function tests, SPTs for allergy and provided a blood sample. Details of the testing are described below.

The 2004 follow-up study was approved by the Human Research Ethics Committee at the University of Melbourne (HREC No. 040375). Written informed consent was provided by all participants when they attended the laboratory visit.

Questionnaires and Definitions

The interviewer-administered questionnaire collected information on sociodemographic characteristics, asthma status, symptoms and medication use, healthcare service utilization and smoking history. Having had asthma at any time in 2004 was defined by a positive response to the question ‘Have you ever had asthma?’ Current asthma was based on self-reported morning, day-time and night-time symptoms and/or flare-ups in the last 12 months. Early-onset asthma was defined as asthma reported at the 1968 or 1974 survey by a parent to the question: ‘Has he/she at any time in his/her life suffered from attacks of asthma or wheezy breathing?’ Late-onset asthma was defined by asthma only reported in the 2004 survey. Using these definitions, participants were then classified into 1 of the following 5 asthma phenotypes: early-onset remitted asthma, early-onset persistent asthma, late-onset remitted asthma, late-onset current asthma and ‘never’ asthma.

SPTs

SPTs were performed using a standard technique with eight aeroallergens (Hollister-Stier, EBOS Groups Pty Ltd., Australia) including *Dermatophagoides pteronyssinus*, cat pelt, *Cladosporium*, *Alternaria tenuis*, penicillium mix, *Aspergillus fumigatus*, mixed grasses No. 7 (which included Kentucky bluegrass, orchard, redtop, Timothy, sweet vernal grass, meadow fescue and perennial ryegrass) and histamine as a positive control. A wheal size of at least 3 mm greater than the negative control was regarded as indicating sensitivity.

Blood Sample Processing and Cytokine Measurement

A whole blood sample was collected from participants and was stored at room temperature until processing. The average time from blood collection to processing and freezing was 1.8 days. The serum samples were collected in serum tubes and were spun at 1,328 RCF (2,500 rpm) for 15 min and aliquoted into four 1-ml fractions and immediately stored at –80°C. For cytokine testing a single, previously unthawed serum vial was thawed at 4°C.

Preliminary experiments were performed to determine which cytokines could be detected in the serum samples. More details

about these experiments are available in the online supplementary material (www.karger.com/doi/10.1159/000346910). Based on these results and on our a priori hypotheses, the cytokines IL-4, IL-5, IL-6, IL-8, IL-10 and tumor necrosis factor- α (TNF- α) were selected for analysis. Cytokines were measured simultaneously according to the manufacturer's instructions. In brief, microspheres, each with a unique red/infrared spectrum signature, covalently coupled to cytokine-specific receptors, were incubated overnight with 50 μ l of standards, controls and blood serum samples in a 96-well microtiter filter plate at 4 °C. Following incubation, the plate was washed to remove excess reagents and incubated with detection antibody, comprising a mixture of biotinylated mouse/rat anti-human cytokine antibodies directed against each of the 6 cytokines. After 1-hour incubation at room temperature, streptavidin-conjugated phycoerythrin was added for 30 min. All incubation steps were carried out in the dark. Fluids were removed by gentle vacuum; the beads were washed and resuspended in sheath fluid before assay on the Luminex instrument (Luminex Corporation, Austin, Tex., USA). The 1,184 samples were run in 8 batches on the Luminex instrument. Cytokine concentrations were predicted to the log base10 using the asymmetrical 5-parameter logistic equation in Graphpad Prism5 software (GraphPad Software, La Jolla, Calif., USA) and were then converted back to obtain concentrations in pg/ml.

The minimum detection limit for the assay was 0.13 pg/ml and the maximum was 2,000 pg/ml. Samples with readings below or above these levels were assigned values of 0.5 times the minimum (0.065 pg/ml) or maximum (2,000 pg/ml) value, respectively. The percentages of samples with levels below the 0.13 pg/ml detection limit were 20.8, 16.7, 5.9, 0.0, 21.2 and 0.4% for IL-4, IL-5, IL-6, IL-8, IL-10 and TNF- α , respectively. The percentages of samples with levels above the 2,000 pg/ml detection limit were 0.5, 0.0, 0.3, 3.5, 0.6 and 0.0% for IL-4, IL-5, IL-6, IL-8, IL-10 and TNF- α , respectively. The samples with an undetectable reading were evenly distributed between the batches and outcome groups. As sensitivity analyses including and excluding these undetectable readings did not materially change the results, the data including the assigned values are presented.

For a subgroup of 79 participants, we measured the cytokines in duplicate and determined the reliability of the measurements by calculating the intraclass correlation coefficient for each cytokine. Intraclass correlation coefficients for all cytokines were greater than 90%: 98.9% [95% confidence interval (CI) 98.4–99.3%] for IL-4, 96.3% (95% CI 94.6–97.9%) for IL-5, 98.6% (95% CI 98.0–99.2%) for IL-6, 97.7% (95% CI 96.6–98.7%) for IL-8, 94.1% (95% CI 91.6–96.6%) for IL-10 and 92.7% (95% CI 89.6–95.8%) for TNF- α . In addition, we had 2 quality controls for each cytokine, run in duplicate in each batch. All readings for quality controls were within the expected range with coefficients of <15% variation. Based on these data, further single cytokine analyses were performed.

Statistical Analysis

All statistical analyses were performed using Stata version 10 (StataCorp, College Station, Tex., USA). As cytokine concentrations were predicted to the log base 10 geometric means (GM) and 95% CI are presented as summary statistics. Multiple linear regression models were used to estimate the associations between the serum cytokine concentrations and longitudinal asthma phenotypes, while adjusting for batch number, gender, current smoking status, SPT positivity to any allergen(s), level of education, BMI, season of

study attendance and inhaled corticosteroid use. As the distribution of some of the cytokine concentrations (log base 10 units) was slightly skewed, all analyses were repeated using bootstrap methods with 1,000 repetitions. The CIs are derived using these methods.

Results

Characteristics of the Asthma Phenotypes

Altogether, 1,079 participants were included in this analysis (fig. 1). Of these, 38% (n = 411) had never had asthma, 36% (n = 393) had early-onset remitted asthma, 11% (n = 122) had early-onset persistent asthma, 6.8% (n = 73) had late-onset noncurrent asthma and 7.4% (n = 80) had late-onset persistent asthma. The proportion of females differed significantly between groups, with the highest proportion observed in the late-onset current asthma group (table 1). Over 80% of the early-onset current asthma participants and approximately 70% of the late-onset current asthma participants were sensitized to at least one aeroallergen. The distribution of smoking status was similar across the groups.

Cytokine Concentrations according to Asthma Phenotypes

The GM serum concentrations (and 95% CI) of each cytokine for all participants were as follows: 3.0 pg/ml (2.6–3.5) for IL-10, 34.7 pg/ml (28.7–42.0) for IL-4, 0.30 pg/ml (0.27–0.34) for IL-5, 13.4 pg/ml (11.8–15.1) for IL-6, 283 (255 to 313) for IL-8 and 6.6 (6.3–6.9) for TNF- α .

The GM concentration of each cytokine was compared between asthma groups (table 2). The GM level of IL-10 was significantly lower for the early-onset current asthma group than in those without asthma (GM ratio 0.50, 95% CI 0.29–0.87, p = 0.014; fig. 2a). There was also evidence for a difference in the IL-10 concentrations between the early-onset persistent and early-onset noncurrent asthma groups (GM ratio 0.56, 95% CI 0.32–0.97, p = 0.038; fig. 2a).

In addition, serum TNF- α concentrations for all the asthma phenotypes were lower than those in the reference group, particularly for the 2 remitted asthma groups. When the analysis was adjusted for batch variability only, reductions in TNF- α concentrations observed for the 2 current asthma phenotypes were statistically significant (early-onset current asthma group: GM ratio 0.79, 95% CI 0.64–0.99, p = 0.041; late-onset current asthma group: GM ratio 0.82, 95% CI 0.69, 0.99, p = 0.034), but these associations attenuated after adjusting for inhaled corticosteroid use (table 2, fig. 2b). IL-6 con-

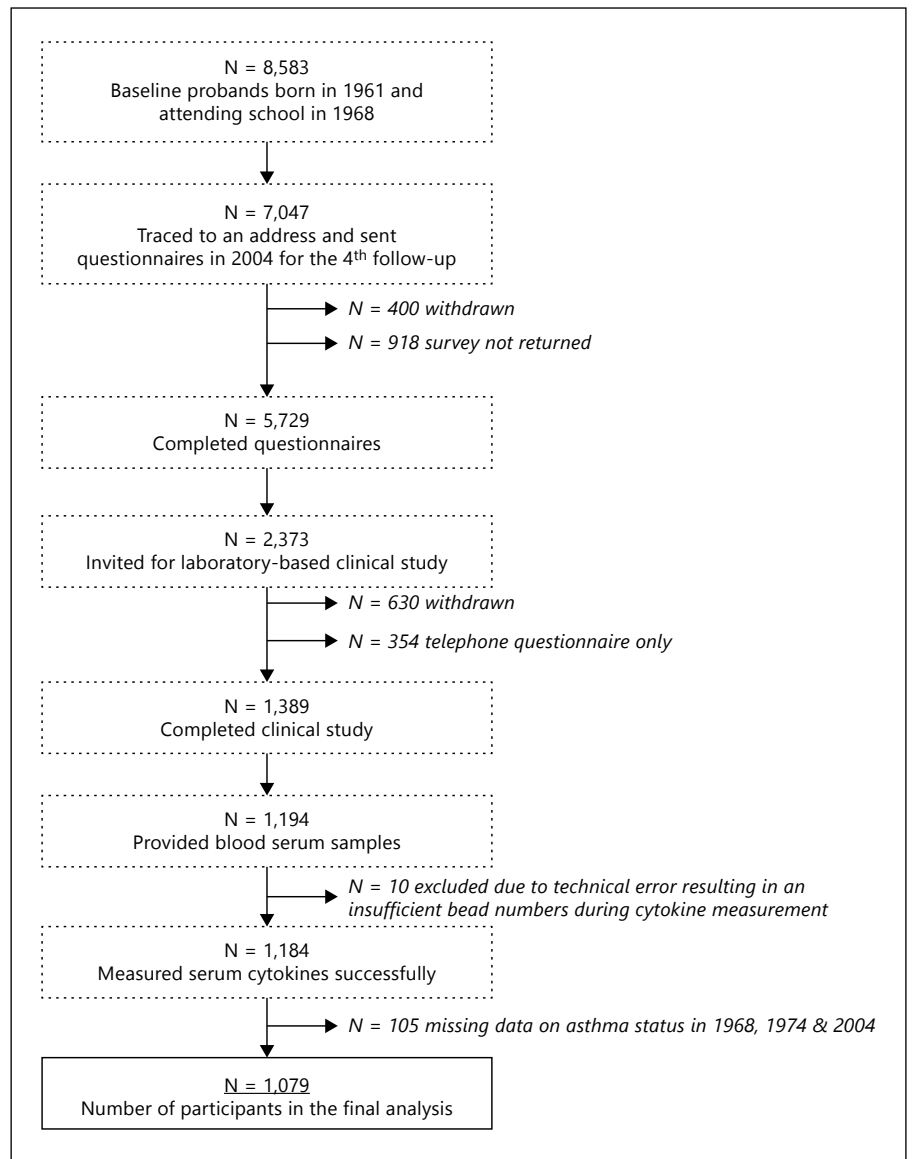


Fig. 1. Overview of the TAHS study group.

centrations were also significantly lower for the late-onset remitted group than for the never asthma group (fig. 2c). IL-6 concentrations for other asthma groups did not differ from that for the reference group. There was no difference in cytokine concentrations between the asthma groups for IL-4, IL-5 or IL-8 (table 2).

Discussion

Using a large population-based study examining peripheral blood cytokine concentrations in relation to asthma phenotypes characterized prospectively over

time, we have demonstrated for the first time that serum concentrations of IL-10 are lower for individuals with early-onset current asthma than for those who have never had asthma and who have had early-onset asthma that has remitted. Our findings suggest that the relative lack of IL-10 may be one of the immune mechanisms contributing to the persistence of asthma over time.

IL-10, a regulatory cytokine produced by regulatory T cells (Treg), inhibits the production of IFN- γ and IL-2 from Th1 cells and IL-4 and IL-5 from Th2 cells, while further autoactivating regulatory T cells [12, 13]. The constitutive expression of IL-10 in the airways has been proposed to mediate protection against allergic condi-

Table 1. Characteristics of the asthma phenotypes

Characteristics	Asthma phenotypes				
	No asthma n = 398 (%)	EO noncurrent asthma n = 393 (%)	EO current asthma n = 122 (%)	LO noncurrent asthma n = 73 (%)	LO current asthma n = 80 (%)
Female gender	211 (53%)	166 (42%)	68 (56%)	40 (55%)	56 (70%)
Atopic ¹	173 (44%)	229 (58%)	97 (82%)	38 (54%)	56 (70%)
<i>Sensitization to perennial allergens</i>					
House dust	124 (31%)	179 (46%)	84 (69%)	26 (36%)	42 (53%)
Cat	34 (8.5%)	58 (15%)	46 (38%)	6 (8.0%)	25 (31%)
Any mould	31 (7.8%)	47 (12%)	37 (30%)	10 (14%)	13 (16%)
<i>Smoking status²</i>					
Never	169 (43%)	156 (40%)	60 (50%)	32 (44%)	28 (35%)
Past	117 (30%)	125 (32%)	37 (31%)	19 (26%)	21 (26%)
Current	110 (28%)	110 (28%)	24 (20%)	22 (30%)	31 (39%)
<i>Asthma severity</i>					
Intermittent			45 (37%)		21 (26%)
Mild			32 (26%)		26 (33%)
Moderate			30 (25%)		20 (25%)
Severe			15 (12%)		13 (16%)
Hay fever	195 (49%)	241 (61%)	108 (89%)	55 (76%)	69 (86%)
Lung function	(n=394)	(n=392)	(n=122)	(n=71)	(n=80)
FEV ₁ , % predicted	103.8±14.9	99.8±16.0	91.6±18.0	101.9±12.9	92.3±28.8
Reversibility	14 (3.5%)	13 (3.3%)	20 (16.4%)	4 (5.5%)	9 (11.3%)
Any inhaled medications	0	25 (6%)	101 (83%)	3 (4%)	66 (83%)
Any oral medications	0	6 (2%)	12 (10%)	2 (3%)	4 (5%)

EO = Early-onset; LO = late-onset.

¹ Data missing for 9 participants; ² data missing for 5 participants.

tions via the activation of Treg residing in the airways [14]. Yuksel et al. [15] found significantly lower baseline serum concentrations of IL-10 in asthmatic children compared to healthy controls. Moreover, Borish et al. [16] reported lower IL-10 concentrations in bronchoalveolar lavage fluid from asthma patients compared to healthy controls. In contrast, Ceyhan et al. [17] found no difference in IL-10 concentrations in serum and sputum samples from 17 adult asthma patients and 5 healthy controls, but this study only examined a small sample of adult asthmatics with no description of asthma age at onset. None of the previous studies of IL-10 and asthma has had detailed, prospectively collected information on each participant's asthma phenotype over time. Our finding suggests that lower serum IL-10 concentration might be an important contributing factor in the persistence of early-onset asthma while early-onset asthma that remits might have IL-10 levels that increase to those of nonasthmatic individuals.

Evidence of an important role for IL-10 in the development of immune tolerance has emerged from clinical

studies of subcutaneous immunotherapy and sublingual immunotherapy. Tolerance induction following subcutaneous immunotherapy has been shown to be mediated by increased production of IL-10 and transforming growth factor-beta from antigen-specific Treg cells [18], and grass pollen immunotherapy resulted in increased mucosal and peripheral T cell expression of IL-10 [19]. A study of sublingual immunotherapy for house-dust mite allergy in asthma patients demonstrated a significant increase in IL-10 production by peripheral blood mononuclear cells after 6 months in treated subjects as opposed to untreated subjects [20]. Furthermore, in vitro studies showed that exogenous IL-10 impaired allergen-specific Th2 responses and downregulated specific IgE production [20]. These studies suggest that IL-10 is important for the downregulation of allergic responses and that a defective IL-10 response may account, in part, for the persistence of allergic inflammation. Interestingly, 83% of the early-onset current asthma group were atopic, providing further support that defective IL-10 responses may be especially important in allergic asthma.

Table 2. Adjusted geometric mean concentrations of IL-4, IL-5, IL-6, IL-8, IL-10 and TNF- α cytokines (in pg/ml) by the asthma phenotypes

Cytokine	Asthma phenotype	GM (95% CI) in pg/ml			Ratios of GM (95% CI)			p value
IL-10	No asthma	3.54	2.72	4.61	1.0			
	EO noncurrent asthma	3.19	2.45	4.15	0.90	0.62	1.30	0.572
	EO current asthma	1.78	1.12	2.81	0.50	0.29	0.87	0.014
	LO noncurrent asthma	2.39	1.39	4.10	0.67	0.37	1.24	0.205
	LO current asthma	3.05	1.68	5.52	0.86	0.44	1.68	0.66
IL-4	No asthma	38.48	28.18	52.54	1.0			
	EO noncurrent asthma	38.88	28.56	52.94	1.01	0.66	1.55	0.962
	EO current asthma	37.21	19.33	71.65	0.97	0.45	2.09	0.932
	LO noncurrent asthma	22.16	10.57	46.47	0.58	0.26	1.29	0.181
	LO current asthma	21.14	9.50	47.04	0.55	0.22	1.36	0.195
IL-5	No asthma	0.32	0.26	0.39	1.0			
	EO noncurrent asthma	0.31	0.25	0.38	0.98	0.73	1.33	0.899
	EO current asthma	0.26	0.18	0.39	0.83	0.52	1.31	0.418
	LO noncurrent asthma	0.28	0.18	0.45	0.90	0.54	1.50	0.681
	LO current asthma	0.36	0.23	0.58	1.14	0.68	1.92	0.618
IL-6	No asthma	15.60	12.73	19.13	1.0			
	EO noncurrent asthma	12.93	10.62	15.74	0.83	0.63	1.09	0.184
	EO current asthma	16.87	11.12	25.59	1.08	0.66	1.77	0.757
	LO noncurrent asthma	7.60	4.61	12.54	0.49	0.28	0.84	0.01
	LO current asthma	10.30	6.30	16.84	0.66	0.38	1.15	0.14
IL-8	No asthma	294.50	244.29	355.03	1.0			
	EO noncurrent asthma	283.26	237.08	338.38	0.96	0.75	1.24	0.762
	EO current asthma	274.92	193.97	389.67	0.93	0.62	1.41	0.744
	LO noncurrent asthma	269.67	183.99	395.18	0.92	0.60	1.39	0.681
	LO current asthma	228.23	152.25	342.14	0.77	0.49	1.24	0.285
TNF- α	No asthma	7.41	6.78	8.11	1.0			
	EO noncurrent asthma	6.21	5.72	6.76	0.84	0.74	0.95	0.005
	EO current asthma	6.27	5.21	7.55	0.85	0.68	1.05	0.128
	LO noncurrent asthma	5.75	5.12	6.45	0.78	0.67	0.90	0.001
	LO current asthma	6.49	5.39	7.81	0.88	0.70	1.09	0.23

Serum cytokine concentrations were adjusted for batch variability, gender, atopic sensitization, current smoking status, level of education, BMI, inhaled corticosteroid use and seasons attended the laboratory study. EO = Early-onset; LO = late-onset.

Mean concentrations of TNF- α were lower in the remitted asthma groups than in healthy controls, after adjusting for corticosteroid use. TNF- α is a proinflammatory cytokine, which has been shown to be elevated in sputum, bronchoalveolar lavage and biopsy samples from asthma patients [21–23]. These observations have led to the suggestion that TNF- α can induce acute inflammation in the airways and is likely to play an important role in driving acute inflammatory responses at local tissue sites, possibly through paracrine actions. However, the relationship between asthma and levels of circulating TNF- α in serum or plasma has not been examined, and it is possible that serum or plasma TNF- α levels do not cor-

relate with airway TNF- α . Moreover, previous studies reporting an association between increased TNF- α and asthma have compared subjects with active asthma and healthy controls [22, 24], so the increased TNF- α levels may reflect disease activity. Indeed, the relationship between TNF- α expression and disease remission has not been examined previously. Given the above considerations, our observation of reduced serum TNF- α in participants with remitted asthma suggests that remission of disease is associated with downregulation of TNF- α . There are several plausible mechanisms for downregulation of TNF- α with disease remission. It was previously reported that resolution of asthma in adults was associ-

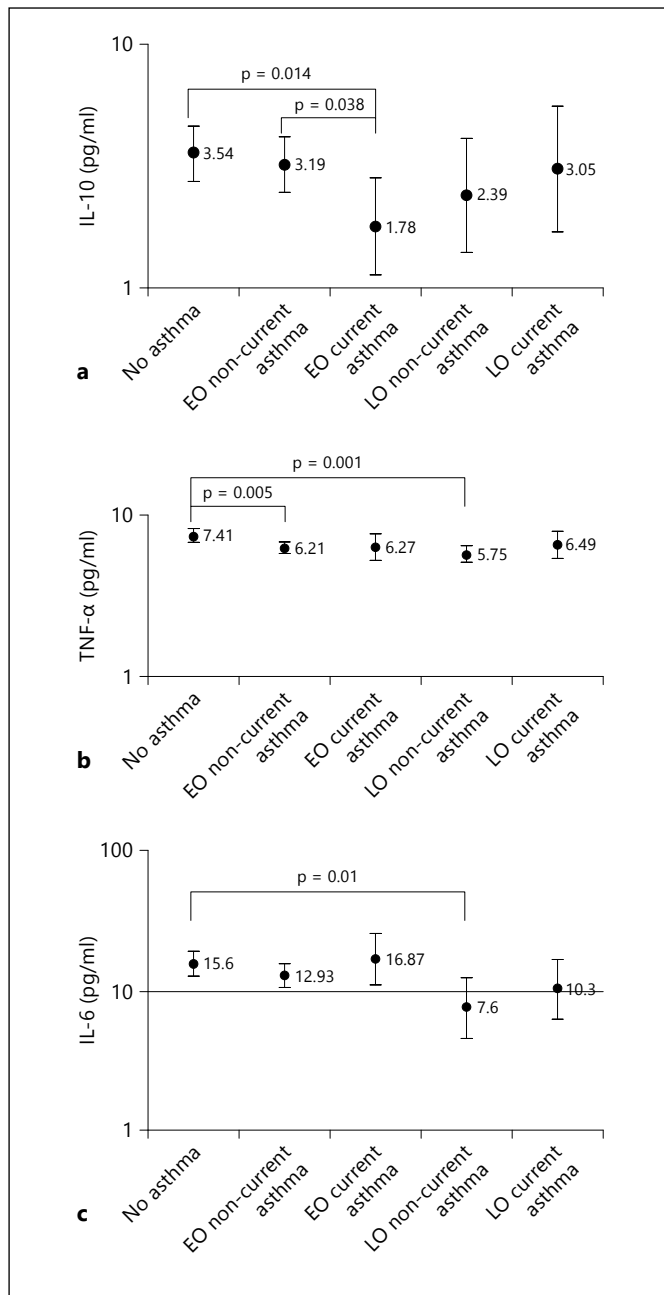


Fig. 2. Serum IL-10 (a), TNF- α (b) and IL-6 (c) concentrations by longitudinal asthma phenotypes Y-scale in the log₁₀ scale. Black filled circles represent geometric mean and bars represent 95% CIs.

ated with increased Th1 responses [10] and Th1 cells have strong immunosuppressive effects on Th17 cells, which are a major source of TNF- α [25]. Therefore, remission of asthma may be associated with upregulation of Th1 responses that, in turn, suppress Th17 cells and TNF- α production. Another plausible mechanism for downreg-

ulation of TNF- α is the use of corticosteroids as these agents are known to suppress acute inflammatory responses including TNF- α production [26]. Further investigation of the immune mechanisms driving disease remission is warranted.

We found a similar downregulation of IL-6, another proinflammatory cytokine in participants with later-onset noncurrent asthma. IL-6 is thought to have both pro- and anti-inflammatory properties. IL-6 is a key mediator of the acute-phase response, is involved in T cell activation, growth and differentiation and plays a crucial role in B cell differentiation and induces immunoglobulin synthesis [27]. In particular, IL-6 is also known as an important cofactor for IL-4-dependent IgE synthesis [28]. Conversely, IL-6 also has anti-inflammatory effects including the inhibition of IL-1 and TNF- α production from macrophages and neutrophil influx in the airways in vivo [29, 30]. Our finding of reduced IL-6 together with reduced TNF- α in noncurrent asthma phenotypes suggests that disease remission is associated with active downregulation of proinflammatory responses.

Despite the well-recognized roles of IL-4 and IL-5 in allergic inflammation, we did not observe any statistically significant associations with longitudinal asthma phenotypes, although IL-4 levels in late-onset asthma phenotypes were lower than in early-onset asthma phenotypes. Similar results for IL-4 have been observed in other studies; in particular, a recent study of 944 individuals of 218 asthma-affected families found that IL-4 levels were lower in adults compared to children with asthma [31]. This observed reduction in IL-4 levels may imply that the inflammatory process associated with adult-onset asthma is different to the inflammatory phenotype associated with early-onset asthma. For IL-5, we found no significant differences in cytokine concentrations between asthma phenotypes. This suggests that an increased IL-5 cytokine response is not necessarily a reflection of asthma activity but rather of the atopic state per se. Other studies have also found similar results, with a study by Smart et al. [10] observing that all asthma patients, irrespective of disease activity, had increased polyclonal or allergen-induced IL-5 production.

We did not observe any association between IL-8 concentrations and our asthma phenotypes. This is in contrast to several studies that have observed increased IL-8 levels in asthma subjects and in particular in patients with severe asthma [31–34]. In our study very few participants had severe asthma and this may explain why we did not observe any association with IL-8.

Overall, the observed lower concentrations of circulating pro-inflammatory cytokines suggest that there may be suppressive mechanisms at play and especially so for those who actually enter remission. Other immune mechanisms may play a greater role in driving active asthma. For example, recent studies suggest a role for natural killer T cells, the presence of which has been observed in severe, poorly controlled asthma [35]. Several studies with different murine models of asthma indicate that the presence of natural killer T cells is required for the development of AHR [36–38] and that these cells can induce AHR dependently [36] or independently of the Th2 pathway [37, 38]. T helper cells type 9 (Th9) may also play an important role in asthma. Studies in murine models [39, 40] and in humans [41] have demonstrated that IL-9, produced by Th9 cells, is associated with enhanced eosinophilic inflammation, mast cell accumulation, increased AHR as well as increased mucus production and fibrosis, which are all features associated with asthma. Since we were limited in the number of cytokines we were able to measure in this study, further research is necessary to address these speculations.

The major strengths of our study are its prospective nature and excellent follow-up. This allowed accurate characterization of phenotypes and changes in asthma activity prospectively. Unfortunately blood samples were not collected in earlier follow-up phases of TAHS and we were therefore unable to investigate change in cytokine expression over time. Also in our preliminary cytokine assay studies we were unable to detect some important cytokines (IFN- γ and IL-12) in peripheral blood serum samples, limiting the cytokines measurable in this study.

Conclusion

Our results suggest that a deficient IL-10 response might contribute to persistence of childhood asthma and, conversely, that normalization of IL-10 might play a role in remission. Further longitudinal studies, with biospecimens collected at multiple time points, will be required to definitively determine whether a change in IL-10 concentrations is associated with a remission of asthma. This would have important implications for monitoring the course of asthma in clinical practice and would provide a new avenue for therapeutic targets aimed at increasing circulating IL-10 concentrations in asthma patients. In addition, we observed the circulating concentrations of the proinflammatory cytokines TNF- α and IL-6 to be lower in asthma, irrespective of disease activity. This suggests that these proinflammatory cytokines may not be directly associated with or required for current asthma, or that they are being actively suppressed by regulatory networks. Further research is needed to fully understand the role of these cytokines in asthma.

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Online Supplementary Material

Methods

Cytokine Selection

Preliminary experiments were conducted to confirm that cytokines could be detected in serum using high sensitivity human cytokine premixed Lincoplex kits (Linco Research, ABACUS ALS Company). The assay detected 13 cytokines simultaneously and included GM-CSF, IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13 and TNF- α . The protocol described by the manufacture.

Samples for n=28 participants were selected for the preliminary detection experiment (n=14 never asthma and n=14 moderate-to-severe persistent asthma) and were analysed in duplicate. The detection range of assay was from 0.13pg/mL to 2000 pg/mL. The number of samples with detectable levels is shown in the Table 1. Based on these results and our *a priori* hypothesis 6 cytokines were selected for the main study and these were IL-4, IL-5, IL-6, IL-8, IL-10 and TNF- α .

Table S1. Number of serum samples with detectable cytokine levels

Cytokine	N (%) of cells with detectable* cytokine levels (n=56)
GM-CSF	50 (89%)
IFN- γ	9 (16%)
IL-1 β	26 (46%)
IL-2	46 (82%)
IL-4	35 (62%)
IL-5	42 (75%)
IL-6	55 (98%)
IL-7	55 (98%)
IL-8	42 (75%)
IL-10	51 (91%)
IL-12 (p70)	22 (39%)
IL-13	44 (79%)
TNF- α	56 (100%)

*within the detection limit of 0.13pg/mL and 2000pg/mL.

Table S2. Proportion of participants with an undetectable reading or reading above the upper limit for the 6 cytokines

	IL-4	IL-5	IL-6	IL-8	IL-10	TNF-α
Number of participants with positive readings (%)	932 (78.7%)	986 (83.3%)	1111 (93.8%)	1143 (96.5%)	926 (78.2%)	1179 (99.6%)
Number of participants with readings outside the range						
Below the lower limit	246	198	70	0	251	5
Above the upper limit	6	0	3	41	7	0
Total	252	198	73	41	258	5