

Interlevkini, povezani s Th17, in parametri, povezani z dušikovim oksidom pri astmi otrok

Th17-Related Interleukins and Parameters of Oxidative/Nitrosative Events in Childhood Asthma

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Izvleček

Namen: Alergična astma je kronična pljučna bolezen s pomembno vnetno komponento, vključno z reaktivnimi spojinami kisika in dušika. Namen raziskave je bil raziskati odnos s Th17 povezanimi interlevkini (IL-6 in IL-10) in markerji vnetja, povezanimi z dušikovim oksidom pri otroški atopični astmi.

Metode: V raziskavo je bilo zajetih 35 otrok z astmo (AG) in 21 zdravih otrok (CG); z NIOX monitornim sistemom za dušikov oksid smo izmerili volumen frakcioniranega izdihanega dušikovega oksida (FE_{NO}). Nivo IL-6 in IL-10 v plazmi je bil analiziran z ELISA, malondialdehid (MDA) s HPLC.

Rezultati: V primerjavi s kontrolno skupino so astmatiki izdihali pomembno večjo količino FE_{NO} ($p < 0,001$) in imeli pomembno višje vrednosti IL-6 ($p = 0,012$). Pomembna korelacija

Abstract

Purpose: Allergic asthma is a chronic lung disease with a major inflammatory component including reactive oxygen and nitrogen species in the background. The objective of this study was to investigate the relationship between the Th17-associated interleukins IL-6 and IL-10, and oxidative/nitrosative markers of stress in childhood atopic asthma.

Methods: For this observational study 35 asthmatic (AG) and 21 healthy (CG) children were recruited; the volume of fractionated exhaled nitric oxide (FE_{NO}) was measured with the NIOX nitric oxide monitoring system. Plasma levels of IL-6 and IL-10 were analysed with ELISA, malondialdehyde (MDA) with HPLC.

Results: Compared to healthy controls, asthmatics exhaled a significantly ($p < 0.001$) higher mean volume of FE_{NO} and had significantly ($p = 0.012$)

je bila med aktivatorjem Th17, ki ga predstavlja IL-6, in FE_{NO} pri astmatikih, ne pa tudi pri kontrolni skupini. Plazemska koncentracija IL-10 je bila pomembno nižja pri astmatikih ($p = 0,002$) v primerjavi s kontrolno skupino. V obeh skupinah smo ugotovili statistično pomembno negativno korelacijo med IL-10 in FE_{NO} (AG: $p = 0,020$; CG: $p = 0,008$). Plazemske vrednosti MDA, ki je lipidni produkt peroksidacije in indikator oksidativnega stresa, so bile pri astmatikih pomembno zvišane ($p < 0,001$). Pri obeh skupinah je bila pomembna korelacija med MDA in IL-6, ne pa z IL-10.

Zaključek: Raziskava je potrdila pomembno povezanost s Th17 povezanimi citokini IL-6 in IL-10, vnetjem pljuč in sistemskim oksidativnim stresom pri otrocih z astmo. Mehanizmi, ki so vpleteni v te procese, še niso popolnoma razjasnjeni. Spremenjeno redoks ravnovesje ima najverjetneje pomemben vpliv na imunopatologijo astme.

enhanced levels of IL-6. A significant correlation between the Th17 activator IL-6 and FE_{NO} could be found in the asthma ($p=0.005$) but not in the control group. The plasma concentration of IL-10 was significantly ($p=0.002$) lower in asthmatics than in controls and correlated significantly negatively with FE_{NO} in both groups (AG: $p=0.020$; CG: $p=0.008$). Plasma levels of MDA, a lipid peroxidation product and indicator of oxidative stress, were significantly ($p<0.001$) enhanced in asthmatics. There was a significant (AG: $p=0.001$; CG: $p=0.005$) correlation between MDA and IL-6 but not of IL-10 in both groups.

Conclusion: This study indicates a significant relationship between the Th17-related cytokines IL-6 and IL-10, pulmonary inflammation, and systemic oxidative stress in asthmatic children. The mechanisms involved in this process have not been fully elucidated an altered redox balance may, however, have considerable influence on asthmatic immunopathology.

INTRODUCTION

Allergic asthma is a chronic inflammatory disorder of the lung characterized clinically by bronchial hyper-responsiveness, recurrent episodes of exaggerated bronchoconstriction and airway obstruction (1). These changes are associated with the development of a CD4+ Th2-type immune response in the lung (2). Th2 cells, driven by interleukin (IL)-4, mediate humoral immunity, secrete the interleukins IL-4, IL-5, IL-13, and IL-25, which are co-regulated transcriptionally, and play an important role in producing and maintaining airway inflammation in asthma (3).

It is, however, evident that Th17 is also an important player in allergic airway inflammation (4, 5). Th17 cells are driven to differentiate by IL-6, transforming growth factor (TGF) β , IL-1, and IL-21, whereas IL-23 is required for Th17 expansion, survival, and pathogenicity. IL-27, Th1 and such Th2 cytokines, including IL-4, interferon (IFN)- γ , IL-2, and IL-10, inhibit the development of Th17 cells (3).

Although the pathogenesis of asthma remains unclear, substantial evidence indicates that the cytokine environment plays an essential role in initiating and maintaining airway inflammation by influencing the fate of effector CD4+ T cells in atopic asthma (6). Because they secrete specific cytokines, lung epithelial cells can contribute locally to the type of immune response being driven. One of the cytokines that is produced by lung epithelial cells, and is often reported to be enhanced in patients with asthma, is IL-6 (7, 8). IL-6 is a biomarker of ongoing inflammation, and has recently been found to modulate the type and intensity of the immune response by influencing the differentiation of CD4+ T cells (9) and inhibiting T regulatory cell development (10). This cytokine has been further considered to promote the generation of Th17 cells (11). The immunopathological role of IL-6 and its effect on Th17 cell differentiation in asthma is, however, controversial and only beginning to be addressed (2).

It has been suggested that IL-10, a Th17 inhibitor, plays a modulatory role in the induction and maintenance of allergen-specific tolerance (12). Data show that chronic allergic inflammation in asthmatic children can be alleviated by increasing IL-10 levels (13). The regulatory impact of IL-10 on Th17 cell differentiation is still controversial but animal models indicate that Th17-mediated allergic lung inflammation can be suppressed effectively by IL-12 through an IL-10-dependent mechanism (14).

Several experimental and clinical data indicate that enhanced formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is an important event in the pathogenesis of asthma, and is associated with increased inflammation, the development of airway hyper-responsiveness (15, 16), and alterations in the antioxidant activity in the lung and blood (17). Because of their participation in the regulation of cellular signaling (18) and their involvement in modulating gene expression of two pivotal inflammatory regulators (15), ROS have been reported to play a central role in airway inflammation and the pathogenesis of asthma (1, 19). Additionally, superoxide anions have been reported to potentiate the production of such proinflammatory cytokines as tumor-necrosis factor (TNF)- α and macrophage-inflammatory protein (MIP)-2 in neutrophils (20). Inflammatory cytokines

(e.g.: TNF- α , INF- γ , and IL-1 β) are known to stimulate inducible NO synthase, expressed in activated macrophages and respiratory epithelium. This, in turn, leads to increased formation of NO and RNS (16, 21), which fuel airway inflammation (22) and enhance the production of proinflammatory cytokines in bronchial asthma (1, 23).

Since the pathogenesis of asthma is still incompletely understood, the objective of this study was to investigate a possible relationship between Th17-associated interleukins and oxidative/nitrosative markers of stress in childhood asthma.

MATERIALS AND METHODS

The clinical part of this observational controlled study was performed at the Allergy Unit of the Svábhegy National Institute for Pediatrics and the National Institute for Child Health, Budapest, Hungary. Prior approval for this study was obtained from the local Ethics Committee of Svábhegy Institute and from the Medical Research Council of Hungary (ETT-TUKEB No: 84-206/2008-1018 EKU). The study was performed in accordance with the guidelines of the Declaration of Helsinki; written informed consent was obtained from the legal guardians and the voluntarily participating children.

Table 1: Data pertaining the study subjects (26)

Parameter	Asthma Group	Control Group	P Value
Number (n)	35	21	n.s.
Age (years)	10 \pm 3	9 \pm 4	n.s.
BMI (kg/m)	19 \pm 3	18 \pm 4	n.s.
Asthma history (years)	\geq 1	–	–
Pulmonary function			
FEV1 (%)	82 \pm 11	–	–
PEF (l/min)	255 \pm 78	–	–
Atopy associated parameters			
IgE (kU/l)	280 \pm 83	45 \pm 32	0.009
Total eosinophils	314 \pm 189	130 \pm 78	<0.001
ECP (μ g/l)	34 \pm 21	12 \pm 6	<0.001
CRP (mg/l)	1.9 \pm 1.7	0.34 \pm 0.3	<0.001

BMI: Body mass index; FEV: Forced expiratory volume in one second; PEF: Peak expiratory flow; IgE: immune globuline, ECP: eosinophil cationic protein, CRP: C-reactive protein; n.s.: not significant

Reporting of the study conforms to the STROBE statement and the broader EQUATOR guidelines (24).

Patients & Design

A total of 35 children with atopic asthma (asthma group: AG), and 21 healthy, age- and sex-matched controls (control group: CG) without allergy, respiratory symptoms and normal lung function living in Budapest, Hungary, participated in this study (Table 1). Patients aged between 5 and 15 years, with a history of asthma of at least 1 year, diagnosed with mild, well-controlled asthma classified according to the GINA (Global Initiative for Asthma) guidelines 2006/07 (25) were included in this study. The patients had received previous treatment with bronchodilators (short beta-2 agonist Salbutamol as needed, but a maximum 4x2 puffs/day which is equal to 4x100 µg/day), and/or inhaled, low-dose corticosteroids (Budesonide/Fluticasone: 100–200 µg/day), but had not been on medication for at least 4 weeks before the investigation, and were not under acute exacerbation at the time of the study. Children younger than 5 and older than 15 years of age, or who had a shorter history of atopic asthma than a year and/or were under acute exacerbation and/or diagnosed with severe asthma were excluded from the study. Moreover, the presence of any acute and/or chronic disorders, including other allergic diseases, was defined as a criterion for exclusion from the study; thus co-morbidities were not present in our study group.

Measurements

The volume of fractionated exhaled NO (FE_{NO}) was analyzed by the NIOX Test System (Aerocrine AB, Solna, Sweden). Forced expiratory volume (FEV_1) and the peak expiratory flow (PEF) were measured with a

Table 2: Markers of airway inflammation and oxidative stress in asthmatics and healthy controls

Parameter	Asthma Group	Control Group	P Value
FE_{NO} (ppb)	40 ± 26	11 ± 7	<0.001
MDA (µmol/l)	1.22 ± 0.38	0.86 ± 0.15	<0.001

FE_{NO} : Fractionated exhaled nitric oxide; MDA: Malondialdehyde

PISTON Medical PDD-301 spirometer (Piston Ltd., Budapest, Hungary).

Venous blood samples were taken from each patient after an overnight fast. Plasma concentrations of immune globulin E (IgE), the total number of eosinophils, eosinophil cationic protein (ECP), and C-reactive protein (CRP) were auto-analyzed (Roche/HITACHI, Basel, Switzerland). IL-6, IL-8 and IL-10 levels were determined by ELISA (Diagnosticum ZRt, Budapest, Hungary).

The plasma concentration of the lipid peroxidation product malondialdehyde (MDA) was analyzed by HPLC (RECIPE, Munich, Germany).

The accuracy of all the measurements included in this manuscript was checked daily with appropriate reference material and calibration, and was always ascertained for all parameters.

Statistical analysis

All data are presented as the mean ± standard deviation (sd). Statistical analyses were performed using SPSS 16 (SPSS Inc, Chicago, IL, USA). Because of statistical calculations an appropriate sample size is given in both investigated groups. Differences between the analyzed parameters of the asthma and the control group were compared using the independent Student's *t*-test. The pair-wise associations between variables were examined by Pearson linear correlation. A *p* value of < 0.05 was considered statistically significant.

RESULTS

Children suffering from allergic asthma had impaired pulmonary function (Table 1), and exhaled a significantly higher mean volume of the airway inflammation marker FE_{NO} than controls ($p < 0.001$) (Table 2). Plasma levels of IgE ($p = 0.009$), total eosinophils ($p < 0.001$), ECP ($p < 0.001$), and CRP ($p < 0.001$) were significantly elevated in asthmatic patients (Table 1). Moreover, there were significant positive correlations between FE_{NO} and IgE, and FE_{NO} and ECP in both the asthmatic and the control groups (26).

Compared to healthy subjects, IL-6 was significantly enhanced in children suffering from atopic asthma ($p = 0.012$) (Figure 1a). A significant correlation be-

tween IL-6 and Fe_{NO} could be found in the asthma ($p = 0.005$), but not in the control group ($p = 0.054$) (Figure 2a). The plasma concentration of IL-10 was significantly lower in asthmatics than in controls ($p = 0.002$) (Figure 1c), and correlated significantly and negatively with Fe_{NO} in both investigated groups (AG: $p = 0.020$; CG: $p = 0.008$) (Figure 2b). Moreover, levels of IL-8 were significantly elevated in children with asthma as compared to controls ($p = 0.017$) (Figure 1b).

The plasma level of MDA, a lipid peroxidation product and indicator of systemic oxidative stress, was

significantly higher in asthmatic children than in controls ($p < 0.001$) (Table 2). There was a significant positive correlation between MDA and Fe_{NO} (AG: $p = 0.001$; CG: $p = 0.001$), as well as between MDA and IL-6 (AG: $p = 0.002$; CG: $p = 0.005$) (26) (Figure 3). Additionally, plasma concentrations of MDA and IL-8 were significantly and positively associated in both asthmatics and controls (AG: $r = 0.604$; $p = 0.005$; CG: $r = 0.609$; $p = 0.027$). There was, however, now significant correlation between plasma levels of MDA and IL-10.

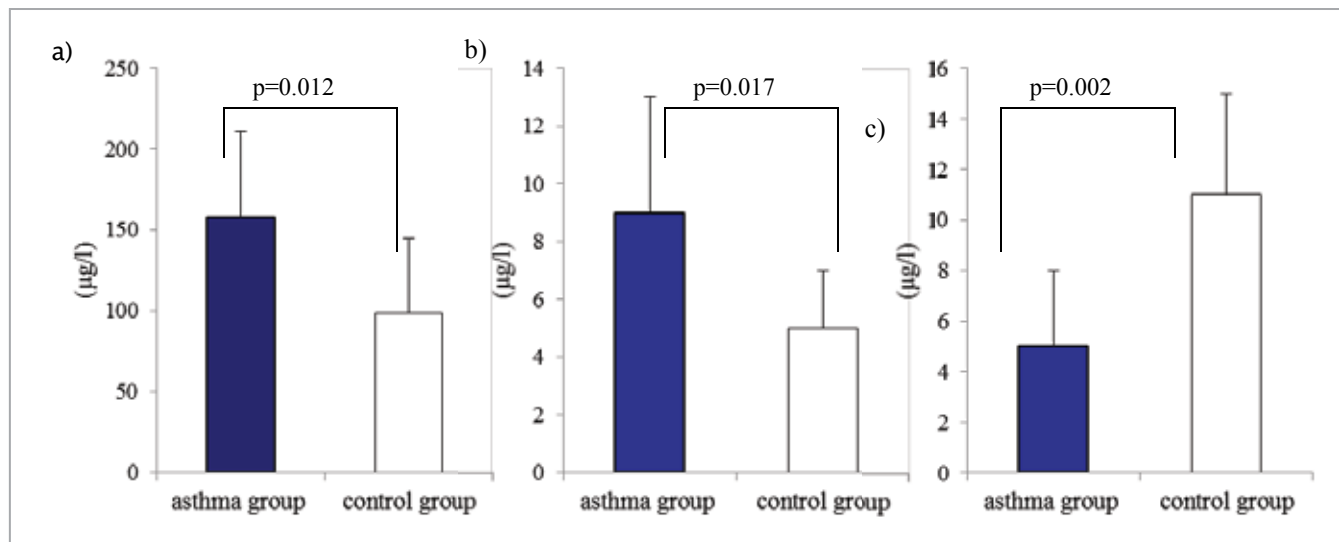


Figure 1: Plasma levels of interleukin (IL)-6 (a), IL-8 (b) and IL-10 (c) in asthmatics and healthy controls

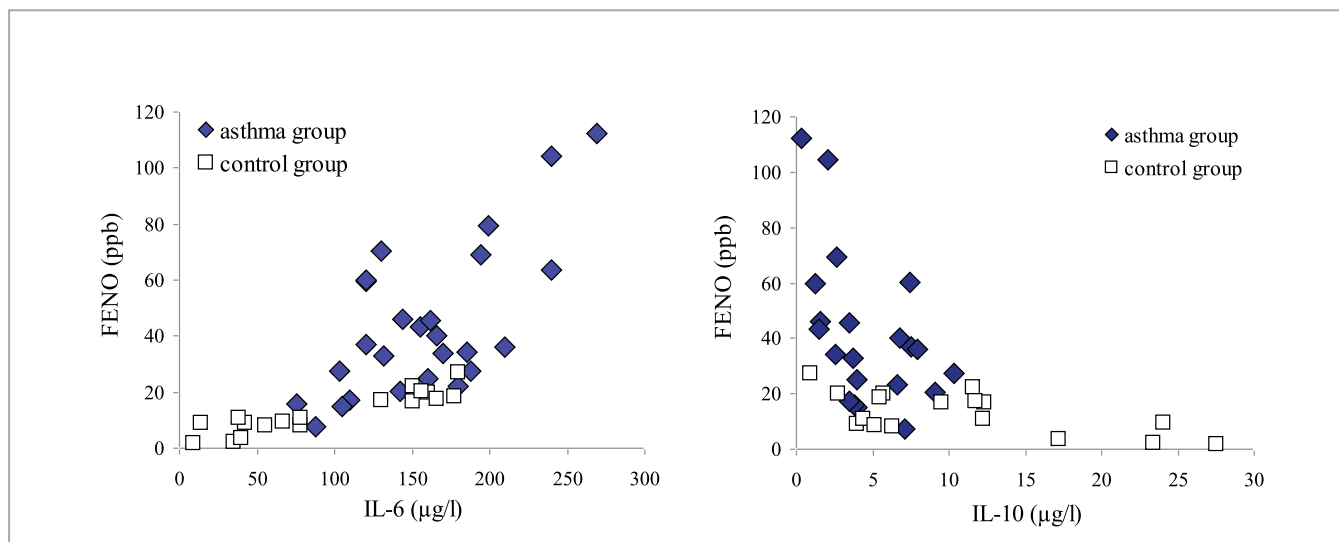


Figure 2: Correlations between fractionated exhaled nitric oxide (Fe_{NO}) and interleukin (IL)-6 (a), and Fe_{NO} and IL-10 (b)
 Asthma group: correlation between: Fe_{NO} and IL-6: $r=0.669$, $p=0.005$; Fe_{NO} and IL-10: $r=-0.503$, $p=0.020$
 Control group: correlation between: Fe_{NO} and IL-6: $r=0.545$, $p=0.054$; Fe_{NO} and IL-10: $r=-0.618$, $p=0.008$

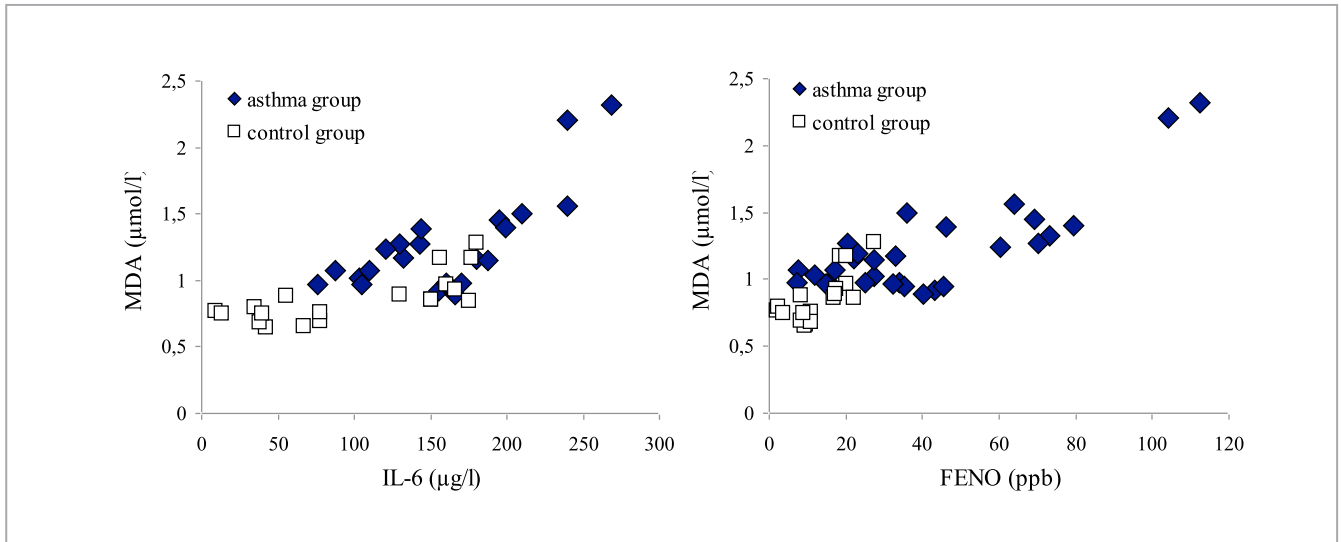


Figure 3: Correlations between malondialdehyde (MDA) and interleukin (IL)-6 (a), and MDA and fractionated exhaled nitric oxide (Fe_{NO}) (b)

Asthma group: correlation between: MDA and IL-6: $r=0.712$, $p=0.002$; MDA and Fe_{NO} : $r=0.605$, $p=0.001$

Control group: correlation between: MDA and IL-6: $r=0.701$, $p=0.005$; MDA and Fe_{NO} : $r=0.694$, $p=0.001$

DISCUSSION

The results of this study indicate a relationship between Th17-associated cytokines, pulmonary inflammation, and enhanced systemic oxidative stress in children suffering from atopic asthma.

Our data show that asthmatic patients exhale a significantly higher mean volume of the airway inflammation marker Fe_{NO} than controls. Increased NO formation in the lung is considered to be a key event in the pathogenesis of asthma. Inflammatory cytokines, such as TNF- α , INF- γ , IL-1 β , and probably IL-6, are known to stimulate inducible NO synthase expressed in activated macrophages and respiratory epithelium, thereby increasing the generation of NO and RNS as nitrogen dioxide and peroxynitrite (16, 21). This further enhances the production of proinflammatory cytokines (1, 23), and fuels airway inflammation in bronchial asthma (22), hence perpetuating the state of inflammation. Asthmatic animal models suggest that peroxynitrite stimulates the release of toxic granules from eosinophiles including ECP and oxidants, and induces airway hyper-responsiveness (27). Thus, RNS may be responsible for asthmatic airway inflammation through eosinophil activation (1), which is substantiated by our

finding of a significant positive correlation between Fe_{NO} and ECP (26). Moreover, antigen hypersensitivity that is prevalent in allergic asthma, and reflected by increased levels of IgE, as also observed in our study (26, 28), facilitates the exaggerated formation of NO and RNS in the sensitized lung (29). Although the lung has a well-developed antioxidant system to protect itself against exposure to endogenous or exogenous noxious oxidants, excessively produced ROS/RNS can fuel inflammation and affect the systemic oxidant/antioxidant balance in asthmatics. A number of studies have clearly demonstrated alterations in the redox status in the lung and blood, resulting in systemic oxidative stress in bronchial asthma (17, 30).

Significantly enhanced plasma levels of MDA, reflecting increased systemic oxidative stress were also found in asthmatics investigated in this study. Moreover, the positive inflammation marker CRP, and the proinflammatory cytokines IL-6 and IL-8, were significantly higher in the asthma group than in the control group. Thus, the significant positive correlations between MDA and Fe_{NO} , MDA and IL-6, and MDA and IL-8 found in this study emphasize a relationship between the severity of the disease, airway inflammation, and systemic redox imbalance in asthma.

The immunopathological mechanisms of allergic asthma are still only partially explained. Substantial evidence indicates that the cytokine environment plays an essential role in initiating and maintaining airway inflammation by influencing the fate of effector CD4⁺ T cells in atopic asthma (6).

In asthmatic pathobiology, Th1 and Th2 cytokines are differentially regulated under conditions of oxidative stress (31). Oxidants have been shown to modulate gene expression of pivotal inflammatory regulators (15), and have been reported to potentiate the production of proinflammatory cytokines (20) that up-regulate inducible NO synthase and NO formation in the lung (21). It is, however, evident that the effect of ROS in transcriptional modulation is determined strongly by the redox balance and the dominating species of ROS. Superoxide anions have been shown to be proinflammatory in that they enhance NF- κ B activation and formation of proinflammatory cytokines, whereas hydrogen peroxide mediates anti-inflammatory effects. This suggests that the balance of these two ROS may be important in modulating inflammatory responses (20), thereby probably affecting the T-cell lineage being driven.

Plasma levels of proinflammatory IL-6 have often been reported to be enhanced under the oxidative stress of asthma, as also observed in our study (8, 32). Apart from other recently discovered immune modulating properties of this cytokine, it is a key cytokine required for driving the differentiation of Th17 cells by recruiting the transcription factor STAT-3. By contrast, IL-10, besides other Th1 and Th2 cytokines and IL-27, constrains Th17 activity (reviewed by 3).

The specific effector functions of Th17 subsets expand beyond previously described effects of Th1 and Th2 immunity that are importantly involved in inflammatory processes (reviewed by 3). The role of Th17 cells and their signature cytokines in asthma has only begun to be investigated. Recent studies have described elevated IL-17 mRNA and protein expression levels in serum and sputum of patients with asthma, and their correlation with sputum neutrophil counts and airway hyper-reactivity to methacholine (32–34); but the exact role of Th17 in Th2-dominated lung inflammation remains undefined.

In our study, plasma levels of the Th17 activator IL-6 were significantly elevated in asthmatics and significantly positively correlated to the extent of airway inflammation (FE_{NO}) and systemic oxidative stress (MDA) (26). By contrast, plasma concentrations of the Th17 inhibitor IL-10 were significantly lower in asthmatic patients than in controls, and were significantly negatively associated with exhaled FE_{NO} . These findings presume that under inflammatory conditions, ROS/RNS contribute to the perpetuation and amplification of airway inflammation, probably by modulating the immune response through cytokine expression, and, subsequently, the propagation of the proinflammatory Th17 lineage in asthma.

Mechanistically, this may occur through the influx of neutrophils, and activation by IL-17 and CXC chemokines, such as IL-8, in the sensitized lung exposed to oxidative stress (35). This suggestion is supported by our findings of significantly elevated levels of IL-8 in asthmatics that were significantly positively related to the extent of systemic oxidative stress. Apart from orchestrating the infiltration of mononuclear cells and evoking a strong neutrophil response by induction of IL-8 and granulocyte macrophage colony-stimulating factor (GM-CSF), IL-17 may also control the migration of various other cell types, leading to the inflammation of sensitized airways after antigen-inhalation challenge (35, 36).

In experimentally induced infection, the Th17 response triggers the production of chemokines like CXCL9, CXCL10, and CXCL11 that attract INF- γ -producing CD4⁺ Th cells into the lung parenchyma, eventually controlling the infection (37). INF- γ stimulates inducible NO synthase, expressed in activated macrophages and respiratory epithelium, thereby increasing the formation of NO (21) as well as airway inflammation (22) and oxidative stress (17, 30), both of which are important features of allergic asthma. Thus, the significant positive correlations between Th17-activating IL-6, and markers of airway inflammation (FE_{NO}) and systemic oxidative stress (MDA), as well as the significant negative associations between the Th17 inhibitor, IL-10 and FE_{NO} observed in this study strongly indicate an engagement of the Th17 lineage in allergic asthma. This is supported by earlier

findings showing that the treatment of sensitized mice with anti-IL17A mAb can reduce significantly peribronchial inflammation and attenuate the increased responsiveness after inhalation challenge. These observations suggest that Th17 cells incite airway inflammation after antigen inhalation, as observed in allergic asthma (35).

Furthermore, our data support the suggestion that ROS and a modified redox balance may have considerable influence on governing the immune response (driving the T-cell subsets) in asthmatic airways through regulation of cellular signaling (18), transcriptional modulation of key inflammatory regulators (15), and potentiation of the production of pro-inflammatory cytokines (20).

Measurement of Th17 cells, and a broader spectrum of Th17-produced ILs in blood and the lung lining fluid would be useful to identify further immunopathological mechanisms in asthma and to substantiate the discussion of our results. Although it is generally accepted that plasma reflects the immunological and oxidative/antioxidative state of the body, how well this applies to lung tissue is, unfortunately, not well established. The lack of such data is a limitation of this study. Thus, our findings may be useful for

further investigations concerning the immunopathological mechanisms in asthma.

CONCLUSIONS

This study indicates a significant relationship between the Th17-related cytokines IL-6 and IL-10, pulmonary inflammation, and systemic oxidative stress in children suffering from allergic asthma. The mechanisms involved in this process have not been fully elucidated. However, an altered redox balance may have a considerable influence on asthmatic immunopathology.

DECLARATION OF INTEREST

The authors declare that they have no conflict of interest.

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