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ORIGINAL ARTICLE

Increased seroprevalence of Enterovirus 71 IgE antibodies in asthmatic compared with non-asthmatic children

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Abstract
Background Asthma is a common pediatric chronic inflammatory airway disease. Respiratory viral infections are frequent infectious triggers for exacerbations of asthma.
Objective We sought to determine whether Enterovirus 71 (EV71), a ubiquitous virus that causes systemic inflammatory responses in children but is not a known respiratory pathogen, can also serve as an infectious trigger for asthma.
Methods Specific EV71 IgE and IgM antibodies (Abs), total serum IgE, and IL-2 and IL-4 cytokine levels in serum of asthmatic and non-asthmatic children ($N = 42$, ages 5–19; $N = 35$, ages 1–20, respectively) were measured (ELISA).
Results Asthmatic children had higher EV71 IgE Ab levels than non-asthmatic ($P < 0.001$). Non-asthmatic children had significantly higher EV71 IgM Ab levels than asthmatic ($P < 0.001$). Despite low serum IgE levels of non-asthmatic, compared with asthmatic ($P < 0.001$), the non-asthmatic children produced significantly more IL-2 and IL-4 than asthmatic ($P < 0.001$; $P < 0.001$). The ages of the asthmatics, but not the non-asthmatics had a significant effect on the levels of EV 71 IgE Abs ($P = 0.02$; $P = 0.356$). A test of difference between these two slopes was significant. However, the ages of the non-asthmatic, but not the asthmatic children had a significant effect on the levels of EV 71 IgM Abs; a test of difference between these two slopes was significant.
Conclusions Increased specific EV71 IgE Ab responses may indicate that EV71 infection may also be an infectious trigger in asthma. However, the role of specific EV71 IgM Abs, Th2 cytokines, and age in non-asthmatic children should be further studied.

Keywords EV71 · IgE · Cytokines

Abbreviations

Ig	Immunoglobulin
EV	Enterovirus
AR	Atopic rhinoconjuctivitis
AD	Atopic dermatitis
Ab	Antibody
HFMD	Hand, foot, mouth disease
ES	Estimated slope
ELISA	Enzyme linked immunosorbent assay
PCR	Polymerase chain reaction
RSV	Respiratory syncytial virus
HIV	Human immunodeficiency virus
HRV	Human rhinovirus
HBV	Hepatitis b virus
OD	Optical density
Th	T helper
TBS	Tris buffered saline

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TMB	3,3′,5,5′-Tetramethylbemzidine
SNP	Single nucleotide polymorphism
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cells
TLR	Toll-like receptor

Introduction

Asthma is a common chronic disease which affects 23 million people in the United States [1]; it is a significant public health concern and poses an economic burden on society [1]. Increased incidence and prevalence of asthma and allergic disease in most areas of the world have been reported over the past two decades [2], perhaps in part, due to changes in the environment (i.e., declining family size, improvements in household amenities, and higher standards of personal cleanliness) [3]. A possible explanation for these trends over time may be due to reduced opportunity for cross infection in young families [3], thus, resulting in possible increased clinical expression of allergic disease [3].

Strachan et al. [3] described the "hygiene hypothesis" in that infections and unhygienic contact in youth may confer protection against allergic rhinoconjunctivitis (AR), atopic dermatitis (AD), and hay fever [3]. Studies of others have explained this hypothesis based on the fact that T helper (Th) 1 responses induced by microbial infection can counterbalance allergen-induced Th2 responses [4–7]. It has been reported that some viral infections, including Hepatitis B Virus (HBV) and Epstein Barr Virus, may inhibit allergic disease [8, 9], and may be associated with lower IgE levels [10, 11], decreased IgE sensitization [12], and asthma [13]. Since IgE plays an important role in asthma, there has been research into the therapeutic potential of targeting this antibody, using the humanized anti-IgE mAb Omalizumab [14]. Omalizumab specifically binds free IgE and interrupts the allergic cascade by preventing binding of IgE with its high-affinity FcRI receptors on mast cells [14]. It has been shown to be effective in preventing asthma exacerbations and improving quality of life in patients with asthma [14].

Previous studies in our laboratory have demonstrated that IgE may play a role in immunity to specific viruses including parvovirus B19 [15], human immunodeficiency virus (HIV)-1 [16], varicella zoster virus [17], influenza virus [18], and respiratory syncytial virus (RSV) [19]. Other studies from our laboratory also identified IgE Abs to *Chlamydia pneumoniae* [20] and *Mycoplasma pneumoniae* [21] in children with reactive airway disease or asthma. Thus, certain viral or bacterial infections may serve as infectious/inflammatory triggers for asthma; IgE (total and/or specific) may also play an important role in allergic inflammation in children with asthma.

The purpose of the current investigation was to investigate whether there was a differential response in the types of Abs to EV71 in asthmatic and non-asthmatic patients. Human EV71 was chosen because of its ubiquitous presence; it is one of the most frequently detected pathogenic human enteroviruses, and is responsible for large-scale epidemics, including hand, foot, and mouth disease (HFMD) [22]. HFMD is an acute viral illness that presents as a vesicular eruption in the mouth, but can also involve the hands, feet, buttocks and/or genitalia, often accompanied with malaise [22]. EV71 is a virus that causes severe systemic inflammatory responses [23] (i.e., increased pulmonary vascular permeability that is similar to acute respiratory distress syndrome), and therefore, may have an association with asthma. We describe a cross-sectional study of asthmatic and non-asthmatic children with regard to specific EV71 IgE and IgM Abs, cytokine levels and total serum IgE.

Materials and methods

Patients and study design

Eligible patients were recruited from the Pediatric Asthma and General Pediatric Clinics at Kings County Hospital Center (Brooklyn, NY, USA). Patient subject inclusion criteria included: physician's diagnosis of asthma or clinically defined persistent asthma symptoms [24], or both, and allergic rhinoconjunctivitis. Subjects were nonsmokers and had no chest infections or antibiotic use within the past month. Subjects were HIV negative. All asthma subjects were classified as having moderate persistent asthma and all were treated with inhaled corticosteroids. No evidence of active EV71 infection was observed; past infection was determined by the presence of positive IgM EV71 titers (ELISA).

Specific exclusion criteria included history of immunodeficiency or autoimmune disorders, use of systemic corticosteroids within the past 30 days, or immunotherapy, personal or family history of cigarette smoking or tobacco use, and incomplete follow-up. Control subjects were recruited from an outpatient pediatric practice (Brooklyn, NY), and defined by absence of asthma based on clinical criteria [25]. The protocol was approved by the SUNY Downstate Medical Center Institutional Review Board, and the procedures followed were in accordance with institutional guidelines involving human subjects. Written informed consent was obtained from either enrolled participants or their guardians.

Immunoglobulin determination

Total serum IgE Blood was collected and IgE levels were determined in serum using the UniCap Total IgE fluroenzyme immunoassay (Phamacia and Upjohn Diagnostics) performed according to the manufacturer's recommendations (reference range for healthy serum: 20–100 IU/mL). All tests were performed in the Clinical Diagnostic Laboratory at SUNY Downstate Medical Center (Brooklyn, NY).

EV 71 serum antibody detection: Enzyme linked immunosorbent assay (ELISA)

a. *IgM* Serum EV71 IgM Abs were determined by ELISA (Immuno-Biological Laboratories, Inc (IBL) America, Minneapolis, MN) according to the manufacturer's recommendations. Data are reported as optical density (O.D.) values (range for EV 71 IgM: positive: >0.159).
b. *IgE* The presence of EV71 IgE Abs was determined by a modification of ELISA using IgM EV71 ELISA kits (IBL America). Briefly, undiluted samples were directly added (100 μL) to the microwell plates (precoated with EV71 antigen) and incubated for 1 h RT. Goat polyclonal anti-human IgE (100 μL) (ICN Biomedicals, Aurora, OH), diluted 1:200 in Tris-Buffered Saline (TBS) wash (and blocking) buffer (TBS—0.05 % Tween) was added to each well, and incubated for 1 h. The wells were washed 3X in wash buffer. Rabbit anti-goat peroxidase-labeled antibody (ICN Biomedicals), diluted 1:1000 in washing/diluting buffer, was then added to each well and incubated for 1 h. The wells were washed again 3X in washing/diluting buffer, and developed in 3,3′,5,5′-tetramethylbemzidine (TMB) substrate solution (100 μL) (Bio-Quant, San Diego, CA) for 10 min. The reaction was stopped by adding 1 N H_2SO_4 (100 μL). Samples were run in duplicate. The plates were read using an automated microplate reader (Model Elx800; Bio-Tek Instruments, Winooski, VT); O.D. measurements were read at 450 nm. For determination of IgE EV71, data are reported as O.D. values (range: positive cutoff value >0. 0627 O.D. Value, positive). Final O.D. value reported was subtracted from chromagen blank O.D. value (background).

Cytokine ELISA: IL-2 and IL-4

Serum cytokine (IL-2 and IL-4) levels were determined by ELISA (BioSource International, Inc., Camarillo, CA) according to manufacturer's recommendations. Lower limit of detection for test was: IL-2 <6.0 pg/mL; IL-4: <2 pg/mL. Cyotkine data are reported as pg/mL.

Statistical methods

Analyses to determine significant differences between groups were determined using 2-tailed Mann–Whitney tests (P values) and data expressed as median [interquartile range]. A two-sided P value <0.05 was taken to indicate statistical significance for all tests. An analysis of covariance was conducted on log-10-transformed IgE and IgM anti-EV71 scores, with predictors asthma status and age ($N = 49$). All statistical analyses were performed in the Statistical Design and Analysis Research Division (College of Medicine, SUNY Downstate Medical Center, Brooklyn, NY) using SAS software version 9.4 (SAS Institute, Cary, N.C.).

Results

Subjects

A total of 77 subjects were recruited for the study; 42 (55 %) were asthmatic patients (mean age 15.3 ± 3.0) and 35 (45 %) were non-asthmatic controls (mean age 14.8 ± 3.0). Gender were similar (50 % male, 50 % female) between asthma and non-asthmatic subjects. Asthmatic patients had higher total serum IgE levels than non-asthmatic subjects (341[365], 82[111], $P < 0.001$).

Differences in EV71 IgE and EV71 IgM Ab levels in asthma compared with no asthma

Asthmatic children had higher EV 71 IgE Ab levels than non-asthmatic children (0.09 [0.05], 0.04 [0.01], $P < 0.001$). However, non-asthmatic children had significantly higher EV71 IgM levels than asthmatic children (2.5[1.6], 1.1[0.8], $P < 0.001$) (Fig. 1a, b)

Differences in IL-2 and IL-4 levels in asthma compared with no asthma

The non-asthmatic children produced significantly more IL-2 and IL-4 than asthmatic children (313 [369], 28 [23] ($P < 0.001$); 4.2 [0.5], 3.9 [0.5] ($P < 0.001$), respectively) (Fig. 2a, b)

Association between age and EV 71 IgE or EV71 IgM Abs

The ages of the asthmatics, but not the non-asthmatics had a significant effect on the levels of EV71 IgE Abs [estimated slope (ES): −0.015 log-units/year, SE = 0.006, $P = 0.021$ versus +0.005 log-units/year, SE = 0.005, $P = 0.356$]. A test of difference between these two slopes was statistically significant (Fig. 3a). However, the ages of the non-

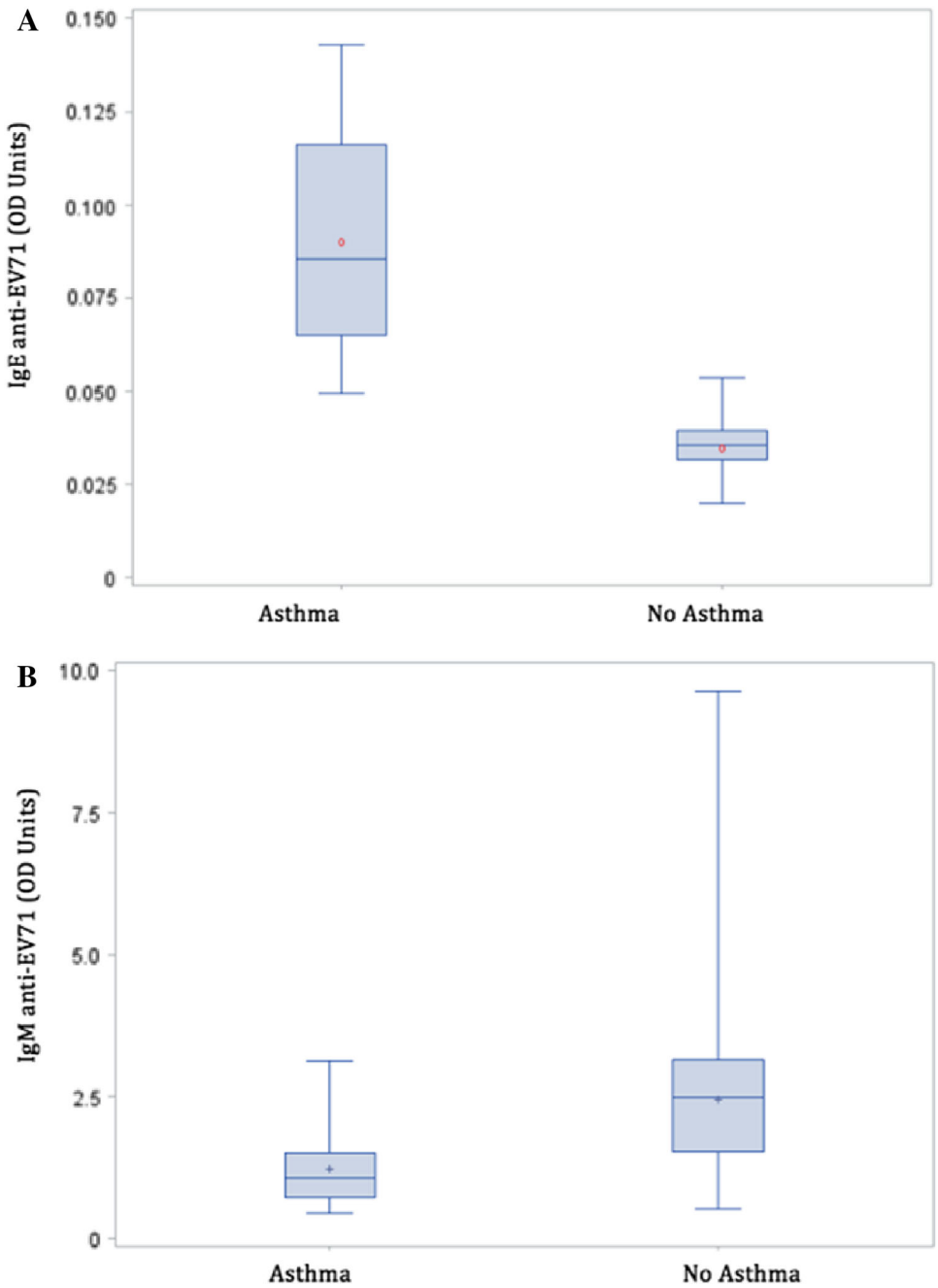

Fig. 1 Differences in EV71 IgE and EV71 IgM Ab levels in asthma compared with no asthma. **a** EV 71IgE Ab levels were higher in asthmatic children ($N = 42$) than non-asthmatic ($N = 34$). **b** EV71 IgM Ab levels were higher in non-asthmatic children than asthmatic

asthmatic, but not the asthmatic children had a significant effect on the levels of EV71 IgM Abs (ES: +0.035 log-units/year, SE = 0.012, $P = 0.006$; ES: −0.007 log-units/year, SE = 0.016, $P = 0.660$, respectively) (Fig. 3b).

Discussion

The results of this study demonstrate that: (1) asthmatic children had higher EV71 IgE Ab levels than non-asthmatic children, (2) but non-asthmatic children had higher EV71 IgM Ab levels than asthmatic children, (3) non-asthmatic children produced more IL-2 and IL-4 than asthmatic children, and (4) in asthmatic children age was related to the levels of EV71 IgE Ab but not levels of EV71 IgM Ab. Overall, our results showed different patterns of association between specific EV71 IgE or EV71 IgM Ab responses and age, stratified according to asthma status. The data from this study reveal important information for the further study of EV71, which is a major pathogen for HFMD [22], and is a health concern in young children.

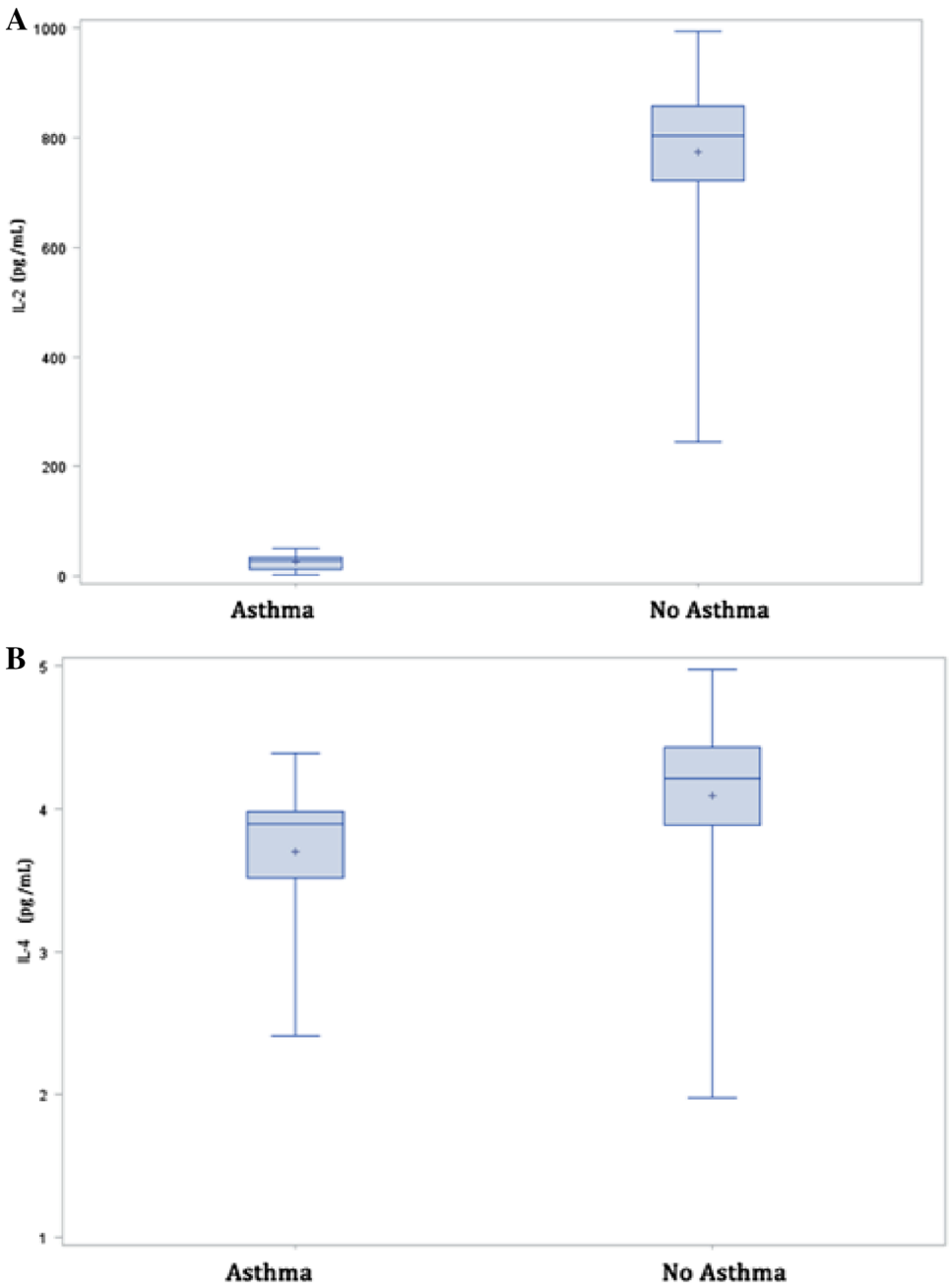

Fig. 2 Differences in IL-2 and IL-4 levels in asthma compared with no asthma. **a** Non-asthmatic children ($N = 34$) produced more IL-2 than asthmatic children ($N = 42$). **b** Non-asthmatic children produced significantly more IL-4 than asthmatic children

The role of microbial infections in the development of atopy and asthma remains controversial [26]. It is well recognized that some viruses induce strong Th1 responses, while suppressing Th2 responses, thus preventing atopic disease [26]. Enterovirus infections in early childhood have been associated with a reduced risk of atopy [26], and may protect from atopic sensitization and atopic diseases [26]. An inverse relationship between IgE sensitization and seropositivity to infectious agents has been observed; enteroviruses showed the strongest protective effect among viruses studied [27]. Seiskari et al. reported that using highly sensitive and specific neutralization assays certain Enterovirus serotypes (i.e., echoviruses) are associated with protection against IgE sensitization [28]. It has been suggested that this protective effect may be biological, since enteroviruses replicate in the cells of the intestinal immune system, which have been implicated in the development of immunoregulatory responses [28]; these viruses also infect white blood cells, including dendritic cells [29, 30], thus impeding regulation of immune responses [29].

Viral infections are also recognized as risk factors for development of asthma [31–37]; synergistic interactions between viral lower respiratory infections and allergic sensitization in early life are important factors in increasing

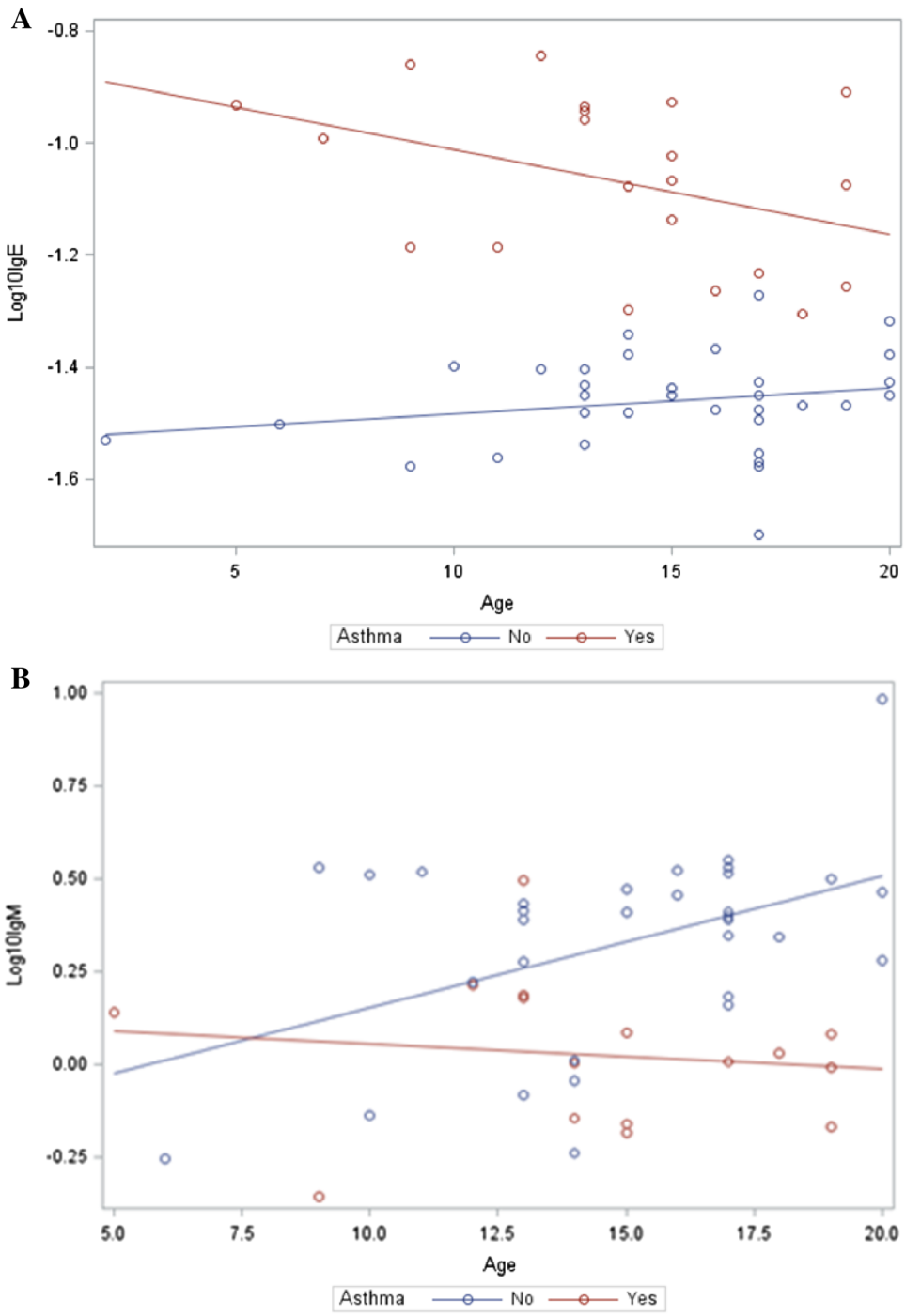

Fig. 3 Association between age and EV 71 IgE or EV71 IgM Abs. **a** Analysis of Covariance for Log_{10}IgE. Ages of the asthmatics ($N = 42$), but not non-asthmatics ($N = 35$) had a significant effect on the levels of EV71 IgE Abs. **b** Analysis of Covariance for Log_{10}IgM. Ages of the non-asthmatic, but not asthmatic children had a significant effect on the levels of EV 71 IgM Abs

the risk of subsequent asthma [32]. The two viruses most frequently associated with asthma susceptibility and exacerbation are RSV and human rhinovirus (HRV) [38, 39]. It is not clear whether the viruses impact or modify the development of the immune system (causal) or if viral infections initiate symptoms in people with impaired lung function [36]. Clinical observations have reported that children with atopy have more severe viral infections and allergic sensitization precedes wheezing with respiratory infection [40–43]. Prior literature has reported that in asthmatic children with elevated levels of allergen-specific IgE titers increased the possibility of wheeze with HRV infection [44]. It has been suggested that viral infections initiate asthma in children genetically predisposed to this disease, which might involve multiple genetic variants and viruses [45].

In the current investigation we sought to explore the relative role and prevalence of humoral immune responses

to EV71 by analyzing specific EV 71 IgM or IgE antibody levels in asthmatic children. We found that levels of specific EV71 IgE Abs were significantly higher in asthmatic children compared with non-asthmatic controls, while levels of specific EV71 IgM Abs were significantly higher in non-asthmatic children, compared with asthmatics. Since IgE is involved in allergic reactions [3, 5, 9], this finding may suggest a Th2 bias due to either: (1) underlying genetics, (2) environmental factors, or (3) heterogeneity in T cell responses to infection. However, higher levels of EV 71 IgM Abs in the non-asthmatics could indicate that asthmatics are not as effective in making high levels of specific IgM compared with the non-asthmatic subjects. It could be that EV71 IgM Abs in non-asthmatics use different target antigens and different mechanisms than EV71 IgE Abs. A possible mechanism involved in this increase may be antibody-dependent cellular cytoxicity (ADCC). Thus, this result demonstrates that EV71 may have a non-specific association with asthma status or reflect a different nonspecific immune response in atopic individuals to viral infection.

Data from the present study demonstrate that prevalence of specific EV 71 IgE Abs correlated with age, whereas the prevalence of specific EV 71 IgM Abs did not correlate with age in asthmatics. It could be that this difference may lie in the humoral specificity of the response or may have different susceptibility to EV71. This difference may also be due to a function of age related immune competence or other factors. However, our finding also suggests that asthmatics develop specific EV71 IgE Abs earlier in life than non-asthmatics. It could be that the virus may contribute to the development of asthma or atopy in susceptible individuals during a vulnerable period; the underlying mechanism is unknown. In contrast, previous studies in our laboratory reported that prevalence of specific HBV IgE or HBV IgG Abs did not correlate with age [46]. However, in those studies HBV IgE or HBV IgG Ab responses were measured against HBV immunization and not viral infection [46].

It is well known that humoral mediators (i.e., cytokines) play an important role in the pathophysiology of viral infection [47]. However, little is known about cytokine patterns that link asthma to EV71 infection. Prior literature has reported that pulmonary edema that occurs in EV71-infected children is caused by abnormal cytokine activation that produces systemic inflammation that in turn, causes pulmonary vascular permeability, which is similar to respiratory distress syndrome [48]. Thus, the last part of our study focused on whether there is an association between cytokine patterns (Th1 versus Th2 responses) in children with EV71 titers who had asthma compared with no asthma. We observed that levels of IL-2 (its secretion precedes Th1/Th2 differentiation) and IL-4 (Th2 response) were higher in children without asthma; children without asthma had lower levels of total serum IgE and specific EV 71 IgE Abs. Thus, it could be that synergistic pro-inflammatory and anti-inflammatory cytokine responses are both necessary to prevent possible development of asthma, following exposure to EV71. Wang et al. did not detect changes in IL-4 levels in EV71-patients, but IL-13 levels were elevated [48]; however, these patients did not have asthma. IL-13 and IL-4 are cytokines produced by T cells that have anti-inflammatory activity and suppresses cytotoxic functions of monocytes/macrophages [48–50]. It should also be mentioned that elevated plasma levels of IL-6 (a cytokine produced by T and B cells and other cells [51]) were detected in patients infected with EV71 [52]; however, in this study, IL-6 was not studied. Our cytokine findings are in contrast to previous studies of Ceyhan et al. who reported that IL-2 levels usually are comparable in asthmatic and healthy subjects [53]. Prior literature has also reported that serum IL-4 levels in asthmatics children are significantly increased compared with healthy controls [54–56]. It could be that these discrepancies exist due to variation of asthma severity, different sample populations or geographical locations evaluated in this study compared with the aforementioned studies.

A potential limitation of this investigation is small sample size. In order to confirm the findings of this pilot study, future larger studies are warranted in order to reveal whether there are significant correlations between IgE and EV-71 in patients with asthma. However, despite this limitation, our study has a number of strengths, including the fact that measurement of specific IgE EV71 Ab levels allows testing of large numbers of subjects from the general population by non-invasive means.

Based on the data presented in this study, we hypothesize that there may exist an indirect association between specific EV71 IgE and EV71 IgM titers and different types of immunity in asthmatic and non-asthmatic children; IgE anti-viral responses might contribute to protective immunity in certain populations. However, further exploration of our preliminary findings in future studies would be of interest, including prospective studies in children infected with EV71 with virologic confirmation of infection. Given the public health significance of EV71, these findings will allow us to develop new prognostic indicators and strategies to prevent infection in children at risk for asthma.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest to disclose.

Research involving human participants was approved by the SUNY Downstate Med Ctr IRB committee (stated in methods).
And we had informed consent from patients or their parents.

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