IP-10 Is Elevated in Virus-Induced Acute Exacerbations in Childhood Asthma

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(Received July 4, 2016; Accepted September 1, 2016)

Objective: Viral infections and sensitization to aeroallergens are major factors in the exacerbation of asthma and its development during early childhood. However, the cytokine profiles and eosinophil activation status linked to the association between viral infections and sensitization to aeroallergens are incompletely understood. Here we investigated respiratory viruses, serum eosinophil cationic protein (ECP), and various cytokines/chemokines in acute exacerbation of childhood asthma.
Methods: We analyzed peripheral eosinophil counts, serum ECP, and 27 cytokines/chemokines in 76 virus-induced acute asthma cases with or without aeroallergen sensitization. Asthma due to sensitization was defined by a positive reaction to at least one aeroallergen in serum specific IgE antibody tests. Virus detection was performed using antigen detection kits and/or RT-PCR, followed by direct DNA sequencing analysis. Serum cytokines/chemokines were measured using a multi-cytokine detection system.
Results: Peripheral eosinophil counts and serum ECP and IL-5 levels were significantly elevated in sensitized cases compared with nonsensitized cases. Conversely, IP-10 values were significantly higher in nonsensitized cases. An inverse correlation between IP-10 and IL-5 production was identified in virus-induced acute exacerbations of asthma but not in controls.
Conclusions: Cytokine profiles and eosinophil activation status might be different between sensitized and nonsensitized cases of virus-induced acute exacerbations of asthma.

Key words: Aeroallergen sensitization, Asthma, Children, IP-10, Virus

INTRODUCTION

Risk factors for subsequent development of asthma at school age and for exacerbations of asthma symptoms include aeroallergen sensitization and virus-induced wheezing episodes during infancy and early childhood [1, 2]. Furthermore, viral respiratory illnesses are more severe after early allergic sensitization [3]. A prospective, birth cohort demonstrated that allergic sensitization precedes rhinovirus infection-induced wheezing but the infection does not induce allergic sensitization. This sequential relationship and the plausible mechanisms by which allergic sensitization can lead to more severe rhinovirus infection-induced lower respiratory illnesses lend support to the causal role of allergic sensitization in this developmental pathway, and indicate that therapeutics for preventing allergic sensitization may modify virus-induced wheezing and the development of asthma in later life [4].

From these findings, it is important to explore the different pathophysiological routes between atopic and non-atopic asthma induced by viral infection. Therefore, the purpose of this study was to investigate differences among the viruses detected, peripheral eosinophil counts, and serum levels of eosinophil cationic protein (ECP) and several cytokines and chemokines in cases of virus-induced acute exacerbations of childhood asthma with or without aeroallergen sensitization.

MATERIALS AND METHODS

Patients and study setting

We investigated 93 outpatients and hospitalized subjects with acute respiratory symptoms (60 boys, 33 girls, mean/median ages 3.7/2.8 years) at the Tokai University Hospital and Gunma Children's Medical Center between January 1, 2008 and December 31, 2015. All patients had a history of three or more different episodes of recurrent wheezing and documented evidence of wheezing by auscultation. Subjects with asthma were diagnosed according to the criteria of the guideline of the Japanese Society of Pediatric Allergy and Clinical Immunology [5]. Briefly, a diagnosis of asthma was confirmed on the basis of a history of recurrent wheezing and dyspnea on at least three independent occasions, and reversible bronchoconstriction. Patients were prescribed short-acting β agonists and/or long-term controller medications and these patients were not using systemic corticosteroids at the time of further examination. We excluded children with obvious bacterial infections, congenital heart diseases, and chronic lung diseases as well as those who showed the presence of a foreign body, had signs of severe infection, or were immunosuppressed, as these complications can interfere with the assessment

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of asthma-related outcome measures. The criteria of 'sensitized asthma' patients was defined as being positive for at least one aeroallergen by serum specific IgE antibody. The severity in subjects with acute exacerbation of asthma such as mild attack, moderate attack, and severe attack was defined according to the Japanese guideline [5]. The symptom severity score was also defined as mild attack=1, moderate attack = 2, severe attack = 3. Exclusion criteria for the controls included immunosuppression, the presence of other respiratory tract symptoms, or a history of previous wheezing and asthma. Controls and patient cases were age- and sex-matched. This study was approved by the Ethics Committees of Tokai University Hospital and Gunma Children's Medical Center. Informed consent was obtained from parents of patients and assent was obtained from the children if they were considered old enough (generally > 9 years old).

Virus detection

Nasal aspirates were obtained from 93 patients during acute exacerbations of asthma and analyzed using antigen detection kits for respiratory syncytial (RS) virus (Becton Dickinson, Fukushima, Japan), influenza virus types A and B (Denka-Seiken, Gosen, Japan), and adenovirus (Tauns, Izunokuni, Japan). The remaining secretions were frozen at −80 °C until examination by reverse transcription-polymerase chain reaction (RT-PCR), followed by direct DNA sequencing analysis as previously reported [6, 7]. Some samples were tested by multiplex PCR (Seeplex RV15 OneStep ACE Detection kit, Seegene, Inc., Seoul, Korea) for the presence of 15 human viral respiratory pathogens (adenovirus A/B/C/D/E, human metapneumovirus, enterovirus, human bocavirus 1/2/3/4, human coronavirus 229E/NL63 and OC43, human parainfluenza virus 1/2/3/4, influenza virus A/B, RS virus A/B, and rhinovirus A/B/C) [6]. The amplified PCR products were analyzed by automatic electrophoresis (MCE-202 MultiNA; Shimazu, Kyoto, Japan) [7].

Serum total IgE and allergen-specific IgE

Automated fluoroenzyme immunoassay (FEIAUnicap® 100, Phadia AB, Uppsala, Sweden) was used to measure serum levels of total IgE and allergen-specific IgE against inhalant allergens, including *Dermatophagoides farinae/pteronyssinus,* house dust, Japanese cedar, orchard grass, ragweed, cat and dog dander, *Alternaria,* and *Cladosporium*, as described previously [8]. Allergen-specific IgE values ≥ 0.35 U_A/mL (Class I) were considered positive, and the sensitivity for the detection of total IgE was 2.00 IU/mL. Allergic sensitization was defined as having one or more positive values for allergen-specific IgE.

Serum cytokines/chemokines, and ECP

We measured total IgE, peripheral eosinophil counts, and the concentrations of serum ECP and 27 cytokines/chemokines (interleukin [IL]-1β, IL-1 receptor antagonist [IL-1ra], IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, interferon [IFN]-γ, IFN-γ-induced protein [IP]-10, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, monocyte chemoattractant protein 1, macrophage inflammatory protein [MIP]-1α, MIP-1β, eotaxin, regulated on activation normal T expressed and secreted chemokines [RANTES], platelet-derived growth factor BB, fibroblast growth factor basic, and vascular endothelial growth factor) in samples from 76 patients with asthma showing the presence of any viruses at the time of examination and from 13 control subjects. A fluoroenzyme immunoassay kit (Pharmacia, Uppsala, Sweden) was used for determination of serum ECP. Serum cytokines/chemokines were measured by using a multi-cytokine detection system, Bio-Plex® (Bio-Rad, Hercules, CA), following the manufacturer's instructions, and a Luminex System (Luminex Corporation, Austin, TX), and calculated using Bio-Plex software (Bio-Rad), as reported previously [6].

Statistical analyses

Patient characteristics were evaluated by the Pearson x^2 test and Fisher's exact test for categorical variables. Unpaired data were analyzed using the Mann-Whitney U test. Correlation coefficients for the parameters were calculated with Spearman rank correlation coefficient analysis. A statistically significant result was indicated by a value of $P < 0.05$ (two-sided). All analyses were performed using statistical software package IBM SPSS for Windows (version 23.0, SPSS Japan Inc., Tokyo, Japan) or GraphPad Prism for Windows (version 6.0, GraphPad Software, San Diego, CA).

RESULTS

Virus detection

Of the 93 samples from patients with acute exacerbations of asthma, rhinovirus was detected in 29 (31.2%), RS virus in 22 (23.7%), enterovirus in 9 (9.7%), human coronavirus in 2 (2.2%), human bocavirus in 1 (1.1%), influenza virus B in 1 (1.1%), human parainfluenza virus type 3 in 1 (1.1%), adenovirus in 1 (1.1%), and ≥ 2 viruses in 10 (10.8%); no viruses were detected in 17 samples (18.3%), respectively.

In this study, we further investigated 76 cases showing the presence of viruses with or without aeroallergen sensitization; patient characteristics are shown in Table 1. Although enteroviruses were dominant in sensitized asthma cases, there was no significant difference among nonsensitized and sensitized cases (Fig. 1). In addition, we evaluated the severity of acute exacerbations of asthma attacks. Among the 76 cases with viral infection, 6 were rated as mild attacks, 66 were moderate attacks, and 4 were severe attacks. There was no significant difference in severity in acute exacerbations of asthma between nonsensitized and sensitized cases; however, 3 of 4 severe attacks were sensitized: 2 were infected by rhinoviruses, and 1 by an enterovirus. One nonsensitized case was infected by coronavirus.

Abbreviations: ECP, eosinophil cationic protein; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; IP, interferon-c-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation normal T expressed and secreted chemokine; RS, respiratory syncytial; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

Table 1 Patient characteristics

	Asthma, All	Asthma, Nonsensitized	Asthma, Sensitized	Control
Number	76	29	47	13
Age, year, mean/median	3.4/2.6	1.6/1.4***	4. 5 /3.8###	3.7/4.2
Gender, % male	67.1	62.1	70.2	61.5
≥1 Positive aeroallergen CAP-RAST	61.8 ***	0.0	100.0***###	0.0

CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test. Data were analyzed using Pearson x^2 test and Fisher's exact test for categorical variables. Unpaired data were analyzed using the Mann-Whitney U test. **$p < 0.05$ or ***$p < 0.001$ vs Control; ###$p < 0.001$ vs Asthma, Nonsensitized.

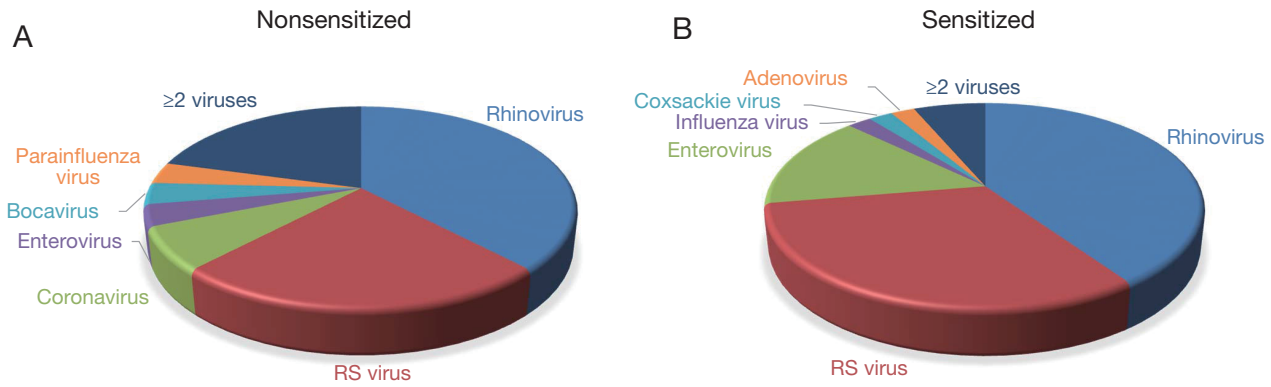

Fig. 1 Virus detection in acute exacerbations of childhood asthma: aeroallergen nonsensitized (A) or sensitized (B) patients.
There were no significant differences among viruses detected in acute exacerbations of childhood asthma between nonsensitized and sensitized states.

Serum total IgE, cytokines/chemokines and ECP

We compared several parameters between nonsensitized and sensitized patients with aeroallergen-specific IgE antibodies. The peripheral eosinophil counts, and concentrations of ECP and IL-5 were significantly elevated in sensitized virus-induced asthma cases compared with nonsensitized cases (Fig. 2). In contrast, among 27 cytokines/chemokines, only IP-10 was significantly higher in nonsensitized asthma cases compared with sensitized cases (Fig. 2). Although the age in nonsensitized cases was significantly smaller than in sensitized cases (Table 1), there was no correlation between the age and values of IP-10 in nonsensitized ($r = -0.529$, $p = 0.077$) and sensitized groups ($r = -0.333$, $p = 0.077$), respectivly. The results of serum total IgE levels, peripheral eosinophil counts, and serum ECP and 27 types of cytokines/chemokines are summarized in Table 2.

We then evaluated the correlation between each parameter in acute exacerbations of asthma. Total serum IgE showed significant correlation with peripheral eosinophils counts, serum ECP, and IL-5 (Fig. 3). As expected, IL-5 correlated significantly with peripheral eosinophil counts. However, IL-5 showed an inverse correlation with IP-10 (Fig. 4). In contrast, these parameters did not correlate among the control subjects (data not shown).

DISCUSSION

The sequences of events that lead to sensitization and virus-induced wheezing during early life are important for the identification of potential causal relationships in the development of atopic asthma [1, 2]. A recent birth cohort study suggested that aeroallergen sensitization is a fundamental risk factor for the development of human rhinovirus wheezing illnesses [4]. Furthermore, rhinoviruses are the most common cause of exacerbations in established asthma and are causative in 30−90% of child and adult cases [9−12]. In a cohort of school-aged children with asthma, aeroallergen sensitization cases were associated with similar numbers of viral infections, but atopic asthma patients experienced more frequent and severe upper and lower virus-induced respiratory illnesses [13]. Furthermore, Heymann *et al.* [14] found that viral infection is the main risk factor for wheezing in children hospitalized before 3 years of age. Conversely, the majority of children aged 3 to 18 years who experience wheezing episodes show prominent atopic characteristics that may represent key risk factors for hospitalization and adverse reactions to viral infections, especially rhinovirus infections. Indeed, our study showed that 3 of 4 severe attacks were in sensitized patients and 2 of 3 cases were infected by rhinoviruses, although the two major viruses, rhinovirus and RS virus, were not significantly associated with aeroallergen sensitization

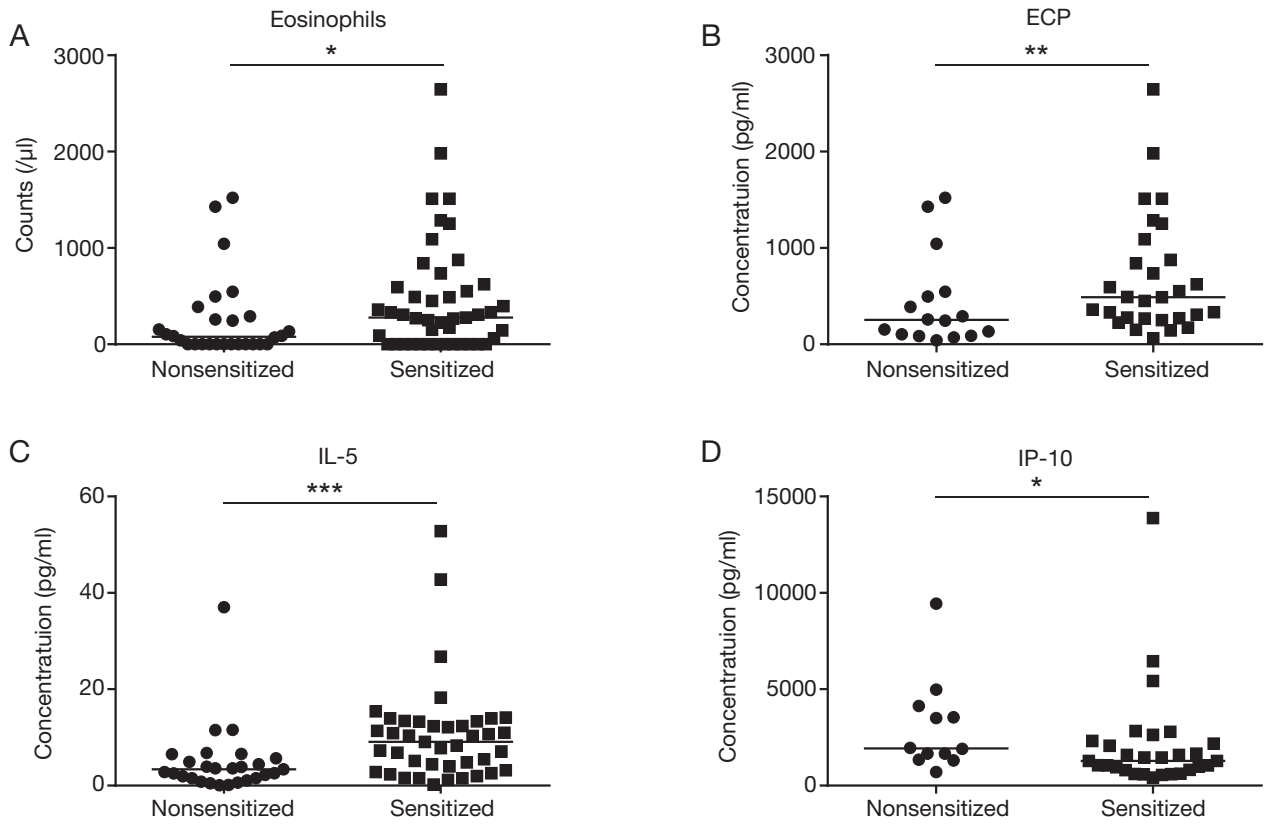

Fig. 2 Peripheral eosinophils counts, serum ECP, and cytokines/chemokines in virus-induced acute exacerbation of asthma in patients who are nonsensitized or sensitized to aeroallergens.
Peripheral eosinophils counts and serum ECP and IL-5 concentrations were significantly elevated in sensitized virus-induced asthma cases compared with nonsensitized cases. In contrast, only IP-10 was significantly lower in sensitized compared with nonsensitized cases. A horizontal bar represents the median. Data were analyzed using the Mann-Whitney U test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

in acute exacerbations of asthma.

However, the role of aeroallergen sensitization on immunological responses during virus-induced asthma are not well clarified. Previous studies suggested that the interferon (IFN)-α, -β, and -λ to rhinovirus or RS virus infection are deficient in both adult and child atopic asthmatic patients [15–17] and that this deficiency correlates with the severity of virus-induced asthma exacerbations [16] and asthma symptoms [18]. Furthermore, anti-IgE antibody, omalizumab improved IFN-α response to rhinovirus, and in the omalizumab group, increases in IFN-α were associated with fewer exacerbations [19]. These results collectively suggest that antigens sensitization is closely related to IFN productions to viral infection and exacerbations of virus-induced asthma. On the other hand, a recent report suggest that rhinovirus type 16-induced IFN-β and -λ productions were significantly reduced in not only atopic asthmatic children but nonatopic asthmatic, and atopic nonasthmatic children compared with nonatopic nonasthmatic children. In addition, IFN-β and IFN-λ induction correlated inversely with the airway Th2 immunopathologic profile such as eosinophilia and IL-4 production and with epithelial damage. Furthermore, total serum IgE levels correlated negatively with rhinovirus-induced IFN-λ mRNA levels and positively with rhinovirus viral RNA levels. These findings suggest that deficient immune responses to viral infections are not limited to patients with atopic asthma but are present in those with other Th2-oriented conditions. We also found that peripheral eosinophil counts and serum concentrations of ECP and IL-5 as the Th2 conditions were significantly elevated in sensitized acute exacerbations of asthma cases compared with nonsensitized cases. The results of eosinophil counts, and ECP and IL-5 concentration measurements were similar to those in previous reports. Zimmerman *et al.* [20] showed that atopic asthmatic children have higher eosinophil counts and greater levels of serum ECP than non-atopic patients. Amin *et al.* [21] compared biopsy samples of patients with atopic and non-atopic asthma and showed that the levels of IL-5-positive cells were higher in atopic cases. Furthermore, IL-5 levels and eosinophilia in induced sputum samples were significantly higher in pediatric patients with atopic asthma compared with non-atopic and control cases [22].

In contrast, this is the first report to show that serum IP-10 values were significantly higher in nonsensitized patients compared with sensitized patients in acute exacerbations of asthma. Furthermore, serum IP-10 and IL-5 production showed an inverse correlation in virus-induced acute exacerbations of childhood asthma but not in controls.

T-helper 2 (Th2)-mediated immune responses are a well-recognized feature of bronchial asthma [23].

Table 2 IgE, Eosinophils, ECP, and cytokines/chemokines in acute asthma compared with control.

	Asthma, All (n = 76)	Asthma, Nonsensitized (n = 29)	Asthma, Sensitized (n = 47)	Control (n = 13)
IgE (RIST)	474.2 (1.9–3150.0)[1]	173.6 (1.9–3150.0)	674.6 (40.2–3018.0)[c]	55.0 (4.0–96.2)
Eosinophils	174 (0–2646)[1]	79 (0–1520)	278 (0–2646)[d]	152 (0–936)
ECP	22.9 (3.0–107.0)[a]	10.1 (4.5–51.4)	32.4 (3.0–107.0)[b, e]	7.3 (3.2–80.6)
IL-1b	5.6 (0.0–52.7)[a]	4.8 (0.0–19.0)[a]	6.3 (0.1–52.7)[a]	8.5 (2.2–31.0)
IL-1ra	393.0 (137.0–4556.2)	337.0 (137.0–1373.7)	413.1 (145.5–4556.2)	196.0 (100.6–1253.9)
IL-2	6.8 (0.4–248.8)	9.8 (3.7–57.5)	6.0 (0.4–248.8)	8.2 (3.6–11.9)
IL-4	13.8 (3.5–151.5)	13.2 (3.5–97.9)	13.9 (4.3–151.5)	18.5 (2.04–73.31)
IL-5	5.3 (0.1–52.8)[b]	3.4 (0.1–37.0)	9.1 (0.2–52.8)[b, f]	3.0 (0.0–4.6)
IL-6	79.8 (6.8–893.8)	97.3 (6.8–893.8)	75.4 (12.5–550.3)	52.7 (14.1–238.5)
IL-7	14.8 (1.5–42.8)	13.3 (1.5–42.8)	15.3 (2.6–38.1)	17.6 (0.8–27.1)
IL-8	13.3 (0.2–236.1)	14.2 (1.3–236.1)	12.2 (0.2–108.7)	9.1 (0.4–17.6)
IL-9	55.8 (5.4–2036.2)	39.9 (8.8–2036.2)	58.6 (5.4–1470.0)	33.8 (29.4–72.9)
IL-10	15.7 (0.1–145.4)	12.2 (0.1–88.1)	18.3 (0.6–145.4)	16.9 (0.4–64.3)
IL-12	9.5 (0.9–110.7)	18.4 (2.3–110.7)	8.3 (0.9–83.5)	6.9 (0.3–52.3)
IL-13	3.4 (0.1–3.7)	6.9 (0.1–23.3)	2.9 (0.5–33.7)	3.6 (0.5–4.0)
IL-15	10.1 (0.0–71.3)	10.1 (5.3–71.3)	10.1 (0.0–66.0)	18.6 (5.9–90.1)
IL-17	11.4 (0.0–217.9)	21.3 (11.7–33.9)	6.5 (0.0–217.9)	0.1 (0.1–0.1)
IFN-γ	132.6 (3.5–570.1)	109.7 (4.5–570.1)	138.0 (3.5–529.6)	152.1 (44.8–1061.3)
IP-10	1592.6 (423.5–13882.2)[c]	1930.8 (702.5–9437.7)[c]	1287.4 (423.5–13882.2)[c, d]	508.7 (330.9–824.4)
TNF-α	20.5 (0.1–682.8)	8.9 (0.2–91.6)	41.4 (0.1–682.8)	36.0 (0.3–392.1)
GM-CSF	33.5 (0.2–962.0)	61.9 (5.3–844.6)	27.1 (0.2–962.0)	22.2 (0.6–72.0)
G-CSF	45.9 (4.2–1586.7)	56.4 (19.2–1586.7)	43.5 (4.2–173.8)	36.0 (19.4–129.6)
MCP-1	48.2 (13.0–223.6)	61.1 (13.5–223.6)	47.8 (13.5–164.1)	57.3 (30.2–81.0)
MIP-1α	10.1 (1.5–46.8)	8.8 (2.6–46.8)	11.8 (1.5–36.9)	12.5 (10.6–14.8)
MIP-1β	80.7 (6.0–228.4)	85.1 (6.0–200.7)	79.3 (14.4–228.4)[a]	112.4 (15.3–168.0)
Eotaxin	77.9 (8.2–440.8)	63.7 (8.2–354.9)	81.4 (35.5–440.8)	127.0 (69.8–577.4)
RANTES	7256.1 (3198.8–114340.4)	9455.9 (7624.3–66245.6)	4903.2 (3198.8–114340.4)	5831.7 (4632.5–7030.9)
PDGF-bb	5012.9 (433.3–14969.3)	4143.6 (789.0–11617.3)	5412.2 (433.3–14969.3)	6200.3 (2622.6–12493.9)
FGF-basic	73.3 (15.4–471.6)	65.5 (25.2–471.6)	74.4 (15.4–215.7)	71.6 (35.2–156.8)
VEGF	124.8 (5.2–729.1)	153.4 (21.6–319.6)[a]	94.1 (5.2–729.1)	55.4 (18.1–171.0)

[1]Median (range), Mann-Whitney U test, [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$ versus Control: [d]$p < 0.05$, [e]$p < 0.01$; [f]$p < 0.001$ versus Asthma, Nonsensitized. IgE: IU/ml; Eosinophils: /mm^3; ECP: ng/ml; cytokines/chemokines: pg/ml.

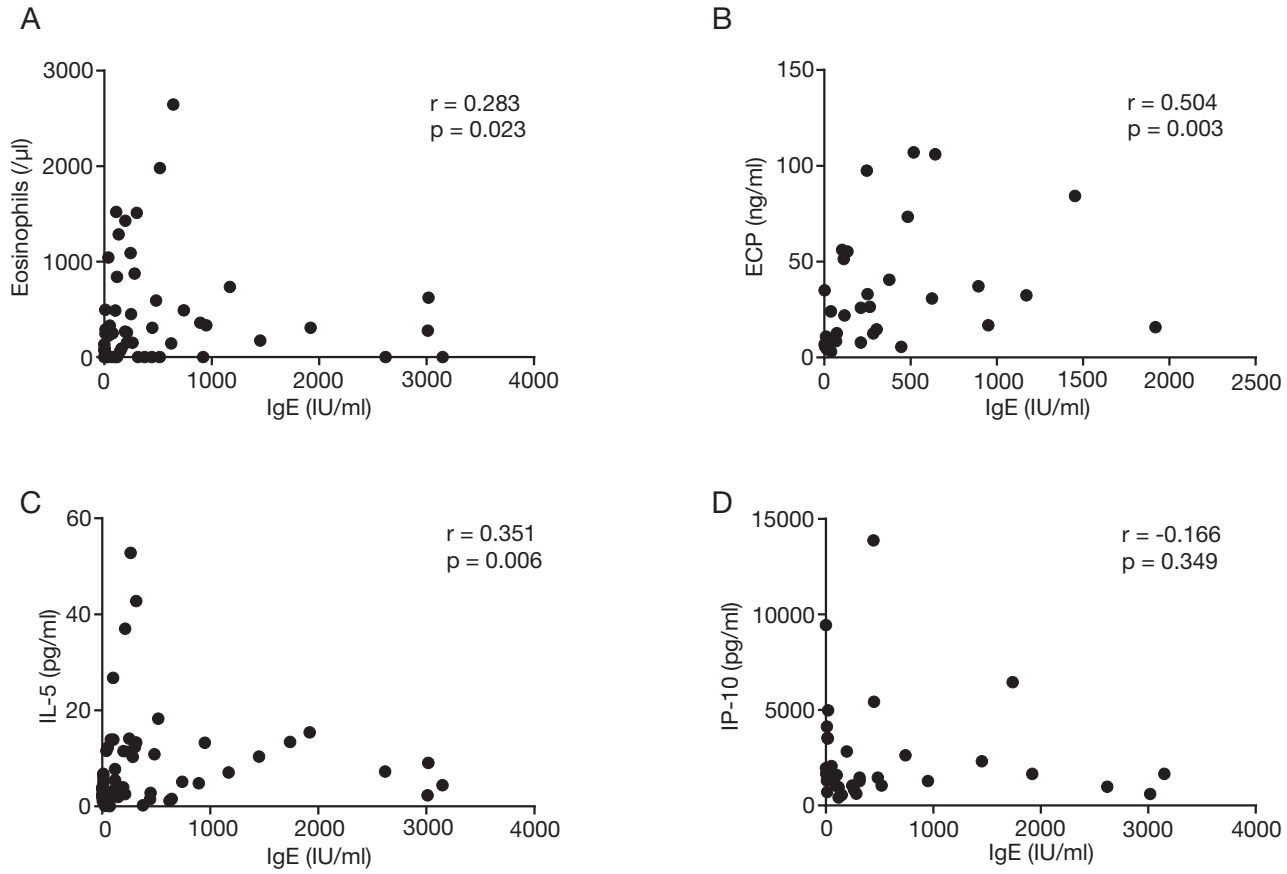

Fig. 3 Comparison between peripheral eosinophils counts, and serum ECP, IL-5, and total IgE in virus-induced acute exacerbations of asthma.
Peripheral eosinophil counts, and serum ECP and IL-5 concentrations correlated significantly with serum total IgE. Data were analyzed using the Spearman rank correlation coefficient analysis.

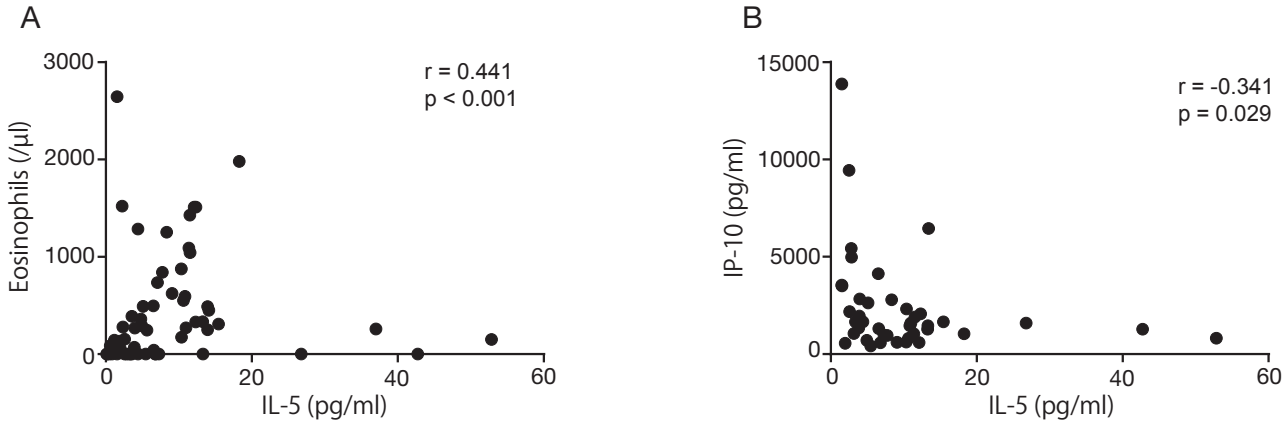

Fig. 4 Comparison between peripheral eosinophils counts, and serum IP-10 and IL-5 concentrations in virus-induced acute exacerbations of asthma.
Serum IL-5 showed significant correlation with peripheral eosinophil counts while IL-5 inversely correlated with IP-10. Data were analyzed using the Spearman rank correlation coefficient analysis.

Th1-associated chemokines have the potential to downregulate allergic inflammation [24]. However, interferon-inducible protein 10 (IP-10)/CXCL10 also contributes to bronchial hyperreactivity and Th2-type allergic inflammation in animal models of asthma [25]. Furthermore, IP-10 is present in bronchial lavage fluid during human allergic pulmonary late-phase reactions [26]. Brightling *et al.* [27] found that IP-10 was expressed more frequently in bronchial biopsies of asthmatic airway smooth muscle compared with tissue from healthy control subjects. T cells recruited during viral infections are predominantly Th1 and characterized by the production of a pattern of cytokines that includes IFN-γ and IL-2 [28]. Th1 chemokine IP-10 attracts Th1 cells through interaction with CXCR3 and plays a prognostic role in viral infection [28]. In hu-

man studies, serum IP-10 levels in virus-induced acute asthma subjects aged 16–74 years are higher than in non-virus-induced acute asthma cases, specifically in cases of rhinovirus infection [29]. Our recent study also suggests that serum IP-10 is elevated in rhinovirus- and RS virus-induced acute childhood asthma exacerbations [6]. Another report showed that the levels of plasma IP-10 in infants are elevated during acute RS virus infection compared with the convalescence period and controls [30]. Furthermore, serum IP-10 is elevated in viral-induced acute exacerbations of asthma compared with stable asthma [6, 29] and acute non-virus-induced asthma [29]. These observations and the present study collectively suggest that Th1 chemokine, IP-10, could be used to predict a virus-induced acute exacerbations, specifically in nonsensitized cases and that the inhibition of the CXCL10/CXCR3 axis may be a novel target for the treatment of virus-induced asthma. Although the exact reasons for the higher production of IP-10 in nonsensitized virus-induced acute exacerbations of childhood asthma remain elusive, they might be explained by Th1-dominant immune responses to, for example, virus infections, but not Th2 immune responses that include aeroallergen sensitization and eosinophil activation.

The limitation of the present study is that we used serum sample to measure 27 cytokines and chemokines. Previous report suggest that the large amounts of RANTES and MIP-1α are released from platelets during *ex vivo* clotting [31], suggesting that the measurement of serum sample, but not plasma sample is not appropriate for some cytokines and chemokines. Further studies are needed to investigate to correct levels of them.

Further work is required to clarify the mechanisms leading to acute exacerbations of asthma and the relationship between allergen sensitization and IP-10 production. These studies might ultimately lead to the prevention and/or treatment of non-atopic/atopic asthma exacerbations caused by both virus infections and aeroallergens.

SOURCE OF SUPPORT

This study was supported in part by Grants-in-Aid for Scientific Research (C) (grant number: 24591565 and 15K09665) from the Japan Society for the Promotion of Science (JSPS) KAKENHI.

AUTHORSHIP

MK conducted the study design. KS and MK collected samples, performed data analysis, wrote first draft, and finalized the manuscript. SK, MN, ME, HT, KH, YY, and KM collected samples and performed data analysis. YH and HM interpreted the results. All authors read and approved the final manuscript.

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