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Peripheral Blood Mononuclear Cells from Patients with Bronchial Asthma Show Impaired Innate Immune Responses to Rhinovirus in vitro

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Key Words
Rhinovirus · Peripheral blood mononuclear cells · IFN-α · Innate immune response · IL-10

Abstract
Background: Asthmatic patients have a higher susceptibility to rhinovirus (RV) infection, and impaired IFN-β and IFN-λ production has been demonstrated in bronchial epithelial cells from asthmatic adults upon exposure to RV. However, the mechanisms underlying the increased susceptibility of asthmatic patients to RV infection remain poorly understood. The present study aimed to elucidate the characteristics of the immune responses of asthmatic patients' peripheral blood mononuclear cells (PBMCs) to RV exposure. ***Methods:*** PBMCs obtained from 3 different age groups (2–6 years: young-children group; 7–19 years: youth group; ≥20 years: adult group) of asthmatic patients and nonasthmatic control subjects were stimulated with RV-14 for 72 h. Healthy adults with a history of childhood asthma were also enrolled. The concentrations of IFN-α, IL-6, TNF-α, IL-10, and soluble Fas ligand (sFasL) in the culture supernatants were measured by ELISA. ***Results:*** When compared with age-matched control subjects, IFN-α production was significantly lower in the asthmatic youth group. IL-6, TNF-α, IL-10, and sFasL productions were significantly lower in both the asthmatic youth group and the adult group. Such impaired responses were not found in healthy adults with a history of childhood asthma. No significantly different responses were found between the asthmatics and controls in the young-children group, whereas young asthmatic children with persistent wheeze during a 2-year follow-up showed significantly lower IL-10 production than those without wheeze. ***Conclusions:*** These results imply the involvement of impaired production of both IFN-α and inflammatory cytokines seen in asthmatic patients' PBMCs upon exposure to RV in the higher susceptibility of those patients to RV infection.

Introduction

Viral respiratory infections are known to have profound effects on many aspects of bronchial asthma, including its development, exacerbation, and probably airway remodeling [1–3]. In particular, rhinovirus (RV) infection is the most frequent trigger of asthma exacerbation in both adults and children [4, 5]. RV is a genus of positive, single-stranded RNA viruses of the family Picornaviridae. Thus far, more than 100 different RV serotypes have been identified. Most of them typically cause upper respiratory symptoms in the common cold in healthy individuals, including rhinorrhea, sore throat, nasal congestion, sneezing, cough, and headache [6]. Ex-

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perimental RV infection, however, induces airway hypersensitivity in asthmatic patients but not in control subjects [7], suggesting that asthmatics are more susceptible to RV infections than are nonasthmatics.

Subsequent studies revealed that bronchial epithelial cells from asthmatics produce less IFN-β and IFN-λ than those from control subjects upon exposure to RV ex vivo [8, 9]. These type I and type III IFNs play critical roles in the synthesis of antiviral proteins such as 2′-5′-oligoadenylate synthetase 1 (OAS-1) and myxovirus resistance-1 (MxA), the release of NK cell- and Th1 cell-attracting CXCR3 chemokines, and the activation of NK cells and cytotoxic T cells [10]. In addition, type I IFN induces virus-inoculated epithelial cell apoptosis [8]. Thus, impaired type I and type III IFNs may cause defective antivirus immune responses and allow invasion of RV into the lower airways [11] and persistence of RV replication [8, 12], which in turn increases the risk of lower airway diseases in asthmatics [13, 14].

The finding that Th2 cytokines such as IL-4 can diminish innate immune responses [15, 16] raised the question of whether such a defective innate immune response in asthmatics is epithelial cell specific or not. A number of previous studies have demonstrated that peripheral blood mononuclear cells (PBMCs) from asthmatic patients showed impaired IFN-γ production [17–20], suggesting that the defective innate immune response in asthmatics is not epithelial cell specific. However, it is still unclear whether or not such impaired innate immune responses can be found in young or older children with asthma. In addition, it also remains unclear whether defective innate immune responses only affect the production of IFNs or whether they also affect the production of inflammatory cytokines because asthmatics tend to have a higher risk of severe lower respiratory diseases upon RV infection [13].

In order to answer these questions, we investigated the innate immune responses of PBMCs from asthmatic patients of different age groups to RV.

Subjects and Methods

Subjects

Thirty-nine currently asthmatic patients (7–35 years of age, median 19 years; 21 males and 18 females) who fulfilled the diagnostic criteria for asthma of the Global Initiative for Asthma (GINA) [21], 7 healthy adults with a history of childhood asthma (15–26 years of age) who had been free of exacerbation for at least 5 years without any medication for asthma (remission group), and 50 age-matched control subjects who had never experienced wheeze were enrolled into this study. In addition, 13 young children (2–6 years of age) who had had at least 3 episodes of wheeze and fulfilled the criteria of the asthma predictive index [22], as well as 11 age-matched young children were also enrolled. The clinical characteristics of the subjects are summarized in table 1. Informed consent was obtained from all subjects or their parents, and the study was approved by the Jikei University ethics committee. All subjects were nonsmokers. None of the patients had received a systemic corticosteroid as daily treatment or for acute exacerbations within 2 months before the study. No subjects had experienced the symptoms of a common cold during the last 2 weeks before the study.

FeNO concentrations in all subjects except a few young children were measured by an offline method using a Sievers collection device (Sievers Instruments, Inc., Boulder, Colo., USA) and a chemiluminescence analyzer (NOA 280, Sievers Instruments, Inc.) or by an online method using a chemiluminescence analyzer (NOA 280), respectively, according to ATS/ERS guidelines [23].

Preparation of RV

RV-14 was originally obtained from Chiba Prefectural Institute of Public Health, and a virus suspension was prepared as previously described with slight modifications [24]. Briefly, viruses were propagated in Ohio HeLa cells (multiplicity of infection 0.001) and cultured in Eagle's MEM (Gibco BRL, Gaithersburg, Md., USA) containing 1% glutamine, 1% nonessential amino acids, 2% FCS (Biowhittaker, Walkersville, Md., USA), 7.5% sodium bicarbonate, 1% tryptose phosphate broth, and 3 M $MgCl_2$ at 33°C in a humidified 5% CO_2 incubator. After 96 h, RV-14 was purified to remove soluble factors of HeLa cell origin via the following steps: (1) the cell culture medium was precipitated by centrifugation (480 *g* for 20 min and 120,000 *g* for 6 h), (2) the supernatant was removed and medium (Eagle's MEM containing 1% glutamine, 0.5% FCS, and 7.5% sodium bicarbonate) was added to suspend the sediment at 4°C, and finally (3) the suspension was condensed (×40) and stored at –80°C.

PBMC Culture

PBMCs were obtained by gradient centrifugation using Lymphocyte Separation Medium (Organon Teknika Corp., Durham, N.C., USA), washed, and resuspended in RPMI1640 medium (GIBCO) supplemented with 2.5% FCS, 2.5% human AB serum (Sigma, St. Louis, Mo., USA), and antibiotics (penicillin-streptomycin) at a cell density of 1×10^6/ml. PBMCs were cultured in 24-well plates to which RV-14 was added to a final concentration of 1 multiplicity of infection. The plates were then placed in a humidified 5% CO_2 incubator at 37°C. Supernatants were harvested at 72 h and stored at –80°C until further assay.

Cytokine Assays

The concentrations of the following cytokines in the culture supernatants were measured using ELISA kits: IFN-α (PBL Biomedical Laboratories, Inc., New Brunswick, N.J., USA), TNF-α (R&D Systems, Minneapolis, Minn., USA), IL-6 (Thermo Fisher Scientific, East Greenbush, N.Y., USA), IFN-γ (R&D Systems), soluble Fas ligand (sFasL; MBL Nagoya, Japan), IL-10 (R&D Systems), IL-4 (Sumitomo Bakelite, Tokyo, Japan), and IL-13 (Ray-Biotech, Norcross, Ga., USA).

According to the manufacturers' instructions as well as our dilution experiments, the minimal detection limits of these assays

Table 1. Clinical characteristics of the subjects

	2–6 years		7–19 years		≥20 years		
	nonwheeze	wheeze	non-BA	BA	non-BA	BA	remission[b]
Subjects, n (M/F)	11 (7/4)	13 (8/5)	17 (7/10)	26 (17/9)	33 (22/11)	13 (4/9)	7 (5/2)
Atopy[a], n	5	9	7	26	28	13	7
Age, years	4 (2–5)	5 (2–6)	15 (7–19)	10 (7–19)	22 (21–35)	26 (20–31)	21 (15–26)
Eosinophil ratio, %	3.6 (2.0–8.7)	4.5 (2.5–8.1)	3.4 (1.0–15.0)	7.4 (0.0–9.0)	3.1 (0.4–10.0)	7.1 (1.0–16.0)	3.7 (1.7–7.0)
p value	n.s.		<0.001		0.03		n.s.
Serum IgE, IU/ml	59.0 (30–249)	685.0 (231–1,381)	108.0 (34–29,800)	1,976.9 (24.1–419)	301.3 (0–2,800)	2,166.5 (7.7–13,300)	236.1 (21.8–758)
p value	<0.001		<0.001		n.s.		n.s.
FEV_1, %	not done	not done	90.3 (79.5–100)	84.5 (59.9–94.9)	88.8 (81.2–95.2)	81.8 (67.8–91.9)	86.4 (83.1–91.3)
p value	–		0.01		<0.001		n.s.
MMF, %	not done	not done	87.1 (40.0–145.0)	91.8 (53–131.0)	93.1 (60.0–131.0)	65.7 (47.0–90.0)	85.4 (61.0–106.0)
p value	–		n.s.		<0.001		0.04
FeNO, ppb	17.0 (5.9–13.4)	21.9 (7.0–77.1)	23.4 (8.5–68.6)	51.3 (2.3–115.6)	40.7 (5.5–121.5)	52.3 (10.3–158.6)	56.0 (16.9–95.0)
p value	n.s.		<0.01		n.s.		n.s.
ICS dose, μg/day	–	200 (100–200)	–	200 (100–800)	–	400 (200–400)	–
Severity of asthma							
Mild intermittent, n		8		8		6	
Mild persistent, n		3		7		2	
Moderate persistent, n		2		10		3	

All numbers are shown as medians (ranges) unless otherwise stated. BA = Bronchial asthma; FEV_1 = forced expiratory volume in the first second; MMF = maximum mid expiratory flow rate; FeNO = fractional exhaled nitric oxide; ICS = inhaled corticosteroids.

[a] Number of subjects having specific IgE to 1 or more common environmental allergens (house dust mite or Japanese cedar pollen) and increased total serum IgE (>80 IU/ml).

[b] Remission asthma subjects were compared with adult asthma subjects (≥20 years).

were 12.5, 15.6, 10.24, 15.6, 100, 15.6, 2.0, and 0.16 pg/ml for IFN-α, TNF-α, IL-6, IFN-γ, sFasL, IL-10, IL-4, and IL-13, respectively.

Statistical Analysis

All of the data are presented as means ± SEM unless otherwise noted. The statistical significance between 2 groups was determined using Welch's t test. $p < 0.05$ was considered statistically significant.

Results

IFN-α

The concentrations of IFN-α in the supernatants of PBMCs from 7- to 19-year-old asthmatics were significantly lower compared with those in the age-matched nonasthmatic control subjects (816.4 ± 128.7 pg/ml vs. 509.8 ± 41.4 pg/ml; fig. 1). A similar tendency was observed in adult asthmatics, although the difference did not reach statistical significance. In contrast, the concentrations of IFN-α in the supernatant were the same for young children with recurrent wheeze and those without wheeze.

TNF-α and IL-6

The concentrations of TNF-α and IL-6 in the supernatants of PBMCs from 7- to 19-year-old and adult asthmatics were significantly lower compared with those of the age-matched nonasthmatic control subjects (TNF-α: 1,085.5 ± 246.9 pg/ml vs. 518.2 ± 66.7 pg/ml and 865.8 ± 158.6 pg/ml vs. 250.5 ± 43.6 pg/ml for age 7–19 years

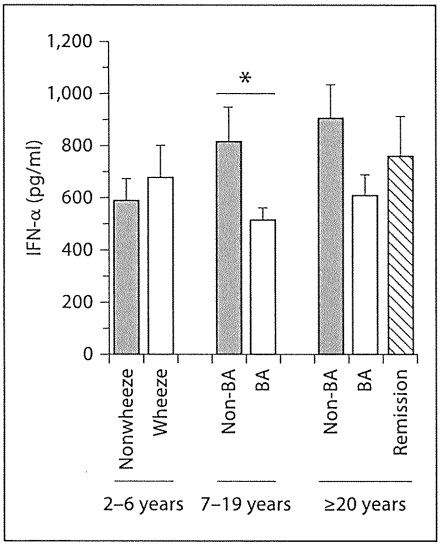

Fig. 1. The concentrations of IFN-α in the supernatant of PBMCs 72 h after exposure to RV-14 are shown. Values represent means ± SEM. The number of subjects in each group is shown in table 1. BA = Bronchial asthma; Remission = healthy adults with a history of childhood asthma. * $p < 0.05$ for asthmatics vs. age-matched nonasthmatic control subjects.

and adults, respectively; IL-6: 4,099.8 ± 550.3 pg/ml vs. 2,700.5 ± 260.3 pg/ml and 3,882.9 ± 464.5 pg/ml vs. 1,745.8 ± 286.0 pg/ml for age 7–19 years and adults, respectively; fig. 2a, b). In addition, the concentrations of TNF-α and IL-6 were significantly higher in healthy adults with a history of childhood asthma than in age-matched asthmatics. However, no significant differences were found between PBMCs from young children with recurrent wheeze and those without wheeze.

IFN-γ and IL-4

No significant differences were found in the concentrations of IFN-γ between asthmatics and the nonasthmatic control subjects in all age groups (527.1 ± 310.5 pg/ml vs. 275.2 ± 61.2 pg/ml and 409.7 ± 104.1 pg/ml vs. 233.6 ± 67.8 pg/ml for age 7–19 years and adults, respectively), including young children (205.0 ± 56.3 pg/ml vs. 121.7 ± 23.4 pg/ml; fig. 3a). In addition, IL-4 was below the detection limit in all PBMC supernatants from all subjects (data not shown).

sFasL

The concentrations of sFasL in the supernatants of PBMCs from 7- to 19-year-old and adult asthmatics were significantly lower compared with the age-matched nonasthmatic control subjects (95.1 ± 36.8 pg/ml vs. 46.8 ± 21.3 pg/ml and 211.3 ± 40.4 pg/ml vs. 0 ± 0 pg/ml for age 7–19 years and adults, respectively; fig. 3b). In addition, the concentrations of sFasL were significantly higher in healthy adults with a history of childhood asthma than in the age-matched asthmatics (70.4 ± 41.9 pg/ml vs. 0 ± 0 pg/ml). However, only trace levels of sFasL were detected in young children both with and without recurrent wheeze.

IL-10

The concentrations of IL-10 in the supernatants of PBMCs from 7- to 19-year-old and adult asthmatics were significantly lower compared with the age-matched nonasthmatic control subjects (504.7 ± 134.3 pg/ml vs. 187.6 ± 30.5 pg/ml and 364.8 ± 68.7 pg/ml vs. 106.9 ± 18.1 pg/ml for 7- to 19-year-olds and adults, respectively; fig. 4a). In addition, the concentrations of IL-10 were significantly higher in healthy adults with a history of childhood asthma than in the age-matched asthmatics. However, no significant differences were found between PBMCs from young children with and without recurrent wheeze.

We conducted a 2-year follow-up of young children with recurrent wheeze (n = 13) who were recruited into the present study. We found that 5 of 13 children had no wheeze, with or without asthma medication, during the follow-up period, whereas 8 children still suffered from recurrent wheeze. Thus, we compared the IL-10 concentrations in the PBMC supernatants from these children and their asthma status during the subsequent 2-year follow-up. We found that IL-10 concentrations were significantly higher in children who had outgrown the wheeze compared with those still experiencing wheeze (345.47 ± 66.0 pg/ml vs. 130.4 ± 22.1 pg/ml; fig. 4b).

Discussion

In the present study, in order to clarify the mechanisms underlying the impairment of anti-RV responses in asthmatic patients, we investigated the innate immune responses of asthmatics' PBMCs to RV. We found that IFN-α production by asthmatics' PBMCs was impaired in children, indicating that such impairment is not adult asthmatic specific. Thus, the impairment of virus-induced type I IFN production by epithelial cells and PBMCs is likely to be a marker of children with a high risk of developing asthma [25].

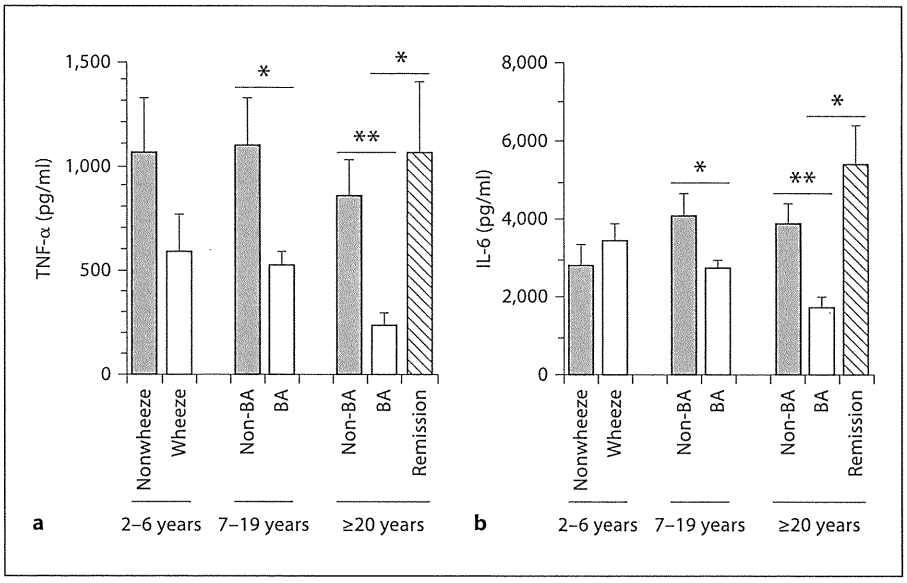

Fig. 2. The concentrations of TNF-α (**a**) and IL-6 (**b**) in the supernatant of PBMCs 72 h after exposure to RV-14 are shown. Values represent means ± SEM. The number of subjects in each group is shown in table 1. BA = Bronchial asthma; Remission = healthy adults with a history of childhood asthma. * $p < 0.05$ and ** $p < 0.01$ for asthmatics vs. age-matched nonasthmatic control subjects or healthy adults with a history of childhood asthma.

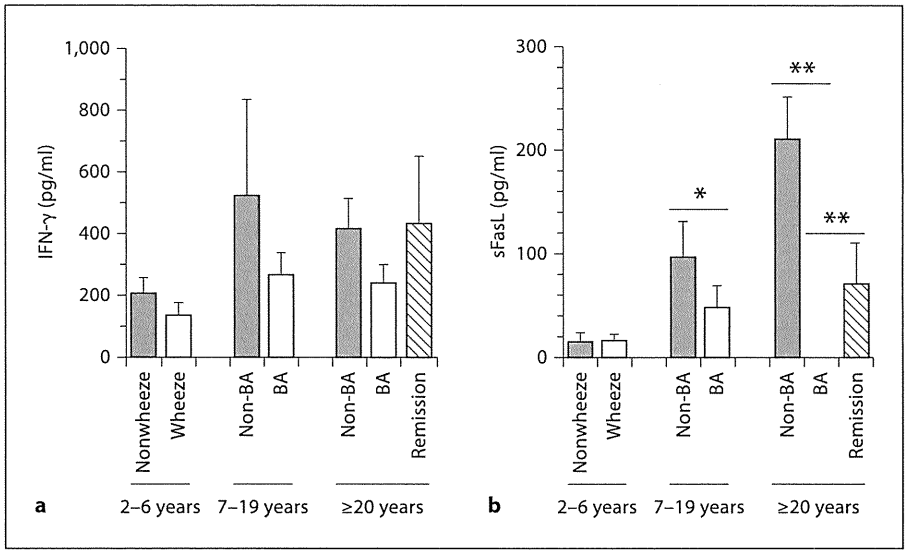

Fig. 3. The concentrations of IFN-γ (**a**) and sFasL (**b**) in the supernatant of PBMCs 72 h after exposure to RV-14 are shown. Values represent means ± SEM. The number of subjects in each group is shown in table 1. BA = Bronchial asthma; Remission = healthy adults with a history of childhood asthma. * $p < 0.05$ and ** $p < 0.01$ for asthmatics vs. age-matched nonasthmatic control subjects or healthy adults with a history of childhood asthma.

Production of both TNF-α and IL-6 was also impaired in PBMCs from asthmatics. It suggests that the increase in lower airway diseases caused by RV infection in asthmatics [13, 14] is not due to an overproduction of inflammatory cytokines (TNF-α and IL-6) but is presumably due to the activation of allergic inflammation in the lower airway. This seems compatible with a previous finding that administration of a leukotriene receptor antagonist prevents wheeze in asthmatic children with respiratory tract infections [26].

As in previous studies [17–20, 27, 28], we observed a tendency towards Th1/Th2 cytokine imbalance (fig. 3a), although it did not reach statistical significance.

One of the most drastic impairments found in asthmatics was that of sFasL production by PBMCs (fig. 3b). Fas-mediated apoptosis is known to be important in the induction of respiratory virus-infected epithelial cell apoptosis [29]. The impaired epithelial cell apoptosis observed in asthmatic airways was thought to be mediated by impaired IFN synthesis and activation of caspases [8], but our results suggest a potential involvement of im-

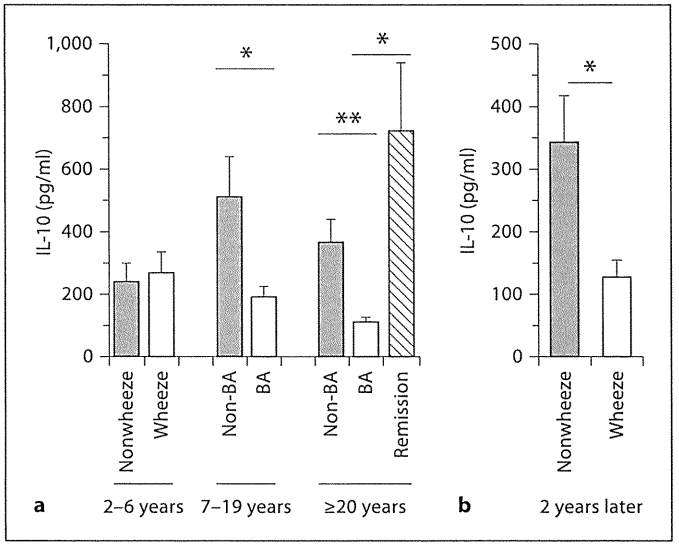

Fig. 4. The concentrations of IL-10 in the supernatant of PBMCs 72 h after exposure to RV-14 are shown. Values represent means ± SEM. **a** The number of subjects in each group is shown in table 1. BA = Bronchial asthma; Remission = healthy adults with a history of childhood asthma. * $p < 0.05$ and ** $p < 0.01$ for asthmatics vs. age-matched nonasthmatic control subjects or healthy adults with a history of childhood asthma. **b** Among 13 young children with wheeze, 5 children had no wheeze with or without asthma medication during the 2-year follow-up period, whereas 8 children still suffered from recurrent wheeze. * $p < 0.05$.

paired sFasL production in the impaired apoptosis of epithelial cells.

Approximately one third of young children develop lower airway diseases regardless of the presence of atopy [30], and that high incidence has been thought to be due to the anatomical characteristics of young children. However, our results suggest that remarkably diminished sFasL production in young children may be involved in their high susceptibility to lower airway infections. In addition, we previously found that Fas triggers apoptosis in both eosinophils [31] and basophils [32], suggesting that impaired sFasL production in asthmatic patients may prolong the survival of eosinophils and basophils.

IL-10 production by PBMCs was also impaired in the asthmatics in our study. A previous study, however, described that overproduction of IL-10 was observed in asthmatics [28]. This discrepancy may be due to the difference in the time kinetics of IL-10 production: the previous study harvested supernatants at 48 h in comparison to our 72 h. It is also possible that IL-10 can be produced by ordinary Th2 cells but not by inflammatory Th2 cells; virus infections induce TSLP production [33] that leads to the development of inflammatory Th2 cells [34]. IL-10 is an immunoregulatory cytokine, and impairment of its production may exacerbate allergic inflammatory reactions in the lower airways of asthmatics.

Interestingly, our findings indicate that impaired IL-10 production predicts the development of asthma in young children with recurrent wheeze (fig. 4b). This fact suggests that IL-10 plays important roles in the development of persistent wheeze in young children [35].

Finally, the healthy adults with a history of childhood asthma in our study showed almost normal cytokine production profiles (fig. 1–4), suggesting that outgrowing asthma may be associated with restoration of innate immune responses, at least to virus infections.

In conclusion, we found that impaired innate immune responses in asthmatic adults were also present in asthmatic children. This impairment may allow invasion of RV into the lower airways [11] and cause allergic inflammatory reactions. A previous study revealed that toll-like receptor (TLR)3 and melanoma differentiation-associated gene (MDA)-5, but not retinoic acid-inducible gene (RIG)-I, are required for maximal sensing of RV replication (double-stranded RNA) and that TLR3 and MDA-5 activate common downstream signaling molecule IFN response factor (IRF)-3 [36]. The precise mechanisms and defective functional molecules in these pathways need to be studied in the future. In addition, restoration of impaired innate immune responses in asthmatic patients is a potential therapeutic approach for bronchial asthma.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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Human Eosinophils Produce and Release a Novel Chemokine, CCL23, in vitro

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Key Words
Eosinophil · CCL23 · MPIF-1 · CK-β8 · CK-β8-1 · Granulocyte-macrophage colony-stimulating factor

Abstract
Background: CCL23 (MPIF1/CK-BETA-8) is a novel CC chemokine that plays important roles in the inhibition of myeloid progenitor cell development, the selective recruitment of resting T lymphocytes and monocytes, and the potentiation of VEGF-induced proliferation and migration of human endothelial cells. Since eosinophils participate in the pathogenesis of airway remodeling, we examined CCL23 production and release by human eosinophils in vitro. ***Methods:*** Using Ficoll and antibody-coated immunomagnetic beads, eosinophils and other blood cells were purified from peripheral blood samples obtained from normal subjects and mildly allergic patients. Eosinophils were cultured in the presence of 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 ng/ml IL-5, 100 ng/ml IFN-γ, 100 ng/ml IFN-α, or immobilized secretory IgA (sIgA). Total mRNA was extracted after 6 h of culture, and mRNA expression was measured using a microarray and RT-PCR. The CCL23 concentrations in the supernatants and cell lysates after 24 and 48 h of culture were measured by ELISA. ***Results:*** CCL23 mRNAs (both CK-β8-1 and CK-β8) were constitutively expressed in fresh eosinophils, and their expression levels were higher than in other types of blood cells. CCL23 mRNAs were significantly increased by stimulation with GM-CSF and IL-5 and slightly by IFN-α and immobilized sIgA. Fresh eosinophils contained trace amounts of CCL23 protein. CCL23 was significantly released into the supernatant when the eosinophils were stimulated with GM-CSF or IL-5 but not with IFN-γ or immobilized sIgA. ***Conclusion:*** Our data suggest that eosinophils produce and release CCL23 and may be involved in some in vivo physiological and pathological conditions.

Introduction

Eosinophils are known to play critical roles in the pathogenesis of helminth infections and allergic diseases. Recent studies have demonstrated the involvement of eosinophils in airway remodeling in bronchial asthma [1, 2]. Eosinophils can act as both effector cells and modulatory cells in allergic inflammatory sites. Upon stimulation, they produce and release not only granule proteins and leukotrienes but also a number of cytokines and chemokines [3]. To date, 12 chemokines, consisting of GRO-A (CXCL1), ENA78 (CXCL5), IL-8 (CXCL8), MIG (CXCL9), IP-10 (CXCL10), I-TAC (CXCL11), MCP-1 (CCL2), MIP-1A (CCL3), RANTES (CCL5), MCP-3 (CCL7), eotaxin 1 (CCL11), and MCP-4 (CCL13), have been reported to be released from eosinophils [3, 4]. However, these chemokines are also known to be released by other types of blood cells and tissue cells [5].

A recent meta-analysis demonstrated that tailored asthma interventions based on sputum eosinophils are most beneficial in reducing the frequency of asthma ex-

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acerbation in adults with asthma [6], suggesting that there must be some eosinophil-specific role(s) in the pathogenesis of bronchial asthma that is probably involved in airway remodeling. In order to elucidate eosinophil-specific function(s), we determined the comprehensive mRNA expression profiles of eosinophils and compared them with those of other blood cells [7, 8]. As a result, we found that CCL23 is exclusively expressed in eosinophils but not in other freshly isolated blood cells. Therefore, in the present in vitro study we further analyzed CCL23 production and release by eosinophils.

Materials and Methods

Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, IFN-γ, TGF-β, and IFN-α were purchased from PeproTech (Rocky Hill, N.J., USA). Eotaxin was purchased from R&D Systems (Minneapolis, Minn., USA). Platelet-activating factor (PAF; 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) was purchased from Sigma (St. Louis, Mo., USA). Secretory IgA (sIgA) was purchased from ICN Biomedicals, Inc. (Aurora, Ohio, USA).

Preparation of Leukocytes

Eosinophils were purified from the peripheral blood of normal subjects and patients with mild allergic diseases (n = 18) by Ficoll-sodium diatrizoate density gradient (lymphocyte separation medium; ICN Biomedicals) sedimentation and negative selection using anti-CD16 antibody-coated immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described [9]. Both the purity and the viability of the eosinophils were confirmed to exceed 97 and 99%, respectively, based on light-microscopic examinations of cytocentrifuged preparations stained with Diff-Quik (American Scientific Products, McGraw Park, Ill., USA) or trypan blue (Sigma) dye exclusion, respectively. All of the allergic donors had been diagnosed as having allergic rhinitis and/or bronchial asthma but exhibited no or only mild symptoms at the time of blood collection.

CD14+ cells, CD4+ cells, CD8+ cells, CD19+ cells, neutrophils, and basophils were purified from the peripheral blood of normal subjects, as described previously [7]. Briefly, CD14+ cells, CD4+ cells, and CD8+ cells were purified by density gradient sedimentation and positive selection using anti-CD14, CD4, and CD8 immunomagnetic beads (Miltenyi Biotec), respectively. CD19+ cells were purified by a combination of negative (antibodies to CD3, CD7, CD14, CD42b, and CD56) and positive (antibody to CD19) selection using MicroBeads (Miltenyi Biotec). Neutrophils were purified by density gradient sedimentation and a combination of positive (anti-CD16) and negative (anti-CD81) selection using MicroBeads (Miltenyi Biotec). Basophils were semipurified by Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and the cells were further purified by negative selection using a MACS Basophil Isolation Kit (Miltenyi Biotec), as described previously [10]. The purity and viability of each cell type exceeded 98 and 99%, respectively.

Written informed consent was obtained from all of the subjects, and the study was approved by the Ethical Review Board of the National Research Institute for Child Health and Development, Tokyo, Japan.

Eosinophil Culture

Purified eosinophils were suspended at a cell density of 1×10^6/ml in Iscove's minimal essential medium (Gibco, Grand Island, N.Y., USA) supplemented with 10% FCS (HyClone Laboratories, Inc., Logan, Utah, USA). They were cultured overnight at 4°C in 24-well microtiter plates (Costar Corp., Cambridge, Mass., USA) previously coated with 2-ml aliquots of PBS containing 1% HSA (heat denatured at 65°C for 1 h) to reduce nonspecific adherence to the plates. The eosinophils were then cultured in the presence and absence of 10 ng/ml GM-CSF, 10 ng/ml IL-5, 100 ng/ml IFN-γ, 100 ng/ml eotaxin, 10^{-8} M PAF, 100 ng/ml TGF-β, and 100 ng/ml IFN-α for up to 48 h at 37°C. For activation with sIgA, 24-well microtiter plates were previously coated with 0.5-ml aliquots of 100 μg/ml sIgA overnight at 4°C and then washed and treated with 2-ml aliquots of PBS containing 1% HSA for at least 2 h at room temperature [11].

Microarray Analysis of Freshly Isolated Leukocytes and Activated Eosinophils

Total RNA samples were extracted from freshly isolated CD14+ cells, CD4+ cells, CD8+ cells, CD19+ cells, neutrophils, and basophils using RNeasy (QIAGEN, Valencia, Calif., USA) and digested with RNase-free DNase I (QIAGEN) according to the manufacturer's instructions. To normalize the results and obtain 5 μg of total RNA for each cell type, equal amounts of total RNA from 3–8 separate donors were mixed. For eosinophils, freshly isolated or activated eosinophils (6 h after activation) were first lysed in ISOGEN (Nippon Gene, Toyama, Japan), and total RNA was extracted using PCI (buffer-saturated phenol:chloroform:isoamyl alcohol, 25:24:1, v/v; Wako Pure Chemical Industry, Saitama, Japan) and RNeasy (QIAGEN) and digested with RNase-free DNase I (QIAGEN) according to the manufacturers' instructions.

Five micrograms of total RNA from each type of leukocyte were used to prepare cRNA. Gene expression was examined using GeneChip Human Genome U133 plus 2.0 probe arrays (Affymetrix, Santa Clara, Calif., USA), which contain the oligonucleotide probe sets for 54,120 full-length genes and expressed sequence tags, according to the manufacturer's protocol [7]. Data analysis was performed using GeneSpring software version 7.2 (Silicon Genetics, Redwood City, Calif., USA). To normalize staining intensity variations among the chips, the average difference values for all of the genes on a given chip were divided by the median expression value for all of the genes on the chip. To eliminate genes whose expressions only represented background noise, genes were selected only if the raw data was >100 and gene expression was judged to be 'present' by GeneChip Analysis Suite 5.0 (Affymetrix). Hierarchical clustering analysis was performed using a minimum distance value of 0.001, a separation ratio of 0.5, and the standard definition of correlation distance.

RT-PCR

The primer sets for CCL23 (sense, 5′-GTTACTGCCCTTGGATCCCAG-3′; antisense, 5′-GATCCGTGTGTCCAGCTTCAG-3′) and β-actin (sense, 5′-CCCAGCCATGTACGTTGCTAT-3′;

antisense, 5′-TCACCGGAGTCCATCACGAT-3′) were synthesized at FASMAC (Kanagawa, Japan). The primer set for CCL23 was designed to detect and distinguish the mRNA of two isoforms of CCL23 (fig. 1b) [12], spanning fragments of 357 and 306 bp for CK-β8-1 and CK-β8, respectively. First-strand cDNA was synthesized using an oligo(dT) (12–18 mers) primer (Invitrogen, Carlsbad, Calif., USA) and an I Script™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif., USA). For RT-PCR, cDNA generated from 5 ng of total RNA was amplified using rTaq polymerase (TOYOBO, Osaka, Japan) and a thermal cycler (GeneAmp PCR System 9700; PE Biosystems, Foster City, Calif., USA) under the following conditions: 94°C for 30 s, 60°C for30 s, and 72°C for 1 min for 35 cycles for CCL23 or for 30 cycles for β-actin. The PCR products were visualized on 0.8% agarose gel (BRL Life Technologies Inc., Grand Island, N.Y., USA) containing 0.05 μg/ml ethidium bromide (Sigma).

CCL23 Concentration in Supernatants and Cell Lysates

Eosinophil culture supernatants were harvested 24 and 48 h after stimulation with cytokines, chemokines, or immobilized sIgA. To measure the amount of prestored CCL23 in the eosinophils, both fresh and stimulated eosinophils were washed with PBS and resuspended at a cell density of 1×10^6/ml in PBS. Cell lysates were obtained by freezing and thawing the cell suspension 3 times. The concentration of CCL23 was measured using ELISA kits (MPIF1; R&D Systems, Inc.) according to the manufacturer's instructions, with a slight modification consisting of the addition of serially diluted standard samples to extensively define the minimal detection limits in each assay. The minimal detection limit of each assay was at least 7.2 pg/ml. According to the manufacturer's instructions, this ELISA kit specifically measures the concentration of the CK-β8 isoform but not of CK-β8-1.

Statistical Analysis

All of the data are presented as means + SEM unless otherwise indicated. Differences between groups were analyzed using a paired t test after a 1-way analysis of variance (ANOVA) test and StatView™ SE+ Graphics software (Abacus Concepts, Inc., Berkeley, Calif., USA). $p < 0.05$ was considered statistically significant.

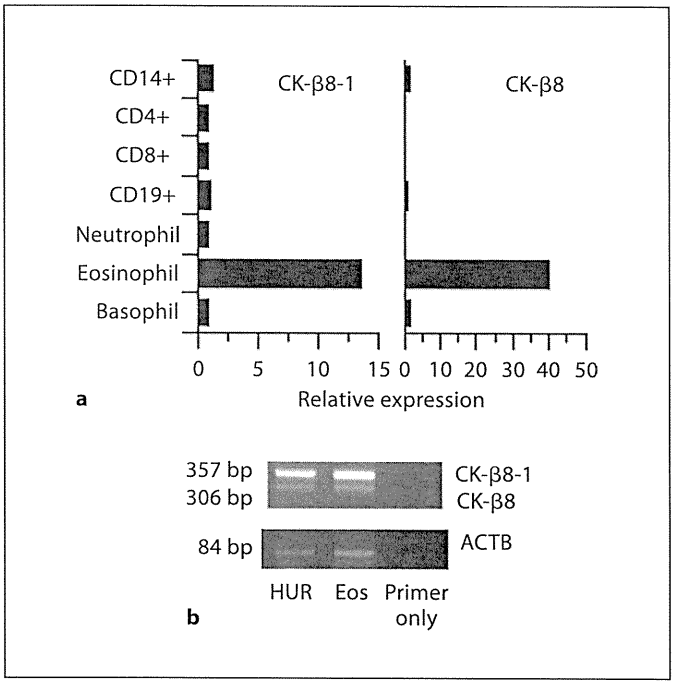

Fig. 1. a Microarray analysis of CCL23 mRNA expression in freshly isolated leukocytes. To normalize the results and to obtain 5 μg of total RNA from each cell type, equal amounts of total RNA from 3–8 separate donors were mixed. CCL23 mRNA expression in various types of freshly isolated blood cells was determined using the GeneChip system. The relative CCL23 mRNA expression levels (CK-β8-1: Affymetrix probe ID 210548_at, left panel; CK-β8: probe ID 210549_at, right panel) are shown after per-chip normalization. **b** RT-PCR analysis of CCL23 mRNA expression in freshly isolated eosinophils. CCL23 mRNA expression in freshly isolated eosinophils was determined using RT-PCR. The figure shows data from a single experiment that was representative of 3 separate experiments using samples from different donors. Lane 1: human universal RNA mixture (HUR; positive control); lane 2: freshly isolated eosinophils (Eos); lane 3: primers only (negative control). ACTB = β-Actin.

Results

CCL23 mRNA Expression in Fresh Eosinophils

The mRNA expressions for two isoforms of CCL23, i.e. CK-β8-1 (Affymetrix probe ID 210548_at) and CK-β8 (probe ID 210549_at), were separately determined in various types of freshly isolated blood cells using the GeneChip system. As a result, both mRNAs were demonstrated to be constitutively expressed in fresh eosinophils (raw data: 486.2 and 560.5 for CK-β8-1 and CK-β8, respectively) at levels that were remarkably higher than in other types of blood cells (fig. 1a). RT-PCR analysis confirmed mRNA expression for both the CK-β8-1 isoform and the CK-β8 isoform of CCL23 as prospective amplicon sizes in fresh eosinophils (fig. 1b).

CCL23 mRNA Expression in Eosinophils after Exposure to Various Cytokines, Chemokines and Immobilized sIgA

The mRNA expressions for the CK-β8-1 and CK-β8 isoforms of CCL23 were separately determined in eosinophils after exposure to various cytokines, chemokines, and immobilized IgA using the GeneChip system. As a result, CCL23 mRNA expression was remarkably increased, i.e. 4- to 5-fold, upon stimulation with IL-5 or GM-CSF, respectively, but not with IFN-γ (fig. 2a).

In order to elucidate potential factors regulating the alternative splicing of CCL23, the gene expression levels (raw data) for CK-β8-1 and CK-β8 in all samples measured with the GeneChip system were compared (fig. 2b).

2

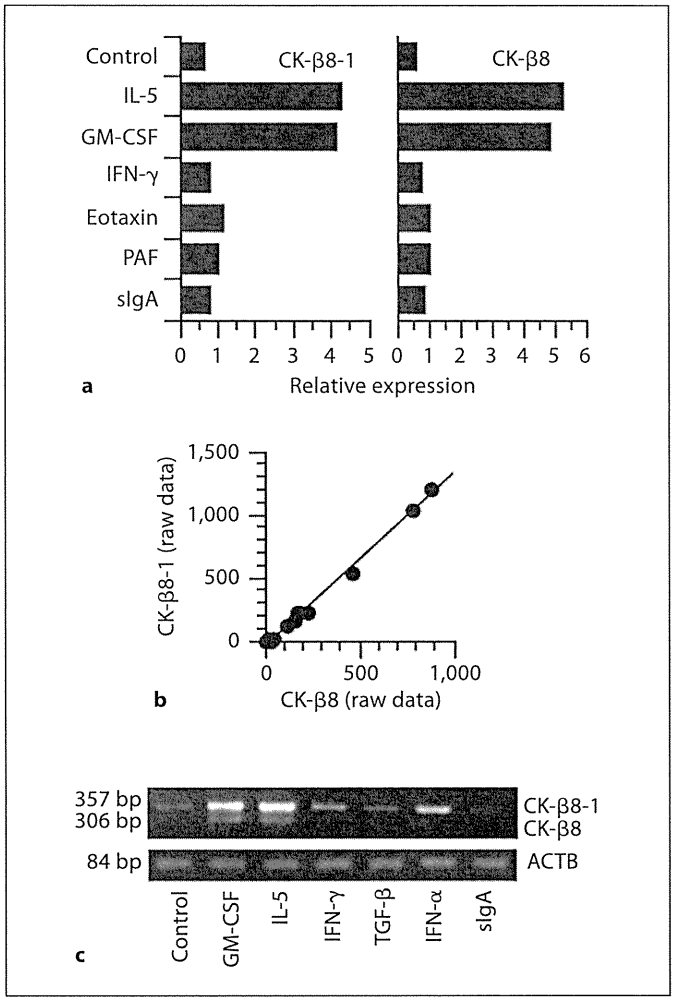

Fig. 2. a Microarray analysis of CCL23 mRNA expression in activated eosinophils. CCL23 mRNA expression in eosinophils after stimulation with vehicle (control), 10 ng/ml GM-CSF, 10 ng/ml IL-5, 100 ng/ml IFN-γ, 100 ng/ml eotaxin, 10^{-7} M PAF, or 100 μg/ml immobilized sIgA was determined using the GeneChip system. The relative CCL23 mRNA expression levels (CK-β8-1, left panel; CK-β8, right panel) are shown after per-chip normalization. **b** The Pearson correlation coefficient of gene expression levels (raw data from GeneChip HG-U133 plus 2.0 array) of CK-β8-1 and CK-β8 was determined (n = 14, r = 0.9976, p = 0.0001). **c** RT-PCR analysis of CCL23 mRNA expression in activated eosinophils. CCL23 mRNA expression in eosinophils after stimulation for 6 h was determined by RT-PCR. The figure shows data from a single experiment that was representative of 3 separate experiments using samples from different donors. Lane 1: vehicle (control); lane 2: 10 ng/ml GM-CSF; lane 3: 10 ng/ml IL-5; lane 4: 100 ng/ml IFN-γ; lane 5: 100 ng/ml TGF-β; lane 6: 100 ng/ml IFN-α; lane 7: 100 μg/ml immobilized sIgA. ACTB = β-Actin.

3

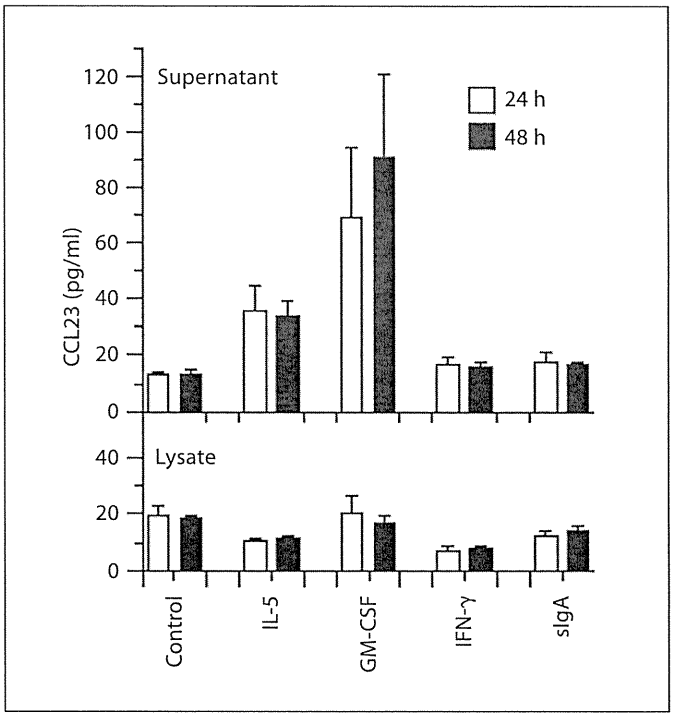

Fig. 3. CCL23 concentration in the supernatants (upper panel) and lysates (lower panel) of eosinophils. CCL23 (CK-β8) concentrations in the supernatant 24 and 48 h after stimulation with the control (vehicle), 10 ng/ml GM-CSF, 10 ng/ml IL-5, 100 ng/ml IFN-γ, or 100 μg/ml immobilized sIgA were determined by ELISA. The data shown are the means + SEM of at least 4 separate experiments. * $p < 0.05$ when compared with the control.

We found an extremely strong positive correlation between the expression levels of these two isoforms (n = 14, r = 0.9976, p = 0.0001).

We further confirmed the real mRNA expression levels for the two CCL23 isoforms by RT-PCR analysis. The effects of TGF-β and IFN-α on CCL23 expression in eosinophils were determined here because these two cytokines are well known to play central roles in airway remodeling and viral infections, respectively (fig. 2c). As a result, mRNA expression for both CK-β8-1 and CK-β8 was markedly upregulated by stimulation with IL-5 and GM-CSF, which confirmed the microarray results. In addition, mRNA expression for both CCL23 isoforms was slightly upregulated by stimulation with IFN-α but not with IFN-γ, eotaxin, TGF-β, PAF, or immobilized sIgA (fig. 2a, c).

RT-PCR analysis confirmed mRNA expression for both the CK-β8-1 isoform and the CK-β8 isoform of CCL23 as prospective amplicon sizes in eosinophils even after various activations. Densitometric analysis dem-

onstrated that CK-β8-1 mRNA expression was approximately 20-fold higher than that of CK-β8 mRNA (fig. 2c).

CCL23 Protein in Culture Supernatants and Cell Lysates

The average concentrations of CCL23 (CK-β8) in the lysates of the control (vehicle) eosinophils were 19.7 ± 3.9 pg/ml and 18.2 ± 1.0 pg/ml at 24 and 48 h, respectively (fig. 3, lower panel). These concentrations were virtually the same even 48 h after exposure to GM-CSF, IL-5, IFN-γ, and immobilized sIgA. However, the CCL23 concentrations in the culture supernatants of eosinophils exposed to IL-5 and GM-CSF were significantly increased (IL-5: 35.5 ± 9.1 pg/ml and 33.72 ± 5.37 pg/ml at 24 and 48 h, respectively; GM-CSF: 68.9 ± 25.3 pg/ml and 91.2 ± 29.3 pg/ml at 24 and 48 h, respectively, $p < 0.05$). No changes were found in the concentrations of CCL23 in the supernatants of eosinophils exposed to IFN-γ or sIgA at either time point (fig. 3, upper panel).

Discussion

Eosinophils produce and release various cytokines and chemokines upon stimulation with cytokines, chemokines, or immobilized immunoglobulins [3]. In the present study, we found that human peripheral eosinophils constitutively expressed mRNA for two isoforms of CCL23 (CK-β8-1 and CK-β8) and that those expression levels were remarkably higher than in the other types of tested blood cells (fig. 1a, b). In addition, microarray and RT-PCR analysis demonstrated that mRNA expression for both of those isoforms in the eosinophils was significantly increased by stimulation with GM-CSF and IL-5 but not IFN-γ (fig. 2a). IFN-α slightly enhanced mRNA expression for both isoforms (fig. 2c).

ELISA revealed that fresh eosinophils contained and released only marginally detectable amounts of CCL23 (CK-β8) even though they constitutively expressed large amounts of CCL23 mRNA. Eosinophil lysates also contained only marginal amounts of CCL23 protein even after stimulation with any of the tested cytokines for up to 48 h. However, after 24 and 48 h of stimulation with GM-CSF and IL-5, the eosinophils produced and released CCL23 into the supernatant, whereas other cytokine milieus did not induce CCL23 release (fig. 3). In addition, the fact that CCL23 was not prestored in either unstimulated or activated eosinophils suggested that the CCL23 was de novo produced by eosinophils; viability in our study conditions did not affect the release of CCL23 because only a marginal release of CCL23 was observed in eosinophils exposed to vehicle or immobilized sIgA, in which eosinophil viability seemed to be very low. Thus, CCL23 production by eosinophils may be allergic inflammation specific and may be involved in the exaggeration of allergic inflammation and in turn facilitate airway remodeling.

Previous studies identified the STAT6, NFAT, NF-κB, and AP-1 binding sites in the 5′ flanking region of the CCL23 gene [13, 14]. However, our results suggest that other transcription factor(s) may be involved in the transcriptional regulation of CCL23 because neither GM-CSF nor IL-5 activates those known transcription factors.

CCL23, also known as MPIF1/CK-β8, is a unique chemokine and is the first example that alternative splicing produces two active CC chemokines from a single gene [12]. CK-β8 is short [51 nucleotides (17 amino acids)] compared to CK-β8-1; the mature proteins of CK-β8-1 and CK-β8 consist of 116 and 99 amino acids with calculated molecular weights of 12,500 and 10,950, respectively. Both CK-β8-1 and CK-β8 are potent agonists at CCR1 and attract dendritic cells, monocytes, and resting T cells but not activated T cells [12]. The fact that CCL23 does not attract activated T cells implies involvement of another receptor or another molecular complex with CCR1 [15]. Both CCL23 isoforms reportedly show similar biological potency, including chemotactic activity and upregulation of cyclins [12, 16]. To date, no studies have described the regulatory mechanisms of the alternative splicing of CCL23 [15]. We examined the correlation coefficiency of the mRNA expression levels for CK-β8-1 and CK-β8 (microarray data; fig. 2b). Surprisingly, the expression levels of these two isoforms showed a strongly positive correlation, suggesting that the alternative splicing occurs in a constant manner. Densitometric analysis of RT-PCR products (fig. 2c) also confirmed this positive correlation (data not shown). When we compared the mRNA expression levels for CK-β8-1 and CK-β8 using RT-PCR and densitometry, that for CK-β8-1 was approximately 20-fold higher than that for CK-β8 (fig. 2c). Unfortunately, however, ELISA for specific measurement of the CK-β8-1 level was not available at the time we performed this study. The difference in mRNA levels between CK-β8-1 and CK-β8 strongly suggests that much larger amounts of CK-β8-1 should be produced and released from human eosinophils upon stimulation.

As with other chemokines, CCL23 has various additional functional properties, including inhibition of myeloid progenitor development [17], induction of matrix metalloproteinase-2 [18], enhancement of VEGF produc-

tion by monocytes [19] and modulation of cell cycles [16]. Those functions support the concept of the involvement of eosinophils in the pathogenesis of airway remodeling in bronchial asthma.

Interestingly, no mouse homolog of CCL23 has been identified. With respect to bronchial asthma, administration of anti-IL-5 mAb drastically improved allergic inflammation in mouse models, whereas administration of anti-IL-5 mAb in humans failed to improve acute symptoms [20]. Further investigations revealed that eosinophils play important roles in airway remodeling [2, 21]. However, it remains unclear why the effects of eosinophil depletion differ between human asthma and the mouse models of asthma. One possible explanation is that eosinophils have human- and/or mouse-specific functions. CCL23 production by human eosinophils may fall within the scope of that concept.

In conclusion, our results demonstrate that human eosinophils produce novel chemokines, i.e. two isoforms of CCL23, upon exposure to GM-CSF and IL-5 and suggest that they may be involved in the pathogenesis of allergic inflammation, especially airway remodeling. In vivo expression of CCL23, such as in bronchoalveolar lavage fluid, will be examined in the future.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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Genome-Wide Association Study Identifies *HLA-DP* as a Susceptibility Gene for Pediatric Asthma in Asian Populations

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Abstract

Asthma is a complex phenotype influenced by genetic and environmental factors. We conducted a genome-wide association study (GWAS) with 938 Japanese pediatric asthma patients and 2,376 controls. Single-nucleotide polymorphisms (SNPs) showing strong associations ($P<1\times10^{-8}$) in GWAS were further genotyped in an independent Japanese samples (818 cases and 1,032 controls) and in Korean samples (835 cases and 421 controls). SNP rs987870, located between *HLA-DPA1* and *HLA-DPB1*, was consistently associated with pediatric asthma in 3 independent populations ($P_{combined}=2.3\times10^{-10}$, odds ratio [OR] = 1.40). *HLA-DP* allele analysis showed that *DPA1*0201* and *DPB1*0901*, which were in strong linkage disequilibrium, were strongly associated with pediatric asthma (*DPA1*0201*: $P=5.5\times10^{-10}$, OR = 1.52, and *DPB1**0901: $P=2.0\times10^{-7}$, OR = 1.49). Our findings show that genetic variants in the *HLA-DP* locus are associated with the risk of pediatric asthma in Asian populations.

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Introduction

Asthma is the most common chronic disorder in children, and asthma exacerbation is an important cause of childhood morbidity and hospitalization. The prevalence of childhood asthma in Japan is 5.0% among school children in 2006 [1], and an estimated 300 million people worldwide have asthma [2]. Asthma is characterized by airway hyperresponsiveness and inflammation, tissue remodeling, and airflow obstruction. Infiltration of lymphocytes, mast cells, and eosinophils in the airways cause airway inflammation, and T helper (Th) type 2 cytokines play crucial roles in orchestrating the inflammatory responses; thus, asthma is considered a Th2-type immune disease.

Previously conducted genome-wide association studies (GWAS) for asthma identified association with the loci on chromosomes 17q21 (*ORMDL3* for Caucasian pediatric asthma, odds ratio (OR) = 1.45, $P=1\times10^{-10}$) [3], 5q21 (*PDE4D* for pediatric asthma, OR = 0.6, $P=4.7\times10^{-7}$) [4], 9q21.31 (*TLE4* for Hispanic pediatric asthma, OR = 0.6, $P=6.8\times10^{-7}$) [5], and 1q31 (*DENND1B* for Europeans and African ancestries [6], OR = 0.77 and 1.41, respectively; combined $P=1.7\times10^{-13}$). A GWAS for severe asthma identified association with the region between

Author Summary

Asthma is the most common chronic disorder in children, and asthma exacerbation is an important cause of childhood morbidity and hospitalization. Here, taking advantage of recent technological advances in human genetics, we performed a genome-wide association study and follow-up validation studies to identify genetic variants for asthma. By examining 6,428 Asians, we found rs987870 and *HLA-DPA1*0201/DPB1*0901* were associated with pediatric asthma. The association signal was stretched in the region of *HLA-DPB2*, collagen, type XI, alpha 2 (*COL11A2*), and Retinoid X receptor beta (*RXRB*), but strong linkage disequilibrium in this region made it difficult to specifically identify causative variants. Interestingly, the SNP (or the HLA-DP allele) associated with pediatric asthma (Th-2 type immune diseases) in the present study confers protection against Th-1 type immune diseases, such as type 1 diabetes and rheumatoid arthritis. Therefore, the association results obtained in the present study could partially explain the inverse relationship between asthma and Th-1 type immune diseases and may lead to better understanding of Th-1/Th-2 immune diseases.

RAD50 and *IL5* on chromosome 5q (OR = 1.64, $P = 3.0 \times 10^{-7}$) and *HLA-DR/DQ* (OR = 0.68, $P = 9.6 \times 10^{-6}$), but they did not include a replication dataset [7]. Recently, Moffatt *et al.* conducted a large-scale GWAS in Caucasian populations and identified 6 loci (*IL18R1*, *HLA-DQ*, *IL33*, *SMAD3*, *GSDMB/GSDMA*, and *IL2RB*) associated with asthma [8].

In the present study, we conducted the first GWAS in Asian population for pediatric asthma by using Illumina HumanHap550/610-Quad BeadChip (Illumina, San Diego, USA).

Results

GWAS analysis

The GWAS flow chart is shown in Figure 1. We analyzed 450,326 SNPs in 938 cases and 2,376 controls, using standard quality control practices (Table S1). The genotypes in cases and controls were compared using the Cochran–Armitage trend test (Figure 2). There was only minor inflation of the genome-wide statistical results owing to population stratification (genomic control (λ_{GC}) = 1.048; Figure 3). Five SNPs (rs3019885, rs987870, rs2281389, rs2064478, and rs3117230) showed strong association with pediatric asthma with $P < 1 \times 10^{-8}$. Of these, rs2064478 and rs3117230 were in complete linkage disequilibrium (LD) ($r^2 = 1$) with rs2281389. In order to validate the results of the GWAS, we tested the remaining 3 SNPs (rs3019885, rs987870, and rs2281389) in 2 independent replication cohorts comprising Asians (Japanese and Koreans), considering $P < 0.05$ as significant replication.

Of these 3 SNPs, significant associations were noted at rs987870 in both cohorts (Table 1). To merge the findings of these studies, we conducted meta-analysis with a fixed-effects model by using the Mantel–Haenszel method. As shown in Table 1, the Mantel–Haenszel P value of 2.3×10^{-10} was noted for rs987870 (OR = 1.40, confidence interval (CI) = 1.26–1.55).

HLA-DP association with pediatric asthma

The rs987870 is located between *HLA-DPA1* and *HLA-DPB1*. Genotype imputation using MACH [9] revealed association between asthma and the SNPs that were in strong LD with rs987870 (Figure 4, Table S2). Moreover, rs987870 C allele was in complete LD with *DPA1*0201* ($r^2 = 1$). We determined *HLA-DPA1* genotypes by using direct sequencing and MACH imputation of the data from 1135 cases and 2376 controls and found that *DPA1*0201* was strongly associated with pediatric asthma ($P = 5.2 \times 10^{-10}$, OR = 1.52, Table 2). Then, we determined the *HLA-DPB1* genotypes in 1135 cases and 2296 controls and found that *DPB1*0901* was associated with pediatric asthma ($P = 2.0 \times 10^{-7}$, OR = 1.49, Table 3). *DPB1*0901* was in strong LD with *DPA1*0201* and rs987870 C allele (*D* prime = 0.93). Because more than 90% of pediatric asthma patients were allergic to house dust mites, it is possible that the association was due to IgE reactivity (sensitization) against mites. We performed an association study for mite sensitization using independent adult subjects without allergic respiratory diseases such as asthma and perennial allergic rhinitis (367 subjects with house dust mite-specific IgE and 1633 subjects without mite-specific IgE). Subjects with house dust mite-specific IgE were non-allergic in terms of symptoms but possessed mite-specific IgE. Subjects without mite-specific IgE did not exhibit allergic symptoms. We did not find an association between rs987870 and mite sensitization ($P = 0.54$, OR = 1.07, Table S3).

Discussion

Our GWAS in Asian populations found HLA-DP as susceptibility gene for pediatric asthma. Majority of pediatric asthmas are atopic (i.e., familial tendency to produce IgE antibodies against common environmental allergens) and possess specific IgE against the house dust mite. Mite sensitization is more prevalent in Asia than in Europe and is observed in 39% of the general adult population in Japan [10]. High prevalence of mite sensitization in asthmatic children has also been reported in Taiwan, where 94.2% of children with asthma are sensitized against *Dermatophagoides pteronyssinus* [11]. However, only a small subset of subjects with house dust allergy develop asthma [12].

We performed an independent association study for mite sensitization in adult subjects without allergic respiratory diseases and did not find an association between rs987870 and mite sensitization without symptoms. If the relative risk for mite sensitization in the individuals carrying a putative risk allele was 1.4 and the allele frequency was 0.15 compared to that in individuals without the allele, the statistical power of the sample size for mite sensitization study was 0.92 at an alpha level of 0.05. These results suggested that *DPA1*0201* and *DPB1*0901* may be associated with asthma rather than IgE production against house dust mite.

The association signal was stretched in the region of *HLA-DPB2*, collagen, type XI, alpha 2 (*COL11A2*), and Retinoid X receptor beta (*RXRB*) (Figure 4). Because of LD in this region, it is difficult to specifically identify causative variants. *HLA-DPB2* is a pseudogene. *COL11A2* encodes a component of type XI collagen called the pro-alpha2(XI) chain. Mutations in *COL11A2* have been associated with non-syndromic deafness, otospondylomegaepiphyseal dysplasia, Weissenbacher-Zweymüller syndrome, and Stickler syndrome (OMIM ID *120290). RXRB belongs to the RXR family and is involved in mediating the effects of retinoic acid. RXRB forms a heterodimer with the retinoic acid receptor and thus preferentially increases its DNA binding and transcriptional activity at promoters containing retinoic acid [13]. All SNPs showing strong association with asthma ($P < 1 \times 10^{-10}$) were located in introns or intergenic regions. LD of these associated SNPs with rs987870 was not strong; therefore, it is likely that the functional effect is due to *DPA1*0201* and *DPB1*0901*.

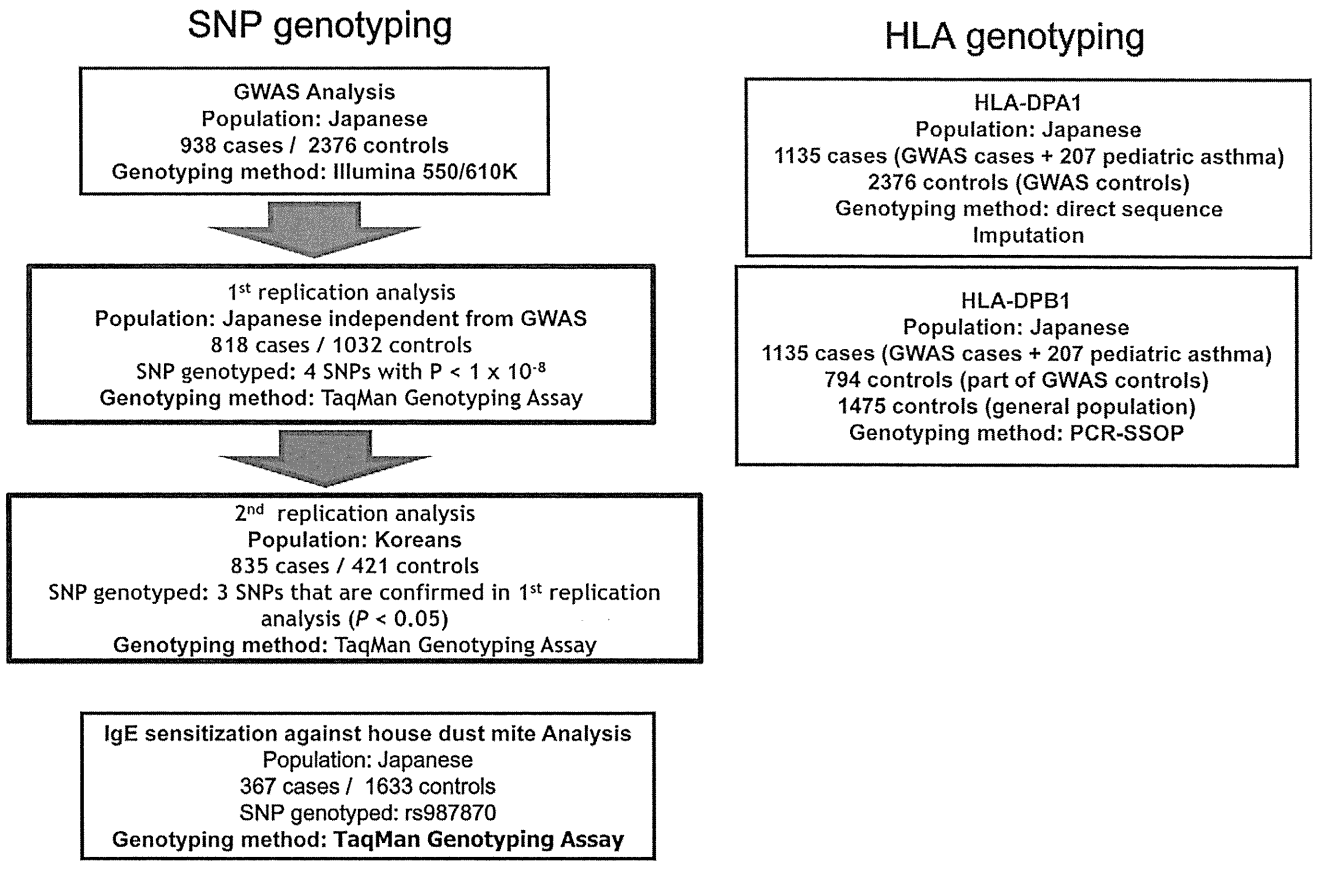

Figure 1. Flow chart of the present study.
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In HLA-DP, Caraballo *et al.* reported that *DPB1*0401* is significantly decreased in patients with allergic asthma in Mulatto population (an admixture population of European and African ancestries) [14]. Apart from the study of Caraballo *et al.*, the association between *HLA-DP* alleles and asthma was restricted to occupational [15] or aspirin-induced asthma [16]. Howell *et al.* reported associations between HLA-DR genotype and asthma and between *HLA-DPA1*0201* and IgE specific to grass pollen mix and the pollen allergen Phl p 5 [17]. Grass pollen allergy is not a major cause of asthma in Japan [18]; therefore, the *HLA-DPA1*0201* association in the present study was less likely to be due to sensitization to grass pollen.

*DPA1*0201* has also been reported to be positively associated with lower levels of rubella-induced antibodies [19], cytokine immune responses against measles vaccine [20], and ulcerative colitis [21], and negatively associated with type 1 diabetes [22].

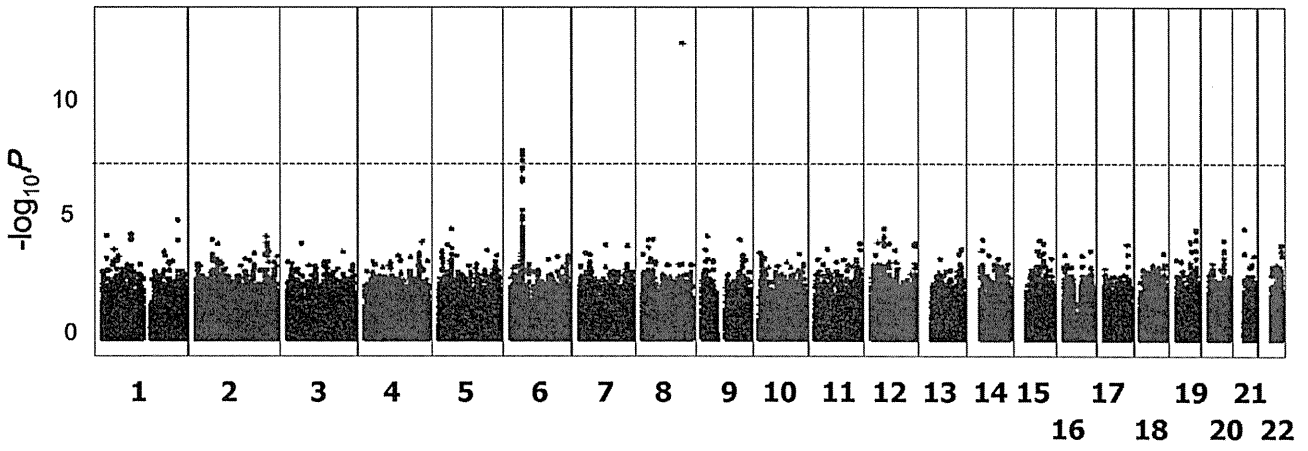

Figure 2. *P* values of GWAS. The Manhattan plot shows the Cochran–Armitage trend test *P* values for 938 cases of asthma and 2,376 controls; 450,326 autosomal SNPs were considered in the study. The dashed line indicates the genome-wide significance level ($P<5\times10^{-8}$).
doi:10.1371/journal.pgen.1002170.g002

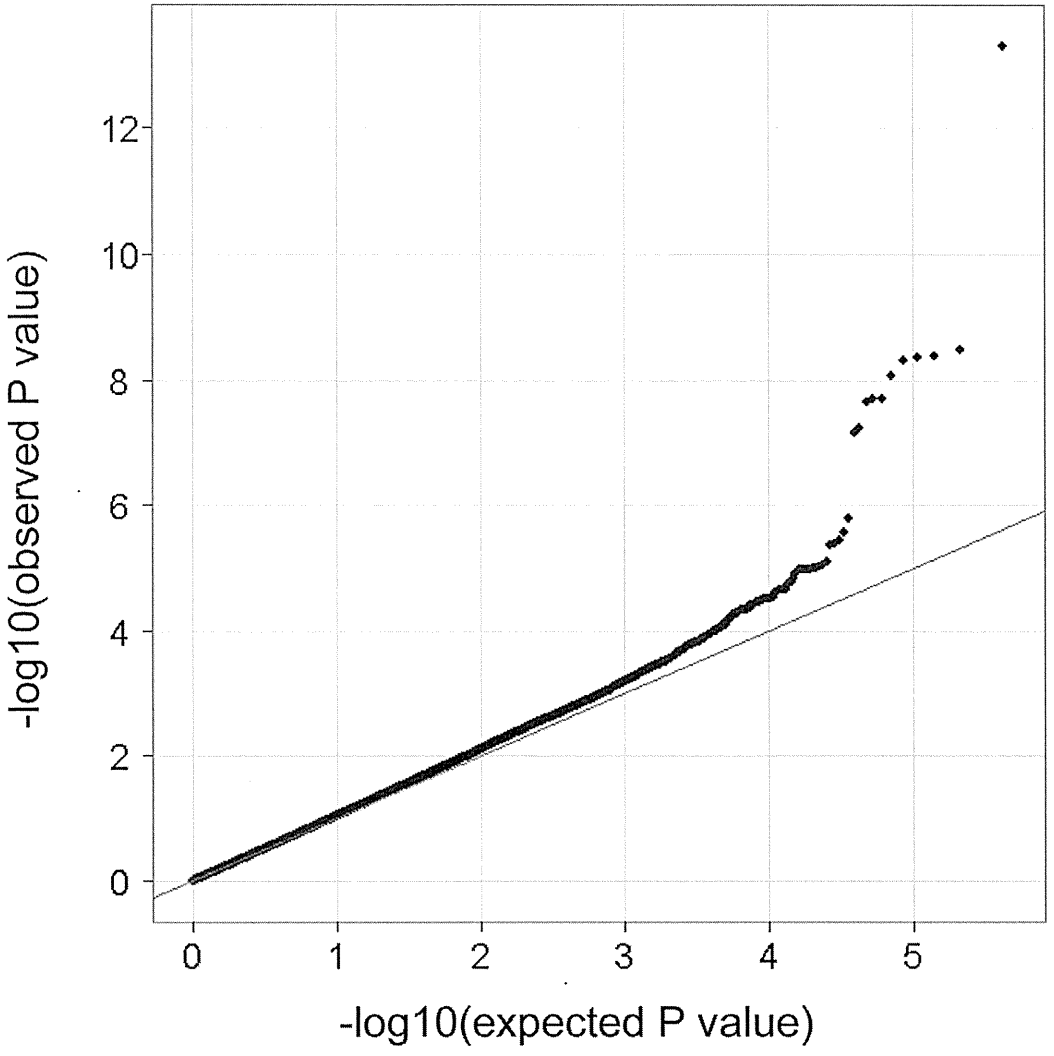

Figure 3. Quantile–quantile (Q–Q) plot of GWAS for pediatric asthma. The results of the Cochrane–Armitage trend *P* are plotted as dots and the line y = x is in red. The horizontal and vertical lines represent expected *P* values under null distribution and observed *P* values, respectively.
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*DPB1*0901* was shown to be associated with systemic sclerosis [23], non-permissive mismatches for hematologic stem cell transplantation [24], ulcerative colitis [21], and Takayasu's arteritis [25]. *HLA-DP* molecules present short peptides of largely exogenous origin to CD4-positive helper T cells and other T cells, leading to subsequent immunological responses. T cells recognize complex formation between a specific HLA type and a particular antigen-derived epitope. Therefore, HLA molecules capable of binding a particular epitope can restrict T cell induced-immune responses, leading to association between particular HLA types and immune-related diseases. Type 1 diabetes is a Th-1 type immune disease. Varney *et al.* studied 1,771 type 1 diabetes multiplex families, analyzing them by the affected family-based control method [26], and found that *DPA1*0201* has a protective effect on the development of type 1 diabetes (adjusted $P = 5 \times 10^{-4}$, OR 0.7) [22]. Epidemiologic studies have associated type 1 diabetes with lower prevalence of asthma and other allergic diseases [26,27]. Also, the previous GWAS of rheumatoid arthritis, other Th-1 type immune disease, has shown that rs987870 C allele confers protection against rheumatoid arthritis [28]. These findings suggest that *HLA-DPA1*0201* could determine Th1/Th2 dominance and could partially explain the inverse relationship between asthma and Th-1 type immune diseases.

Previous GWAS involving European, Mexican, and African populations showed association of asthma with SNPs located in several newly discovered genes. Our GWAS dataset supported an association between identical SNPs reported in *ORMDL3/GSDMB/GSDMA*, *IL5/RAD50/IL13*, *HLA-DR/DQ*, and *SMAD3* and pediatric asthma ($P<0.05$, Table S4). Two asthma GWA studies revealed an association of HLA-DQ with pediatric/adult asthma in Caucasians [7,8]. HLA-DQ, like HLA-DP, is an αβ heterodimer of the MHC Class II type. Like HLA-DP, HLA-DQ recognizes and presents foreign antigens, but is also involved in recognizing common self-antigens and presenting those antigens to the immune system.

We failed to replicate the top SNPs of *PDE4D*, *TLE4*, *DENND1B*, *IL18R1*, and *IL2RB* that were reported in the original articles, but several SNPs in the regions surrounding *PDE4D* and *IL2RB* showed significant association when we set the significance level at $P = 0.05$ (Table S4). The different LD patterns/allele frequencies observed in *PDE4D* and *IL2RB* in Asians and Caucasians may explain the different SNP associations observed

Table 1. Results of GWAS and replication studies for 4 SNPs.

SNP	Nearest Gene	Allele[a]	Samples	MAF (asthma)	MAF (control)	OR (95%CI)[b]	P^c	P^e
rs3019885	SLC30A8	T/G	GWAS	0.41	0.31	1.55(1.39–1.73)	1.3×10^{-14}	
			First replication (Japanese)	0.34	0.30	1.21(1.05–1.39)	8.7×10^{-3}	
			Second replication (Koreans)	0.27	0.26	1.075(0.88–1.31)	4.7×10^{-1}	
			Meta analysis (HM)[d]			1.34(1.24–1.45)	5.0×10^{-13}	0.0011
rs987870	HLA-DPB1	T/C	GWAS	0.19	0.14	1.51(1.31–1.74)	7.5×10^{-9}	
			First replication (Japanese)	0.17	0.14	1.26(1.05–1.50)	1.2×10^{-2}	
			Second replication (Koreans)	0.12	0.10	1.34(1.01–1.76)	4.1×10^{-2}	
			Meta analysis (HM)[d]			1.40(1.26–1.55)	2.3×10^{-10}	0.33
rs2281389	HLA-DPB1	T/C	GWAS	0.23	0.17	1.47(1.29–1.68)	8.5×10^{-9}	
			First replication (Japanese)	0.20	0.17	1.20(1.02–1.42)	2.9×10^{-2}	
			Second replication (Koreans)	0.08	0.08	1.085(0.80–1.48)	6.1×10^{-1}	
			Meta analysis (HM)[d]			1.33(1.20–1.47)	1.4×10^{-8}	0.076

[a]The former allele represents the major allele.
[b]Odds ratio and 95% confidence interval (CI) of minor allele.
[c]*P* values of allelic model.
[d]Meta-analysis using Mantel-Haenszel approach.
[e]*P* values for heterogeneity test.
doi:10.1371/journal.pgen.1002170.t001

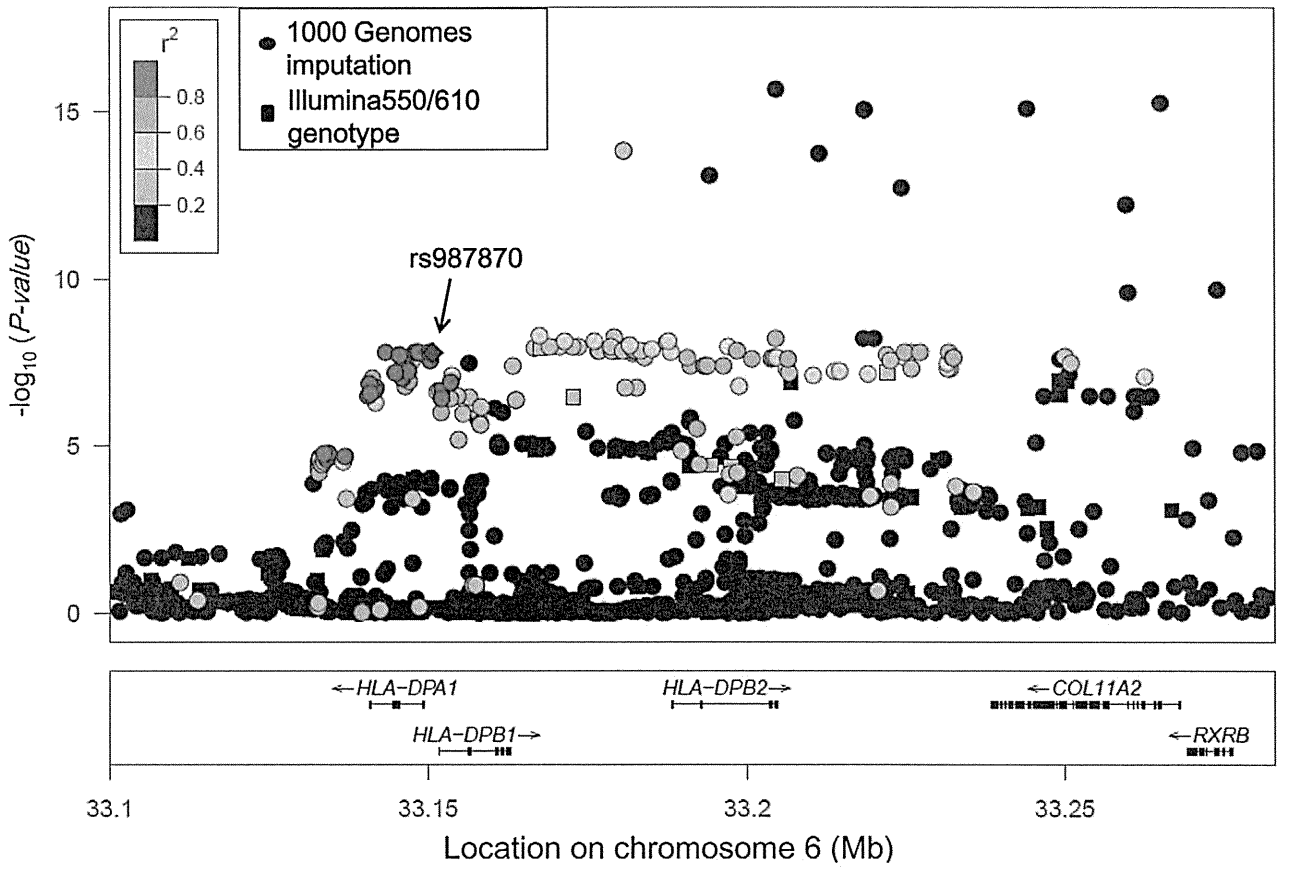

Figure 4. Association findings of genotyped (squares) and imputed (circles) SNPs in the *HLA-DP* region. SNP rs987870, which consistently showed an association with pediatric asthma in 3 independent populations, is located in the LD block between *HLA-DPA1* and *HLA-DPB1*. The color intensity of each symbol reflects the extent of LD with rs987870: from red ($r^2>0.8$) to blue ($r^2<0.2$). The physical positions are based on NCBI build 36 of the human genome.
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Table 2. HLA-DPA1-rs987870 Haplotype analysis of pediatric asthma.

DPA1	rs987870	asthma	control	Odds ratio (95%CI)	*P* values
*DPA1*0103*	T	858(38%)	1866(39%)	0.98 (0.85–1.05)	0.29
*DPA1*0201*	C	439 (19%)	650 (14%)	1.52 (1.33–1.74)	5.5×10^{-10}
*DPA1*0202*	T	957(42%)	2223(47%)	0.83 (0.75–0.92)	0.00046
*DPA1*0401*	T	3 (0.1%)	5 (0.1%)	1.26 (0.30–5.28)	0.75

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in different ethnic populations. rs1342326 in *IL33* was not polymorphic in the Asian population.

There were several limitations of the present GWAS. The controls for the GWAS and 1st replication samples were from adult populations. Information regarding history of asthma in early childhood or other asthma-related information (i.e., status of allergic sensitization and lung function) was not collected for these controls. Therefore, we cannot exclude the possibility that our control samples may include subjects who outgrew asthma. The prevalence of pediatric asthma in Japan is around 5%; therefore, our GWAS samples have reduced power compared with that of selected controls. In the 1st replication Japanese controls, subjects with present and past history of allergic diseases were excluded, and Korean controls in the 2nd replication were non-allergic pediatric controls (Table S5).

The genomic control value in the present study was 1.053, indicating minor population stratification. The Japanese population comprises 2 clusters (Hondo and Ryukyu; Hondo is the mainland of Japan and Ryukyu is the name of the island south of Japan). We performed principal component analysis using EIGENSTRAT software [29] to identify subjects belonging to Ryukyu. Because 2nd or 3rd generation Chinese live in Japan, and the genetic population structure in Chinese differs from that in Japanese, we also performed principal component analysis to exclude Chinese subjects. Although hidden population stratification may exist, its influence on the final results is not expected to be significant.

rs3019885 is located in intron 2 of solute carrier family 30 (SLC30A8), and showed strong association in the GWAS population. The association was replicated in the independent Japanese samples, but not in the Korean population. SLC30A8 is a zinc efflux transporter expressed at high levels only in the pancreas; the GWAS revealed that variants of *SLC30A8* are associated with type 2 diabetes [30]. Japanese and Koreans are genetically close but we cannot exclude the possibility that the association of rs3019885 with pediatric asthma is population specific.

In conclusion, we performed the first GWAS in Asian population for pediatric asthma and found that *DPA*0201/DPB1*0901* is strongly associated with pediatric asthma. The association with the HLA-DP locus emphasizes the importance of the HLA-class II molecules on the biological pathways involved in the etiology of pediatric asthma, and suggests that HLA-DP can be a therapeutic target for asthma.

Materials and Methods

Ethical statement

The study was approved by the institutional review board and the ethics committee of each institution. Written informed consent was obtained from each participant in accordance with institutional requirements and the Declaration of Helsinki Principles.

Subject participants

Characteristics of pediatric asthma cases and controls are summarized in Table S5.

GWAS population. All subjects with asthma were child or child-onset (<15 years old) asthmatics in Japan. Patients were recruited from 3 pediatric hospitals and 1 pediatric clinic, and the diagnosis of the asthma in all patients was confirmed by specialists in pediatric allergology on the basis of the criteria of the National Institutes of Health, USA, with minor modifications.

The control cases for the GWAS were healthy Japanese adult subjects from Osaka (n = 964), Tokyo (n = 660), and Ibaraki

Table 3. HLA-DPB1 allele frequency in pediatric asthma and controls.

Allele	Asthma	Control 1	Control 2	Asthma vs Control 1		Asthma vs Control 2		Asthma vs Control 1+2	
	n = 1135	n = 794	n = 1475	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)
DPB1*05:01	34.4%	36.5%	38.0%	0.18	0.91(0.80–1.04)	0.007	0.85(0.76–0.96)	0.013	0.87(0.79–0.97)
DPB1*02:01	22.4%	24.2%	24.3%	0.19	0.90(0.78–1.05)	0.11	0.89(0.79–1.02)	0.09	0.90(0.80–1.02)
DPB1*09:01	14.5%	10.1%	10.3%	5.5×10^{-5}	1.51(1.23–1.84)	3.4×10^{-6}	1.48(1.25–1.75)	2.0×10^{-7}	1.49(1.28–1.74)
DPB1*04:02	10.0%	9.2%	9.6%	0.38	1.10(0.89–1.37)	0.57	1.05(0.88–1.27)	0.43	1.07(0.90–1.27)
DPB1*04:01	4.8%	7.1%	5.0%	0.0019	0.65(0.50–0.86)	0.64	0.94(0.73–1.21)	0.08	0.82(0.65–1.03)
DPB1*03:01	4.9%	4.7%	4.0%	0.76	1.05(0.78–1.41)	0.11	1.24(0.95–1.62)	0.21	1.17(0.92–1.48)
DPB1*02:02	3.7%	3.2%	3.4%	0.46	1.14(0.80–1.63)	0.61	1.08(0.80–1.45)	0.49	1.10(0.84–1.45)
DPB1*13:01	1.6%	1.5%	2.1%	0.85	1.05(0.62–1.77)	0.18	0.75(0.50–1.14)	0.38	0.84(0.57–1.24)
DPB1*14:01	1.8%	1.7%	1.4%	0.88	1.04(0.63–1.70)	0.258	1.28(0.83–2.01)	0.39	1.19(0.80–1.76)

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(n = 778) who had no current history of asthma [31]. In the GWAS, we genotyped 978 cases with pediatric asthma and 2402 controls using Illumina HumanHap550v3/610-Quad Genotyping BeadChip (Illumina, San Diego, USA). Subjects from Osaka and Ibaraki were randomly selected from residents of Suita city and Tone town, respectively. Subjects from Tokyo were hospital workers from Keio University Hospital, Tokyo. We excluded samples considered duplicated, related (first- or second-degree relatives), or belonging to Han Chinese or Ryukyu. In total, 938 cases and 2376 controls were considered for further analysis.

First replication population (Japanese). We recruited 818 subjects with childhood atopic asthma from the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Dokkyo University School of Medicine, National Research Institute for Child Health & Development, National Sagamihara Hospital, and Chiba University Hospital. All subjects with bronchial asthma were diagnosed according to the criteria of the National Institutes of Health (National Heart, Lung, and Blood Institute, National Institutes of Health, 1991) by physicians who were asthma specialists [32,33]. After the exclusion of individuals who had been diagnosed with asthma, atopic dermatitis, or nasal allergies by physicians' interviews, 825 healthy individuals were recruited from the Midousuji Rotary Club [32,33]. Two hundred and seven control subjects who never had the symptoms of allergic rhinitis/asthma and did not show any sensitization to 7 common aeroallergens were recruited from Fukui [34].

Second replication population (Korean). Patients with pediatric asthma were enrolled at Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea. The control subjects were age-matched children with no history of asthma or other allergic diseases, negative skin prick test, and normal total IgE values (<100 IU/mL) recruited from the same district (Seoul). Total of 835 cases and 421 controls participated in this study. The details of the patients and controls were described in a previous study [35].

Subjects for IgE sensitization against house dust mite. General populations for mite sensitivity study were recruited from Fukui [10] and Tsukuba in Japan. Total and specific IgE levels (produced in response to Japanese cedar, *Dermatophagoides*, *Dactylis glomerata*, *Ambrosia artemisiifolia*, *Candida albicans*, and *Aspergillus*) were measured using the CAP-RAST method (for Fukui samples; Pharmacia Diagnostics AB, Uppsala, Sweden) or MAST-26 (for Tsukuba samples; Hitachi Chemical Co. Ltd., Tokyo, Japan). Positive sensitization against house dust mite was defined as specific IgE levels against the house dust mite (*Dermatophagoides farinae* or *Dermatophagoides pteronyssinus*) greater than or equal to 0.70 IU/ml (class 2) or lumicount greater than 2.76 (class 2). Subjects with asthma (current or past) or perennial allergic rhinitis were excluded from the analysis. Sensitized subjects (Mite-positive) were non-allergic in terms of symptoms but possessed mite-specific IgE. Non-sensitized subjects (Mite-negative) did not show any allergic symptoms and did not have mite-specific IgE.

Subjects for *HLA-DPA1* typing. Cases with asthma included 938 subjects used in GWAS analysis and 207 Japanese subjects with child- or child-onset (<15 years) asthmatics recruited in Tsukuba. The diagnosis of asthma in all patients was confirmed by specialists in pediatric allergology on the basis of the criteria of the National Institutes of Health, USA, with minor modifications. The control subjects were 2378 subjects that were used in GWAS analysis. Because most of the DNA from the GWAS controls was not available for genotyping, and we found that imputation of the *HLA-DPA1* allele using GWAS results was highly accurate (error rate, 0.003), we decided to genotype the *HLA-DPA1* allele by direct sequencing and imputation. Among the subjects for *HLA-DPA1* genotyping (1135 cases and 2376 controls), genotyping of 383 subjects was performed by direct sequencing and genotyping of the remaining 3128 samples was performed by imputation.

Subjects for *HLA-DPB1* typing. Cases with asthma included 938 subjects used in GWAS analysis and 207 Japanese subjects with child- or child-onset (<15 years) asthmatics; the same as those used in *HLA-DPA1* typing. The control 1 subjects for *HLA-DPB1* typing were 794 healthy adult subjects from Tokyo and 399 subjects were the same as those in GWAS. The control 2 subjects (n = 1475) were general datasets from Japanese population samples publically available at http://www.hla.or.jp/hapro/top.html. Because most of the DNA from the GWAS controls was not available for genotyping, and the imputation of the DPB1 allele using the GWAS results was not possible, we used 794 healthy adult subjects from Tokyo and 399 subjects from the GWAS for *DPB1* genotyping (Control 1). The control 2 subjects (n = 1475) were general datasets from Japanese population samples publically available at http://www.hla.or.jp/hapro/top.html. The status of asthma or other allergic diseases for these samples is not available.

Genotyping

Genotyping for GWAS was performed using the Illumina HumanHap550v3/610-Quad Genotyping BeadChip (Illumina), as per manufacturer's instruction.

In replication analyses, genotyping of each individual was performed with the TaqMan genotyping system (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). PCR was performed on a 384-well format, and automatic allele calling was performed using ABI PRISM 7900HT data collection and analysis software, version 2.2.2 (Applied Biosystems).

HLA-DPB1 genotyping of 1135 cases, 794 controls (control 1) and 1475 controls (control 2) were performed with the WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan), as per manufacturer's instruction. First, the target DNA was amplified by polymerase chain reaction (PCR) with biotinylated primers specifically designed for each HLA-DPB1 locus. Then, the PCR product was denatured and hybridized to complementary oligonucleotide probes immobilized on fluorescent-coded microsphere beads. Concurrently, the biotinylated PCR product was labeled with phycoerythrin-conjugated streptavidin and immediately examined with the Luminex 100 system (Luminex, Austin, TX). Genotype determination and data analysis were performed with the WAKFlow typing software (Wakunaga).

HLA-DPA1 genotyping was performed with direct sequencing of exon 2 with forward primer 5′-TCAGGATGCCCAGACTTTCAA-3′ and reverse primer 5′-CAGGGGGCACTTAGGCTTCC-3′, and with the sequencing primer 5′-TCAGGATGCCCAGACTTTCAA-3′ using the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Statistical analysis

In the GWAS, we examined the potential genetic relatedness on the basis of pairwise identity by state for all of the successfully genotyped samples using the EIGENSTRAT software [29]. In the GWAS, we genotyped 978 cases with pediatric asthma and 2402 controls using Illumina HumanHap550v3/610-Quad Genotyping BeadChip (Illumina, San Diego, USA). Samples of duplicated (identical individual or monozygotic twin), first-, second-, and third-degree pairs were detected, and the individual with a lower call rate was excluded from further analysis. PCA was performed, and the results were combined with those obtained for our in-