TABLE III. Haplotype frequency in *NLRP3*

	rs4612666	rs10754558	No. Case	No. Control	Frequency Case	Frequency Control
Haplotype_1	C (susceptible)	G (susceptible)	89	130	0.45	0.30
Haplotype_2	T (nonsusceptible)	G (susceptible)	11	27	0.06	0.06
Haplotype_3	C (susceptible)	C (nonsusceptible)	39	92	0.20	0.21
Haplotype_4	T (nonsusceptible)	C (nonsusceptible)	56	181	0.29	0.42
	***P* value**	**OR**	**95% CI**			
Haplotype_1 vs haplotype_4	.000098	2.21	1.48-3.31			

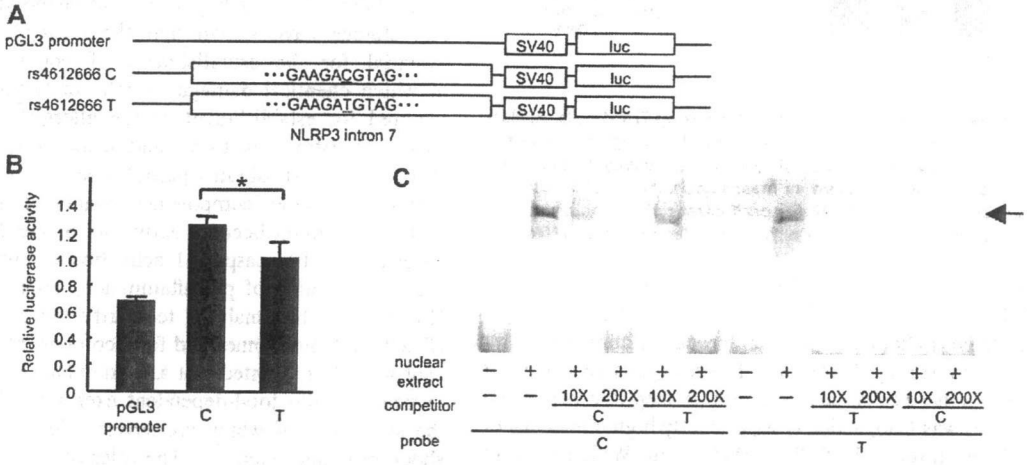

FIG 2. Functional analyses of *NLRP3* rs4612666 using THP-1 cells. **A**, Plasmid constructs used for transfection. **B**, Transcriptional enhancer activities of rs4612666 measured by luciferase *(luc)* activity 24 hours after transfection. Values of relative luciferase activity are shown as means±SDs. **P* < .05, Student *t* test. **C**, Electrophoretic mobility shift assays of rs4612666. An unlabeled probe was used as a competitor. C, C allele; T, T allele.

Influence of rs4612666 on differential expression of *NLRP3* mRNA

We next conducted functional analysis of the rs4612666 SNP. Because rs4612666 was located in intron 7, we examined whether the genomic region around rs4612666 had enhancer activity. At first, to survey the *NLRP3* mRNA expression levels in cells and tissues, we conducted real-time quantitative RT-PCR. *NLRP3* mRNA was dominantly expressed in peripheral leukocytes (see Fig E1*A*, in this article's Online Repository at www.jacionline.org), and among the cell lines of leukocytes, higher expression of *NLRP3* mRNA was detected in the monocyte cell line THP-1 (see Fig E1*B*). We subsequently performed a luciferase assay to test whether the allelic difference contributed to the efficiency of expression of *NLRP3* mRNA using THP-1 cells. The allele-specific constructs containing the food-induced anaphylaxis risk allele rs4612666 showed 1.2-fold higher transcriptional enhancer activity than the other constructs containing the T allele of rs4612666 (Fig 2, *A* and *B*). These experiments were performed 4 times with similar results. We next searched for nuclear transcription factors that might bind to oligonucleotide sequences containing rs4612666 by using TRANSFAC and TFSEARCH, and the genomic region containing the C allele of rs4612666 was found to create a novel consensus sequence corresponding to the putative binding element of GATA-2. Therefore we examined the allelic differences in the binding of nuclear proteins by using the electrophoretic mobility shift assay. The signal intensity of the DNA-protein complex derived from the C allele was higher than that from the T allele in the presence of THP-1 nuclear extract, and the complex was diminished by excess amounts of a nonlabeled allele-specific competitor probe (Fig 2, *C*). Four independent experiments were performed with similar results. However, the band was not supershifted by the addition of antibodies to GATA-2 in the present study (see Fig E2 in this article's Online Repository at www.jacionline.org).

Contribution of the 3′ untranslated region rs10754558 SNP to *NLRP3* mRNA stability

Like the *NLRP3* rs4612666 in intron 7, rs10754558 was significantly associated with susceptibility to food-induced anaphylaxis. Because of the location of rs10754558 in the 3' untranslated region, we further examined whether the risk allele of rs10754558 affected the stability of the *NLRP3* mRNA. RNA secondary structure prediction with the mfold Web server showed that the risk allele contributed to the mRNA stability more than the other allele of rs10754558 (see Fig E3 in this article's Online Repository at www.jacionline.org). First, we performed a luciferase assay using THP-1 cells. The allele-specific construct containing the G allele of rs10754558 showed 1.3-fold higher activity than the other constructs containing the C allele of rs10754558 (Fig 3, *A* and *B*). These experiments were performed 4 times

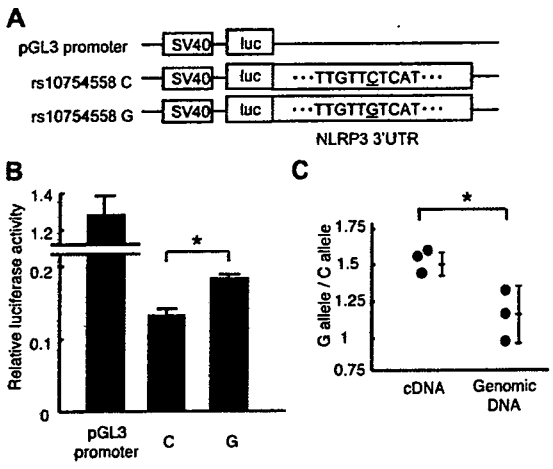

FIG 3. Functional analyses of *NLRP3* rs10754558 using THP-1 cells. **A**, Plasmid constructs used for transfection. *UTR*, Untranslated region. **B**, Effect of the SNP on mRNA stability as measured by luciferase activity. Values of relative luciferase activity are shown as means±SDs. *$P < .0005$, Student *t* test). *C*, C allele; *G*, G allele. **C**, Allele-specific transcript quantification of rs10754558 is shown as means ± SDs. *$P < .05$, Mann-Whitney *U* test.

with similar results. To further investigate the effect of rs10754558 on transcription of mRNA, we performed allele-specific quantitative PCR with a TaqMan probe on human primary monocytes from healthy donors with heterozygous genotypes of rs10754558. In these cells the mean ratio (susceptible vs nonsusceptible allele) was 1.50, which is significantly higher than that of DNA amplicons (ratio = 1.15; $P = .0495$, Mann-Whitney *U* test; Fig 3, *C*). Three independent experiments were performed with similar results. These results indicated that the higher expression of *NLRP3* mRNA was a component of the pathologic mechanisms leading to food-induced anaphylaxis.

DISCUSSION

The common feature of food-induced anaphylaxis and AIA is the immediate hypersensitivity reaction. In the present study we identified significant associations between human *NLRP3* polymorphisms and susceptibility to food-induced anaphylaxis and AIA. Because the 2 *NLRP3* SNPs rs4612666 and rs10754558 were not in strong LD (r^2<0.20), it is possible that the SNPs could contribute susceptibility to food-induced anaphylaxis independently. We further found a significant association between AIA and the rs4612666 variant, and the direction of association was similar to the finding in food-induced anaphylaxis. Functional analyses of the 2 related *NLRP3* polymorphisms showed that both variants influenced higher mRNA expression by altering expression enhancer activity or mRNA stability. These observations suggest that human NLRP3 appears to be involved in the hypersensitive immune reaction in allergy through gain-of-function variants.

Several recent studies have shown that nonsynonymous substitutions of *NLRP3* are associated with rare, severe autoinflammatory diseases, such as familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and chronic infantile neurological, cutaneous, and articular syndrome.[19] However, these nonsynonymous substitutions and polymorphisms were not in LD with either of the SNPs associated with susceptibility to food-induced anaphylaxis and AIA in the present study ($r^2 < 0.20$).

In this study the 2 *NLRP3* SNPs associated with food-induced anaphylaxis did not show any association with susceptibility to food allergy. Food-induced allergic reactions exhibit various symptoms, ranging from localized urticaria to severe life-threatening anaphylaxis. In subjects with anaphylaxis caused by insect stings or food, many recent studies have shown no clear relationship between the levels of allergen-specific IgE and the presence, absence, or severity of the clinical response to the allergen.[16] Different genetic factors might be involved in the diverse immunologic responses to foods, and innate immune activation through NLRP3 inflammasomes sensing food components might be one of the immunologic mechanisms in anaphylaxis.

In the present study we found *NLRP3* polymorphisms that increased the risk of the hypersensitive phenotype of allergy. Murine studies have shown that the NLRP3 inflammasome is essential for the establishment of contact hypersensitivity, in which chemical damage to cells or tissues by a hapten is required for establishment of the allergy.[22,30] Recent reports have demonstrated that uric acid, calcium pyrophosphate dehydrate, silica, and asbestos particles activate the NLRP3 inflammasome.[31] Some components contained in food or food ingredients might become activators of the NLRP3 inflammasome and lead to caspase-1 activation to promote the processing and secretion of proinflammatory inflammatory cytokines. However, further analyses to clarify the relationship between NLRP3 inflammasomes and food components are needed. A recent study has reported that aspirin enhances allergic symptoms in patients with food-dependent exercise-induced anaphylaxis, the symptoms of which are severe allergic reactions, such as shock or hypotension.[32,33] The roles of aspirin and nonsteroidal anti-inflammatory drugs in the NLRP3 inflammasome response also remain unexplored.

NLRP3 is in the NLR family of proteins, and other NLR family genes have been shown to be associated with susceptibility to various inflammatory diseases: polymorphisms of nucleotide-binding oligomerization domain containing 1 *(NOD1)* with asthma[34] and inflammatory bowel disease,[35] nucleotide-binding oligomerization domain containing 2 *(NOD2)* with Crohn disease,[36] and *NLRP1* with vitiligo-associated multiple autoimmune disease.[37] Genetic studies on whether the polymorphisms of other NLR family genes are associated with food allergy, food-induced anaphylaxis, and AIA susceptibility remain to be conducted.

Further investigation of the roles of NALP3 inflammasomes in food-induced anaphylaxis and AIA might contribute to our understanding of the pathophysiology of these severe and potentially life-threatening systemic allergic reactions and to the development of novel diagnostic methods for risk assessment of patients with anaphylaxis or AIA.

We thank all the participants in the study. We also thank Dr Akari Suzuki for valuable suggestions and Makiko Shimizu-Terada, Hiroshi Sekiguchi, Nami Kawaraichi, and Aya Jodo-Ito for technical assistance.

Key messages

- Gain-of-function variants of the *NLRP3* gene are associated with food-induced anaphylaxis and AIA.
- The NLRP3 inflammasome might play an important role in the hypersensitivity phenotype of allergy.

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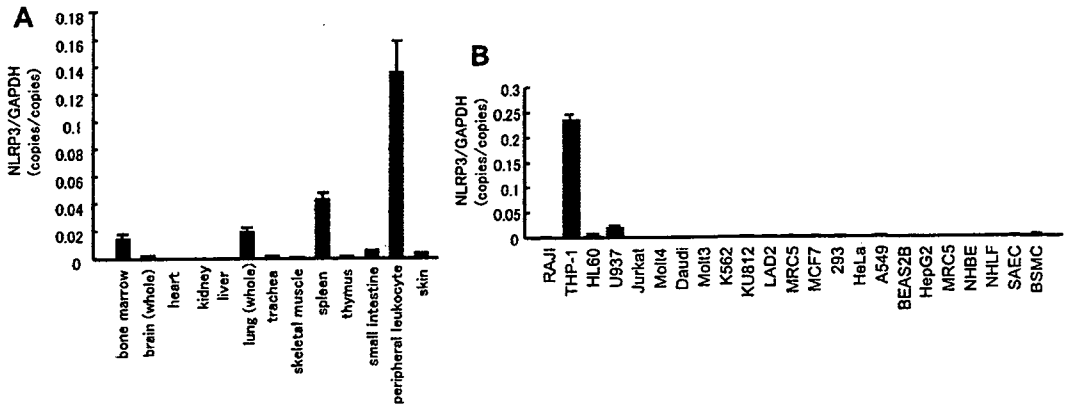

FIG E1. Comparison of relative mRNA expression of *NLRP3* in different tissues **(A)** and cell lines **(B)**. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* transcripts. *NHBE,* Normal human bronchial epithelial cells; *NHLF,* normal human lung fibroblasts; *SAEC,* normal human small airway epithelial cells; *BSMC,* bronchial smooth muscle cells. Results are means ± SDs of triplicate assays.

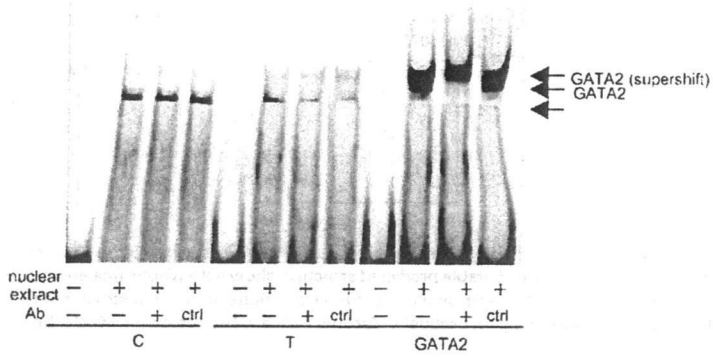

FIG E2. Electrophoretic mobility shift assay of rs4612666. Normal rabbit IgG and a GATA-2 consensus probe were used for control. Two independent experiments were performed with similar results. *Ab*, Antibody; *C*, C allele; *T*, T allele.

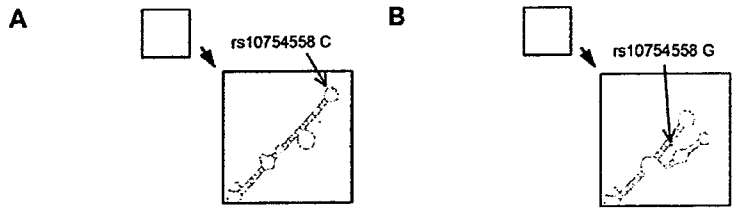

FIG E3. Prediction of the RNA secondary structure. Possible conformations of rs10754558-C **(A)** and rs10754558-G **(B)** and the most stable predicted structure show a dG (Gibbs free energy), which is a free energy increment related to the permissible structural transitions from the unstructured to structured state of −130.3 and −133.1 kcal/mol, respectively. *Arrows* indicate the region in which the rs10754558 SNP is located.

TABLE E1. Clinical information about patients with AIA and control subjects

Characteristics	Patients with bronchial asthma		Control subjects, n = 730 (%)
	AIA (+), n = 79 (%)	AIA (−), n = 470 (%)	
Age (y)	37.7 ± 14.2	37.0 ± 19.8	49.4 ± 14.5
Sex			
Male	25 (31.6)	205 (43.6)	532 (72.9)
Female	54 (68.4)	265 (56.4)	198 (27.1)
Log serum total IgE (IU/mL)	2.3 ± 0.5	2.3 ± 0.6	—

TABLE E2. *NLRP3* polymorphisms and susceptibility to food allergy

			Patients with food allergy, n = 320 (%)			Control subjects, n = 254 (%)			Frequency of allele 1		
Allele 1/2	dbSNP ID	Location	1/1	1/2	2/2	1/1	1/2	2/2	Case	Control	*P* value
−3656 C/T	rs2027432	5′ Flanking	247 (77.2)	69 (21.6)	4 (1.3)	197 (77.6)	53 (20.9)	4 (1.6)	0.88	0.88	NS
−2189 G/A	rs12079994	Intron 1	225 (71.2)	79 (25.0)	12 (3.8)	192 (75.6)	56 (22.0)	6 (2.4)	0.84	0.87	NS
−1529 C/T	rs4925648	Intron 1	247 (77.2)	71 (22.2)	2 (0.6)	193 (76.0)	56 (22.0)	5 (2.0)	0.88	0.87	NS
1979 G/A	rs4925650	Intron 3	89 (27.8)	169 (52.8)	62 (19.4)	68 (26.8)	123 (48.4)	63 (24.8)	0.54	0.51	NS
2495 A/G	rs12048215	Intron 3	181 (56.6)	124 (38.8)	15 (4.7)	148 (58.5)	88 (34.8)	17 (6.7)	0.76	0.76	NS
2547 C/G	rs10754555	Intron 3	119 (37.4)	162 (50.9)	37 (11.6)	101 (39.9)	120 (47.4)	32 (12.6)	0.63	0.64	NS
4240 T/C	rs3806265	Intron 3	105 (33.2)	158 (50.0)	53 (16.8)	95 (37.4)	121 (47.6)	38 (15.0)	0.58	0.61	NS
13754 C/T	rs10925019	Intron 6	163 (50.9)	131 (40.9)	26 (8.1)	145 (57.1)	89 (35.0)	20 (7.9)	0.71	0.75	NS
13961 G/A	rs4925654	Intron 6	211 (65.9)	96 (30.0)	13 (4.1)	154 (60.6)	87 (34.3)	13 (5.1)	0.81	0.78	NS
16974 C/T	rs4612666	Intron 7	100 (31.5)	155 (48.9)	62 (19.6)	95 (37.5)	119 (47.0)	39 (15.4)	0.56	0.61	NS
22162 A/C	rs10925026	Intron 8	110 (34.6)	160 (50.3)	48 (15.1)	100 (39.7)	106 (42.1)	46 (18.3)	0.60	0.61	NS
27232 C/T	rs12565738	Intron 10	257 (80.3)	59 (18.4)	4 (1.3)	197 (78.2)	55 (21.8)	0 (0.0)	0.90	0.89	NS
29231 T/C	rs4378247	Intron 10	265 (82.8)	53 (16.6)	2 (0.6)	206 (81.1)	47 (18.5)	1 (0.4)	0.91	0.90	NS
29940 C/G	rs10754558	Exon 11 (3′UTR)	108 (34.3)	155 (49.2)	52 (16.5)	92 (36.4)	114 (45.1)	47 (18.6)	0.59	0.59	NS
31792 C/T	rs10733112	3′ Flanking	81 (25.3)	168 (52.5)	71 (22.2)	73 (28.7)	118 (46.5)	63 (24.8)	0.52	0.52	NS

NS, Not significant; *UTR*, untranslated region.

TABLE E3. Association between *NLRP3* polymorphisms and susceptibility to AIA

	Allele 1/2	dbSNP ID	Location	AIA (+), n = 79 (%)			AIA (−), n = 470 (%)			Control, n = 730 (%)		
				1/1	1/2	2/2	1/1	1/2	2/2	1/1	1/2	2/2
1	1979 G/A	rs4925650	Intron 3	22 (27.8)	38 (48.1)	19 (24.1)	108 (23.3)	240 (51.8)	115 (24.8)	204 (28.1)	346 (47.6)	177 (24.3)
2	4240 T/C	rs3806265	Intron 3	36 (45.6)	34 (43.0)	9 (11.4)	174 (38.1)	209 (45.7)	74 (16.2)	290 (39.9)	331 (45.5)	106 (14.6)
3	16974 C/T	rs4612666	Intron 7	41 (51.9)	30 (38.0)	8 (10.1)	174 (37.7)	215 (46.6)	72 (15.6)	268 (36.9)	341 (46.9)	118 (16.2)
4	29940 C/G	rs10754558	Exon 11 (3'UTR)	22 (28.2)	35 (44.9)	21 (26.9)	146 (31.4)	231 (49.7)	88 (18.9)	229 (31.5)	360 (49.5)	139 (19.1)
5	31792 C/T	rs10733112	3' Flanking	32 (41.0)	30 (38.5)	16 (20.5)	136 (29.5)	218 (47.3)	107 (23.2)	211 (29.0)	346 (47.6)	170 (23.4)

	Frequency of allele 1			AIA (+) vs AIA (−)				AIA (+) vs control			
	AIA (+)	AIA (−)	Control	*P* value	OR	95% CI		*P* value	OR	95% CI	
1	0.52	0.49	0.52	NS				NS			
2	0.67	0.61	0.63	NS				NS			
3	0.71	0.61	0.60	.018	1.55	1.08	2.24	.0096	1.60	1.12	2.29
4	0.51	0.56	0.56	NS				NS			
5	0.60	0.53	0.53	NS				NS			

UTR, Untranslated region; *NS*, not significant.

17

Large scale genotyping study for asthma in the Japanese population.

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Short Report

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Large scale genotyping study for asthma in the Japanese population

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Abstract

Background: Asthma is a complex phenotype that is influenced by both genetic and environmental factors. Genome-wide linkage and association studies have been performed to identify susceptibility genes for asthma. These studies identified new genes and pathways implicated in this disease, many of which were previously unknown.

Objective: To perform a large-scale genotyping study to identify asthma-susceptibility genes in the Japanese population.

Methods: We performed a large-scale, three-stage association study on 288 atopic asthmatics and 1032 controls, by using multiplex PCR-Invader assay methods at 82,935 single nucleotide polymorphisms (SNPs) (1st stage). SNPs that were strongly associated with asthma were further genotyped in samples from asthmatic families (216 families, 762 members, 2nd stage), 541 independent patients, and 744 controls (3rd stage).

Results: SNPs located in the 5' region of *PEX19* (rs2820421) were significantly associated with $P < 0.05$ through the 1st to the 3rd stage analyses; however, the *P* values did not reach statistically significant levels (combined, $P = 3.8 \times 10^{-5}$; statistically significant levels with Bonferroni correction, $P = 6.57 \times 10^{-7}$). SNPs on *HPCAL1* (rs3771140) and on *IL18R1* (rs3213733) were associated with asthma in the 1st and 2nd stage analyses, but the associations were not observed in the 3rd stage analysis.

Conclusion: No association attained genome-wide significance, but several loci for possible association emerged. Future studies are required to validate these results for the prevention and treatment of asthma.

Findings

Asthma is the most common chronic disorder in children, and asthma exacerbation is an important cause of childhood morbidity and hospitalization. Currently, approximately 300 million people worldwide have asthma, and this disease claims the lives of 180,000 people every year [1].

Asthma and atopy are complex phenotypes that are influenced by both genetic and environmental factors. Twin studies have supported the role of a strong genetic contribution with a heritability of 0.71 [2], and asthma shows a familial risk higher than that of many common diseases such as stroke, epilepsy, and most types of cancer [3]. Atopy is characterized by increased levels of immunoglobulin E (IgE) against common environmental allergens, and is considered the strongest predisposing factor for asthma. Majority of children with asthma develop specific IgE against house dust mites, and dust mite allergy is strongly associated with asthma [4,5], however, only a small subset of subjects with dust mite allergy develops asthma [6]. These data suggest that other factors are involved in the development of asthma, and genome-wide linkage and association studies have been used to find novel asthma genes and their associated pathways [7]. Using family and case-referent panels of European populations and based on the genome-wide association study conducted on asthma, Moffatt *et al.* identified that the cis-acting single nucleotide polymorphisms (SNPs) in *ORMDL3* were associated with asthma [8], and the results were replicated in independent populations [9].

In order to identify novel asthma susceptibility genes, we performed a large-scale, 3-stage association study using the Japanese population. No association attained genome-wide significance, but several loci for possible association emerged. Further studies are required to validate these results in the future.

Subjects and genotyping results

The childhood asthmatics in the case-control study (1st and 3rd stage analysis) were atopic asthmatic children diagnosed by pediatricians on the basis of clinical examination. Probands of the asthmatic families (2nd stage) were atopic asthmatic children who visited the Pediatric Allergy Clinic of the University Hospital of Tsukuba. Two hundred and sixteen families (762 members), provided informed consent and participated in this study. The clinical details of the families are shown in Table 1. The criteria used for the diagnosis of asthma in case-control study and families were the same, and have been previously described [10]. The control group comprised 1032 adult Japanese individuals from the general population (1st stage) and 744 healthy adults (ages, 19–78 years, mean 46.2 years) with no history of any allergic disease (3rd stage). Cases and controls as well as asthmatic families were recruited from the mainland of Japan. This study was approved by the Committee of Ethics of the University of Tsukuba. The details of the study populations are shown in Table 2.

Table 1: Clinical details of the asthmatic families

No. of Families	216
No. of children	346
No. of affected children	315
Mean age (yeas ± SD)	10.9 ± 2.4
Male:Female ratio	1.8:1
Log(total IgE) (IU/ml ± SD)	2.8 ± 0.6
No. of parents	416
Mean age (yeas ± SD)	40.7 ± 7.5
Log(total IgE) (IU/ml ± SD)	2.0 ± 0.7

Large-scale genotyping using 82,935 randomly selected gene-based SNPs was carried out using the high-throughput multiplex PCR-Invader assay method as described previously [11]. The population frequency of asthma in Japan was 0.065 [12], and the statistical power of the 1st stage analysis was 0.93 and 0.44 at the alpha level of 0.001 and 0.000001, respectively if the relative risk for asthma in those persons carrying a putative risk allele is 2 and the high risk allele frequency is 0.3 compared with that in persons without the allele. Therefore, our sample size may not be enough to detect a low risk allele.

The SNPs genotyped in the 1st stage analysis were those identified in the JSNP project [11]. There are 2 approaches to the construction of SNP databases: one is genome-wide screening, and the other is gene-based screening. Although SNPs around genes are likely to be functional SNPs, it should be noted that SNPs outside genes have also been found to be associated with diseases.

In the present study, the distribution of allelic frequencies was largely even, with an average minor allele frequency of 24%. The SNPs with problematic genotyping in the 1st stage were flagged, and we excluded these SNPs from the analysis (n = 4683). Moreover, Hardy-Weinberg equilibrium was calculated using the χ^2 test with 2 degrees of freedom on the basis of the observed and expected genotype frequencies; SNPs with $P < 0.001$ (n = 2160) were excluded from the analysis. After the exclusions, 76,092 autosomal SNPs were available for analysis. The significance of the differences in the allele frequencies in case-control comparisons was determined by the χ^2 test with 1 degree of freedom. The distribution of the observed P values was as follows: $P < 0.0001$, 20 SNPs (0.0263%); $P < 0.001$, 146 SNPs (0.192%); and $P < 0.01$, 1111 SNPs (1.46%). The genomic inflation factor of the study population was calculated using the method described by Devlin et al [13] and was found to be 1.13. A previous study

Table 2: Study design

	Case	Control	Analysis	No of SNPs
1st stage	Childhood atopic Asthma: 288	General Japanese population: 1032	χ^2 test (allelic)	82,935
2nd stage	Family with childhood atopic asthma (216 families, 762 members)		PDT	125
3rd stage	Childhood atopic Asthma: 541	Non atopic control: 752	χ^2 test (allelic)	3

has reported that the inclusion of different proportions of individuals from different regions of Japan in case and control groups can lead to an exaggerated number of false-positive results when the sample sizes are large, and it has recommended the exclusion of subjects belonging to the Ryukyu (southern island of Japan) cluster [14]. The patients of the present study were from the Kanto and Kinki regions, and the controls were from the Kinki region alone. On the basis of the results of a previous simulation study, we can state that the subjects from the Kanto region are not genetically different from those from the Kinki region [14]; moreover, we did not include cases or controls from the Ryukyu island. However, we cannot exclude the possibility that population stratification exists in our case-control samples.

We used a cut-off *P* value of 0.002 (corrected $P = 0.0036$) and minor allele frequency of 0.2 for allelic association for the 1st stage analysis. There were 262 SNPs with *P* values < 0.002, and among them, 138 SNPs had minor allele frequencies > 0.2. We further chose 125 SNPs that were not in tightly linked with other SNPs for the 2nd stage analysis. SNP typing for 2nd and 3rd stages was performed using the TaqMan Assay-on-Demand™ and Assay-by-Design SNP Assay Systems (Applied Biosystems, Foster City, CA) as per the manufacture's instructions. The pedigree disequilibrium test (PDT) [15] for the family-based association study (2nd stage) was performed using the UNPHASED program version 2.404 http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/. The PDT can use data from related nuclear families from extended pedigrees with multiple offspring and is valid even when there is population substructure. Eight SNPs (rs1045487, rs2288601, rs3773265, rs2273188, rs2041125, rs3213733, rs3771140, and rs2820421) were observed to be associated with asthma at the significance levels of $P < 0.05$, however, the risk alleles for asthma in 5 SNPs (rs1045487, rs2288601, rs3773265, rs2273188, and rs2041125) differed from the ones observed in the 1st stage analysis. Therefore, we further genotyped the remaining 3 SNPs in a larger replication panel comprising children with atopic asthma and healthy adult controls without atopic disease (3rd stage). The results of these 3 SNPs are shown in Table 3. Although the SNPs located in the 5' region of peroxisome biogenesis factor 19 (*PEX19*, rs2820421) were significantly associated with $P < 0.05$ through the 1st to the 3rd stage analyses, the *P* values did not reach statistically significant levels (combined, $P = 3.8 \times 10^{-5}$, calculated by the method described by Kirov et al [16]; statistically significant levels with Bonferroni correction, $P = 6.57 \times 10^{-7}$).

Our results revealed a few candidate genes of pediatric asthma. Though the *P* values did not reach statistically significant levels, SNPs in the 5' region of *PEX19* were consistently associated with asthma in the 1st to the 3rd stage analyses. On the contrary, SNPs on hippocalcin-like 1 (*HPCAL1*, rs3771140) and on interleukin (*IL*)*18R1* (rs3213733) were associated with asthma in the 1st and 2nd stage analyses, but the associations were not observed in the 3rd stage analysis.

Pairwise linkage disequilibrium (LD) plots using HapMap data of the Japanese and Chinese population revealed that

Table 3: Association results of three SNPs

7			1st stage			2nd stage	3rd stage		
Gene	rs number	Allele	Case*	Control*	*P* value (Corrected P)	PDT *P* value	Case*	Control*	*P* value
HPCAL1	rs3771140	A	0.84	0.77	0.0011 (0.0021)	0.0258	0.78	0.78	0.86
PEX19	rs2820421	A	0.55	0.48	0.0013 (0.0025)	0.045	0.53	0.47	0.0306
IL18R1	rs3213733	G	0.89	0.84	0.0019 (0.0035)	0.0246	0.83	0.82	0.62

*Allele frequencies

the SNPs were located in a tight LD region, spanning approximately 150-kb between rs822450 and rs6668576, and 4 genes (*PEA15*, *WDR42A*, *PEX19*, and *COPA*) were located in the LD region (Figure 1).

PEX19 is a human ortholog of the *Saccharomyces cerevisiae* gene, Pex19p, which encodes an oleic acid-inducible, farnesylated protein essential for peroxisome biogenesis [17]. Peroxisomes function to rid cells of toxic substances, such as hydrogen peroxide, or other metabolites and are essential for human survival. It has been reported that, in mice cells, Pex19p interacts with p19ARF in the cell cytoplasm and excludes p19ARF from the nucleus, leading to a concurrent inactivation of p53 function [18]. p19ARF is encoded by the cyclin-dependent kinase inhibitor 2a (Cdkn2a), and the human ortholog of Cdkn2a (CDKN2A) has been extensively examined in relation to cancer and aging [19]. Down regulation of Pex19p by its antisense expression resulted in increased levels of p19ARF, increased p53 function, and a p53/p21WAF1-mediated senescence [18]. p19ARF proteins regulate p53 pathways, and the disruption of these proteins results in aberrant cell cycle regulation and perturbation of apoptotic response [20]. Recently, it has been shown that p19Arf overexpression resulted in impaired transition from CD4(-)CD8(-) (double negative stage) to CD4(+)CD8(+) (double positive stage), leading to impaired thymocyte expansion and development [21]. Functions of other 3 genes, *WDR42A*, *PEA15* and *COPA* for the immune systems are currently not well understood.

IL-18 was initially identified as a potent interferon gamma (IFNγ)-inducing factor, and was later shown to have the potential to induce IL-4 production. Therefore, IL-18 can induce both IFNγ and IL-4 responses depending on its cytokine environment [22]. Recently, polymorphisms in *IL18* receptor 1 (*IL18R1*) have been reported to be associated with asthma and bronchial hyperresponsiveness in the European population [23,24]. Therefore, *IL18R1* is a good candidate for asthma, and further replication studies are required to determine the causal variants.

In the present study, we did not detect statistically significant associations of asthma with SNPs. This may be because of the limited statistical power, considering the sample size and extent of multiple testing. Our statistical power in the 3rd-stage analysis was 95%, but the powers

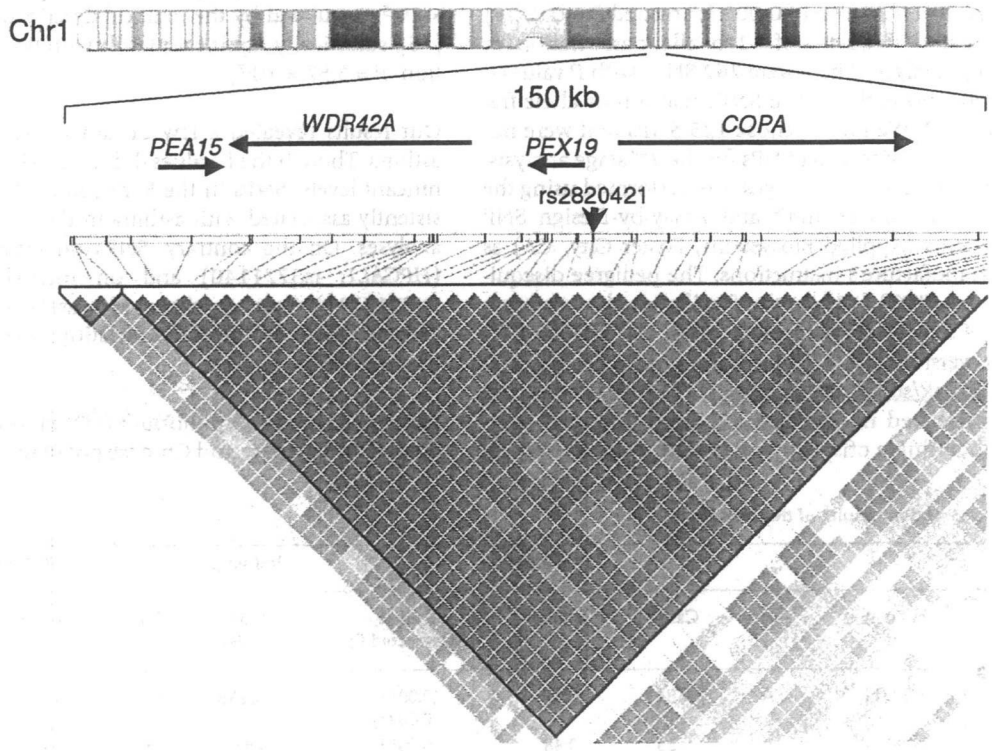

Figure 1
LD map around rs2820421. Top, locations of the genes in the 150 kb LD region. Bottom, Haploview plot of LD blocks. Red color indicates strong LD, expressed as r^2. SNPs with an allele frequency > 0.2 are shown. An arrow indicates the location of rs2820421.

of the 1st- and 2nd-stage analyses were 0.95 and 0.85 with a genotypic relative risk of 2.0, and 0.41 and 0.45 with a genotypic relative risk of 1.5, respectively. Another reason is population stratification in the 1st-stage analysis. Although we collected the case and control samples from geographic regions wherein people are considered to be genetically similar, population stratification may exists in our case-control samples, leading to the inflated test statistics.

In summary, we performed a large scale genotyping study to identify the susceptibility genes for pediatric asthma. Although no SNPs attained genome-wide significance, we identified several loci with a possible association with asthma. Further studies are required to validate these results for the prevention and treatment of asthma.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YI, MF, KH, and TH carried out molecular genetic study. YS, HS, KM, AA, TK, SY, ME, and MS prepared the samples and participated in the study design and coordination. TA, MT and EM participated in the design of the study, performed the statistical analysis and prepared the manuscript. All authors read and approved the final manuscript.

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2007, at the patient's request. After approximately 1½ years of normal eosinophil levels, the patient's eosinophilia worsened during late 2008 (Fig 1). A single osseous plasmacytoma was found and irradiated but progressed to multiple lytic lesions and multiple myelomas confirmed by means of bone marrow biopsy. The patient died in December 2008 as a result of this disease.

HES can occur as a myeloproliferative, lymphoproliferative, or, most frequently, idiopathic variant. Some myeloproliferative patients respond to imatinib mesylate[3] and possess a mutant gene on chromosome 4.[4] Deletion of approximately 800 kB on chromosome 4 results in a *FIP1L1/PDGFRA* fusion gene and formation of a kinase potently inhibited by imatinib mesylate. In our patient the *FIP1L1/PDGFRA* fusion gene was not detected, and imatinib mesylate failed to control eosinophilia. In the lymphoproliferative HES variant, T-lymphocyte clones produce cytokines, especially IL-5, that stimulate eosinophil production in the bone marrow. Mepolizumab, an anti–IL-5 drug, is used to inhibit eosinophil proliferation stimulated by IL-5; however, for unknown reasons, 16% of patients do not respond to mepolizumab.[5] In this case mepolizumab had no effect, suggesting that eosinophilia was not IL-5 dependent or that other cytokines, such as IL-3 or GM-CSF, were supporting eosinophil growth. Alternatively, the patient might have been producing so much IL-5 that the levels might have outpaced mepolizumab injections. IFN-α treatment is often effective because of a shift in the cytokine milieu from T_H2, which is supportive of eosinophil growth, to a T_H1-type response. In this case IFN-α caused a decrease in eosinophil counts, although not to normal levels, and the patient experienced the side effects of IFN-α.

Alemtuzumab is an anti-CD52 antibody that can bind to both eosinophils and T cells, potentially inhibiting either the myeloproliferative, lymphoproliferative, or idiopathic variant.[6] CD52 is a glycosylphosphatidylinositol-anchored molecule expressed on human eosinophils, lymphocytes, macrophages, and monocytes but not on neutrophils.[7] Alemtuzumab is approved for the treatment of B-cell chronic lymphocytic leukemia and is also used to treat small lymphocytic lymphoma and mantle cell lymphoma in conjunction with other treatments. Side effects include infusion reactions (often severe), lymphopenia, anemia, thrombocytopenia, and infections. Alemtuzumab was used successfully as a treatment for HES in 2 prior cases, 1 lymphoproliferative and 1 myeloproliferative, both of which did not respond to imatinib mesylate or IFN-α and that were not tested for the *FIP1L1/PDGFRA* fusion.[8,9] Alemtuzumab controlled our patient's eosinophilia for 1½ years, and the patient's quality of life appeared improved. Our patient most likely had the idiopathic HES variant. However, the occurrence of thromboembolism and an increased B12 level point to a possible myeloproliferative HES variant. The patient had a plasmacytoma and then multiple lytic lesions and multiple myelomas, suggesting involvement of 2 cell lineages by a single mutation or possibly independent mutations. Overall, the results in our patient and the previously reported cases suggest that alemtuzumab might be an effective treatment for the myeloproliferative, idiopathic, and lymphoproliferative HES variants.

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CD14 and *IL4R* gene polymorphisms modify the effect of day care attendance on serum IgE levels

To the Editor:

The cause of atopy is generally traced to the interplay of genetic and environmental factors.[1] Day care appears to be one of the most frequently investigated environmental factors. Although the results of studies investigating the association between day care attendance and atopy, as assessed by skin prick test responses, were inconsistent, all studies[2-4] measuring serum IgE levels have thus far shown a constant decreasing effect on serum IgE levels.

Among the genes that show a gene-environment interaction for the development of atopy or allergic diseases, the most frequently investigated is the *CD14* gene.[1] However, there is no report that investigates interaction of this gene and day care attendance. CD14 is a pattern-recognition receptor involved in the clearance of bacterial endotoxin and is also known as a receptor of respiratory syncytial virus. We investigated *CD14*−159C/T (rs2569190) and *CD14*−550C/T (rs5744455) polymorphisms in Japanese patients with severe respiratory syncytial virus–induced bronchiolitis and found that *CD14*−550C/T but not *CD14*−159C/T was significantly associated with the condition.[5]

The IL-4 receptor α gene *(IL4R)* is also one of the most frequently investigated genes and has been shown to be associated with atopy and atopic diseases.[6] The Ile50Val polymorphism (rs1805010) of the *IL4R* gene is a functional polymorphism and has been reported to be strongly associated with atopy and atopic asthma in the Japanese population. To date, only one study has reported the interaction of the *IL4R* Ile50Val polymorphism and day care attendance in the first year of life.[7] The result showed a

TABLE I. Characteristics of the subjects

Total no. of participants	473
Age (mo)	
Mean ± SD	111.1 ± 19.9
Range	76-147
Sex ratio (male:female)	1.00:1.01
Day care attendance before age 2 y (%)	14.5
Total IgE (IU/mL), mean ± SD	
Male	254 ± 340
Female	241 ± 469
Prevalence of atopy (%)	
Male	76.9
Female	68.0
Prevalence of allergic disorders (%)	
Asthma	
Male	14.1
Female	6.6
Atopic dermatitis	
Male	11.5
Female	9.7
Allergic rhinitis	
Male	42.1
Female	31.2
Food allergy	
Male	3.0
Female	3.4

significant gene-environment interaction for IFN-γ production at 1 year of age. However, it is not known whether this modified cytokine response affects the chance of having atopy or allergic diseases in the later period of life.

Here we report a relationship between serum total and specific IgE levels in Japanese elementary school children and day care attendance during earlier life. Our results suggest that day care attendance is associated with serum IgE levels, and this effect is modified by *CD14*−550C/T and *IL4R* Ile50Val polymorphisms. This is the first report that suggests an interaction between early-life day care attendance and genetic variations on IgE levels in later life.

Children attending an elementary school located in the central area of Chiba city (population of approximately 930,000) were recruited for this study. We first asked all (n = 843) children for participate in the survey. We then sent a detailed questionnaire to those who had a positive response (n = 582). Children with congenital heart diseases and lung diseases caused by immature birth were excluded. A total of 473 school children aged 6 to 12 years were enrolled. Blood samples were collected from 411 children on 2 separate days (July 3 and 12, 2006) for serum and DNA preparation. A complete set of information on total and 8 specific IgE levels, genotypes, and environmental factors was obtained from 375 children. All parents provided written informed consent. The study protocol was approved by the Ethics Committee of Chiba University Graduate School of Medicine.

The status of allergic diseases was evaluated by using questions based on the International Study of Asthma and Allergies in Childhood. We asked whether the child regularly attends a day care center where time is spent with other children at or before 2 years of age. For parents who responded yes to this question, the age of entry of their child to the day care center was obtained. The questionnaire also included the following items to assess possible confounding factors: number of siblings; number of older siblings; allergic diseases of parents and siblings (family history: scored as positive if parents, siblings, or both had any of 4 allergic diseases [asthma, allergic rhinitis, atopic eczema, and food allergy]); residential area (6 categories), type of house structure (5 categories), and floor type of bedroom (5 categories); yogurt/fermented food consumption; pet ownership; and smoking among family members.

Genotyping of the *CD14*−550C/T polymorphism was performed as described previously,[5] whereas that of the *IL4R* Ile50Val (rs1805010) polymorphism was carried out with the TaqMan allele-specific PCR method.[8] Primer sequences were as shown in this article's Online Repository at www.jacionline.org.

Table I shows the characteristics of the investigated population. The percentage of children who had regularly attended day care before 2 years of age was 14.5%. Atopy was defined as the presence of positive (≥0.35 IU/mL) specific IgE level against at least 1 of the 8 allergens. Although the prevalences of asthma, atopic dermatitis, and food allergy were compatible with those in a recent large study,[9] prevalences of allergic rhinitis and atopy were about 10 to 20 points higher, suggesting that children who had allergic rhinitis were more likely to attend this study.

Table II shows the association between day care attendance and serum IgE levels or atopy after being stratified with the *CD14*−550C/T genotype. Day care significantly decreased total IgE levels ($P = 9.7 \times 10^{-5}$), mite-specific IgE levels ($P = .0016$), and rate of atopy ($P = .00041$) in individuals with the C/T or T/T genotype, whereas the effect of day care was not observed in those with the C/C genotype. Numbers of children with the C/T+T/T genotype and those with the C/C genotype were similar, suggesting that the difference is not likely due to the statistical power for detecting association. Multivariate analyses with confounding factors were performed to evaluate the significance of this gene-environment interaction. The interaction between the *CD14*−550C/T polymorphism and day care was significant for $\log_{10}$(total IgE) ($P = .0046$), mite-specific IgE classes ($P = .00047$), and atopy ($P = .0097$) after adjusting for age, sex, family history, and number of siblings.

Table III shows the association between day care attendance and serum IgE levels or atopy after being stratified with the *IL4R* Val50Ile genotype. The effects of day care on total and some specific IgE levels were significant in Val/Ile heterozygotes but not in Val/Val or Ile/Ile homozygotes. In Val/Ile individuals day care significantly decreased total IgE levels ($P = .0012$), mite-specific ($P = .011$) and cedar pollen–specific ($P = .034$) IgE levels, and rate of atopy ($P = .018$). No such trend was observed in Val/Val or Ile/Ile individuals. The numbers of Val/Val and Val/Ile individuals were similar. It is therefore unlikely that the lack of significant association in Val/Val individuals was due to smaller statistical power for detecting association. When the significance of gene-environment interaction was assessed with the confounding factors, the interaction term between *IL4R* and day care attendance was significant for $\log_{10}$(total IgE) ($P = .019$) and mite-specific ($P = .0025$) and cedar pollen–specific ($P = .040$) IgE classes but not for atopy.

Total IgE levels in 4 genotype groups (group 1: *CD14* C/C, *IL4R* Ile/Ile+Val/Val; group 2: *CD14* C/C, *IL4R* Val/Ile; group 3: *CD14* C/T+T/T, *IL4R* Ile/Ile+Val/Val; and group 4: *CD14* C/T+T/T, *IL4R* Val/Ile) were compared to evaluate the combined effect of 2 polymorphisms on total IgE levels. Fig 1 shows the box

TABLE II. Effects of day care attendance on IgE levels when stratified by *CD14*−550C/T genotype

	C/C				C/T + T/T				
	Day care attendance		Effect size or odds ratio (95% CI)	*P* value	Day care attendance		Effect size or odds ratio (95% CI)	*P* value	Gene-environment interaction *P* value*
	No	Yes			No	Yes			
No. of subjects	169	22			157	28			
Log_{10}(total IgE)									
Mean	1.88	1.98	0.094 (−0.21 to 0.39)¶	0.54†	2.09	1.58	−0.50 (−0.26 to −0.76)¶	9.7×10^{-5}†	**.0046****
SD	0.77	0.76			0.63	0.51			
Specific IgE (positive‡ rate)									
Mite	0.49	0.59	1.50 (0.61 to 3.69)#	.51§	0.61	0.32	0.30 (0.13 to 0.71)#	**.0016**§	**.00047**††
Cedar pollen	0.45	0.46	1.02 (0.42 to 2.45)#	.92§	0.57	0.32	0.35 (0.15 to 0.83)#	**.032**§	**.116**††
Atopy (rate)	0.77	0.68	1.60 (0.56 to 4.55)#	.38‖	0.81	0.50	0.24 (0.10 to 0.55)#	**.00041**‖	**.0097**‡‡

Boldface indicates statistically significant values.
*Adjusted for age, sex, number of siblings, and family history.
†Analysis of variance for log_{10}(total IgE [in international units per milliliter]).
‡Class ≥1 (≥0.35 IU/mL).
§Kruskal-Wallis test for IgE value (in international units per milliliter).
‖χ^2 Test of independence.
¶Effect size.
#Odds ratio.
**General liner model.
††Generalized linear model (Poisson distribution, log link function).
‡‡Logistic regression.

TABLE III. Effects of day care attendance on IgE levels when stratified by *IL4R* Val50Ile genotype

	Val/Val				Val/Ile				Ile/Ile				
	Day care attendance		Effect size or odds ratio (95% CI)	*P* value	Day care attendance		Effect size or odds ratio (95% CI)	*P* value	Day care attendance		Effect size of odds ratio (95% CI)	*P* value	Gene-environment interaction *P* value*
	No	Yes			No	Yes			No	Yes			
No. of subjects	125	18			152	27			49	5			
Log_{10}(total IgE)													
Mean	1.94	1.91	−0.058 (−0.38 to 0.27)¶	.72†	1.88	1.55	−0.44 (−0.71 to −0.18)¶	**.0012**†	1.99	2.32	0.33 (−0.31 to 0.97)¶	.12†	**.019****
SD	0.64	0.72			0.57	0.56			0.69	0.52			
Specific IgE (positive‡ rate)													
Mite	0.57	0.56	0.95 (0.35 to 2.57)#	.51§	0.52	0.30	0.39 (0.16 to 0.94)#	**.011**§	0.59	0.80	2.76 (0.29 to 26.5)#	.36§	**.0025**††
Cedar pollen	0.50	0.50	1.01 (0.38 to 2.73)#	.93§	0.51	0.30	0.41 (0.17 to 0.99)#	**.034**§	0.55	0.40	0.54 (0.083 to 3.54)#	.91§	**.040**††
Atopy (rate)	0.74	0.72	0.93 (0.31 to 2.82)#	.90‖	0.74	0.52	0.37 (0.16 to 0.86)#	**.018**‖	0.76	0.80	1.30 (0.13 to 12.8)#	.82‖	.118‡‡

Boldface indicates statistically significant values.
*Adjusted for age, sex, number of siblings, and family history.
†Analysis of variance for log_{10}(total IgE [in international units per milliliter]).
‡Class ≥1 (≥0.35 IU/mL).
§Kruskal-Wallis test for IgE value (in international units per milliliter).
‖χ^2 Test of independence.
¶Effect size.
#Odds ratio.
**General liner model.
††Generalized linear model (Poisson distribution, log link function).
‡‡Logistic regression.

plot of log_{10}(total IgE) in 4 genotype groups. Among children who attended day care compared with group 1, the mean log_{10} (total IgE) values of groups 2, 3, and 4 decreased by 0.41, 0.35, and 0.69, respectively. This magnitude of change suggests that the effects of *CD14* and *IL4R* were additive. The children in group 4 showed significantly (P = .0046) lower total IgE levels than those in group 1. On the other hand, among children who did not attend day care, the log_{10}(total IgE) levels of children in groups 3 (P = .031) and 4 (P = .036) were significantly higher than those of children in group 1. The *CD14* C/T and T/T genotypes appeared to show the opposite effect on the serum total IgE level in children who did not attend day care compared

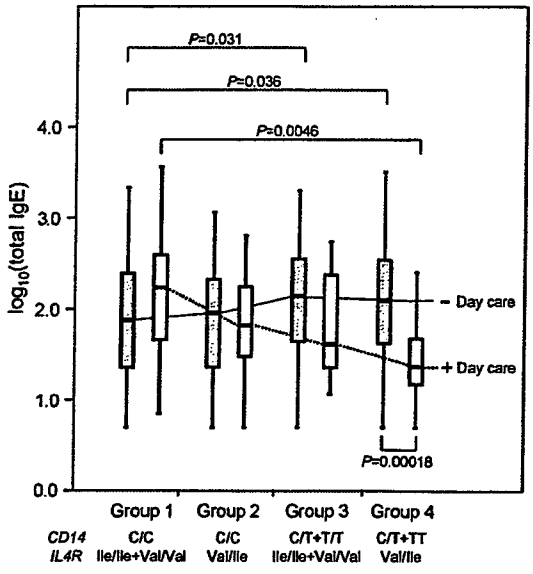

FIG 1. Total IgE levels in 4 groups of children classified based on a combination of *IL4R* and *CD14* genotypes. Box plot of $\log_{10}$(total IgE) values is shown for children who attended day care *(+Day care)* and for those who did not *(−Day care)*. Results are presented as medians and interquartile ranges. Only significant *P* values (<.05) are shown.

with those who did attend day care. When we examined the effect of day care in each genotype group, the effect was not sufficiently large to show a significant change in IgE level in groups 2 and 3, in which individuals had only 1 IgE level–decreasing genotype. However, in group 4, in which individuals had 2 IgE level–decreasing genotypes, the effect was sufficiently large to show a significant difference (P = .00018). Significance of interaction between the *CD14* and *IL4R* genotypes was also evaluated by using general linear models in which age, sex, family history, number of siblings, and day care were included as variables. The interaction term of the 2 genes was not significant, suggesting an independent effect of the *CD14* and *IL4R* genes.

The interaction of the *CD14* gene with day care attendance suggests that the mechanism of the effect of day care involves at least in part a response to infection, environmental endotoxin exposure, or both. The interaction of the *IL4R* gene with day care attendance suggests that the mechanism also involves those related to T_H2 cell proliferation and IgE production. These results suggest that the complex nature of mechanisms underlies the effect of day care attendance on serum IgE levels.

Environmental factors investigated in the present study were determined based on a questionnaire on past day care attendance, and therefore recall bias can be a potential problem. The number of subjects investigated in this study was not so large and might be the acceptable minimum for investigating gene-environment interactions. The subjects evaluated were children who attended a single school and lived in a medium-populated city, thus representing those living in rather small regional environments in Japan. Nevertheless, these characteristics of the present sample might have contributed to minimizing the variances of background and outcome parameters and might have resulted in the positive findings obtained from a relatively small number of subjects. It is necessary to perform a cohort study to follow children with or without day care attendance until they reach school age to validate the current observations.

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Mucormycosis in chronic granulomatous disease: Association with iatrogenic immunosuppression

To the Editor:

Chronic granulomatous disease (CGD) results from mutations in either X-linked ($gp91^{phox}$) or autosomal ($p47^{phox}$, $p67^{phox}$, and $p22^{phox}$) genes encoding the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Impaired generation of reactive oxygen species predisposes to recurrent life-threatening bacterial and fungal infections. Septated hyaline molds (particularly *Aspergillus* species) are the primary fungal pathogens in CGD. Fungi of the order Mucorales (pauciseptated molds) are environmentally ubiquitous and cause mucormycosis in select immunocompromised patient populations, such as those with diabetic ketoacidosis and hematologic malignancy and recipients of transplants or deferoxamine. We investigated the prevalence of mucormycosis in patients with CGD.

PRIMERS FOR *IL4R* GENOTYPING (5′ TO 3′)

TaqMan probe (FAM-TACAGGTGACCAGCCTAACCCAGC CCCTGT-TAMRA); common primer (TGGAGGCATGTCCCG GACAC); Ile (A) allele primer (CGCCTCCGTTGTTCTCAG GGGT); and Val (G) allele primer (CGCCTCCGTTGTTCTC AGGGGC).