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Polymorphisms in the interleukin 4 receptor and interleukin 13 genes in immediate allergic reactions to beta-lactam antibiotics: A case-control study

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Abstract

Background: Immediate hypersensitivity reactions to beta-lactams are IgE-mediated and constitute the most common adverse reactions to antibiotics mediated by a specific immunologic mechanism.

Objective: We investigated the association between four functional polymorphisms of IL13 (R130Q variant) and IL4RA (I50V, S478P and Q551R variants) genes and susceptibility to immediate allergic reactions to beta-lactams in the Algerian population.

Methods: We determined these gene variants in 199 patients and 99 healthy controls from Algeria. In a case-control study using the TaqMan method, we genotyped four single nucleotide polymorphisms (SNPs) including Arg130Gln in IL13, and Ile50Val, Ser478Pro as well as Gln551Arg in IL4RA.

Results: IL4RA I50V variant was more significantly connected with the risk of beta-lactam allergy ($P = 0.0144$) and the total serum IgE level in patients ($P = 0.0136$). A significant correlation was observed between IL13 R130Q and beta-lactam allergy ($P = 0.0384$). Also, a significant gene-gene interaction was detected between the predominant allele of the IL13 R130Q polymorphism and the three polymorphisms of IL4RA ($P < 0.0001$, $P = 0.0163$, and 0.0301, respectively). Haplotype analysis of IL4RA revealed that GTA haplotype had a significant correlation in patients with beta-lactam allergy ($P = 0.0123$).

Conclusions: Our results indicate that IL4RA (I50V) and IL13 R130Q are associated with beta-lactam allergy. The combination of IL13 and IL4RA variants markedly increases an individual's susceptibility to beta-lactam allergy in the Algerian population.

Key words: Allergy, Beta-lactam, IgE, Interleukin-13, Interleukin-4 receptor, Polymorphism.

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Introduction

Allergic reactions to beta-lactams are the most common cause of drug reactions mediated by specific immunological mechanisms, where immunoglobulin E (IgE) and T-cells play a role in the onset of allergic reactions.[1] Hypersensitivity reactions are classified as either immune-mediated reactions or non-immune mediated reactions. Immediate hypersensitivity reactions are usually induced by an IgE-mediated mechanism and occur within the first hour following the last drug administration. These reactions typically appear as urticaria, angioedema, rhinitis, bronchospasm, or anaphylaxis.[2,3] However, the mechanism by which allergic reactions are induced by beta-lactam antibiotics remains unclear.[4]

IgE-mediated reactions also called immediate hypersensitivity reactions (Type-I hypersensitivity reactions) are classified as humoral mediated reactions. When exposed for the first time to an immunogenic drug, T-cells specifically T-helper-2

(Th2) cells, initiate an allergic reaction by releasing interleukin-4 and interleukin-13 (IL4, IL13), which activate and induce proliferation of B-cells. Then, activated B-lymphocytes produce antigen-specific Ig-E. There is a cross-link between multivalent antigen and basophils or mast cells by Ig-E specific for that antigen which leads to the degranulation of basophils and mast cells and release of inflammatory mediators.[5] Interleukins secreted by Th2 cells, predominantly IL4 and IL13, are critical cytokines in the pathogenesis of allergic disorders. These interleukins share many biological and biochemical characteristics.[6] Both IL4 and IL13 use the IL4 receptor α chain (IL4RA) as a component of their receptors and transmit their signals through IL4RA.[7] Several studies reported in Europe, United States of America (USA), and China have also shown that immediate-type allergic reactions to beta-lactams are influenced by three genes that affect IgE production, IL13, IL4, and IL4 receptor α (IL4RA).[8–14] In the present study, we thus aimed to evaluate the correlation between IgE-mediated reactions to beta-lactams and polymorphisms of IL13 (R130Q) and IL4RA (I50V, S478P, and Q551R variants) in the Algerian population.

Methods

Patients' samples

Samples were taken from Allergy Unit at the Faculty of Medicine of Batna University in Algeria. The study was performed in 199 Algerian patients with immediate-type reaction to beta-lactams (penicillin or cephalosporins) occurring within 1 hour after drug administration, with positive skin tests and/or serum-specific IgE assays. The 99 healthy controls showed negative skin test to beta-lactam and had no history of allergic, dermatologic, or respiratory diseases, or autoimmune diseases such as asthma, eczema, allergic rhinitis, and urticaria. They have no family relationship with cases. Informed consent was obtained from all subjects and the study was conducted according to the declaration of Helsinki Principles, and the ethics committee of Centre Hospitalo-Universitaire de Batna (CHUB, Algérie) approved the study.

IgE levels measurments and TaqMan method

Five mL of blood was taken from each participant under complete aseptic conditions and divided into two portions; 1.5 mL of whole blood was collected in sterile EDTA-containing tubes for DNA extraction, and the rest was left for 30 to 60 minutes for spontaneous clotting at room temperature and then centrifuged at 3000 rpm for 10 minutes. Serum samples were separated into another set of tubes and kept frozen at -20°C for determination of total IgE. Total serum IgE levels were measured by sandwich enzyme-linked immunosorbent assay ELISA (Innovative research Inc, Novi, Michigan, USA) following the manufacturer's protocol. "Non enzymatic salting out" method was used to isolate genomic DNA from peripheral blood.[15] All the polymorphisms were genotyped by allelic discrimination polymerase chain reaction assays (5' nuclease assay) using predesigned TaqMan SNP Genotyping Assays (Applied Biosystems, USA). Both PCR primers and MGB TaqMan probes are shown in **Table 1**. Primers and probes annealing temperatures for all allele-discriminating assays were optimized using a standard PCR setup on a Bio-Rad CFX connect real-time PCR instrument (Bio-Rad Laboratories, Hercules, CA, USA). The program consisted of 3 minutes of polymerase activation at 98°C, followed by 40 cycles of collective annealing and elongation steps at 52-64°C (temperature gradient) for 30 seconds, and denaturation at 98°C for 15 seconds. For the optimization of the primer concentration, a titration series of each pair was prepared from 200 to 600 nM, with 300 nM of each of the two probes added, and using a heterozygotic sample as template DNA. Optimal annealing temperature, concentrations of primers and probes were selected based on the efficiency of the real-time PCR amplification. The main advantages of the direct approach for genotyping are less hands-on time during setup, and that the PCR is performed in a closed system, hereby minimizing the risk of contamination.

Reactions were performed in a 12 µL volume, consisting of six µL Bio-Rad SsoAdvenced Universal Probes Supermix, 500 nM of unlabeled PCR primers, 300 nM of TaqMan MGB probes, and 10 ng of template DNA. Thermal cycling was initiated with a denaturation step of 3 min at 98°C, followed by 40 cycles of 15 s at 98°C and 30 s at 60°C. After PCR were completed, allelic discrimination was analyzed using the Bio-Rad CFX Manager Software (Version 3.1, Bio-Rad). Genotype assignment was determined by plotting the endpoint relative fluorescent units (RFU) for one fluorophore (allele one on the

Table 1. Primers and probes for genotyping screening by TaqMan allelic discrimination.

SNP	NCBI rs No	Base change	Primers	Probes
IL13 Arg130Gln	rs20541	G > A	F: 5'-CTGCAAATAATGATGCTTTCGA-3'	A allele: 5'-FAM-GAGGGACAGTTCAACTG-MGB-3'
			R: 5'-CCAGTTTGTAAAGGACCTGCTCT-3'	G allele: 5'-HEX-GAGGGACGGTTCAACT-MGB-3'
IL4RA Ile50Val	rs1805010	A > G	F: 5'-CTACAGGTGACCAGCCTAAC-3'	G allele: 5'-FAM-ACGTGTGTCCCTG-MGB-3'
			R: 5'-CCCACAGGTCCAGTGTATAGT-3'	A allele: 5'-HEX-ACGTGTATCCCTG-MGB-3'
IL4RA Ser478Pro	rs1805015	T > C	F: 5'-CGCAGGCAACCCTGCTTA-3'	C allele: 5'-FAM-CAGCAACCCCCTGAG-MGB-3'
			R:5'-GCATCTCGGGTTCTACTTCCTC-3'	T allele: 5'-HEX-TTCAGCAACTCCCTGAG-MGB-3'
IL4RA Gln551Arg	rs1801275	A > G	F: 5'-CTCCGCCGAAATGTCCTC-3'	G allele: 5'-FAM-GGCTATCGGGAGTTT-MGB-3'
			R: 5'-GCCTTGTAACCAGCCTCTCC-3'	A allele: 5'-HEX-TGGCTATCAGGAGTTTG-MGB-3'

x-axis) against the RFU for the other fluorophore (allele two on the y-axis) on the allelic discrimination plot. All samples were set up in triplicate. PCR reactions were performed in a dedicated PCR area with dedicated PCR pipettes and reagents. For quality control purposes, each real time-PCR included negative as well as positive controls for all the genotypes. For validation, about 10% of the samples were re-genotyped. The results were reproducible with no discrepancies in genotyping.

Statistical analysis:

We used SNPstats software to test Hardy-Weinberg (HW) equilibrium of alleles frequencies.[16] This software was also used to estimate haplotype frequencies in cases and controls. The chi-square test was used to test for significant association between beta-lactam allergies and alleles or genotypes. Odds ratio (OR), used as a measure of association strength, and the corresponding 95% confidence interval (CI) was calculated. Kruskal-Wallis test was used to assess whether the distribution of a categorical variable is the same between genotype groups. A *P*-value of less than 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA).

Results

In the present case-control study, we explored the association between the IL13, IL4RA polymorphisms and beta-lactam allergy in a sample of Algerian population. The association between the immediate allergic reaction to beta-lactams and polymorphisms of IL13 (R130Q), IL4RA (I50V, S478P and Q551R) was evaluated in 199 patient and 99 healthy controls from Algeria. There were no significant differences in the distribution of age ($P = 0.1023$) and sex ($P = 0.5554$) between the cases and controls (**Table 2**). Patients with immediate allergic reactions had a significantly higher concentration of total serum IgE than controls (**Table 2**). All genotyped distributions of control subjects were consistent with those expected from the Hardy-Weinberg equilibrium ($P > 0.05$). Besides, the minor allele frequency (MAF) of all the four SNPs was consistent with that reported in the HapMap database (**Table 3**). No linkage disequilibrium was found between IL13 and IL4RA polymorphisms.

Table 2. Clinical characteristics and genotypes and allele frequencies of IL13 and IL4RA of patients and controls.

Characteristic	Patients, n = 199, Mean ± SD and number of cases (%, 95% confidence interval)	Controls, n = 99, Mean ± SD and number of cases (%, 95% confidence interval)	*P*-value
Age	39.48 ± 15.72	35.78 ± 11.78	0.1023
Male gender	65 (32.7, 26.5–39.4)	29 (29.3, 21.2–38.9)	0.5554
Total serum IgE	187 ± 94.55	41 ± 35.7	< 0.0001
IgE >100	152 (76.3, 70.02–81.75)	11 (11.11, 6.31–18.81)	< 0.0001
Personal history of allergy	53	None	
Urticaria	19	None	
Anaphylactic shock	15	None	
Asthma	19	None	
IL4RA I50V			
II (AA)	44 (22.1, 16.5–28.5)	32 (32.3, 23.3–42.5)	0.0144
IV (AG)	86 (43.2, 36.2–50.4)	48 (48.5, 38.3–58.7)	
VV (GG)	69 (34.7, 28.0–41.7)	19 (19.2, 11.9–28.3)	0.0031
Predominant allele I	174 (43.7, 38.9–48.6)	112 (56.6, 49.6–63.3)	
Less frequent allele V	224 (56.3, 51.3–61.0)	86 (43.4, 36.7–50.4)	
IL4RA S478P			
SS (TT)	139 (69.9, 63.1–75.8)	74 (74.7, 66.4–83.1)	0.1925
SP (TC)	54 (27.1, 21.4–33.7)	25 (25.2, 17.0–33.5)	
PP (CC)	06 (3.0, 1.4–6.4)	00 (0, 0–3.7)	0.2059
Predominant allele T	332 (83.4, 79.4–86.7)	173 (87.9, 82.6–87.8)	
Less frequent allele C	66 (16.6, 13.2–20.5)	25 (12.1, 8.30–17.4)	

Table 2. (Continued)

Characteristic	Patients, n = 199, Mean ± SD and number of cases (%, 95% confidence interval)	Controls, n = 99, Mean ± SD and number of cases (%, 95% confidence interval)	*P*-value
IL4RA Q551R			
QQ (AA)	121 (60.8, 53.9–67.3)	61 (61.6, 51.8–70.6)	0.1378
QR (AG)	73 (36.7, 30.3–43.6)	31 (31.3, 23.0–41.0)	
RR (GG)	05 (2.5, 1.1–5.7)	07 (7.0, 3.5–13.9)	0.6000
Predominant allele Q	315 (79.1, 74.9–82.8)	153 (77.3, 70.9–82.5)	
Less frequent allele R	83 (20.9, 17.15–25.1)	45 (22.7, 17.4–29.0)	
IL13 R130Q			
RR (GG)	152 (76.4, 70.01–81.7)	87 (87.9, 79.9–92.9)	0.0384
RQ (GA)	42 (21.1, 16.0–27.3)	12 (12.1, 7.1–20.0)	
QQ (AA)	05 (2.5, 1.1–5.7)	00 (0, 0–3.7)	0.0093
Predominant allele R	346 (86.9, 83.3–89.9)	186 (93.9, 89.7–96.5)	
Less frequent allele Q	52 (13.1, 10.1–16.7)	12 (6.1, 3.5–10.3)	

Table 3. Primary information of genotyped SNPs in the IL13 and IL4RA genes.

SNP	NCBI rs No	Location	Base change	MAF			*P* for HWE[b]
				HapMap[a]	Case	Control	
IL13 Arg130Gln	rs20541	exon 4	G > A	0,130	0.13	0.07	0,991
IL4RA Ile50Val	rs1805010	exon 5	A > G	0,425	0.51	0.43	0,990
IL4RA Ser478Pro	rs1805015	exon 12	T > C	0,152	0.17	0.13	0,350
IL4RA Gln551Arg	rs1801275	exon 12	A > G	0,207	0.21	0.23	0,260

[a] MAF from the HapMap database
[b] HWE P value in the control group

Genotype distributions and allele frequencies of all analyzed polymorphisms for the patients and control group are shown in **Table 2**. The frequency of the predominant alleles of IL4RA I50V and IL13 R130Q was significantly higher in patients than in controls, whereas no difference was observed for the IL4RA S478P and IL4RA Q551R (**Table 2**). We observed a significant association between IL13 R130Q and total serum level of IgE in patients as well as controls (P = 0.0002). The association of IL4RA I50V and S478P with total IgE was more significant when restricting the analysis to patients (**Table 4**).

Table 4. Serum total IgE levels in patients with beta-lactam allergy.

Polymorphism	Total IgE (IU/ml) Median (25th - 75th)	*P*-value	Polymorphism	Total IgE (IU/ml) Median (25th - 75th)	*P*-value
IL4RA I50V		0.0136	IL4RA Q551R		0.2011
II	168.3 (81.03-253)		QQ	219.2 (110.7-273.1)	
IV	216.5 (82.75-259.6)		QR	213.4 (90.75-261.6)	
VV	252.4 (181.6-269.1)		RR	112 (63.3-180.9)	
IL4RA S478P		0.0492	IL13 R130Q		0.0460
SS	218.3 (100.5-276)		RR	214.4 (92.38-261.1)	
SP	197.4 (97.78-258.5)		RQ	213.7 (146.6-277.5)	
PP	261.6 (260.7-264.2)		QQ	270.2 (240-301)	

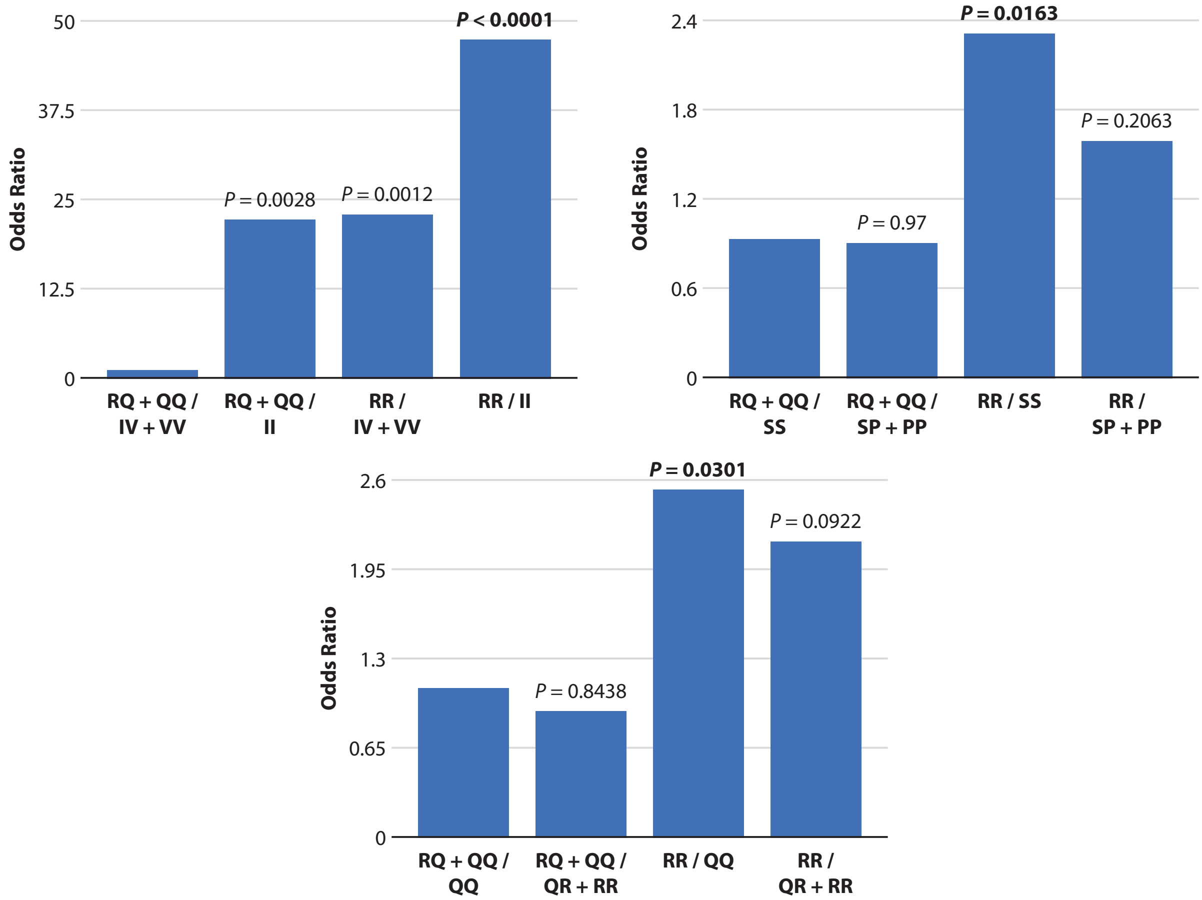

Figure 1. Interaction of IL4RA and IL13 Genotypes.
Bars indicate the odds ratio between the different combinations of genotypes for IL4RA (I50V, S478P, and Q551R) and IL13 R130Q. The non-risk genotype for each gene was used as the reference odds ratio.

Table 5. Major haplotype frequencies of IL4RA in the case and control groups.

Genotype	Haplotype	Frequency		*P*-value	OR (95% CI)
		Case	Control		
IL4RA					
rs1805010	ATA	150 (0.378%)	96 (0.486%)	< 0.0001	0.45 (0.31–0.65)
rs1805015	GTA	152 (0.383%)	54 (0.275%)	0.0123	1.61 (1.11–2.35)
rs1801275	GCG	95 (0.240%)	45 (0.230%)	0.3099	1.23 (0.82–1.83)

OR: Odds Ratio, CI: Confidence Interval

Because of the biological relationship of IL4RA and IL13, an analysis was performed to determine if individuals with the risk genotypes for both genes were at higher risk of developing beta-lactam allergy. The data are summarized in **Figure 1** and showed that IL13 130RR combined with any of the predominant homozygous genotypes of IL4RA was a risk factor in allergy to beta-lactams. A similar analysis was performed examining total serum IgE levels. Our results showed IL13/IL4RA variant combination: P = 0.0220, 0.0002, 0.0020, respectively and each variant P = 0.0002 0.2224, 0.6978, 0.1237, respectively. A linkage disequilibrium (LD) analysis was performed to study the relationships between the three SNPs of IL4RA and beta-lactam allergy. The LD showed that rs1805010 and rs1805015 had linkage disequilibrium with D’ of 0.5195, rs1805015 and rs1801275 had a score of D’ = 0.7977. However, rs1805010 and rs1801275 did not show linkage disequilibrium. Three haplotypes were found in the three SNPs of IL4RA gene: ATA, GTA, and GCG (**Table 5**). These haplotypes were observed in the case and control groups (P < 0.0001, P = 0.0123, and 0.3099, respectively). The haplotype GTA is correlated with beta-lactam allergy in Algerian population. Indeed, the haplotype GTA was significantly more frequent in patients with immediate allergic reactions to beta -lactams than in control subjects (P = 0.0123). Interestingly, the haplotype ATA was significantly more frequent in controls subjects than in patients (P < 0.0001).

Table 6. Genetic predictors in association with beta-lactam allergy.

Author	Geographical region	Study design and approach	Cases (n)	Controls (n)	Gene variant	Effect size	Functional validation
Guéant-Rodriguez, 2006 [8]	Italy	Case-Control (candidate gene)	210	265	IL13 R130Q IL4RA I50V IL4RA S478P IL4RA Q551R	130 (RQ+QQ); OR = 1.44(0.95–2.18); *P* = 0.0881 50II; OR = 1.65 (1.06–2.57); *P* = 0.0272 478SS; OR = 1.82 (1.07–3.12); *P* = 0.0271 551QQ; OR = 1.67(1.02–2.74); *P* = 0.0426	Serum IgE levels
Guglielmi, 2006 [9]	France	Case-Control (candidate gene)	44	44	IL4RA Ile75Val IL10 -819C>T IL10 -592C>A	OR = 5.4(1.16–27.7); *P* = 0.012 OR = 17.5(1.26–533.07); *P* = 0.023	None
Apter, 2008 [10]	USA	Case-Control (candidate gene)	23	39	IL4 IL4R LACTB	rs2070874; OR = 3.33(1.09–10.21); *P* = 0.035 rs10062446; OR = 3.61(1.21–10.71); *P* = 0.021 rs11740584; OR = 4.08(1.35–12.30); *P* = 0.012 rs1805010; OR = 1.35(0.40–4.62); *P* = 0.63 rs2729835; OR = 2.99 (0.96–9.28); *P* = 0.058	Penicillin metabolism (LACTB)
Cornejo-Garcia, 2012 [12]	Spain	Case-Control (candidate gene)	340	340	IL4RA I50V IL4RA Q551R	NR	Specifc IgE against prevalent allergens; Prevalence of atopy
Qiao, 2005 [13]	China	Case-Control (candidate gene)	245	101	IL4R Q576R	NR	Specific IgE to penicillins (eight types); serum levels of IL-4, IL-13, and IFN-gamma
Huang, 2009 [14]	China	Case-Control (candidate gene)	242	240	IL4R Q576R IL4R I75V	Q576; OR = 1.67(1.17–2.38); *P* = 0.003 I75; OR = 1.21(0.93–1.57); *P* = 0.19	Specif IgE (eight types)
This study	Algeria	Case-Control (candidate gene)	199	99	IL13 R130Q IL4RA I50V IL4RA S478P IL4RA Q551R	130 RR; OR = 3.56(1.78–7.12); *P* = 0.0002 50II; OR = 0.65 (0.37–1.13); *P* = 0.2224 478SS; OR = 1.07(0.61–1.87); *P* = 0.6978 551QQ; OR = 0.65(0.39–1.10); *P* = 0.1237	Serum IgE levels

NR: not reported

Discussion

Several studies suggested that allergic reaction to beta-lactams are influenced by genes involved in IgE production, including IL13 and IL4 pathways.[8–14,17,18] Besides, recent population studies have reported an association between IL13 and IL4RA with atopy and asthma.[19–21] In this study, we found for the first time in the Algerian population, an association of rs1805010 polymorphism in IL4RA gene and rs20541 in IL13 with an allergic reaction to beta-lactams.

In Algerian patients with allergic reaction to beta-lactams, we observed a higher concentration of total serum IgE than non-allergic patients suggesting the involvement of a genetic mechanism related to IgE class switching. Supporting our data, a relationship was found among IL4RA I50V and IL13 R130Q polymorphisms, the risk of immediate reaction to beta-lactams, and total serum IgE level.[8] However, Apter *et al*. reported that the IL4RA I50V polymorphism had no relationship with penicillin allergy based on a series of 23 self-reported penicillin -allergic patients from USA.[10] One possible explanation for this discrepancy is the difference in the genotype frequency of ILR4A I50V between different populations. This explanation is supported by the research of Gueant *et al*. who showed that the IL4RA I50V of the AA genotype was more significantly associated with the risk of penicillin allergy than with the risk of cephalosporin allergy.[17] This study also demonstrated that a difference in the AA genotype frequency of IL4RA I50V existed between two Europeans populations.[17]

The IL4RA gene is located on chromosome 16p11−16p12. It is a subunit that plays a key role in allergic disease by promoting the IgE production.[22] In our study, the I50V and S478R were correlated with IgE production in patients, whereas the Q551R was not associated with the IgE level (**Table 4**). However, Cornejo-Garcia *et al*. found that total IgE was affected by Q551R polymorphism as well as IL13 130RQ/QQ and IL4RA 551QQ epistatic genotype in Spanish Caucasians.[12] In our series, the two symmetrical combinations (IL13 130RR and IL4RA 50II, IL13 130RR and IL4RA 551QQ) are significantly correlated with total IgE level, but less than the effect of IL13 R130Q alone ($P = 0.0002$), confirming the critical role of IL13 in the initiation of IgE production.[23–26] These gene-gene interactions were consistent with the complementary role of both molecules in IgE switching.[8] Another interesting finding of our study, is the combination of the predominant allele of IL13 R130Q polymorphism with any of the predominant homozygous genotypes of the three polymorphisms of IL4RA (I50V, S478P, and Q551R) was more significantly associated with the risk of beta-lactam allergy ($P < 0.0001$, $p = 0.0163$, 0.0301, respectively) than any polymorphism considered alone ($P = 0.0093$, 0.0031, 0.2059, 0.6000, respectively). Also, the symmetrical combinations (IL13 130RQ/QQ and IL4RA 50II), and (IL13 130RR and IL4RA 50IV/VV) were significantly associated with the risk of beta-lactam allergy, while the other combinations were not significant (**Figure 1**). **Table 6** shows genetic association studies that reported genetic predictors in association with beta-lactam allergy compared with our study. These studies suggested that pro-inflammatory cytokine genes such as IL4R, IL4, IL13 are involved in IgE mediated beta-lactam reactions.

Computer modelling of the rs20541 variant has shown that this substitution affects the signal strength between interleukin 13 and its receptor.[27] This polymorphism encodes an amino acid residue, which is located within the D helix, close to the C-terminal region of IL13.[28] IL13 is a ligand of the IL4RA subunit; it is thus possible that the R130Q polymorphism influences the interaction between D helix and the IL4RA subunit. The underlying molecular mechanisms of this association need to be clarified because the computer modelling of the IL13/IL4RA interaction suggests that the arginine of the 130RR variant repulses the histidine 131 of IL4RA.[27] The S478P and Q551R variants of IL4RA may intensify the downstream signalling, because of their position close to a STAT6-recruiting domain.[28] Therefore, additional genes related to the signalling pathways of IL4RA, such as IL4, STAT6, and JAK1, could also account for an additional risk of IgE mediated allergy to beta-lactams, as previously suggested in probands with asthma susceptibility.[21]

In the haplotype analysis of the IL4RA gene, the GTA haplotype frequency in patients with beta-lactam allergy was found to be significantly higher than the control group suggesting an interaction between the three polymorphisms regarding susceptibility to beta-lactam allergy. In other words, the results indicate that GTA haplotype could be associated with the susceptibility to beta-lactam allergy in the Algerian population. The association of G50, T478 and A551 combination with beta-lactam allergy was higher than each allele alone, suggesting that haplotype analysis can provide more information than the single SNP alone. Moreover, it is interesting to observe that the haplotype ATA seems to have a protective effect against beta-lactam allergy, although the reason is unclear. Thus further studies should be undertaken to analyse the putative relevance of haplotypes of IL4RA Ile50Val, Ser478Pro and Gln551Arg polymorphisms in the development of beta-lactam allergy.

Conclusion

In summary, our study suggests that IL4RA I50V and IL13 R130Q polymorphisms are related to beta-lactam allergy. Our data demonstrate that IL13 is a more potent predictor of beta-lactam allergy than IL4RA. In the Algerian population, a significant association of IL13/IL4RA polymorphism combinations with beta-lactam allergy and IgE levels is observed. However, additional studies are needed to confirm these results in other populations. Also, our data suggest that the haplotype GTA from rs1805010, rs1805015, and rs1801275 of IL4RA may be related somehow to beta-lactam allergy. This relationship needs to be further studied using a larger sample.

Our results have a certain clinical implication. The identification of genetic risk factors may improve the diagnosis and understanding of the pathophysiology of beta-lactam allergy. Therefore, having a clear view of the genetic factors involved can lead us to develop better preventive methods and strategies as well as effecting better drug design and treatment strategies in the future.

Conflicts of Interests

The authors have not declared any conflict of interests

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ORIGINAL ARTICLE

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Prevalence of allergic rhinitis comorbidity with asthma and asthma with allergic rhinitis in China: A meta-analysis

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Abstract

Background: Allergic rhinitis (AR) and asthma are the most common inflammatory diseases of the airways. The relationship between asthma and AR is widely and clinically recognised. The concept "one airway, one disease" has been gradually accepted. However, in China, we could not find any systematic review and meta-analysis on the prevalence of AR with asthma and asthma with AR.

Objective: The aim of this research was to carry out a meta-analysis on the results of all conducted studies to present valid information about the co-occurrence rate of AR with asthma and asthma with AR in China.

Methods: Pubmed/Medline, Science, Springer, Elsevier, Embase, Wanfang data, VIP, CBM, and CNKI were searched systemically and data were extracted from eligible studies by two independent reviewers. Meta-analysis, study quality assessment, and publication bias assessments were all done using Stata 12.1 software.

Results: The results of this meta-analysis showed that pooled prevalence estimates of AR with asthma ranged from 6.69% to 14.35%, asthma with AR from 26.67% to 54%. Furthermore, an overall prevalence of 10.17% (95% CI 9.08–11.27%) was ascertained for AR with asthma, and 38.97% (95% CI 34.42–43.53%) for asthma with AR.

Conclusions: The present meta-analysis comprehensively provided the first quantitative summary of the prevalence of AR with asthma and asthma with AR in China. Our study demonstrated that, in China, asthma and AR are often comorbid diseases and co-exist in the same patients. There is a close correlation between AR and asthma from an epidemiological standpoint.

Key words: allergic rhinitis, asthma, comorbidity, prevalence, China

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Introduction

Allergic rhinitis (AR) and asthma are the most common inflammatory diseases of the airways. The prevalence of AR is 10-40% worldwide.[1] Our previous epidemiological investigations showed that in Western China, the prevalence of self-reported AR was 32.3% (Chongqing), 34.3% (Chengdu), 37.9% (Urumqi), and 30.3% (Nanning).[2] Globally, the prevalence of asthma has more than doubled over the past 20 years.[3] The prevalence of asthma has been reported to vary in different countries: 10% in the United Kingdom, 4.8% in France, 4.8% in Germany, 4.7% in Italy, and 4.8% in Spain.[4,5]

The relationship between asthma and AR is widely and clinically recognised. Grossman first described the concept "one airway, one disease" in 1997, mainly from the pathophysiological roles of leukotriene inflammation in the upper and lower airways.[6] Research showed that many patients with asthma, particularly those with allergic asthma, also have AR. The mucosa of the upper and lower airways is continuous, and the types of inflammation in AR and asthma are very similar, involving T helper type 2 cells, mast cells, and eosinophils. Both diseases have characteristic symptoms and are strongly influenced by environmental factors. Previous studies demonstrated that among patients with asthma and concomitant AR, those who received treatment for AR had a significantly lower risk of subsequent asthma-related events (emergency care visits /hospitalisations) than those who did not receive treatment.[7] Ohta et al. found that in Japan, AR is a common comorbidity (67.3%) in asthma and that it impairs asthma control.[8]

The data about the prevalence of allergic rhinitis, asthma among the Chinese population may affect the decision of policy makers, insurance organisations, and health authorities. Although, there are a few studies about the prevalence of AR and asthma in China, we could not find any systematic review and meta-analysis on the prevalence of asthma and AR among the Chinese population, especially the prevalence of AR with asthma and asthma with AR. Thus, the aim of this research was to carry out a meta-analysis on the results of all conducted studies to present valid information about the prevalence of AR with asthma. In addition, we aimed to investigate the co-occurrence rate of AR with asthma and asthma with AR in China.

Materials and Methods

Preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines were followed while performing this meta-analysis and associated systematic review.[9]

Literature search

Sensitive, systematic searches were separately conducted by two trained researchers to find studies on allergic rhinitis and asthma. Several electronic databases including Pubmed/Medline, Science, Springer, Elsevier, Embase, Wanfang data, VIP, CBM, and CNKI were searched for relevant articles. The major medical subject headings (MeSH) and keywords used in different logical combinations and phrases included "allergic rhinitis", "asthma", "epidemiology/prevalence/morbidity/incidence/attack rate", and "comorbidity". The search encompassed original research papers published from 2006 to 2016.

Inclusion and exclusion criteria

We included population-based studies that reported the prevalence of allergic rhinitis and asthma among Chinese populations. The inclusion criteria were: (1) studies reporting the prevalence of allergic rhinitis, asthma, allergic rhinitis with asthma, and/or asthma with allergic rhinitis; (2) studies reporting the exact diagnostic criteria; (3) cross-sectional studies; and (4) study reports with data in forms that were able to be utilised in the meta-analysis. The exclusion criteria were: (1) repeated publications; (2) reviews; (3) studies providing insufficient data; and (4) a methodological quality score less than 5.

Data extraction

Initially, two researchers independently reviewed all the titles and abstracts that were selected using the keywords. In the second phase, full texts of the articles, which were selected in the first phase, were reviewed; finally, the researchers selected the articles whose contents were suitable for data extraction. Disagreements between the two reviewers about selecting articles were resolved by a third reviewer via discussion and consensus. Extracted information included name of the first author, year of publication, type of study (local study or survey), total sample size, number of patients, point prevalence, and 95% confidence interval (CI) of point prevalence.

Study quality assessment

The global burden of disease quality assessment checklist was used to assess the quality of the studies. Total study quality score was achieved by summing the sampling method (1–4 score), the sample size (0–3), and the response rate (0–6).[10]

Statistical analysis

The AR with asthma and asthma with AR prevalences were calculated using the random effects model with 95% CI. To evaluate heterogeneity, we estimated the proportion of between-study inconsistency using the I^2 statistic, with values of 25%, 50%, and 75% considered low, moderate, and high, respectively. If the heterogeneity was significant and $I^2 > 50\%$, the random-effect model was adopted; otherwise, the fixed-effect model was used. All statistical tests were performed using Stata software version 12.1 (Stata Corporation, College Station, TX, USA).

Results

Literature search

Following the development of our search strategy, a total of 783 relevant articles were selected from primary research in electronic databases. After deleting duplicate articles and reviews, 325 potential articles were obtained. Then, 278 articles were excluded due to irrelevance to the study subject after evaluation of titles and abstracts, so 47 articles were included into the study for reviewing full-text. Finally, 26 articles were excluded after reviewing full-texts due to inappropriate study design and/or outcome. Thus, 21 studies that met inclusion criteria were included in the meta-analysis and summarised in **Figure 1** and **Table. 1**.

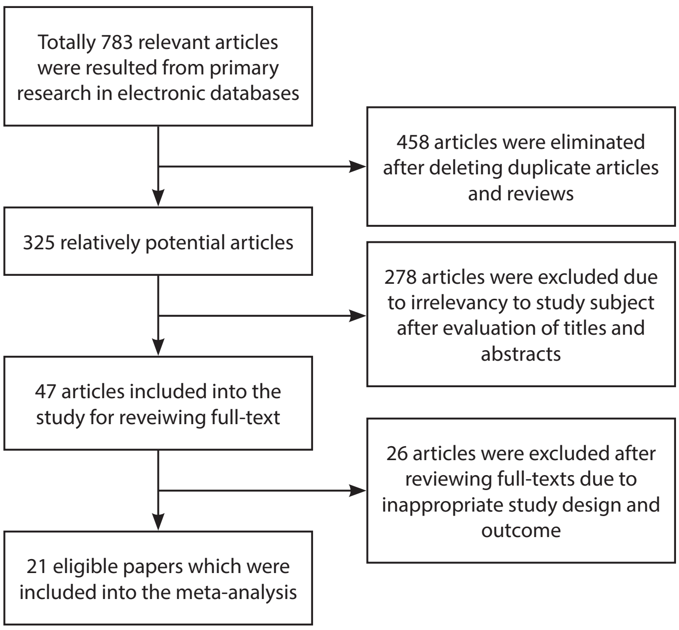

Figure 1. Flowchart for identification of studies selected.

Table 1. Characteristics of the included studies on prevalence of AR with asthma and asthma with AR in China from beginning to 2006.

AR with asthma								
Year	Author	Study	Age (y)	Diagnosis	AR	Asthma	Sample	Rate
2015	Gao Rongli	Cross-sectional study	5-70	ARIA	248	20	2052	8.06%
2015	Zhang Liangran	Cross-sectional study	5-80	ISAAC	690	76	2778	11.01%
2015	Yang Li	Cross-sectional study	2-81	ARIA	324	22	8716	6.79%
2015	Chen Xing	Cross-sectional study	18-70	ARIA	425	61	2580	14.35%
2014	Liu Xiaoling	Cross-sectional study	5-66	ARIA	266	19	266	7.14%
2014	Wang Wenya	Cross-sectional study	≥ 14	ARIA	3859	355	3859	9.20%
2012	Fu Jingming	Cross-sectional study	7-75	ARIA	164	20	916	12.20%
2011	Zhu Xiuqing	Cross-sectional study	7-75	ARIA	672	78	2516	11.61%
2010	Yin Rong	Cross-sectional study	2-81	ARIA	2267	238	2267	10.50%
2009	Dou Xiuli	Cross-sectional study	> 15	ISAAC	901	101	6026	11.21%
2008	Yin Haihong	Cross-sectional study	18-24	ISAAC	226	26	1954	11.50%
Asthma with AR								
Year	Author	Study	Age (y)	Diagnosis	Asthma	AR	Sample	Rate
2015	Li Jipeng	Cross-sectional study	≥ 4	ARIA	174	79	14412	45.40%
2015	Feng Qiuyue	Cross-sectional study	0-99	ARIA	45	12	20000	26.67%
2014	Pan Huiming	Cross-sectional study	16-82	ARIA	212	78	212	36.79
2014	Li Jiaowu	Cross-sectional study	7-92	ARIA	72	21	6909	29.17%
2013	Wang Wenya	Cross-sectional study	> 14	ARIA	687	226	57647	32.90%
2013	Li Seng	Cross-sectional study	12-78	ARIA	300	162	300	54.00%
2011	Qian Juanjuan	Cross-sectional study	≥ 4	ARIA	95	43	4956	45.26%
2010	Ma Li	Cross-sectional study	0-85	ISAAC	731	296	731	40.49%
2009	Zhou Lin	Cross-sectional study	> 15	ARIA	73	23	5216	31.51%
2007	Yu Qihong	Cross-sectional study	14-82	ARIA	793	316	793	37.85%

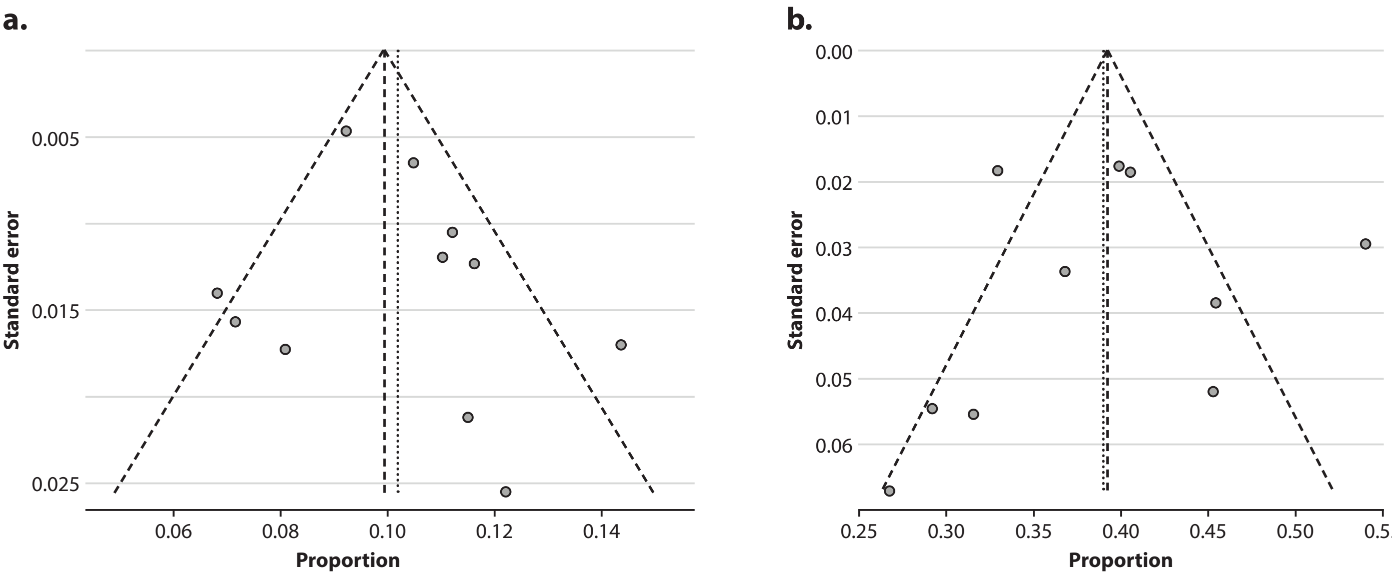

Figure 2. A funnel plot of the overall meta-analysis of metabolic and endocrine comorbidities reflecting publication bias. (a. AR with asthma; b. asthma with AR)

Study characteristics

The selected studies were published from 2006 to 2016 and all the included articles were carried out as cross-sectional surveys, including 133813 participants and 10042 AR patients and 3182 asthma patients in the articles that comprised this meta-analysis. Publication bias assessment was made by visual examination of the funnel plot symmetry. (**Figure 2**)

Estimated prevalence of AR comorbid with asthma

Eleven studies[11-21] about AR with asthma in China were selected in this research. Based on the results of random effect method, the overall prevalence of AR cmorbid with asthma in China was 10.17% (95% CI 9.08–11.27%). In total, 10042 AR patients with an average of 913 AR patients per study were evaluated. The highest prevalence was reported by Chen Xing et al. in 2015 (14.35%) and the lowest by Yang Li et al. in 2015 (6.79%). (**Figure 3**, **Table 1**)

Estimated prevalence of asthma comorbid with AR

Ten studies[22-31] about asthma with AR in China were selected. The overall prevalence of asthma comorbid with AR in China was 38.97% (95% CI 34.42–43.53%). In total, 3182 asthma patients with an average of 32 asthma patients per study were evaluated. The highest prevalence was reported by Li Seng et al. in 2013 (54%) and the lowest by Feng Qiuyue et al. in 2015 (26.67%). (**Figure 4**, **Table 1**)

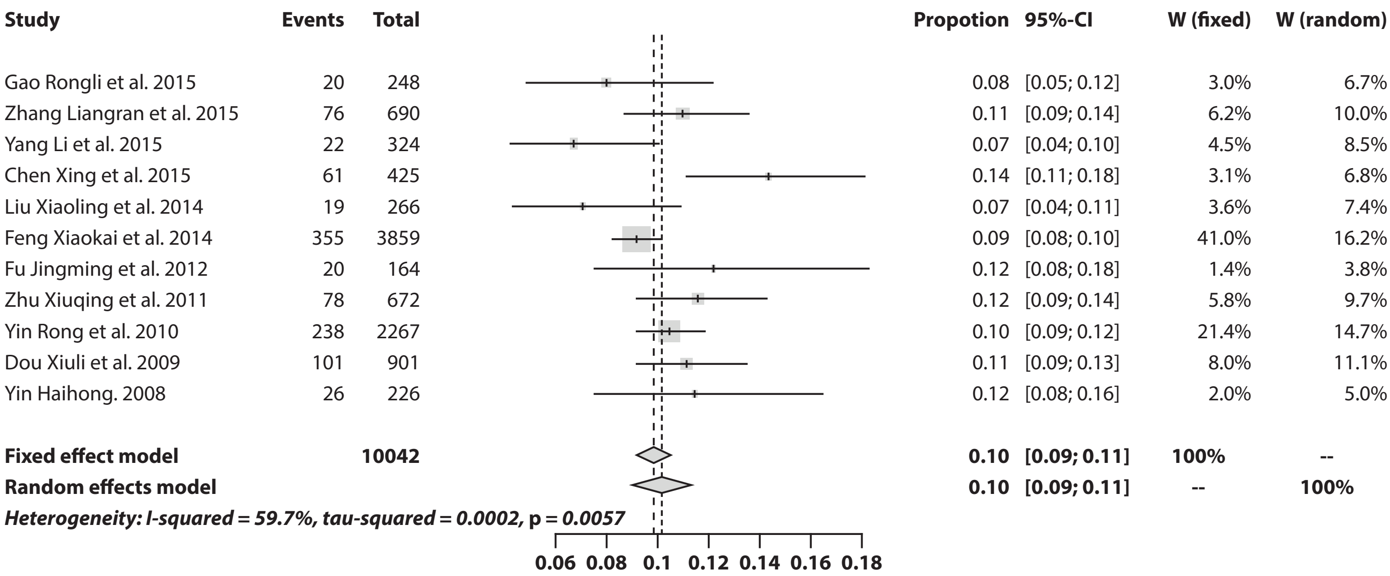

Figure 3. Forest plot of the rate of AR patients with asthma.

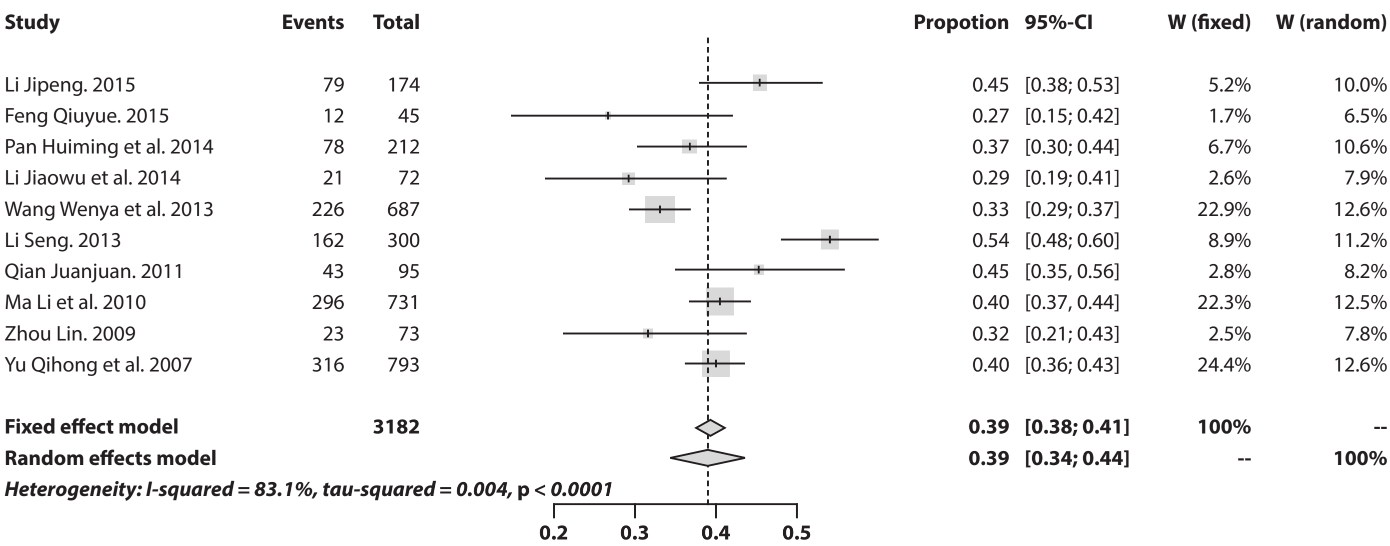

Figure 4. Forest plot of the rate of asthma patients with AR.

Discussion

Allergic rhinitis and asthma are both caused by an inappropriate immunological response to antigens compared to the response elicited in most individuals. Our study presented a comprehensive report about the prevalence of AR with asthma and asthma with AR. The results of this meta-analysis showed that pooled prevalence estimates of AR with asthma ranged from 6.69% to 14.35% and asthma with AR from 26.67% to 54%. Furthermore, an overall prevalence of 10.17% (95% CI 9.08–11.27%) was determined for AR with asthma, and 38.97% (95% CI 34.42–43.53%) for asthma with AR. This study presented a comprehensive report that is the first quantitative summary of the prevalence of AR with asthma and asthma with AR in China. The results of this meta-analysis demonstrated a close correlation between AR and asthma from an epidemiological perspective.

AR and asthma, rather than being considered two distinct diseases, can be unified by the concept of a "united airway," where allergic symptoms of the upper and lower airways can be thought of as manifestations of a common atopic entity.[6,32] Both diseases, which are IgE mediated, can be triggered by similar allergens, including mold, animal dander, and house-dust mites. Epidemiological studies have shown that the majority of patients with asthma have concomitant rhinitis and the presence of rhinitis is an increased risk factor for the development of asthma.[33,34] The prevalence of asthma is < 2% in subjects without rhinitis while it varies from 10% to 40% in patients with rhinitis.[35] Meanwhile, AR occurs in > 75% of patients with asthma, whereas asthma affects up to 40% of patients with AR.[36] In a 10-year longitudinal study of children with AR, asthma was eventually found in 19% of the cases, and in 25% of the sample size asthma and AR developed simultaneously.[37] In a 23-year follow-up study of almost 2000 college students, patients with AR, when compared with controls without AR, were about three times more likely to develop asthma.[38] Pefura-Yone et al. reported that the prevalence of rhinitis was 27.3% among subjects with current wheezing and 25.4% of participants with asthma had rhinitis in Cameroon.[39] Furthermore, in Japan, a nationwide survey of asthmatic patients revealed that 67.3% of asthmatic patients had AR.[8] In addition to the epidemiological evidence, several clinical reports point to a common pathophysiological relationship between AR and asthma.[40] Our meta-analysis demonstrated the prevalence of AR with asthma and asthma with AR in China. The results supported that asthma and AR are often comorbid diseases and co-exist in the same patients. Meanwhile, our data showed the prevalence of asthmatic patients with AR in China to be lower than in Japan. On the one hand, we think the difference may partly be ascribed to regional disparity. On the other hand, environmental factors and different allergens may aos play roles.[41]

Based on the results of previous research and our meta -analysis, we know that there is a close correlation between AR and asthma; AR is highly comorbid with asthma and is a risk factor for asthma. These studies indicate that establishing the overall concept of upper and lower airway is particularly important for AR and asthma treatment. Thus, on the one hand, we should pay attention to the evaluation of the lower airway of AR patients, using pulmonary function tests, bronchial provocation experiment, chest radiograph, and so on. On the other hand, in the process of asthma treatment, we should note to control the symptoms of AR.

Nevertheless, there are some several limitations to the present meta-analysis. First, the number of studies included was comparatively small. Second, the lack of detailed descriptions of AR and asthma features (such as atopic status, age of onset, and disease severity) constrained further subgroup analyses. Third, our study only included the studies from the last 10 years. As we all know, the environment has changed greatly during this time span. Thus, the changes in environmental risk factors for AR may have partially biased the results of this meta-analysis. Meanwhile, in this research, only published studies were reviewed; as a result, unpublished studies and gray literature were not included in our analyses because they were not accessible. Such sets of data could have greatly impacted our results.

In conclusion, the present meta-analysis comprehensively provided the first quantitative summary of the prevalence of AR with asthma and asthma with AR in China. The results of this study showed that the overall prevalence of AR with asthma and asthma with AR was 10.17 % and 38.97 %, respectively. Our study demonstrated that asthma and AR are often comorbid diseases and co-exist in the same patients. There is a close correlation between AR and asthma from an epidemiological perspective. These results can fill the knowledge gaps about the prevalence of respiratory diseases in China, and it can help policy makers, specialists, insurance companies, and all stockholders to make plans and evaluate the medical services required to reduce the prevalence of respiratory diseases.

Disclosure statement

The authors declare no financial or other conflicts of interest regarding the content of this article.

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ORIGINAL ARTICLE

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Prevalence and severity of asthma, rhinoconjunctivitis and eczema in children from the Bangkok area: The Global Asthma Network (GAN) Phase I

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Abstract

Background: As noted in the reports of ISAAC phase I and III, allergic diseases are very common in Thailand, especially among younger children.

Objective: The objectives of this project are to study the prevalence and severity of the most common allergic diseases. i.e. asthma, rhinoconjunctivitis and eczema among children living in Bangkok.

Methods: A cross-sectional multi-centers survey using GAN Core questionnaires on asthma, rhinoconjunctivitis and eczema symptoms were completed by parents of children aged 6–7 years and children aged 13–14 years.

Results: The total of 6,291 questionnaires were eligible for the analysis. The cumulative vs. 12-month period prevalence of the three conditions for all children were: 24.4% vs. 13.5% for wheezing, 51.1% vs. 43.6% for rhinitis and 15.8% vs. 14.2% for eczema, respectively. The period prevalence of wheezing for younger children (14.6%) was higher than for older children (12.5%). Prevalences of severe wheeze and exercise wheeze were more common among older children (2.9% and 14.8%). The 12-month prevalences of rhinitis (43.6%) and rhinoconjunctivitis (16.3%) were higher in both age groups. Eczema, as the same to the other conditions, occurred more frequently in both groups (period prevalence of 14.3% and 14.0%) comparing to ISAAC phase III.

Conclusion: Allergic conditions are very common diseases among children residing in Bangkok. There is an urgent need for an in-depth study to define epidemiological factors responsible for this increase.

Key words Asthma, rhinoconjunctivitis, eczema, ISAAC, GAN

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Introduction

Allergic diseases are among the most common chronic diseases in children and adolescents leading to a substantial health and socioeconomic burden. The International Study of Asthma and Allergy in Childhood (ISAAC) phase I and III surveys reported an overall increase in the prevalence of eczema and allergic rhinoconjunctivitis worldwide. However, no changes in the prevalence of asthma among 13-14-year-old children over a mean period of 7 years was observed.[1-3]

The ISAAC phase I study in Thailand was conducted in 1995-1999 in 3 cities namely; Bangkok,[4] Chiang Mai[5] and Khon Kaen.[6] In Bangkok, the prevalences of three conditions were: asthma 18.3%, rhinitis 44.2% and eczema 15.4%. The ISAAC phase III studying in Bangkok shown that there is a trend of increasing prevalence of all atopic diseases among children.[7]

The Global Asthma Network (GAN), established in 2012, was formed by scientists from the International Study of Asthma and Allergies in Childhood (ISAAC) 1991–2012 (phases

I,[8-13] II[14] and III[1-3,15]) and from the International Union Against Tuberculosis and Lung Disease (The Union[16-19]) following production of the first Global Asthma Report (GAR) 2011,[20] launched in New York (NY, USA) in 2011 at the time of the United Nations high-level meeting on non-communicable diseases. GAN phase I, builds on the ISAAC findings by collecting further information on asthma, rhinitis and eczema, prevalence, severity, diagnoses, asthma emergency room visits, hospital admissions, management and the use of asthma essential medicines.

The objectives of our project are to study the prevalence and severity of the most common allergic diseases. i.e. asthma, rhinoconjunctivitis and eczema in children living in Bangkok. We, herein, report the results of our GAN phase I study in 6,291 children from the two age groups living in the Bangkok area

Methods

Study Design

This study is a cross-sectional, multi-center, study design.

Participants

Seven primary schools and six secondary schools in Bangkok were randomly mapped, stratified and had chosen to represent the population of the entire Bangkok Metropolitan area. In addition, equal numbers of governmental and private schools were selected to avoid an over representation of any predominant socioeconomic classes. Subjects were selected in the same manner as ISAAC phase III. The same age groups were used: 13-14 years old adolescents (self-completed questionnaires) and 6-7 years old children (parental completed questionnaires) and GAN phase I adds their parents as an adult group. Students of both age groups were selected either by grade/level/year or by age group. The questionnaires were sent out to 6,824 children (3,544 for 6-7 years and 3,280 for 13-14 years). Although participation rates for both age groups from these schools were exceptionally high (92.18%), many questionnaires were incompletely answered and were therefore excluded from the analysis. This left a grand total of 6,291 children (3,074 for 6-7 years and 3,217 for 13-14 years) for the inclusion of the analysis. The study was approved by the Human Research Ethics Committee of Thammasat University (054/2560) and the Human Research Ethics Committee of Bhumibol Adulyadej Hospital. The clinical trial number was MTU-EC-ES-4-013/60. Inform consents/assents were obtained by children and by the parents.

GAN Core Questionnaires

GAN Standardized Written Core Questionnaires developed from ISAAC Questionnaires for use in phases I and III, were used in GAN. Demographic questionnaires includes the participant's name, age, date of birth, school (for the adolescents and children), sex and date of interview. Questionnaires were coded by using a unique number for each center, school and participant to ensure confidentiality and to link the questionnaires between the adults, adolescents and children.[21] The written core questionnaires, that was used in ISAAC, have had a question about doctor-diagnosis about asthma, rhinitis and eczema. The core questions were both sensitive and specific, had good content, constructive and concurrent and predictive validity.[22] As in ISAAC, a video of asthma questionnaires was an optional tool: the international version that is being used in ISAAC.[23] This 6-minute non-verbal video showed the clinical signs of asthma symptoms and was developed by the Wellington Asthma Research Group, in order to avoid the problems of translation and understanding of terms of "wheeze" or "whistling" and their uses in culturally heterogeneous population.[24] The video has the advantage of obtaining data from many students quickly and efficiently. The questionnaires were translated into Thai and back translated by a three linguistic proficient individuals and were reviewed and approved by the investigators.

Sample Size

As in ISAAC, a sample size of 3,000 participants per age group (and therefore potentially 6,000 adults of each group) was used. The sample size provided greater than 99% ability (at the 1% level of significance) to detect differences in the prevalence of wheezing of 30% in one center and 25% in another center.[22] As sampling was done by schools, and the information gained from the school pupils and adults, is likely to be a cluster effect. Like ISAAC, the analysis incorporated adjustments in cluster sampling using the design effect,[25] which is important for large studies where clusters of different sizes may be used in different regions. High participation is sought for GAN phase I: at least 80% for 13-14 years old and 70% for 6-7 years old and 70% for adults/parents.

Data Collection and Analysis

Data were collected from July 2017 up to February 2018. Information on the questionnaires was entered in the GAN Epi-Info data entry packaged by GAN Global Center in Auckland, New Zealand (info@globalasthmanetwork.org). Such data were analyzed by using STATA version 14 and expressed in the prevalence of three diseases in both the younger and older groups, separately.

Results

Positive response to wheezing modules for younger and older age groups as well as for all children surveyed are tabulated in **Table 1**. All participants are Thai. The prevalence of ever-wheeze in the younger age group was slightly higher than in the older age group (26.0% vs. 22.9%, $p = 0.004$). This was also true for percentage of current wheeze or wheeze in the past 12 months (14.6% vs. 12.5%, $p = 0.016$) and for attacks within the past 12 months (14.4% vs. 12.6%, $p = 0.029$). Percentages for severe wheeze (1.9% vs. 2.9%, $p = 0.019$) and exercise wheeze (3.0% vs. 14.8%, $p < 0.001$) were much higher among older children. Percentages of night awakening were slightly higher among the younger age group (6.7% vs. 4.2%, $p < 0.001$). Percentages of night cough were noticeably high in both groups (24.2% and 29.9%, $p < 0.001$). The prevalence for diagnosed asthma (asthma-ever, 6.1% and 8.8%, $p < 0.001$) were much lower than wheezing-ever for both groups (26.0% and 22.9%). As for male: female ratio, there was no predominance for males over females other than responses for question of 'asthma ever' (1.36).

Table 1. Percent of positive response of questions in wheezing module.

Symptoms	All (n = 6,291) (95%CI)	6-7 years (n = 3,074) (95%CI)	13-14 years (n = 3,217) (95%CI)	P Value
Current wheeze	13.5 (12.7, 14.3)	14.6 (13.4, 15.9)	12.5 (11.4, 13.7)	0.016
Wheezing ever	24.4 (23.4, 25.5)	26.0 (24.5, 27.6)	22.9 (21.5, 24.4)	0.004
Asthma ever	7.4 (6.8, 8.1)	6.1 (5.2, 6.9)	8.8 (7.8, 9.7)	< 0.001
Symptoms in past 12 months				
- attacks	13.5 (12.6, 14.3)	14.4 (13.2, 15.7)	12.6 (11.4, 13.7)	0.029
- night waking	5.4 (4.9, 6.0)	5.4 (4.9, 6.0)	4.2 (3.5, 4.9)	< 0.001
- severe wheeze	2.4 (2.0, 2.8)	2.4 (2.0, 2.8)	2.9 (2.3, 3.4)	0.019
- exercise wheeze	9.0 (8.3, 9.8)	9.0 (8.3, 9.8)	14.8 (13.6, 16.0)	< 0.001
- night cough	27.1 (26.0, 28.2)	27.1 (26.0, 28.2)	29.9 (28.3, 31.5)	< 0.001

Current wheeze: wheeze in the past 12 months
Symptoms of severe asthma: respondents with current wheeze who had > 4 attacks of wheeze in the last year or had > 1 nights per week sleep disturbance from wheeze in the last year or had wheeze affecting speech in the last year.
P Value for Chi square test of positive response symptom between age groups

Table 2. Percent of positive response to video questionnaires for wheezing

Description of video sequences:	13-14 years (n = 3,217)	
	Cumulative (95%CI)	12 month Prevalence (95%CI)
Wheezing at rest	11.9 (10.8, 13.1)	8.9 (7.9, 9.9)
Exercise wheeze	13.5 (12.3, 14.5)	9.0 (8.1, 10.0)
Night wheeze	6.6 (5.8, 7.5)	5.6 (4.8, 6.4)
Night cough	23.4 (21.9, 24.8)	17.9 (16.6, 19.3)
Severe wheeze	8.1 (7.2, 9.1)	5.8 (5.0, 6.6)

Current wheeze: wheeze in the past 12 months

Table 3. Percent of positive response of questions in rhinitis modules.

Symptoms	All (n = 6,291) (95%CI)	6-7 years (n = 3,074) (95%CI)	13-14 years (n = 3,217) (95%CI)	P Value
Current rhinoconjunctivitis or Current AR	16.3 (15.4, 17.2)	15 (13.8, 16.3)	17.5 (16.2, 18.8)	< 0.001
Current nose symptom	43.6 (42.4, 44.8)	38.2 (36.5, 39.9)	48.8 (47.0, 50.5)	< 0.001
Current eye symptom	16.6 (15.6, 17.5)	15.0 (13.8, 16.3)	18.0 (16.7, 19.4)	0.001
Nose ever	51.1 (49.9, 52.4)	47.3 (45.5, 49.0)	54.9 (53.1, 56.6)	< 0.001
Hay fever ever	27.4 (26.3, 28.5)	24.5 (23.0, 26.0)	30.1 (28.5, 31.7)	< 0.001
Severe rhinoconjunctivitis	1.5 (1.2, 1.7)	1.0 (0.6, 1.3)	1.9 (1.4, 2.4)	< 0.001

Current rhinoconjunctivitis or Current AR: Current nose symptom and current eye symptom
Severe rhinoconjunctivitis: Current rhinoconjunctivitis and answer A LOT to question " In the past 12 months, how much did this nose problem interfere with your (child) daily activities?
P Value for Chi square test of positive response symptom between age groups

The self-reported video questionnaires completing by the 13-14-year-old group revealed a cumulative vs. current prevalence of: wheezing at rest (11.9% vs. 8.9%), exercise wheeze (13.5% vs. 9.0%), night wheeze (6.6% vs. 5.6%), night cough (23.4% vs. 17.9%) and severe wheeze (8.1% vs. 5.8%) (**Table 2**). Percentages for night wheeze (5.6%) was slightly higher than that derived from the written questionnaires (4.2%). The video responses to exercise question (9.0%) was lower than that from the written ones (14.8%). The prevalence of severe wheeze from video responses was 5.8%, which is twice of the written questionnaire (2.9%).

In **Table 3**, prevalences of rhinitis and other associated symptoms are shown. An exceptionally high number of children from both age groups (47.3% and 54.9%) reported nasal

Table 4. Percent of positive response of questions in eczema module.

Symptoms	All (n = 6,291) (95%CI)	6-7 years (n = 3,074) (95%CI)	13-14 years (n = 3,217) (95%CI)	P Value
Rash ever	15.8 (14.9, 16.7)	16.3 (15.0, 17.6)	15.2 (14.0, 16.5)	< 0.001
Eczema ever	22.8 (21.8, 23.9)	28.6 (27.0, 30.2)	17.3 (16.0, 18.7)	< 0.001
Flexural area	10.8 (10.1, 11.6)	11.7 (10.6, 12.9)	10.0 (8.9, 11.0)	0.024
Symptoms in past 12 months				
- rash	14.2 (13.3, 15.0)	14.3 (13.1, 15.6)	14.0 (12.8, 15.2)	0.684
- rash clear	9.6 (8.9, 10.3)	9.1 (8.1, 10.2)	10.0 (9.0, 11.1)	0.226
- night waking	4.7 (4.2, 5.2)	5.6 (4.8, 6.4)	3.8 (3.2, 4.5)	0.001

Severe eczema: Current eczema associated with sleep disturbance 1 or more nights per week
P Value for Chi square test of positive response symptom between age groups

symptoms. Approximately 43.6% experienced nasal symptoms within the past 12 months: whereas, 16.6% reported from concomitant eye symptoms. These children indicated that their symptoms were bothersome at some point. The prevalence of current AR (current rhinoconjunctivitis) of both age group (15% vs. 17.5%). The prevalence of severe AR in children aged 6-7 years and 13-14 years were 1.0% and 1.9% respectively. The prevalence of severe AR in all children was 1.5%. Although the term 'hay fever' does not exist in the Thai language, 27.4% indicated that they suffered from 'allergy to the air', a common term denoting hay fever in Thai.

Positive responses to questions in the eczema module are shown in **Table 4**. The percentage of younger children reported 'rashes within the past 12 months' was 14.3% and up to 11.7% indicated rashes localized in areas typical diagnosis of atopic dermatitis. Slightly lower numbers were reported in older children (14.0% and 10.0%). Many children with a rash indication had mostly cleared within the past twelve months (9.1% and 10.0%). and was not bothersome to them. The prevalence of severe eczema in children aged 6-7 years and 13-14 years were 5.6% and 3.8% respectively. The prevalence of severe eczema in all children was 4.7%. It can be suggested that the degree of eczema was mild among Thai children. Male to female ratio suggested that slightly more females than males were affected with these rashes.

In our study, there were strong associations with other allergic diseases: in asthma patients: 32.5% had AR and 21.8% had eczema, AR patients: 27.1% had asthma and 24.6% had eczema, eczema patients: 37.1% had asthma and 27.4% had AR.

Discussion

As noted in the reports of ISAAC phase I and III, asthma was very common in Thailand, especially among younger children.[4,7] In this study, prevalence rates of current wheeze based on the written questionnaire in the 6–7 years is similar to the prevalence in the ISAAC study phase III; in Bangkok[7] (14.6% vs. 15.0%, p = 0.541). Meanwhile, the prevalence rate in the 13–14 years age group is slightly lower than prevalence in the ISAAC study phase III; in Bangkok[7] (12.5% vs. 13.9%, p = 0.024). Slightly higher than the ISAAC phase III: the mean global prevalence for current wheeze (11.5% and 4.9%) and the Asia-pacific prevalence (9.5% and 8.8%).[9]

The cumulative prevalence of wheezing based on the video questionnaires from this study (11.9%) is closed to the prevalence of the ISAAC study phase III from Bangkok (11.5%).[7] This is much higher than the Asia-Pacific prevalence (5.5%) and, also the global prevalence (8.7%) of the ISAAC study phase III.[9] The prevalence of severe asthma (written questionnaires) in the 13-14 years age group is 2.9%. This is lower than the prevalence of severe asthma from the ISAAC study phase III: globally (6.9%) ranging from 3.8% in Asia-Pacific, Northern and Eastern Europe to 11.3% in North America (compared to Bangkok 4.0%).[9]

The Asthma Insight and Management (AIM) survey (2011) reported the asthma exacerbations in the past 12 months: Thailand (36%), South Korea (47%), Australia (54%), and China (67%).[26] Thai patients that uses controller medication is 54% in previous month. Pill controller medication is the most common form among those reporting controller medication used (67%), whereas 57% reported taking an inhaler.[27]

The new GAN phase I survey, however, portrayed a differing epidemiological outlook than from what has been felt among practitioner caring for asthmatic patients. These preliminary data have shown that prevalence of asthma in younger and older children is still over 10% of the population surveyed. Moreover, the prevalence for those with severe wheeze is roughly 2%. The Chest and Allergy Societies in Thailand have regularly updated asthma guidelines for adults and children. Besides, social media has made it easier for parents/patients to find appropriate professional care. An increase in the availability of asthma controllers throughout the country may help lessen the severe asthma attacks presented to emergency rooms and requiring hospital admissions in this country. Among these drugs, inhaled steroids are very popular. Since generic versions of these controllers are cheaper than original version, they were included in Essential Drug List subsidized by the Government for those eligible for medical supports (governmental employees, those under the social security program and universal health coverage). Effective advocacy by non-governmental organizations, smoking in homes and public places is now rare event. Thailand has enforced stricter regulations to reduce outdoor air pollution, such as cleaner air emissions and vehicle fuels.

Ecological economic analyses also revealed that although the high-income centers tended to have a higher prevalence of current wheeze, a reverse trend was found in the prevalence of symptoms of severe asthma among current wheezers. There may be several reasons underlying this observation. First, asthma care is likely to be poorer in these developing countries, although a recent epidemiological survey showed that suboptimal asthma management was a global phenomenon.[28,29] Secondly, there may be less awareness of wheeze being a symptom of asthma, even in those with frequent wheezing, similar to the situation amongst ethnic minorities in developed countries.[30] This notion is further supported by the finding that undiagnosed asthma among those current wheezers with severe asthma symptoms was most commonly seen in these lower income countries. Children with undiagnosed frequent symptoms are also more likely to receive inadequate care for their asthma and may fall into a vicious downward spiral of asthma control.[30] Thirdly, differences in the levels of environmental exposure, including air pollutants and infective agents, may also contribute to the greater severity observed in these countries.

GAN phase I has provided the most comprehensive estimate of the worldwide symptom prevalence of asthma to date. This global map of asthma is invaluable not only for public health planning, but also for generating hypotheses in explaining the etiological factors for this common disorder.

In our study, the prevalence of current AR or current rhinoconjunctivitis in the 6–7 year and 13–14-year age groups are 15.0%, 17.5% respectively. As the ISAAC study phase III, the prevalence of current AR of Thai children from the Bangkok area were 13.4% and 23.9% respectively.[7] It is slightly higher than the mean of global prevalence (9.1%, 16%), and the Asia-Pacific prevalence (5.8% and 14.5%).[10]

In our study, the prevalence of current eczema symptoms in the 6–7 years and 13–14 years age groups are 14.3%, 14.0% respectively. These values are slightly higher than those from the ISAAC study phase III study in Bangkok (13.3% and 10.4%).[7] However, our GAN results on eczema is much higher than the ISAAC study phase III study elsewhere: the mean global prevalence (7.9%, 7.3%) and the Asia-pacific prevalence (4.7% and 5.3%).[12]

For developing countries, Thailand has been noted to has an increased in the number of patients with food allergy and atopic dermatitis. The reason for this worrisome and unusual increase is uncertain at this point. Similarly, results of GAN phase I survey substantiate the increasing numbers of children in both age groups. If a phenomenon of allergic march operates in this part of the world, one should witness an increase in the number of asthmatic patients rather than a decrease in the next decade.

Strengths and Weaknesses of the Study

The major strengths of our study included a standardized written core questionnaires (GAN 2016) developed from ISAAC Questionnaires, well-established standardized protocol and high response rate. The establishment of GAN 2016 questionnaires allows an excellent opportunity for different countries to establish their own basic epidemiological data for allergic diseases that can be compared internationally. A video asthma questionnaire (6-min non-verbal video) shows clinical signs of asthma symptoms to avoid problems of translation and comprehension of terms such as "wheeze" or "whistling" and their use in culturally heterogeneous population. One limitation of our study is that symptoms of allergic rhinitis were self-reported in the questionnaire, therefore, we could not confirm with physical examination and laboratory investigations.

In conclusion, the result of GAN phase I in Bangkok showed a slightly increase of prevalence of eczema in both age groups, while prevalences of asthma and allergic rhinitis have become stabilized in both age groups. Most Thai children with asthma had coexisting rhinitis, and a portion of patients with rhinitis also had asthma. Allergic conditions are very common among children residing in Bangkok. There is an urgent need for an in-depth study to define epidemiological factors responsible for this increase.

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ORIGINAL ARTICLE

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Prevalence and risk factors of allergic rhinitis in children in Bangkok area

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Abstract

Background: Allergic rhinitis (AR) is a disease with a high global disease burden and significant morbidity and expense. Risk factors are not well understood.

Objective: The objective of our project is to study the prevalence and risk factors of AR in children living in the Bangkok area.

Methods: A cross-sectional, multi-center survey using new GAN core questionnaires on current AR and risk factors was completed by 3,074 parents of children aged 6–7 years and by 3,217 children aged 13–14 years, directly.

Results: The prevalence of current AR in children aged 6–7 years and 13–14 years was 15.0% (95% confidence interval [CI]:13.8–16.3%) and 17.5% (95% CI: 16.2–18.8%), respectively. The prevalence of severe AR in children aged 6–7 years and 13–14 years was 1.0% (95% CI: 0.6–1.3%) and 1.9% (95% CI: 1.4–2.4%), respectively. Co-morbidity with asthma and eczema was 27.1% and 24.6%, respectively. Significant factors associated with AR include parental history of asthma ($p = 0.025$), parental history of AR ($p < 0.001$), parental history of eczema ($p < 0.001$), lower respiratory tract infection in the first year of life ($p < 0.001$), breastfeeding ($p = 0.019$), current use of paracetamol ($p < 0.001$), exercise ($p < 0.001$), current cat exposure ($p = 0.008$), and truck traffic on the street of residence (< 0.001).

Conclusion: AR is a common disease among children residing in Bangkok. This study confirms that a family history of atopy (asthma, AR, and eczema), antibiotics given in the first year of life, current paracetamol use, exercise, current cat exposure, and truck traffic on the street of residence are important and significant risk factors for AR symptoms.

Key words: allergic rhinitis, atopy, asthma, ISAAC, GAN

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Introduction

Allergic rhinitis (AR) is characterized by paroxysms of sneezing, rhinorrhea, and nasal obstruction, often accompanied by itching of the eyes, nose, and palate. Postnasal drip, cough, irritability, and fatigue are other common symptoms.[1,2] AR is associated with significant morbidity and expense.[3,4]

The increase in the prevalence of AR began to attract attention from epidemiologists in the late 1980s. The International Study of Asthma and Allergies in Childhood (ISAAC) was initiated to establish the prevalence of allergic diseases in 257,800 school children aged 6–7 years and in 463,801 children aged 13–14 years using standardized and validated questionnaires.[7] Phase I of ISAAC, which began to enroll patients in 1992, sought to establish prevalence rates in nearly 60 countries on every continent; phase II investigated variables contributing to AR (e.g., environmental exposures); and phase III provided follow-up data on the patients at least five years after entry into the study. In phase I, prevalence rates for AR collected across all centers ranged from 0.8% to 14.9% (median, 6.9%)

in the 6–7-year-olds and from 1.4% to 39.7% (median, 13.6%) in the 13–14-year-olds.[5] The highest prevalence rates for AR were observed in parts of Western Europe, North America, and Australia, whereas the lowest rates were found in parts of Eastern Europe and South and Central Asia. The phase III analyses revealed that the prevalence rates had increased, with 12-month prevalence rates of 1.8% to 24.2% in children aged 6–7 years (median, 8.5%) and 1.0% to 45% (median, 14.6%) in children aged 13–14 years.[6] These findings strongly indicate that the prevalence of AR has increased over a relatively short period of time, mostly in Westernized countries with a higher standard of living.

According to phase I of ISAAC in Bangkok (1995–1999), the prevalence of AR was 10.0% in the children aged 6–7 years and 15.4% in the children aged 13–14 years.[7] In phase III of the study in Bangkok (2001), the prevalence of AR in children aged 6–7 years and 13–14 years was 13.4% and 23.9%, respectively.[8] There was an increase in the prevalence of rhinitis in both age groups.

Phase III of ISAAC included new questions on risk factors that identified several environmental associations.[9] Risk factors for AR include paracetamol, antibiotics, truck traffic, breast-feeding, farm animals, cats and dogs, air pollution, tobacco, body mass index (BMI), diet, cooking fuels, birth weight, migration, and siblings. Despite the considerable research efforts, the risk factors of AR remain poorly understood. A family history of atopic diseases seems to be a major risk factor, but various environmental factors and lifestyle are also considered important elements in the evolution of the disease.[3,10]

The objective of our project is to study the prevalence and risk factors of AR in children living in Bangkok, Thailand.

Methods

Study Design

This study has a cross-sectional, multi-center design.

Participants

Seven primary schools and six secondary schools in Bangkok were randomly mapped, stratified, and chosen to represent the population of the entire Bangkok metropolitan area. Subjects were selected in the same manner as ISAAC phase III.[9] The same age groups were recruited: 13–14-year-old children (self-completed questionnaires) and 6–7-year-old children (parental completed questionnaires). Of 6,834 questionnaires sent to children, 6,291 were completed (95.05%). There were 3,074 (86.49%) questionnaires of children aged 6–7 years and 3,217 (98.08%) questionnaires of children aged 13–14 years available for analysis. The study was approved by the Human Research Ethics Committee of Thammasat University (054/2560) and the Human Research Ethics Committee of Bhumibol Adulyadej Hospital. The clinical trial number was MTU-EC-ES-4-013/60. Informed consents/assents were obtained from the children and parents.

GAN Core Questionnaires

GAN 2016 standardized written core questionnaires for AR modifying from ISAAC questionnaires were used in this study.[11,12] The questionnaires were translated and back-translated into the Thai language by three independent linguistic-proficient individuals. Demographic questions included the participant's name, age, date of birth, school (for the adolescents and children), sex, and date of interview. Questionnaires were coded by using a unique number for each center, school, and participant to ensure confidentiality and to link the questionnaires between the adults and children.[13] The written core questionnaires, used in GAN, had a question about doctor-diagnosed asthma, rhinitis, and eczema added. The core questions were both sensitive and specific, and they had good content, construct, concurrent, and predictive validity.[14] The environmental risk factor questionnaires, developed for ISAAC phase III, were expanded for use in this study. Height and weight measurements were taken by the fieldworkers in schools.

Definitions of AR, Rhinitis, and Hay Fever

The standardized core symptom questionnaire was the same as that used in ISAAC phase I and comprised of six questions on symptoms relating to rhinitis or rhinoconjunctivitis.[11,12] These questions were as follows:

1. Have you (has your child) ever had a problem with sneezing or a runny or blocked nose when you (he or she) DID NOT have a cold or "the flu"?
2. In the past 12 months, have you (has your child) had a problem with sneezing or a runny or blocked nose when you (he or she) DID NOT have a cold or "the flu"?
3. In the past 12 months, has this nose problem been accompanied by itchy/watery eyes?
4. In which of the past 12 months did this nose problem occur? (Month names listed)
5. In the past 12 months, how much did this nose problem interfere with your (child's) daily activities? (Not at all, a little, a moderate amount, a lot)
6. Have you (has your child) ever had hay fever?

Question 2 was used to estimate the prevalence of current rhinitis; question 3 was used to estimate the prevalence of current conjunctivitis; and question 6 was used to estimate the prevalence of "hay fever ever." Questions 2 and 3 were combined to assess current rhinoconjunctivitis symptoms or current AR. Questions 2 and 3 and the answer "A LOT" to question 5 were used to assess the prevalence of severe rhinoconjunctivitis symptoms or severe AR.

Sample Size

A sample size of 2,654 is needed to estimate the prevalence of questionnaire-based AR of 10% for children of each age group with margin errors of ±1.5% and type one error of 0.01. The total sample size of 6,834 was accounted for the non-response rate of 30%.

Data Collection and Analysis

Data were collected from July 2017 to February 2018. Statistical analyses were carried out using STATA/SE software (Stata/SE 14 for Windows, StataCorp LP, College Station, TX, USA). Binomial confidence intervals (CIs) on proportions with rhinitis and rhinoconjunctivitis were calculated. The multivariable logistic regression model was used to conduct exploratory analysis for risk factors of AR. The model included age, sex, family history of allergy, birth weight, paracetamol, antibiotics, truck traffic, breastfeeding, farm animals, cat and dog exposure, air pollution, tobacco, BMI, diet, cooking fuels, migration, and number of older and younger siblings to estimate the magnitude of the association by calculating adjusted odds ratios with their 95% CIs.

Results

The prevalence of questionnaire-based symptoms of rhinitis stratified by age group is shown in **Table 1**. The prevalence of current rhinitis in children aged 6–7 years and 13–14 years was 38.2% (95%CI: 36.5–39.9%) and 48.8% (95%CI: 47.0–50.5%), respectively. The prevalence of current rhinitis in all children was 43.6% (95%CI: 42.4–44.8%). Concomitant eye symptoms were reported at 16.3%. The prevalence of current AR in children aged 6–7 years and 13–14 years was 15.0% (95%CI: 13.8–16.3%) and 17.5% (95%CI: 16.2–18.8%), respectively. The prevalence of current AR in all children was 16.3% (95%CI: 15.4–17.2%).

Although the term so-called "hay fever" does not exist in the Thai language, 27.4% indicated that they suffered from "allergy to the air," a common term denoting hay fever in Thailand. Patterns of rhinitis symptoms of children in Bangkok were of the perennial type. The prevalence of severe AR in children aged 6–7 years and 13–14 years was 1.0% (95%CI: 0.6–1.3%) and 1.9% (95%CI: 1.4–2.4%), respectively. The prevalence of severe AR in all children was 1.5% (95%CI: 1.2–1.7%). There were strong associations with other allergic diseases: 27.1% of children with AR had asthma and 24.6% had eczema.

A parental history of atopy including asthma (p = 0.025, OR = 1.50, 95%CI = 1.05–2.13), AR (p < 0.001, OR = 1.43, 95%CI = 1.10–1.71), and eczema (p < 0.01, OR = 1.56, 95%CI = 1.29–1.88) was significantly related to current AR. Current use of paracetamol was associated with current AR (p < 0.001, OR = 1.64, 95%CI = 1.30–2.08). Exercise was associated with current AR (p < 0.001, OR = 1.49, 95%CI = 1.29–1.71). Only current cat exposure was associated with current AR (p = 0.008, OR = 1.28, 95%CI = 1.07–1.54). The frequency of truck traffic on the street of residence was positively associated with current AR; comparison of both the occasional truck traffic group (p = 0.002, OR = 1.28, 95%CI = 1.10-1.50) and the always truck traffic group (p < 0.001, OR = 1.73, 95%CI = 1.41-2.11) to the never truck traffic group is shown in **Tables 2 and 3**.

Table 1. Prevalence of questionnaires-based symptoms of rhinitis stratified by age group

Symptoms	All (n = 6,291)		6-7 years (n = 3,074)		13-14 years (n = 3,217)	
	N	Prevalence 95%CI	N	Prevalence 95%CI	N	Prevalence 95%CI
Current AR or ARC	1,042	16.3% (15.4%, 17.2%)	462	15.0% (13.8%, 16.3%)	580	17.5 (16.2%, 18.8%)
Current rhinitis	2,744	43.6% (42.4%, 44.8%)	1,175	38.2% (36.5%, 39.9%)	1,569	48.8% (47.0%, 50.5%)
Hay fever (allergic to air)	1,722	27.4% (26.3%, 28.5%)	754	24.5% (23.0%, 26.1%)	968	30.1% (28.5%, 31.7%)
Severe AR	91	1.5% 1.2%, 1.7%)	30	1.0% (0.6%, 1.3%)	61	1.9% (1.4%, 2.4%)

Current AR or Allergic rhinoconjunctivitis (ARC)- positive to question number 2 and 3
Current rhinitis - positive to question number 2
Hay fever ever- positive to question number 6
Severe AR - positive to question number 2 and 3 and the answer "A LOT" to question 5

Table 2. Characteristics of children with AR stratified by age group

Factors	Total (n = 6,291)			6-7 Years old (n = 3,074)			13-14 Years old (n = 3,217)		
	N	n (%)	P-value	N	n (%)	P-value	N	n (%)	P-value
Age (years)			0.009						
6-7	3,074	462 (15.0)		-	-	-	-	-	-
13-14	3,217	562 (17.5)		-	-	-	-	-	-
Sex			0.143			0.023			0.760
Female	3,013	468 (15.6)		1,559	211 (13.6)		1,454	257 (17.7)	
Male	3,278	555 (16.9)		1,515	250 (16.5)		1,763	305 (17.3)	
BMI			0.137			0.172			0.445
< P85	5,360	857 (16.0)		2,619	384 (14.7)		2,471	473 (17.3)	
≥ P85	931	167 (17.9)		455	78 (17.1)		476	89 (18.7)	

Table 2. (Continued)

Factors		Total (n = 6,291)			6-7 Years old (n = 3,074)			13-14 Years old (n = 3,217)		
		N	n (%)	P-value	N	n (%)	P-value	N	n (%)	P-value
Paternal allergy history										
Asthma	No	6,107	976 (16.0)	< 0.001	2,965	434 (14.6)	0.002	3,142	542 (17.3)	0.034
	Yes	184	48 (26.1)		109	28 (25.7)		75	20 (26.7)	
AR	No	5,234	775 (14.8)	< 0.001	2,442	303 (12.4)	< 0.001	2,792	472 (16.9)	0.031
	Yes	1,057	249 (23.6)		632	159 (25.2)		425	90 (21.2)	
Atopic	No	5,434	811 (14.9)	< 0.001	2,595	331 (12.8)	< 0.001	2,839	480 (16.9)	0.021
	Yes	857	213 (24.9)		479	131 (27.3)		378	82 (21.7)	
Sibling	No	2,013	327 (16.2)	0.961	1,034	140 (13.5)	0.100	979	187 (19.1)	0.107
	Yes	4,278	697 (16.3)		2,040	322 (15.8)		2,238	375 (16.8)	
Only 6-7 Years old										
LBW	No	-	-	-	2,830	423 (14.9)	0.664	-	-	-
	Yes	-	-	-	224	39 (16.0)		-	-	-
Breast Feeding (6 months)	No	-	-	-	1,810	246 (13.6)	0.008	-	-	-
	Yes	-	-	-	1,264	216 (17.1)		-	-	-
Antibiotics (first 1 year)	No	-	-	-	1,936	225 (11.6)	< 0.001	-	-	-
	Yes	-	-	-	1,138	237 (20.8)		-	-	-
Paracetamol (first 1 year)	No	-	-	-	1,099	138 (29.9)	0.004	-	-	-
	Yes	-	-	-	1975	324 (70.1)		-	-	-
LRTI (first 1 year)	No	-	-	-	2,383	286(12%)	< 0.001	-	-	-
	Yes	-	-	-	691	176 (25.5%)		-	-	-
Farm animal	No	-	-	-	2,962	435(14.7%)	0.006	-	-	-
	Yes	-	-	-	112	27 (24.1)		-	-	-
Paracetamol	No	893	99 (11.1)	< 0.001	415	40 (9.6)	0.001	478	59 (12.3)	0.001
	Yes	5,398	925 (17.1)		2,659	422 (15.9)		2,739	503 (18.4)	
Exercise	No	4,032	558 (13.8)	< 0.001	2,264	308 (13.)	< 0.001	1,768	250 (14.1)	< 0.001
	Yes	2,259	466 (20.6)		810	154 (19.0)		1,449	312 (21.5)	
Parent Smoke	No	6,025	982 (16.3)	0.826	2,927	438 (15.0)	0.652	3,098	544 (17.6)	0.493
	Yes	266	42 (15.8)		147	24 (16.3)		119	18 (15.1)	
Pet										
Dog Now	No	4,275	728 (15.0)	0.030	2,477	366 (15.0)	0.978	2,248	362 (16.5)	0.020
	Yes	1,566	283 (18.1)		597	90 (15.1)		969	193 (19.9)	
Cat Now	No	5,317	813 (15.5)	< 0.001	2,759	403 (14.8)	0.271	2,558	410 (16.3)	0.001
	Yes	974	197 (20.2)		315	54 (17.1)		659	143 (21.7)	
Truck Traffic				< 0.001			<0.001			<0.001
Never		3,410	459 (13.5)		1,988	251 (12.6)		1,422	208 (14.6)	
Sometime		2,114	384 (18.2)		751	131 (17.4)		1,363	253 (18.6)	
Always		767	181 (23.6)		335	80 (23.9)		432	101 (23.4)	
Fire Cooking	No	6,036	979 (16.2)	0.545	2,928	442 (15.1)	0.645	3,108	537 (17.3)	0.126
	Yes	255	45 (17.6)		146	20 (13.7)		109	25 (22.9)	
Env Factors										
Cockroach	No	4,273	664 (15.5)	0.021	1,973	281 (14.2)	0.102	2,300	383 (16.7)	0.053
	Yes	2,018	360 (17.8)		1,101	181 (16.4)		917	179 (19.5)	
Air Conditioner	No	3,993	619 (15.5)	0.028	1,820	259 (14.2)	0.136	2,173	360 (16.6)	0.052
	Yes	2,298	405 (17.6)		1,254	203 (16.2)		1,044	202 (19.3)	
Tree or Flower	No	2,238	343 (15.3)	0.129	796	106 (13.3)	0.116	1,442	237 (16.4)	0.164
	Yes	4,053	681 (16.8)		2,278	356 (15.6)		1,775	325 (18.3)	
Perfume	No	3,591	557 (15.5)	0.058	1,536	199 (13.0)	0.001	2,055	358 (17.4)	0.923
	Yes	2,700	467 (17.3)		1,538	263 (17.1)		1,162	204 (17.6)	
School Type				0.575			0.763			0.207
Public		4,170	671 (16.1)		1,957	125(10.5)		1,370	226 (16.5)	
Private		2,121	353 (16.6)		1,117	165 (14.8)		1,004	188 (18.7)	

Table 3. Factor Associate with AR of all children

	All				6-7 Years old				13-14 Years old			
	Crude Odds Ratio		Adjusted Odds Ratio		Crude Odds Ratio		Adjusted Odds Ratio		Crude Odds Ratio		Adjusted Odds Ratio	
	Point (95%CI)	P Value	Point (95%CI)	P Value	Point (95%CI)	P Value	Point (95%CI)	P Value	Point (95%CI)	P Value	Point (95%CI)	P Value
Age (years)												
6-7	Ref.	-	Ref.	-	-	-	-	-	-	-	-	-
13-14	1.20 (1.05, 1.37)	0.009	1.11 (0.96 1.29)	0.155	-	-	-	-	-	-	-	-
Sex Male	1.11 (0.97, 1.26)	0.143	-	-	1.26 (1.03, 1.54)	0.023	1.21 (0.98, 1.48)	0.084	0.97 (0.81, 1.17)	0.760	-	-
Paternal allergy history												
Asthma	1.86 (1.33, 2.60)	< 0.001	1.50 (1.05, 2.13)	0.025	2.02 (1.30, 3.14)	0.002	1.41 (0.88, 2.26)	0.157	1.74 (1.04, 2.93)	0.034	1.58 (0.91, 2.72)	0.102
Allergic rhinitis	1.77 (1.51, 2.08)	< 0.001	1.43 (1.20, 1.71)	< 0.001	2.37 (1.91, 2.95)	< 0.001	1.71 (1.35, 2.17)	< 0.001	1.32 (1.03, 1.70)	0.031	1.18 (0.90, 1.57)	0.236
Atopic dermatitis	1.89 (1.59, 2.24)	< 0.001	1.56 (1.29, 1.88)	< 0.001	2.58 (2.04, 3.25)	< 0.001	1.83 (1.42, 2.35)	< 0.001	1.36 (1.05, 1.77)	0.021	1.18 (0.93, 1.64)	0.146
Only 6-7 Years old												
Antibiotics (first 1 year)	-	-	-	-	1.31 (1.07, 2.44)	< 0.001	1.17 (1.45, 2.20)	0.304	-	-	-	-
Paracetamol (first 1 year)	-	-	-	-	1.37 (1.10-1.69)	0.004	0.97 (0.76, 1.23)	0.794	-	-	-	-
LRTI (first 1 year)	-	-	-	-	2.50 (2.03, 3.09)	< 0001	1.86 (1.34, 2.59)	< 0.001	-	-	-	-
Farm animal	-	-	-	-	1.85 (1.18, 2.88)	0.006	1.42 (0.89, 2.27)	0.142	-	-	-	-
Breast feeding	-	-	-	-	1.31 (1.07, 1.60)	0.008	1.28 (1.04, 1.57)	0.019	-	-	-	-
Paracetamol Now	1.66 (1.33, 2.07)	< 0.001	1.64 (1.30, 2.08)	< 0.001	1.77 (1.26, 2.49)	0.001	1.44 (1.01, 2.05)	0.039	1.60 (1.20, 2.13)	0.001	1.57 (1.16, 2.14)	0.004
Exercise	1.62 (1.41, 1.85)	< 0.001	1.49 (1.29, 1.71)	< 0.001	1.49 (1.21, 1.84)	< 0.001	1.29 (1.03, 1.61)	0.025	1.67 (1.39, 2.00)	< 0.001	1.64 (1.36, 1.97)	< 0.001
Pet												
Dog Now	1.18 (1.02, 1.38)	0.030	1.07 (0.91, 1.26)	0.389	1.00 (0.78, 1.29)	0.978	-	-	1.26 (1.04, 1.53)	0.020	1.17 (0.96, 1.44)	0.119
Cat Now	1.38 (1.16, 1.64)	< 0.001	1.28 (1.07, 1.54)	0.008	1.19 (0.87, 1.63)	0.271	-	-	1.42 (1.15, 1.76)	0.001	1.32 (1.05, 1.64)	0.015
Truck Traffic												
Never	Ref.	-	Ref.	-	Ref.	-	Ref.	-	Ref.	-	Ref.	-
Sometime	1.43 (1.23, 1.66)	< 0.001	1.28 (1.10, 1.50)	0.002	1.46 (1.16, 1.84)	0.001	1.39 (1.09, 1.76)	0.007	1.33 (1.09, 1.63)	0.005	1.25 (1.02, 1.54)	0.032
Always	1.99 (1.64, 2.41)	< 0.001	1.73 (1.41, 2.11)	< 0.001	2.17 (1.63, 2.88)	< 0.001	1.92 (1.42, 2.58)	< 0.001	1.78 (1.36, 2.33)	< 0.001	1.62 (1.24, 2.13)	0.001
Env Factors												
Cockroach	1.18 (1.03, 1.36)	0.021	1.11 (0.88, 1.41)	0.385	1.19 (0.97, 1.45)	0.102	-	-	1.21 (1.00, 1.48)	0.053	1.06 (0.76, 1.47)	0.743
Air Conditioner	1.17 (1.02, 1.34)	0.028	1.05 (0.83, 1.32)	0.705	1.16 (0.95, 1.42)	0.136	-	-	1.21 (1.00, 1.46)	0.052	1.14 (0.83, 1.57)	0.424
Perfume	1.14 (1.00, 1.30)	0.058	1.07 (0.93, 1.23)	0.371	1.21 (0.95, 1.52)	0.116	-	-	1.14 (0.95, 1.37)	0.164	-	-

*** Multivariable logistic regression mod**
Point: Point Estimate

Concerning the age group of 6–7 years, parental history of AR and eczema was significantly related to current AR (AR: p < 0.001, OR = 1.71, 95%CI = 1.35–2.17; eczema: p < 0.001, OR = 1.83, 95%CI = 1.42–2.35). Lower respiratory tract infection (LRTI) in the first year of life was positively associated with current AR (p < 0.001, OR = 1.86, 95%CI = 1.34–2.59). Parental reported breastfeeding (six months) was positively associated with current AR (p = 0.019, OR = 1.28, 95%CI = 1.04–1.57). The frequency of truck traffic on the street of residence was positively associated with the prevalence of current AR for both the occasional truck traffic group (p = 0.007, OR = 1.39, 95%CI = 1.09–1.76) and the always truck traffic group (p < 0.001, OR = 1.92, 95%CI = 1.42–2.58), as shown in **Tables 2 and 3**.

In the children aged 13–14 years, parental history of atopy was not significantly related to an increased risk of current AR. Current use of paracetamol, however, was associated with increased risk of current AR (p = 0.004, OR = 1.57, 95%CI = 1.16–2.14). Only current cat exposure was associated with increased risk of current AR (p = 0.015, OR = 1.32, 95%CI = 1.05–1.64). The frequency of truck traffic on the street of residence was also positively associated with the prevalence of current AR in both the occasional truck traffic group (p = 0.032, OR = 1.25, 95%CI = 1.02–1.54) and the always truck traffic group (p < 0.001, OR = 1.62, 95%CI = 1.24–2.13), as shown in **Tables 2 and 3**.

Discussion

The results from our study showed the prevalence of current AR in the children aged 6–7 years to be 15.0%. When compared to ISAAC phase III in the Bangkok area at 13.4%, there was a slightly but significantly increased prevalence in the younger age group (p = 0.006). In this study, the prevalence of current AR in the 13–14-year age group was 17.5%. This decrease was significant when compared to ISAAC phase III in Bangkok (23.9%, p = 0.006). The mean global prevalence of current AR in both age groups was 9.1% and 16%, respectively, in which the Asia-Pacific prevalence was 5.8% and the ISAAC phase III prevalence was 14.5%. The results of our study so far show a higher percentage in both prevalences.

Our study confirms that parental atopy is a risk factor for the development of AR. These results are consistent with the findings of other studies.[15,16] Both genetic and environmental factors play important roles in the etiology of AR. It is likely that there is a multilevel interaction between genetic and environmental factors.[17]

This study did not find any association between antibiotic use in the first year of life and later AR. We found a positive relation between current consumption of paracetamol and the prevalence of current AR. There is a dose-related association between acetaminophen use and AR in children.[18] The association of paracetamol with allergic disease is possible due to the depletion of glutathione. This is a result of the pharmacokinetics of this drug, leaving the respiratory mucosa with inadequate antioxidant protection.[19] This mechanism could explain the possible association between paracetamol consumption and the prevalence of the symptoms of rhinitis in our patients.

Our results show that LRTI in the first year of life was positively associated with current AR. Respiratory infections are among the major causes of hospitalization and pediatric medical consultation, and they are directly associated with mortality in children.[20] Allergic children showed a significantly higher number of respiratory infections in comparison with the non-allergic group.[21] Epidemiological studies have investigated significant relationships between AR and LRTI.[22]

In phase III of ISAAC, there was no consistent association between breastfeeding in the first year of life and rhinoconjunctivitis in 6–7-year-old children. However, breastfeeding was associated with reduced prevalence of current symptoms of severe rhinoconjunctivitis.[23] Our results suggest that breastfeeding (six months) was associated with current AR. Several studies have shown that breastfeeding in developing countries is associated with protection against infections, particularly gastric infection and diarrhea.[24] The immunological properties of breast milk are significant contributing factors to infant health in poor countries. Breastfeeding is therefore rightly promoted by authorities such as the World Health Organization.[25]

ISAAC phase III showed that early-life exposure to cats is a risk factor for symptoms of rhinoconjunctivitis in 6–7-year-old children. Current exposure to cats and dogs combined, and only to dogs, is a risk factor for symptom reporting by 13–14-year-old adolescents worldwide.[26] The Melbourne Atopy Cohort study (MASC) showed no evidence that exposure to cats and dogs at birth increases the risk of allergic disease in high-risk children.[27] The Childhood Origins of ASThma (COAST) showed associations between allergen-specific sensitization and rhinitis. At one year, sensitization to cats was the only aeroallergen associated with an increased risk of rhinitis at 6 years of age. At age 6 years, sensitization to all allergens tested except cockroach was associated with concurrent rhinitis.[28]

In this study, we found a positive global relationship between childhood symptoms of current AR and self-reported frequency of truck traffic on the street of residence. The associations were remarkably similar in different parts of the world in the two age groups studied and when using a self-completed questionnaire and a parent-completed questionnaire for 6–7-year-old children.[29] A recent study from Italy found that self-reported traffic density in the area of residence was clearly associated with nitrogen dioxide, which was 39 $\mu g/m^3$ when self-reported traffic was "absent," 44 $\mu g/m^3$ when "low," 48 $\mu g/m^3$ when "intermediate," and 52 $\mu g/m^3$ when "high."[30] First, there are now several published studies that have used objective measures of exposure and effect and found similar relationships between truck traffic exposure or other measures of exposure to vehicular traffic and respiratory and allergic symptoms in children.[31,32] Second, these studies were conducted mostly in Western Europe and North America, and in ISAAC phase III the associations found in these regions were not different from those found in other parts of the world. One could argue that concern about possible adverse effects on respiratory health by traffic fumes is different in different parts of the world, so one would not expect to see a universal association if responder bias played much of a role. Third, the associations were similar for the

13–14-year-olds and the 6–7-year-olds, despite the fact that the teenagers completed the questionnaires themselves, whereas the parents completed the questionnaires for the 6–7-year-olds. We can only speculate about what factors influence the remaining heterogeneity of exposure–response relationships between participating centers. There is experimental evidence to support that diesel particles may enhance allergic sensitization to common inhalant allergens.[33]

The major strengths of our study included standardized written core questionnaires (GAN 2016) for AR modified from ISAAC questionnaires, a well-established and standardized protocol, and a high response rate. One limitation of our study is that it is cross-sectional, which limits our ability to determine causation. Another limitation is that symptoms of AR were self-reported in the questionnaire; therefore, we could not confirm with physical examination and laboratory investigations.

In conclusion, our study shows that the prevalence of AR remained high in both age groups. Our data confirm that a family history of atopy, LRTI in the first year of life, breastfeeding (six months), current paracetamol use, exercise, current cat exposure, and truck traffic on the street of residence are important and significant risk factors for AR symptoms. This study may serve as evidence-based health education for parents to reduce the prevalence of AR by proper management of common disease (current use of paracetamol, LRTI in the first year of life, asthma, eczema) and environmental control (pets and truck traffic on the street of residence). More detailed studies are needed on the risk factors of AR.

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ORIGINAL ARTICLE

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A novel allergen-specific therapy with regulatory T cells induced by CD40-silenced dendritic cells

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Abstract

Background: We previously reported that dendritic cells (DCs) transfected with CD40 siRNA and pulsed by ovalbumin (OVA) (CD40-silenced OVA DCs) inhibited allergic responses through facilitation of regulatory T cells (Tregs). However, to our knowledge, no prior study has examined allergen-specific therapy by administration of siRNA-induced Tregs for the control of allergy.

Objective: We aimed to investigate the effect of Tregs induced in vitro on allergic responses and symptoms in vivo.

Methods: Mice were treated with Tregs (OVA DCs-induced Tregs) induced by CD40-silenced OVA DCs or Tregs (nonantigen DCs-induced Tregs) induced by DCs transfected with CD40 siRNA and pulsed with no antigen, and the effects of these Tregs on allergic responses were estimated.

Results: Administration of nonantigen DCs-induced Tregs prevented not only OVA-induced allergy but also keyhole limpet hemocyanin-induced allergy. Administration of OVA DCs-induced Tregs significantly reduced the number of sneezes and nasal rubbing movements, eosinophilia in the nasal mucosa, and the level of OVA-specific IgE in mice with OVA-induced allergy, compared with CD40-silenced nonantigen DC-induced Tregs in numbers 20 times greater, even in mice with established allergic rhinitis. Furthermore, Tregs induced by CD40-silneced DCs pulsed with Cry j 1, a major allergen of Japanese cedar pollen, inhibited Japanese cedar-induced allergy.

Conclusions: This study shows for the first time that both antigen-independent Tregs and antigen-specific Tregs can be induced by siRNA, and that therapy with siRNA-induced Tregs inhibits allergic responses and symptoms. It also shows that antigen-specific Tregs have more potent effects in inhibiting allergic responses than antigen-nonspecific Tregs.

Key words: Regulatory T cells, Allergy, CD40, siRNA, Dendritic cells.

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Introduction

CD40 is an integral membrane protein in dendritic cells (DCs) that activates T cells. Blockade of the CD40-CD40L interaction is a potent tolerance-inducing strategy,[1,2] while the inhibition of this interaction suppresses T cell responses[3] and generates regulatory T cells (Tregs).[4]

RNA interference using small interfering RNA (siRNA) induces specific silencing of gene expression, and is a potent, selective, and easy method.[5] Andrew Fire and Craig Mello received the Nobel Prize in Medicine for this discovery. Silencing gene expression by siRNA is more useful and promising than conventional silencing strategies by gene or antibody, such as blocking antibody, blocking protein, antisense oligonucleotide, and ribozymes.[6-8]

We previously reported that vector expressing siRNA specific for CD40 (CD40 siRNA) inhibits allergic responses not only as a means of prevention[9] but also as treatment.[10] However, direct administration of vector expressing siRNA may induce complications, because it is an antigen-nonspecific therapy and the vector or siRNA may change immune responses in vivo. We also showed that administration of CD40-silenced antigen-specific dendritic cells (DCs), transfected with CD40 siRNA but not vector CD40 siRNA and pulsed by antigen in vitro, inhibited allergic responses and symptoms antigen-specifically.[11] However, CD40-silenced antigen-specific DCs may lead to unexpected complications in vivo, since siRNA in CD40-silenced DCs may cause unexpected problems. We additionally documented that CD40-silenced DCs induce facilitation of $CD4^+CD25^+$ Tregs in vivo.[11] Furthermore, induction of Tregs by CD40-silenced DCs is not always the same by the conditions in vivo. Considering this, direct administration of antigen -specific $CD4^+CD25^+$ Tregs, induced by siRNA in vitro, is an attractive strategy for safer and more effective control of allergic diseases. To our knowledge, however, therapy with antigen -specific $CD4^+CD25^+$ Tregs induced by siRNA in vitro has not been reported for the control of allergy, and its usefulness is not known.

The generation of Tregs with anti-CD3/CD28 antibodies in vitro has been reported.[12] However, these are not antigen-specific Tregs. Antigen-specific Tregs are attractive for the treatment of allergy, since antigen-nonspecific Tregs may affect various immune responses and contribute to a range of diseases, including cancer.[13] It has been also reported that induced-Tregs generated by anti-CD3/CD28 antibodies differ from those induced by physiological-like activation with antigen/APC.[14]

In this study, we examined the effect on allergic diseases of $CD4^+CD25^+$ Tregs induced by antigen-specific DCs transfected with siRNA in vitro. The results showed that administration of ovalbumin (OVA)-specific $CD4^+CD25^+$ Tregs, induced by DCs transfected with CD40 siRNA and pulsed with OVA in vitro, inhibited allergic responses and symptoms in mice with allergic rhinitis, and that CD40-silenced DCs pulsed without antigen induced antigen-nonspecific Tregs. It was also shown that antigen-specific Tregs were more potent in inhibiting allergic responses and symptoms than antigen-nonspecific Tregs.

Methods

Generation of bone marrow-derived DCs and gene silencing by siRNA

DCs were generated from bone marrow progenitor cells, as previously described.[9,10,11] These DCs were transfected with transfection reagent alone (No siRNA DCs), siRNA (Control siRNA) specific to the Luciferase gene GL2 Duplex siRNA (Control DCs), or siRNA (CD40 siRNA, UUCUCAGCCCAG UGGAACA) specific to CD40. DCs transfected with CD40 siRNA were pulsed with OVA (CD40-silenced OVA DCs) or without OVA (CD40-silenced nonantigen DCs), as described previously.[9,10,11] DCs transfected with CD40 siRNA were also pulsed with Cry j 1, a major allergen of Japanese cedar (*Cryptomeria japonica*) pollen, (CD40-silenced Cry j 1 DCs) by the same method. Cry j 1 was purified by the method previously reported.[15,16]

Generation of Tregs in vitro

Mouse naïve $CD4^+$ T cells were isolated from splenic cells of six to eight week-old male BALB/c mice using a Mouse Naïve $CD4^+$ T Cell Isolation Kit (R&D Systems, CA). Mouse naïve $CD4^+$ T cells (3×10^5/mL) were co-cultured with 6×10^5/mL No siRNA DCs, Control DCs, CD40-silenced nonantigen DCs, CD40-silenced OVA DCs, or CD40-silenced Cry j 1 DCs for 5 days in 2 mL of complete medium, RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-ME, and 10% FCS supplemented with TGF-ß (5 ng/mL) and IL-2 (50 IU/mL). $CD4^+CD25^+$ T cells were collected using a MACS negative CD4 isolation kit and anti-CD25 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany).[9]

Immunization and Treatment

Six to eight week-old male BALB/c mice (Japan SLC Inc., Shizuoka, Japan) were injected intravenously with PBS alone, Tregs (4×10^5, 4×10^6, or 8×10^6 cells/mouse) induced by CD40-silenced nonantigen DCs, or Tregs (4×10^5 cells/mouse) induced by CD40-silenced OVA DCs on day 1. Mice were also injected intraperitoneally (i.p.) with 4 mg $Al(OH)_3$ and 10 μg ovalbumin (OVA) twice on days 2 and 15. Each group consisted of five mice. The same mice were challenged intranasally (i.n.) on days 21 through 27 with OVA (100 μg). Samples were collected on day 28.

In the second experiment, the protocol was the same as in the above experiment except that mice received PBS alone, Tregs (4×10^5 or 4×10^6 cells/mouse) induced by CD40-silenced nonantigen DCs, or Tregs (4×10^5 or 4×10^6 cells/mouse) induced by CD40-silenced OVA DCs and that mice were injected i.p. with 4 mg $Al(OH)_3$ and keyhole limpet hemocyanin (KLH), but not OVA, on days 2 and 15 and challenged i.n. on days 21 through 27 with KLH.

In the third experiment, mice were sensitized with OVA (10 μg) and 2 mg $Al(OH)_3$ intraperitoneally on days 1 and 14, and then the same mice were challenged intranasally with OVA (100 μg) on days 18 through 24. Intravenous administration of PBS alone, Tregs induced by CD40-silenced nonantigen DCs (4×10^6 or 8×10^6 cells/mouse), or Tregs by CD40-silenced OVA DCs (4×10^5 cells/mouse), was performed on day 26. These mice were then re-challenged intranasally on days 27 through 32 with OVA (100 μg).

In the fourth experiment, mice were sensitized with Cry j 1 (3 μg) and 2 mg $Al(OH)_3$ intraperitoneally on days 1 and 14, and then the same mice were challenged intranasally with Cry j 1 (2 μg) on days 18 through 24. Intravenous administration of PBS alone, Tregs induced by CD40-silenced nonantigen DCs (8×10^6 cells/mouse), or Tregs by CD40-silenced Cry j 1 DCs (4×10^5 cells/mouse), was performed on day 26. These mice were then re-challenged intranasally on days 27 through 32 with Cry j 1 (3 μg).

This study was approved by Research Ethics Committee in Nagoya City University. Mice were housed in an environmentally-controlled animal facility at Nagoya City University in Japan. The protocols were in accordance with the Guidelines for Care and Use of Animals of Nagoya City University. Every effort was made to minimize the discomfort of the animals.

Cry j 1- specific T cell response

$CD4^+CD25^-$ T cells and CD11c cells were isolated from spleen using MACS beads (Miltenyi Biotech). Spleen $CD4^+CD25^-$ T cell (2×10^6 cells/mL) and DC (2×10^5 cells/mL) suspensions were cultured for 72 h and stimulated with 10 μg/mL Cry j 1 antigen.

OVA- specific T cell response

Splenic cells isolated by gradient centrifugation over Ficoll -Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) were cultured in 96-well plates at a concentration of 4×10^5 cells/well for 72 h in the presence of 100 μg/mL OVA antigen.

Measurement of IL-2 production

Spleen $CD4^+CD25^-$ T cell (2×10^6 cells/mL) and DC (2×10^5 cells/mL) transfected with or without CD40 siRNA suspensions were cultured for 72 hours, stimulated with 10 μg/mL Cry j 1. Quantities of IL-2 cytokines in the culture supernatants were determined by using a sandwich ELISA. Plates were coated with anti-mouse IL-2 (BioLegend, San Diego, CA). The culture supernatant was then added, and the plates were incubated with the second antibody of biotinylated anti-mouse IL-2 (BioLegend). Standard curves were generated by using recombinant cytokines.

Measurement of OVA-specific, KLH-specific, and Cry j 1-specific IgE in sera

Titers of specific IgE were measured by ELISA. Briefly, ELISA plates were coated with anti-mouse IgE monoclonal antibody (Yamasa, Tokyo, Japan). Non-specific binding was blocked and sera were added. After washing with wash buffer, biotinylated OVA, KLH, or Cry j 1 was added to the well. The plates were then incubated with avidin-peroxidase at 37°C for an hour after washing. The TMB microwell peroxidase substrate system (KPL, Gaithersburg, MD) was used, and optical density (O.D.) was measured at 450 nm.

Nasal allergic symptoms

Immediately after the last nasal challenge, the number of sneezes and nasal rubbing movements was counted for 20 min according to the method previously reported.[11]

Pathology

The heads were decalcified and sectioned. Three micrometer thick sections of nasal tissue were stained with Luna staining. The number of eosinophils in the nasal mucosa of the nasal septum was counted microscopically in a field of view at 400× magnification. The observer was blinded to treatment when counting the number of eosinophils.

Statistical analysis

Data are expressed as means ± SEM. Statistical comparisons between groups were performed using one-way *ANOVA* followed by the Newman-Keuls Test. Differences with *P*-values less than 0.05 were considered significant.

Results

Prevention of OVA-induced allergy with CD40-silenced DC-induced OVA Tregs

We investigated whether Tregs induced by CD40-silenced OVA DCs in vitro could prevent OVA-induced allergy. Mice that received PBS, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells, or CD40-silenced OVA DC-induced $CD4^+CD25^+$ cells were sensitized and challenged with OVA as described in Methods (treatment on day 1, sensitization on days 2 & 15, challenge on days 21-27, sample collection on day 28). The number of sneezes and nasal rubbing movements was counted immediately after the last nasal challenge to examine the effect of these T cells on nasal allergic symptoms. CD40-silenced OVA DC-induced Tregs significantly decreased the number of sneezes and nasal rubbing movements compared with the other groups (**Figure 1A and B**). Although CD40-silenced nonantigen DC-induced T cells at a concentration of 4×10^5 cells/mouse did not reduce these symptoms, CD40-silenced nonantigen DC-induced T cells at levels 10 times greater and more (4×10^6 cells/mouse and 8×10^6 cells/mouse) significantly inhibited these symptoms. However, there were no significant differences in symptom inhibition between CD40-silenced nonantigen DC-induced Tregs at levels of 4×10^6 cells/mouse and 8×10^6 cells/mouse.

Next, the number of eosinophils in the nasal septum was counted to evaluate eosinophilia, which is associated with allergic symptoms and allergic responses in the nose. The number of eosinophils infiltrating the nasal mucosa in mice injected with Tregs induced by CD40-silenced OVA DCs was

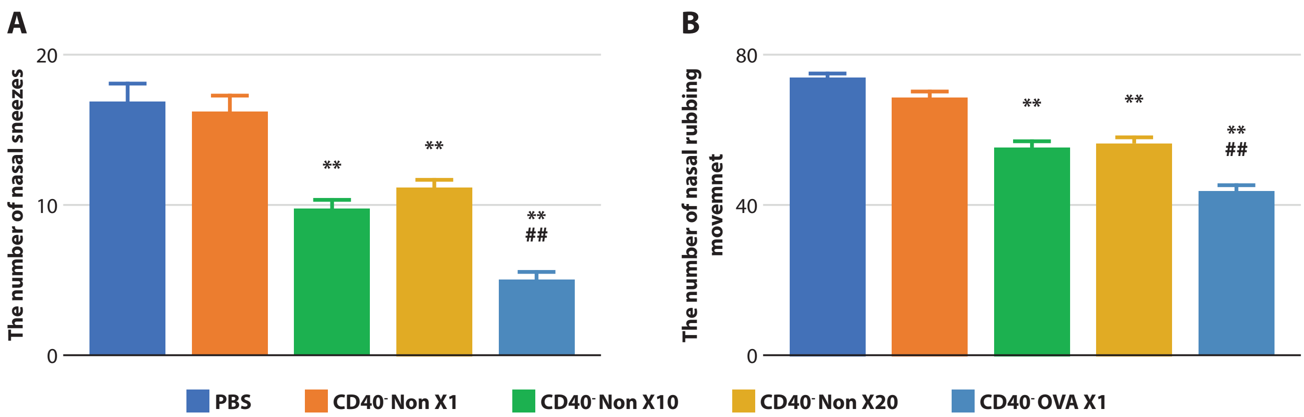

Figure 1. Prevention effects of allergy by $CD4^+CD25^+$ cells induced by CD40-silenced OVA DCs.
Five mice were injected intraperitoneally and challenged intranasally with OVA after treatment of PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells ($CD40^-$ Non, 4×10^5 “X1”, 4×10^6 “X10”, or 8×10^6 “X20”, cells/mouse), or CD40-silenced OVA DC-induced $CD4^+CD25^+$ cells ($CD40^-$ OVA, 4×10^5 cells/mouse). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge.

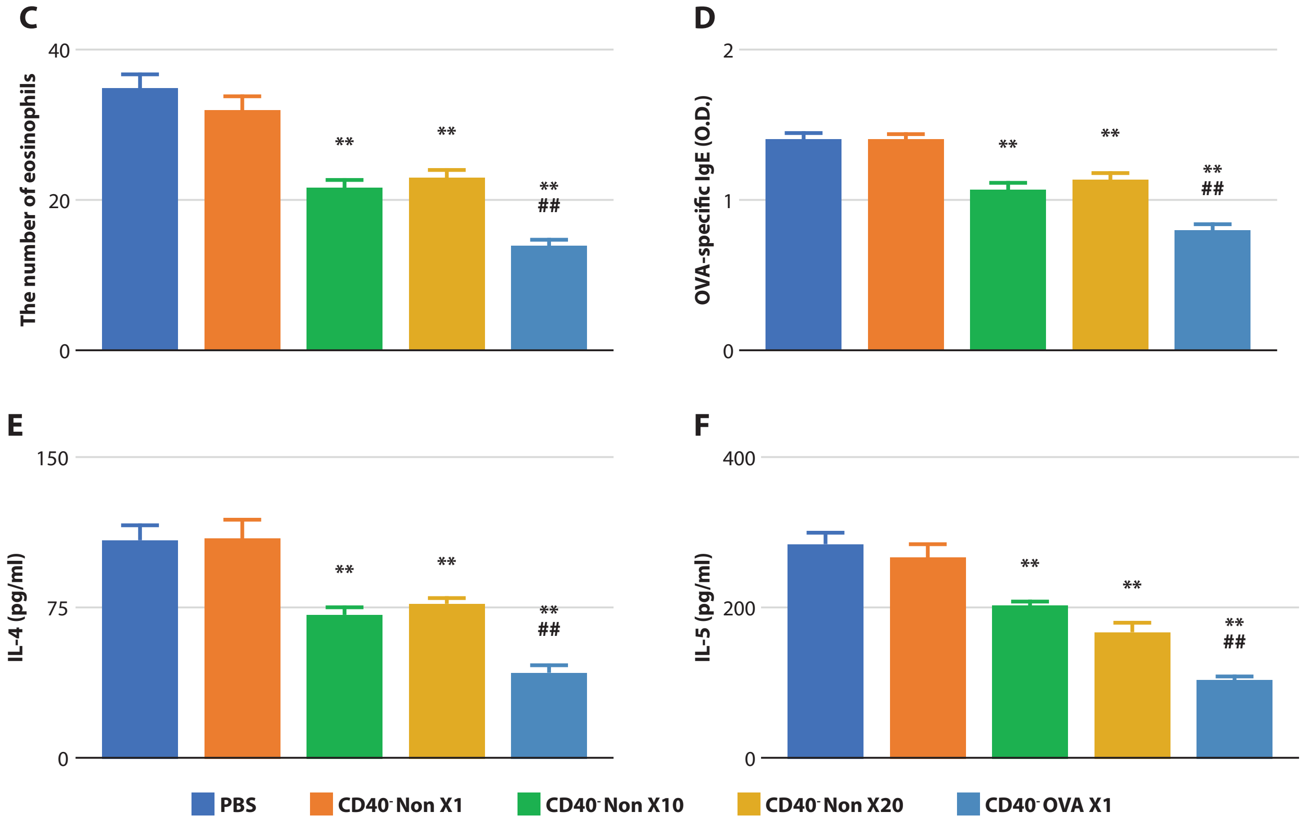

Figure 1. (Continued)
(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. The level of IL-4 (E) and IL-5 (F) production from splenic splenocytes stimulated by OVA was measured by ELISA. ** P < 0.01 versus groups of PBS alone and CD40- Non X1. ##P < 0.01 versus groups of CD40- Non (X10, X20). Experiments were repeated 3 times with similar result.

significantly fewer than that in mice with PBS alone or Tregs induced by CD40-silenced nonantigen DCs (**Figure 1C**). CD40-silenced nonantigen DC-induced Tregs at levels of 4 × 10^6 cells/mouse or 8 × 10^6 cells/mouse also significantly inhibited this eosinophilia, whereas CD40-silenced nonantigen DC-induced Tregs at the level of 4 × 10^5 cells/mouse did not (**Figure 1C**).

We also measured OVA-specific IgE in sera by ELISA, since IgE is associated with allergic reactions. CD40-silenced nonantigen DC-induced Tregs at levels of 4 × 10^6 or 8 × 10^6 cells/mouse also significantly suppressed the level of OVA-specific IgE, although CD40-silenced nonantigen DC-induced Tregs at the level of 4 × 10^5 cells/mouse cells/mouse did not. Tregs produced by CD40-silenced OVA DCs inhibited OVA-specific IgE significantly more than the other groups (**Figure 1D**). These data suggest that Tregs induced by CD40-silenced OVA DCs prevent production of OVA-specific IgE.

IL-4 and IL-5 play important roles in the development of allergic diseases. In order to investigate the effect of Tregs induced by CD40-silenced OVA DCs on cytokine production, we measured the production of IL-4 and IL-5 from splenic T cells stimulated with OVA in vitro. There were no significant differences between mice received PBS alone and CD40-silenced nonantigen DC-induced Tregs at levels of 4 × 10^5 cells/mouse in the productions of IL-4 and IL-5. The levels of IL-4 and IL-5 produced in mice that received Tregs induced by CD40-silenced OVA DCs were significantly lower than those in mice that received PBS or Tregs induced by CD40-silenced nonantigen DCs (**Figure 1E and F**). This suggests that OVA-specific Tregs suppress the production of Th2 cytokines, which may contribute to the prevention of allergy.

No preventive effect of Tregs induced by CD40-silenced OVA DCs on KLH-induced allergy

To investigate antigen specificity, we examined whether Tregs induced by CD40-silenced OVA DCs in vitro can inhibit allergic responses and symptoms caused by KLH. Mice received PBS, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced OVA DC-induced Tregs were sensitized and challenged with KLH as described in Methods (treatment on day 1, sensitization on days 2 & 15, challenge on days 21-27, sample collection on day 28). Administration of Tregs induced by CD40-silenced OVA DCs did not significantly inhibit the number of nasal sneezes, nasal rubbing movements, or eosinophils at the nasal septum and the level of KLH-specific IgE in sera compared with mice that received PBS alone (**Figure 2A-D**). These findings suggest that Tregs induced by CD40-silenced OVA DCs inhibit allergen reactions and symptoms in an antigen-specific manner.

Administration of CD40-silenced nonantigen DC-induced Tregs (4 × 10^6 cells/mouse) inhibited the number of nasal sneezes, nasal rubbing movements, and eosinophils at the nasal mucosa and KLH-specific IgE levels in sera compared with the other groups (**Figure 2A-D**). These results suggest

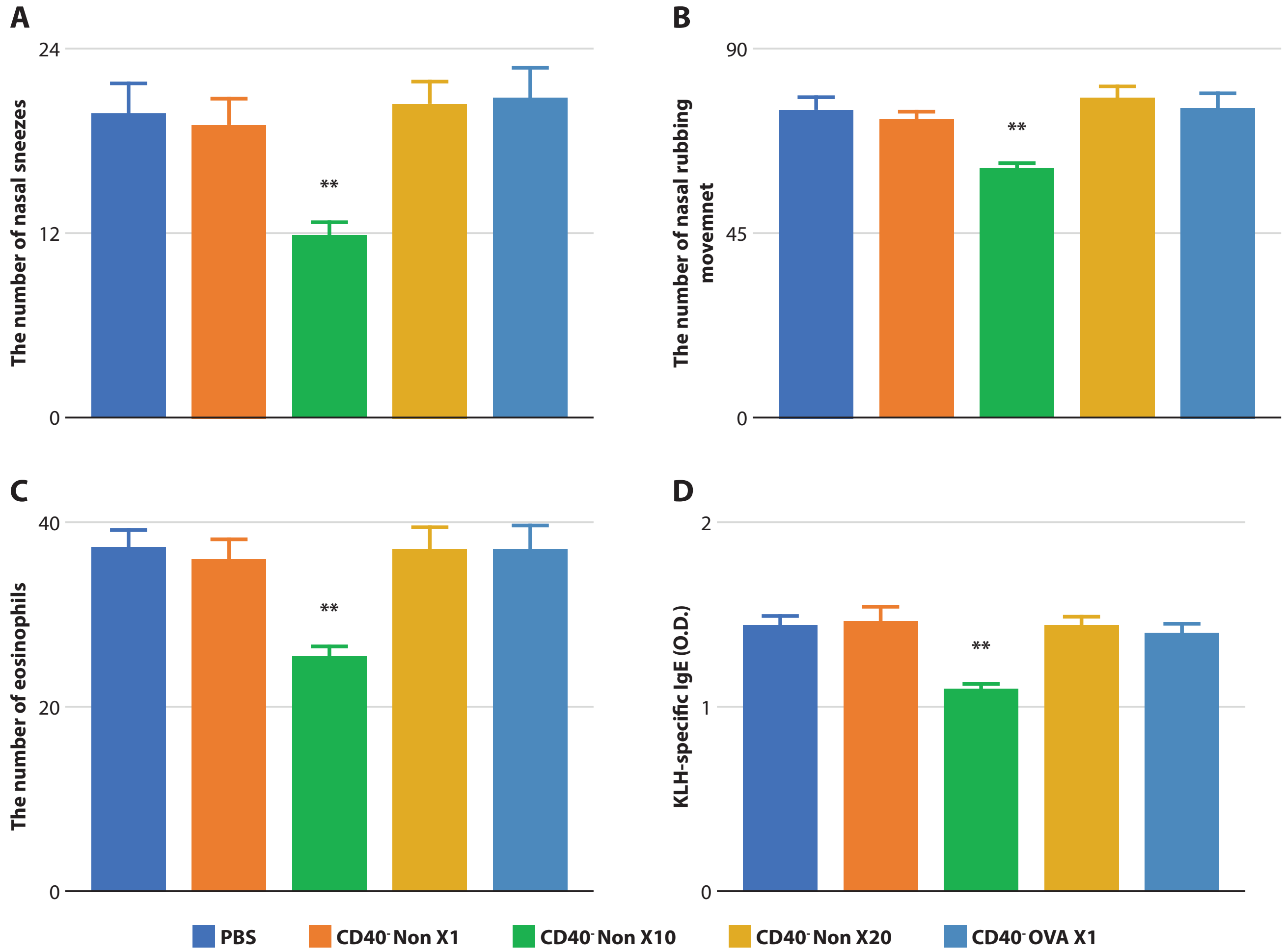

Figure 2. No allergy prevention effect from $CD4^+CD25^+$Tregs induced by CD40-silenced OVA DCs.
Five mice were injected intraperitoneally and challenged intranasally with KLH after treatment with PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells ($CD40^-$ Non, 4×10^5 "X1" or 4×10^6 "X10" cells/mouse), or CD40-silenced OVA DC-induced $CD4^+CD25^+$ cells ($CD40^-$ OVA, 4×10^5 "X1" or 4×10^6 "X10" cells/mouse). The numbers of sneezes (A) and nasal rubbing movements (B) were counted after the last nasal challenge. (C) Eosinophilia of the nasal septum. (D) The level of KLH-specific IgE in sera. ** $P < 0.01$ versus groups of PBS alone, $CD40^-$ Non X1, and $CD40^-$ OVA (X1, X10). Experiments were repeated 3 times with similar result.

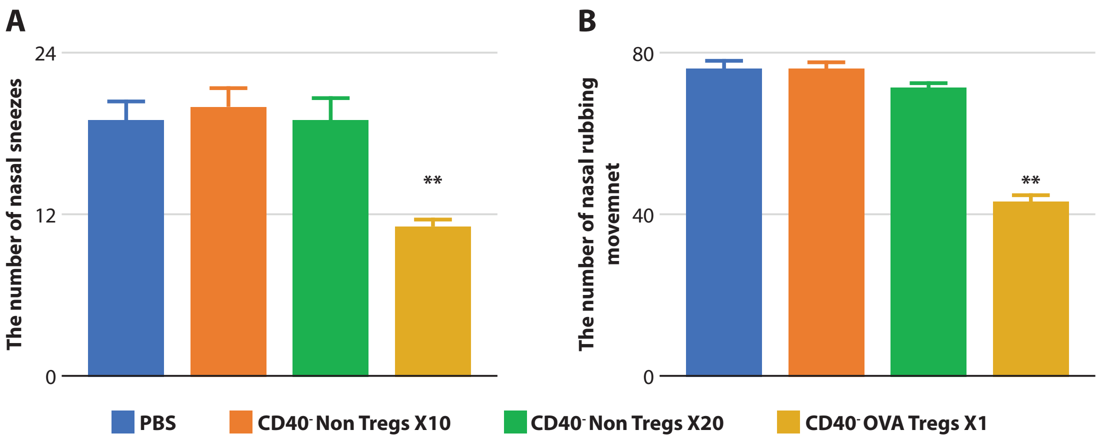

Figure 3. Therapeutic effects of $CD4^+CD25^+$Tregs induced by CD40-silenced OVA DCs in vitro on established allergic rhinitis.
Five mice with OVA-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells ($CD40^-$ Non, 4×10^6 "X10" or 8×10^6 "X20" cells/mouse), or CD40-silenced OVA DC-induced $CD4^+CD25^+$ cells (4×10^5 "CD40-OVA X1" cells/mouse). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge.

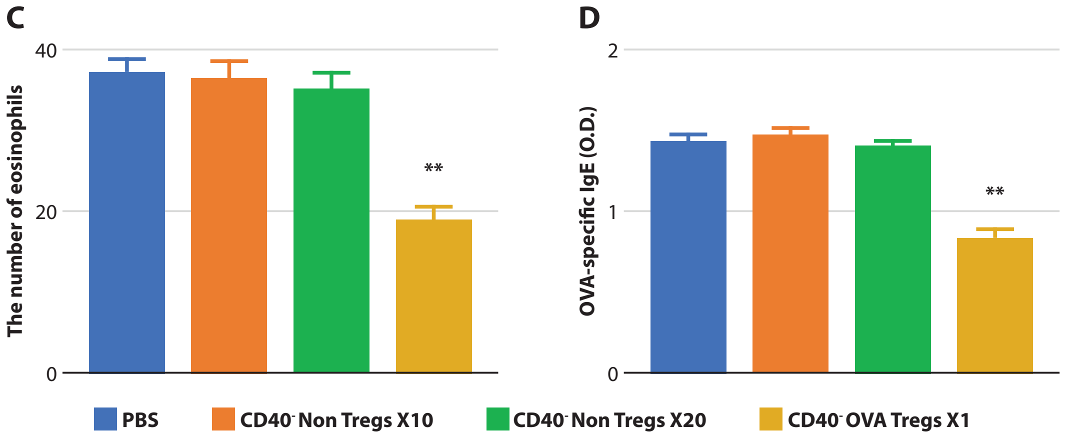

Figure 3. (Continued)
(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. ** P < 0.01 versus group of PBS alone, $CD40^-$ Non X10, and $CD40^-$ Non X20. Experiments were repeated 3 times with similar result.

that CD40-silenced nonantigen DC-induced Tregs are not antigen-specific.

Therapeutic effects of Tregs induced by CD40-silenced OVA DCs on mice with established OVA-induced allergic rhinitis

Mice with established allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced OVA DC-induced Tregs. After treatment, nasal re-challenge with OVA was performed (sensitization on days 1 & 14, nasal challenge on days 18-24, treatment with Tregs on day 26, nasal re-challenge on days 27-32, sample collection on day 33). The number of sneezes and nasal rubbing movements on day 24 was significantly higher than on day 17 (data not shown). Eosinophils in the nasal septum were seen on day 24, although no eosinophilia was found on day 17 (data not shown). These results suggest that mice were suffering from allergic rhinitis on day 24. There were no significant effects on the number of sneezes, nasal rubbing movements, or eosinophils in the nasal mucosa, or the level of OVA-specific IgE in sera, even when CD40-silenced nonantigen DC-induced Tregs (8×10^6 cells/mouse) were injected (**Figure 3A-D**).

Tregs induced by CD40-silenced OVA DCs in vitro significantly reduced the number of sneezes, nasal rubbing movements, and eosinophils in the nasal mucosa, and the level of OVA-specific IgE in sera, compared with the other groups, PBS alone, and Tregs induced by CD40-silenced nonantigen DCs (**Figure 3A-D**). These findings suggest that Tregs induced by CD40-silenced OVA DCs are therapeutically useful even for mice with established allergic rhinitis.

Immune regulatory properties of Tregs induced by DCs (CD40-silenced Cry j 1 DCs) transfected with CD40 siRNA and pulsed with Cry j 1

Next, we investigated Tregs induced by CD40-silenced DCs (CD40-silenced Cry j 1 DCs) pulsed with Cry j 1 but not OVA, because OVA is a food allergen but not aeroallergen. Cry j 1 is one of the major allergens of Japanese cedar pollen which cause severe allergic diseases in Japan.[15-19] Bone marrow-derived DCs were transfected with CD40 siRNA or Control siRNA (Control DCs). DCs transfected with CD40 siRNA were pulsed

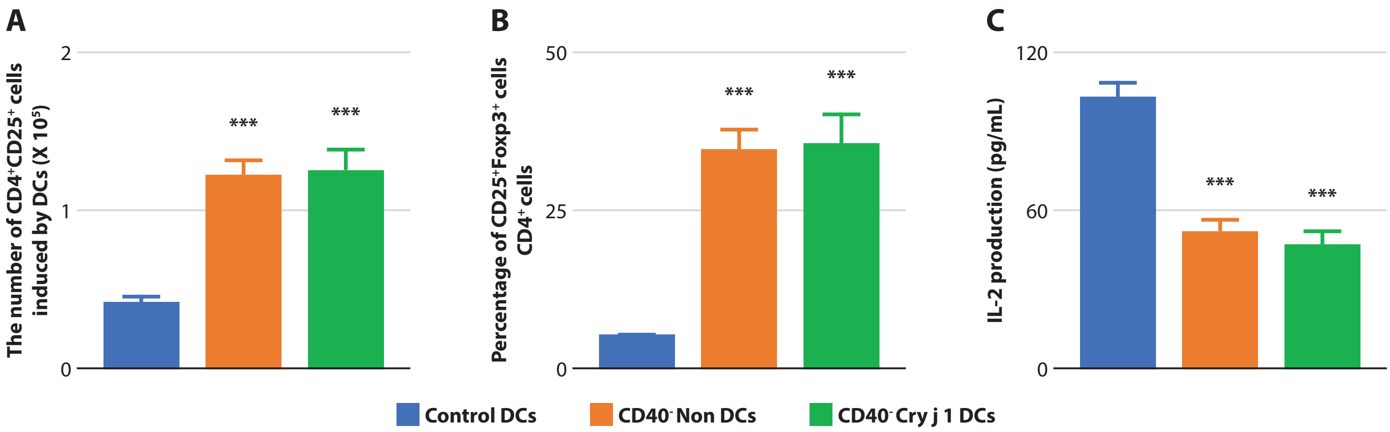

Figure 4. Modulation by CD40 siRNA in vitro. (A) DCs were transfected with Control siRNA (Control DCs) or CD40 siRNA. DCs transfected with CD40 siRNA were pulsed without Cry j 1 ($CD40^-$ Non DCs) or with Cry j 1 ($CD40^-$ Cry j 1 DCs). The numbers of $CD4^+CD25^+$ cells induced from 3×10^5 naïve $CD4^+$ cells by Control DCs, $CD40^-$ Cry j 1 DCs, and $CD40^-$ Non DCs were examined. (B) The percentage of $CD25^+Foxp3^+$ T cells in $CD4^+$ T cells after co-culture of T cells and DCs. (C) Quantity of IL-2 production after co-culture of T cells and DCs. ***P < 0.001 versus group of Control DCs. Experiments were repeated 3 times with similar result.

with Cry j 1 (CD40-silenced Cry j 1 DCs) or no antigen (CD40-silenced nonantigen DCs). Naïve T cells, separated from splenic T cells in naïve mice as described in Methods, were co-cultured with Control DCs, CD40-silenced nonantigen DCs, or CD40-silenced Cry j 1 DCs. Although we assessed the number of CD4$^+$CD25$^+$ cells were induced from 3 × 10^5 naïve CD4$^+$ cells, the number of CD4$^+$CD25$^+$ cells induced by CD40-silenced Cry j 1 DCs or CD40-silenced nonantigen DCs were significantly higher than that by Control DCs. (**Figure 4A**). The percentage of CD25$^+$Foxp3$^+$ cells in CD4$^+$ T cells induced by CD40-silenced nonantigen DCs and CD40-silenced Cry j 1 DCs were significantly higher compared with those induced by Control DCs (**Figure 4B**). And we investigated whether CD4$^+$CD25$^+$ cells induced by CD40-silenced Cry j 1 DCs could affect IL-2 production in order to examine the mechanism of Treg induction, since the association between IL-2 production and Treg expansion has been reported.[20,21] Cry j 1-specific T cell response was generated by a co-culture of DCs and CD4$^+$CD25$^-$ T cells isolated from the spleen in mice sensitized with Cry j 1 antigen. Quantity of IL-2 in the supernatant was measured by ELISA. Consequently, IL-2 production was significantly inhibited by CD40-silenced nonantigen DCs or CD40-silenced Cry j 1 DCs (**Figure 4C**).

Therapeutic effects of Tregs induced by CD40-silenced Cry j 1 DCs on mice with established Cry j 1-induced allergic rhinitis

We assessed the effects of siRNA-induced Tregs on allergic diseases caused by aeroallergen, Japanese cedar pollen. Mice with allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced Cry j 1 DC-induced Tregs. After treatment, nasal re-challenge with Cry j 1 was performed (sensitization on days 1 & 14, nasal challenge on days 18-24, treatment with Tregs on day 26, nasal re-challenge on days 27-32, sample collection on day 33). No eosinophilia in the nasal septum was found on day 17, whereas eosinophilia was seen on day 24 (data not shown). The numbers of sneezes and nasal rubbing movements on day 24 were significantly higher than those on day 17 (data not shown). These suggest that allergic rhinitis was established on day 24. After treatment with CD40-silenced nonantigen DC-induced Tregs, there were no significant effects on the number of sneezes, nasal rubbing movements, eosinophilia in the nasal mucosa, and

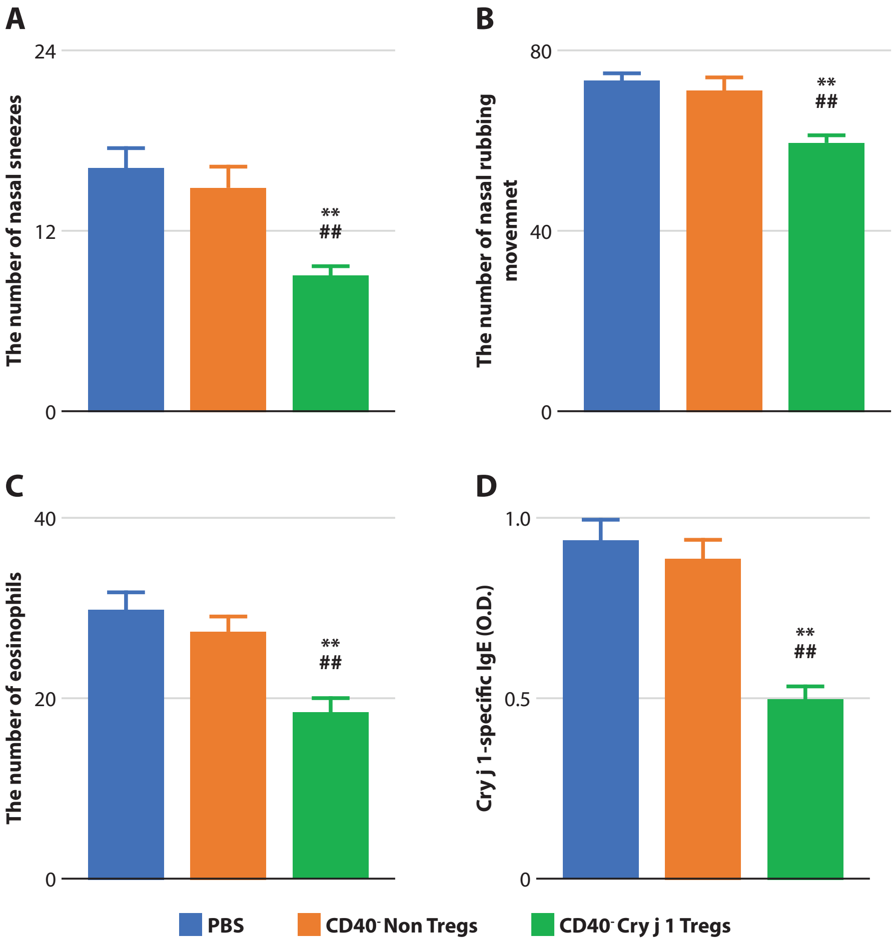

Figure 5. Therapeutic effects of CD4$^+$CD25$^+$Tregs induced by CD40-silenced Cry j 1 DCs in vitro on established allergic rhinitis. Five mice with Cry j 1-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced CD4$^+$CD25$^+$ cells (8 × 10^6 cells/mouse, CD40$^-$ Non Tregs) or CD40-silenced Cry j 1 DC-induced CD4$^+$CD25$^+$ cells (4 × 10^5 cells/mouse, CD40$^-$ Cry j 1 Tregs). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge. (C) Eosinophilia of the nasal septum. (D) The level of Cry j 1-specific IgE in sera. ** P < 0.01 versus group of PBS alone, ## P < 0.01 versus group of CD40$^-$ Non Tregs. Experiments were repeated 3 times with similar result.

the level of Cry j 1-specific IgE in sera (**Figure 5A-D**). However, Tregs induced by CD40-silenced Cry j 1 DCs in vitro significantly reduced the number of sneezes, nasal rubbing movements, and eosinophilia in the nasal mucosa, and the level of Cry j 1-specific IgE in sera, compared with other groups, PBS alone, and Tregs induced by CD40-silenced nonantigen DCs (**Figure 5A-D**). These findings suggest that Tregs induced by CD40-silenced Cry j 1 DCs are therapeutically useful for mice with allergic rhinitis caused by Japanese cedar pollen.

Discussion

Administration of Tregs induced by CD40-silenced nonantigen DCs before sensitization significantly reduced allergic responses and symptoms not only in OVA-induced allergy but also in KLH-induced allergy. These results suggest that Tregs induced by CD40-silenced nonantigen DCs are antigen-nonspecific Tregs. Patients who suffer from sensitization to multiple allergens are increasing.[22] Antigen-specific therapy for these patients is not easy, nor is it applicable for patients with an unknown causative allergen. Thus, CD40 silenced nonantigen DC-induced Tregs may be an alternative, antigen-independent therapy for the prevention of allergic diseases.

Although blockade of CD40-CD40L interaction induce Tregs,[4,23] the underlying mechanism of Treg expansion by blockade of CD40-CD40L is not known.[24] However, low-dose IL-2 expands $CD4^+$ regulatory T cells with a suppressive function in vitro.[21] Both blockade of B7-CD28 and CD40-CD40L also activated $Foxp3^+$ regulatory T cells and reduced IL-2 production.[20] When $CD25^+$ $CD4^+$ T cells compete with other cells for IL-2, $CD4^+CD25^+$ T cells further up-regulate the CD25 (IL-2R alpha chain).[25] And Vogel et al.[20] assumed that the low amount of IL-2 is enough for the survival of $CD4^+Foxp3^+$ cells, but not enough for the survival of $CD4^+Foxp3^-$ cells. This study showed that blockade of only CD40-CD40L pathway inhibited IL-2 productions. These suggest that blockade of CD40-CD40L induces expansion of $CD4^+Foxp3^+$ Tregs through reduction of IL-2 production.

We previously reported that CD40-silenced OVA DCs inhibited allergic reactions and symptoms. However, CD40-silenced OVA DCs may induce unexpected problems in vivo. CD40 siRNA may go out of DCs and induce problems such as inhibition of CD40 gene on other cells, interferon response, and off-target effect, although these have been not reported. If deficiency of CD40-CD40L interaction occurs in vivo, this may lead susceptibility to infection[26,27] like hyper IgM syndrome.[28] dsRNA, less than 30 bp in length, are generally believed to avoid interferon responses.[29] However, interferon response should be paid attention to even in siRNA, since siRNA could interferon response[30,31] and since the threshold of dsRNA length to induce interferon responses varies by cell types.[29] In future, various Treg phenotype may be revealed. Even if siRNA-induced Tregs include various Treg phenotype, it may be possible to collect only specific phenotype before administration in time to come. The advantages of this novel therapy with siRNA-induced Tregs presented herein include: 1) no interferon responses caused by siRNA; 2) no off-target effects by siRNA; 3) no inhibition of CD40 gene expression in vivo by CD40 siRNA; 4) no unexpected problems by siRNA or siRNA-transfected DCs; 5) higher stability in the numbers of siRNA-induced Tregs administered (induction of Tregs by CD40-silenced DCs is not always the same by the conditions in vivo), and 6) possibility to select specific Treg phenotype before administration, compared with therapy with siRNA-transfected DCs. On the other hand, the advantages of therapy with siRNA-transfected DCs presented herein include: 1) less time for preparation in vitro, 2) less cost, and 3) possibilities of tolerance, anergy, and apoptosis by modified DCs,[32-34] compared with therapy with siRNA-induced Tregs.

In this study, we report a novel antigen-specific therapy for the control of allergic diseases, using Tregs induced by CD40-silenced antigen-specific DCs transfected with CD40 siRNA in vitro, and siRNA-induced antigen-nonspecific Tregs for the prevention of allergic diseases. Furthermore, antigen -specific Tregs induced by siRNA-modulated DCs are attractive since they have more potent inhibiting effects on allergic responses and symptoms than antigen non-specific Tregs.

Financial disclosure

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Conflict of interest

None

Authors' contributions

Motohiko Suzuki and Yoshihisa Nakamura designed the study. Motohiko Suzuki and Makoto Yokota wrote the manuscript. Makoto Yokota and Shinya Ozaki contributed to data collection. Shinya Ozaki and Yoshihisa Nakamura performed the statistical analysis and interpretation of the results. All authors read and approved the final manuscript.

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- Figures/Images should be in TIFF, GIF, JPG, PDF, Postscript, or EPS format.

Submission Process

The four steps of the submission process are: Files, Manuscript Information, Validate, and Submit. The four steps each contain sub-steps that can be accessed by clicking on their respective tabs. Navigating through this "Tab View" will save any entered information each time a new tab is clicked (or the boxes "Save and Continue" and "Next" are clicked). Each step and sub-step is listed below:

1. **Files**
   - **Upload Files**

     A screen asking for the actual file locations (via an open file dialog) will appear. After completing this screen, your files will be sent to be converted to PDF for the peer review process.
   - **Remove Files**

     Allows the user to remove previously uploaded files.
   - **Replace Files**

     Allows the user to replace any previously submitted files with another file.
   - **File Type**

     This tab prompts the user to choose the "file type" that corresponds to the upload document. Though the file types can vary from journal to journal, the five basic types of files are, Author Cover Letter, Article File, Figure, Table, Supplemental Material.

- **File Description**

  When uploading a file type labeled "Figure", "Table", or "Supplemental Material" it is required to give a brief description of the content that is included in the file.
- **File Order**

  This tab allows the user to rearrange files to be displayed at the author's discretion. This tab also gives the option to merge PDF files into a single PDF file to display to the Editor and Reviewers. Upon completion the user must check the checkbox indicating completion of the ordering and selection process.

2. **Manuscript Information**
   - **Title, Abstract**

     It is require for the user to provide a Title for manuscript as well as a Running Title and an Abstract. The Title, Running Title, and Abstract all have word or character limits. (See details in Manuscript Format)
   - **Authors**

     This tab prompts the user to submit General Information about the author. The fields marked with an asterisk (*) are required, and need to be completed to continue the submission process.
   - **Keywords & Subject Areas**

     A screen where the author provides at subject areas of the manuscript from the list provided. If needed, the author can provide keywords for the manuscript by typing it in any boxes that might be provided.
   - **Detailed Information**

     This screen asks for more detailed information regarding the manuscript. Though the questions in this tab may vary from journal to journal, typical questions include "Conflict of Interest" and "Dual Publication".
   - **Author Review Suggestions**

     This screen allows the user to provide "suggested reviewers" to include for the revision process. The author can also provide reviewers to exclude from the revision process.

3. **Validate**
   - **Approve Files**

     The screen allows the user to verify that the manuscript has been uploaded and converted to the PDF format correctly.
   - **Approve Manuscript**

     This screen provides the user with all the information gathered from the submission process. It will provide a summary of all of the data entered so far, with the option to change any of those items.

4. **Submit**

   This screen is the final step of the submission process. The system will check to make sure everything is completed before the manuscript is submitted. If the manuscript is ready for submission, then there will be text that reads: "Your manuscript is ready to be submitted. Click the link below to finalize your submission." Otherwise, it will ask that you modify your submission to fulfill all of the submission requirements.

5. **Submission Fee**

   A nonrefundable processing fee of USD $40 is due upon submission. No submission fee is required for invited review article. If a fee is required, you will be asked to pay it online using credit card at the time of submission. Please note that purchase orders and bank wire transfers cannot be accepted for the processing fee. Manuscript will not be processed further unless the submission fee is received by APJAI editorial office.

6. **Manuscript Format**

   Manuscripts should be type-written in English with font style Times New Roman, font size 12. All pages should be numbered consecutively at the top right-hand corner, beginning with the title page. The manuscript must display continuous line numbers (1, 2, 3, and so forth) in the left margin, beginning with the title page. (Line numbering can be added from the Page Setup or Format menu of word processing programs.) All sections of the manuscript should be typed, double-spaced with margins of at least one inch on all sides and arranged in the following order:

   6.1 The title page MUST have the following information
   - Title of the manuscript
   - first and last names of the authors; no initials allowed unless it is a middle name
   - authors and their perspective highest academic degree(s)

     example: Jane S Doe, MD, PhD [1], John K Watson, MSc [2], Katherine Gibson, BSc [3,4]
   - Authors' affiliation(s)
   - Short running title
   - Name of the corresponding author
   - Address of the corresponding author including telephone, fax number and email address
   - Clinical trial registration number (if applicable)
   - word count for abstract
   - word count for text
   - Indicate total number of references
   - Indicate total number of tables and figures (no more than a total of 2 figures and tables combined).

     Example: 250 abstract; 3500 text; 35 references; 2 tables; 4 figures

   6.2 Structured abstract with the following subheadings and not more than 250 words total (including the subheadings)

   Abstract must be written in a structured format with the following headings: background; objective; methods; results; and conclusion. The major points of the article should be summarized in 150 (case reports) to 250 words (original research and review articles), in the order of their appearance in the manuscript. Abbreviations should be kept to an absolute minimum. References are not allowed in the abstract.

   Keywords (at least 5 words or key phrases)

   A minimum of 5 key words or brief phrases should be listed below the abstract for indexing purposes. The Medical Subject Headings (MeSH) used by the US National Library of Medicine's Index Medicus (MEDLINE) are preferred.

6.3 Main text

This section must have the following headings: Introduction, Methods, Results, Discussion, and Conclusion. In the text, cite references sequentially in superscript arabic numerals, e.g., [1,2,3] .Tables must be numbered sequentially in the text with Arabic numerals (1, 2, 3, 4, etc). Figures must be numbered sequentially in the text with Arabic numerals (1, 2, 3, 4, etc).

Introduction

This section should state the specific purpose, research objective, or hypothesis of the study and should provide a context or background information for the study. The aims of the manuscript should be clearly stated. Papers most closely related to the issue of the study may be mentioned. The introduction should not contain either findings or conclusions.

Methods

This section should be concise but provide sufficient detail to allow the work to be repeated by others. The source of material should be given in detail, where possible. Describe the design, subjects, setting, interventions, and main outcome measures. The explanation of the experimental methods provides technical information, apparatus details, and procedures. Describe statistical methods with sufficient detail to enable a reader with access to the original data to verify the reported results. For all research studies including human subjects (excluding Case Reports) the specific IRB that has approved the research must be indicated. Additionally a statement that informed consent was obtained from all research participants must be included. The clinical trial registration number and place of registry should be informed for clinical trial studies.

Results

Describe the experimental data and results as well as the particular statistical significance of the data. Results should be presented in a logical sequence in the text, tables and figures. Excessive repetition of the same data in different forms should be avoided. The Consolidated Standards of Reporting Trials (CONSORT) statement is a set of guidelines for reporting on the methods and results of randomized and nonrandomized medical research studies and is available at the following Website: http://www.consort-statement.org.

Discussion

Provide and quantify the main outcomes of the study. The data should be interpreted concisely, without repeating data already presented in the results section. Identify limitations of the presented data including plausible explanations for discrepancies between the data and the literature, any differences not expected from the initial hypothesis presented in the introduction and a measured description of the conclusions of the study with implications for future research, biological understanding and/or clinical applications.

6.4 Acknowledgements

Conflict of interest (in the past 3 years)
Source of funding with grant numbers (if applicable)
Author contributions

6.5 References

not more than total of 35 for original research papers
not more than 70 for review papers
Vancouver style (you can download the APJAI endnotes style here (URL)

Examples

1 Rose ME, Huerbin MB, Melick J, Marion DW, Palmer AM, Schiding JK, et al. Regulation of interstitial excitatory amino acid concentrations after cortical contusion injury. Brain Res. 2002;935:40-6.
2 Corporate Author Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. Hypertension. 2002;40:679-86.

Books and other monographs

1 Personal Author(s) Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.
2 Chapter in a Book Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. p. 93-113.

6.6 Figure legends

Figure legends should be typewritten, double -spaced, and listed on a separate page after the tables. They should not appear on the figures. List all of the figure titles in the figure legend. The legends should identify the data or subject being presented and its legend are understandable without reference to the text. Figures should be professionally drawn and photographed. Colored photographs may be published and additional expense will be paid by the authors. Titles and detailed explanations belong in the figure legends, not on the figures themselves. Photomicrographs must have internal scale markers. Symbols, arrows, or letters used in the photomicrographs should contrast with the background. If a figure has been published, acknowledge the original source and submit written permission from the copyright holder to reproduce the material.

6.7 Tables

Tables should be numbered in the order in which they are first cited in the text with Arabic numerals (1, 2, 3, 4, etc). They should be on separate pages, one table per page. Each table should have a concise heading that makes it comprehensible without reference to the text of the article. Use horizontal lines only at the top and bottom of the table and between column headings and the body of the table. Use no vertical lines. Explain any nonstandard abbreviations in the footnote of the table, e.g., Abbreviations: CT, computed tomography; MRI, magnetic resonance imaging; OR, odds ratio. Footnotes in captions should appear at the bottom of the table

Please use the program's page break function to begin each section on a new page.

6.8 Figure

Figures (graphs, charts, photographs, and illustrations) should be numbered in the order in which they are first cited in the text.

All figures must be numbered sequentially with Arabic numerals (1, 2, 3, 4, etc). . Graphics should be saved in CMYK (cyan, magenta, yellow, black) rather than RGB (red, green, blue). The resolution specification for TIFF and EPS files is 800 dpi for monochrome, figures that are black and white only and line shots; 250-300 dpi for gray/ CMYK or color photographs, and 600 dpi for combinations, such as photographs labeled with letters or other markings. One figure per page

Manuscripts should be written in proper and clear English so that they are understandable to any reader who is not a specialist in the field. Authors may be requested to have the English of the manuscript checked and improved by language editing services before submission. All measurements must be given in SI units as outlined in the latest edition of Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors (Royal Society of Medicine Press, London). However, liter and molar are permitted. Abbreviations should be used sparingly and only where they reduce repetition of long, technical terms. Initially use the word in full, followed by the abbreviation in parentheses. Thereafter use the abbreviation. All manuscripts must be submitted via online at the following address: http://www.apjai-journal .org/.

Article Types

The APJAI publishes original articles, review articles, and case reports. Topics of interest include all subjects that relate to the basic and clinical aspects of allergy and immunology.

- **Original Research Articles:** The text of original articles should be divided into sections with the following headings in this order: Introduction, Methods, Results, Discussion, and Conclusion. The total text should not exceed 3,500 words (excluding the Abstract, References, and Figure/Table Legends). These should describe fully, but as concisely as possible, the results of original clinical and/or laboratory research. Original articles should have a structured abstract with the following headings: Background, Objective, Methods, Results and Conclusions (maximum 250 words). A minimum of 5 keywords for indexing, and no more than 35 references are required. Text should not exceed 3,500 words. Advice on appropriate sectioning of original articles can be found in the ICMJE's Uniform Requirements. Each original article may be accompanied by a combination of no more than 6 figures and tables. Original article manuscripts that are determined to significantly exceed these limits may be returned to the authors for shortening prior to review. The manuscript should be organized in the following order: title page WITH the names of the authors and affiliations (please see title page requirement mentioned above); abstract and key words; main text; acknowledgements; references; figure legends; tables (each table complete with title and footnotes), and figures. Figures should look sharp and crisp when viewed at 100% magnification. Please note that should your manuscript be accepted, the journal may request for higher resolution TIFF or EPS files.
- **Review Articles:** Review articles are mostly invited by the Editors. Authors interested in submitting a review article should contact the Editor-in-Chief in advance to determine the appropriateness of any proposed review prior to submitting a full manuscript. Review articles address a specific question or issue that provide an evidence-based, review on a focused topic, either clinical or basic science. Review articles should have a structured abstract (250 words or less) with the following headings Objective, Data Sources, Study Selections, Results and Conclusion, a minimum of 5 keywords, and no more than 70 references. Text should not exceed 5,000 words and should be organized into the following sections: Introduction, Body, Discussion and Conclusions.
- **Case Reports:** Case Reports should have an unstructured abstract of no more than 150 words, a minimum of 5 keywords, a maximum of 2 tables or figures and 20 references. The main text should not exceed 1,500 words and should be organized into the following sections: Introduction, Report of Case and Discussion. A fully structured abstract is not necessary for a Case Report. For guidance on acceptable handling of photographs and other safeguards of patient confidentiality and anonymity, refer to section II.E.1 of the ICMJE's Uniform Requirements: Patients and Study Participants.
- **Short Communications:** Short communications are short research articles intended to present exciting finding. Short communications are limited to 1000 words for the body of the text, 8 references and may include no more than 1 figure or 1 table. Manuscripts should be organized as described for original research article and abstract.

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