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Proteomic identification of allergenic proteins of *Morus alba* L. pollen

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Abstract

Background: Tree pollens are well-known aeroallergens all over the world. Little is known about the allergenicity of *Morus alba* (white mulberry) pollen.

Objective: We aimed to explore the potential allergens of this pollen and its clinical relevance in tree pollen allergic patients living in Istanbul, Turkey.

Methods: Twenty three seasonal allergic rhinitis patients with a confirmed tree pollen allergy and 5 healthy control subjects underwent skin prick and nasal provocation tests with *M.alba* pollen extract. The pollen extract was then resolved by gel electrophoresis, and immunoblotted with sera from patients/control individuals to detect the potential allergenic proteins. The prevalent IgE binding proteins from 1D-gel were analyzed by MALDI-TOF/TOF.

Results: Eleven out of 23 patients were reactive to the extract with skin prick tests. Seven of those patients also reacted positively to the nasal provocation tests. The most common IgE-binding pollen proteins were detected between 55-100 kDa, and also at molecular weights lower than 30 kDa for some patients. Mass spectrometry analyses revealed that the principal IgE-binding protein was methionine synthase (5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase), which is then proposed as a novel allergen in *M.alba* pollen.

Conclusion: This study provides the first detailed information for the potential allergens of *Morus alba* pollen of Istanbul. Methionine synthase with an apparent molecular weight of 80 to 85 kDa has been recognized as one of the allergens in *Morus alba* pollen for the first time.

Key words: IgE-binding proteins, methionine synthase (MetE), Moraceae, *Morus alba*, pollen allergy, white mulberry

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Introduction

Tree pollens are well-known aeroallergens all over the world. When they are released into the atmosphere in sufficient amounts, they can cause various allergic diseases such as asthma, rhinitis and conjunctivitis in sensitive individuals, especially in their pollen spreading periods.[1,2] Pollen proteins and glycoproteins with molecular weights of 5-150 kDa are primarily responsible for allergenicity.[3] Proteomic studies represent a quick and efficient way of identifying protein(s) of interest and characterizing complex allergen sources. The combination of immunoblotting with 1-D or 2-D gel electrophoresis provides

an opportunity to detect Ig-E binding proteins in more detail after protein separation depending on their isoelectric points and/or molecular weights. After the detection of IgE binding proteins, they can be identified efficiently by mass spectrometry (MS).[4] Such an effort may help to extend the efficiency of both diagnostic and therapeutic tools for allergic diseases.[5] The protein family distribution of pollen allergens is regarded as up to 29 families[6] while tree pollen allergens are mostly found in pathogenesis-related group 10 (PR-10 or Bet v I-related) proteins, profilins, calcium binding proteins (polcalcins), expansins and pectate lyases.[7]

Mulberry (*Morus*) is a genus of the Moraceae family, which comprises native or cultivated trees in mild regions of the world. The fruits are consumed by humans as food and as traditional medicine, and the leaves are used as animal feed for both silkworms in silk production and for farm animals.[8] The *Morus* genus contains widespread species such as *M. nigra* (black mulberry), *M. rubra* (red mulberry), *M. microphylla* (Texas mulberry), *M. papyrifera* (paper mulberry) and *M. alba* (white mulberry). However, there are a limited number of studies on the allergenic proteins of these species. A non-specific lipid transfer protein (ns-LTP), Mor n 3 from the black mulberry with a molecular mass of 9246 Da, the first isolated and completely characterized fruit allergen was shown to cross-react with other plant-derived LTPs.[9] More recently a 10-kDa protein has been proposed by Micheal et al. as an unidentified pollen allergen from the paper mulberry.[10] The authors suggested that paper mulberry pollen allergens show no homology with nsLTPs or birch pollen allergens.

Some clinical studies indicate that *M.alba* pollen induces allergic diseases, such as asthma, allergic rhinitis, allergic conjunctivitis and urticaria, especially in pollen-spreading periods (April-May).[11-13] Although white mulberry pollen is regarded as an important aeroallergen, there are a limited number of reports on its allergenicity and allergenic proteins. Navarro and coworkers demonstrated that IgE antibodies were produced against 10- and 18-kDa allergens from white mulberry fruit in a 46-year-old female patient.[13] The latter allergen (18 kDa) is also present in white mulberry pollen and leaves, and has been found to cross-react with birch pollen.

The present study aimed to investigate the allergenicity of white mulberry pollen extract and to identify its allergenic proteins using the immunoproteomic approach called Serological Proteomic Analysis (SERPA). As this study was the first clinical report on Turkish population, our results have been expected to contribute both to clinical data and to pollen proteomics.

Methods

Pollen collection

Pollen samples from *Morus alba* L. were collected from the garden of Faculty of Forestry, Istanbul University (Bahçeköy -Istanbul/Turkey) during the pollen-spreading period (April/2012). The plants were identified by means of rigorous botanical criteria, and pollen was collected from the mature flowering plants by using, at a close distance, a filter-equipped vacuum device to avoid contamination. Pollen purity (> 99%) was assessed by microscopic analysis performed by a well-trained specialist. Pollen grains were separated with different pore size (250, 180 and 90 µm) sieves, dried at room temperature, and kept at -80ºC until protein extraction.

Preparation of pollen extract

Pollen extraction was performed with some modifications according to Iacovacci et al.[14] Ten grams of dried pollen was suspended in 125 mM NH_4HCO_3 at a ratio of 1:12 (w/v) for 12 hours at 4ºC with constant stirring. Insoluble materials were removed by centrifugation at 13000x*g* for 1 hour at 4ºC. Afterwards the extract was filtered through a Whatman No. 1 filter paper with 0.45 µm pore size and 125 mm Whatman filter paper with a Millipore vacuum filtration system. The filtrate was dialyzed for 24 hours at 4ºC with distilled water using 43 mm dialysis tubing. The final dialysate was lyophilized and stored at -80ºC.

The lyophilized pollen extract was solubilized in distilled water and the protein concentration was determined by using a Bicinchoninic Acid (BCA) Protein Assay Kit. The final absorbance of the assay mixture was measured by VarioScan Flash Image System (Bio-Rad) at a 562 nm wavelength.

Clinical Studies

Patient Selection: After receiving ethical approval from the Ethics Committee of Istanbul Faculty of Medicine, Ethical Committee and written informed consents from the subjects, 23 seasonal allergic rhinitis patients (16 female, 7 male; 21-56 year old) who displayed positive prick test results for common tree pollens and 5 healthy control subjects were included into the study. A pollen allergy was established by means of positive skin prick test (SPT) and nasal provocation test (NPT) results.

Skin Prick Test (SPT): A skin prick test was performed with the commercial allergen extracts from different tree pollens (*Betula verrucosa*, *Platanus acerifolia*, *Quercus ilex*, *Cupressus arizonica*, *Cupressus sempervirens*, *Corylus avellana*, *Alnus glutinosa*, *Fagus sylvatica*, *Quercus robur*), as well as with the prepared *M.alba* pollen extract. The prepared *M.alba* pollen extract was used for skin prick-testing in four different concentrations starting with a 1/1000 diluted suspension to 1/1 undiluted (5 mg/mL lyophilized powder) raw extract. The test was repeated with a tenfold increase in the extract concentration if the previous test was found negative. A positive response was defined as a wheal measuring at least 3 mm in diameter when compared with serum physiologic that was used as a negative control.

Nasal Provocation Test (NPT):Each nasal cavity was evaluated separately. Airflow was measured under 150 Pa pressure and resistance was calculated using an anterior rhinomanometer (Jaeger brand Masterscope Rhino Carefusion, Germany). Patients were challenged first with 2 puffs (100 µL) of saline in each nostril to exclude nasal hyperreactivity. If no reaction to the physiological saline solution occurred, NPT was initiated with increasing concentrations of *M.alba* pollen extract in 15-minute intervals. Two puffs (100 µL) of the solution at room temperature were applied to each nostril. If a positive reaction did not occur with the previous concentration, the concentration of pollen extract was incrementally increased until the final concentration of a 1/1 undiluted form. Symptom scores and nasal resistance with anterior rhinometry were recorded before and after each provocation. Positivity criteria in the nasal provocation test consisted of both symptom score

positivity according to the Lebel symptom score scale and changes in the measurement of rhinometry, which included a fall in peak inspiratory flow (PIF) of ≥ 40% post-NPT and/or increase in airflow resistance by 100%.[15-17]

Electrophoresis

SDS- PAGE for Western blotting was carried out as described earlier with a slight modification in sample buffer.[18] Lyophilized pollen extract in distilled water was mixed with sample buffer containing 200 mM Tris-HCl, 8% SDS, 40% glycerol and 0.04% bromophenol blue in a proper ratio, heated in a hot plate at 95°C for 5 minutes and 20 microgram of each sample were loaded onto the electrophoresis system (Mini -PROTEAN 3 Cell, Bio-Rad).

Protein samples were migrated on a discontinuous gel consisting of a stacking part (5% acrylamide) and a resolving part (10% acrylamide) under 200 V until the dye front reached the bottom of the gel. Proteins were visualized with Imperial Protein Stain (Thermo Scientific) based on Coomassie R-250 dye. The SDS-PAGE gel was scanned by a Chemidoc TM XRS+System (Bio-Rad).

Western blotting

Separated proteins on 1D-gel were transferred onto a PVDF membrane by a semidry blotting system (Bio-Rad) at 0.5 mA/gel and 25 V for 90 minutes. The membrane was blocked with 5% skimmed milk in phosphate buffered saline (PBS) containing 0.5% Tween 20 for 1 hour. After washing with PBS-0.5% Tween 20, the membrane was incubated overnight with a 1:4 dilution of sera from patients or healthy control subjects at 4°C. IgE-binding proteins were detected using a 1:1000 dilution of HRP (horse radish peroxidase)-conjugated mouse anti-human IgE (Fc) antibody (Southern Biotech). Revelation was carried out using ECL Western Blotting Detection Reagents (GE-Healthcare), and the membrane was scanned by the Chemidoc ™ XRS+System (Bio-Rad).

Proteomic Analysis

In Gel Digestion: Common IgE-binding protein bands from 1D-gel were excised into small pieces and destained in 100 mM ammonium bicarbonate and in 100% acetonitrile (ACN), alternatively, and then dried at 37°C. In-gel digestion was performed as following: the dried gel pieces were reduced with 65 mM DTT for 1hour at 37°C and alkylated with 135 mM iodoacetamide (IAA) for 30 minutes at room temperature in the dark. After removing the solution, the gel pieces were washed with 100 mM ammonium bicarbonate; and an equal volume of 100% ACN was added and incubated for 10 minutes, and then dried at 37°C. For gel digestion, MS grade trypsin (Trypsin Gold, Promega) was added to the gel pieces at 125 ng in 0.01% surfactant (ProteaseMAX™ Surfactant, Trypsin Enhancer, Promega) and incubated for 2 hours at 37°C. The digestion was stopped by adding 0.4 µL of 10% trifluoroacetic acid (TFA). The resulting peptides were concentrated by vacuum centrifuge and maintained at -20°C until further analysis.

Mass spectrometric analysis: In order to remove salt and contaminants from the peptide mixture, it was purified and condensed with Zip Tip C18 tips (Millipore) and mixed with α-cyano-4 hydroxy-cinnamic acid (Sigma-Aldrich) and spotted onto target MALDI plates. The peptides were identified by the 4800 MALDI TOF/TOF mass spectrometer (ABSciex, Les Ulis, France). Data acquisition was carried out using 4000 Series Explorer software, V3.5.3 (ABSCiex) in positive reflector ion mode for both MS and MS/MS analyses. The mass spectrometer was calibrated before each analysis with Peptide Calibration Standard II (Bruker Daltonics, Bremen, Germany). MS analyses were performed within a range of *m/z* 700 - *m/z* 4000. MS/MS experiments were performed on the 30 most abundant ions with a threshold of S/N higher than 30 by using CID (Collision Induced Dissociation) activation mode.

Protein Identification: Post-analysis data processing was performed using Protein Pilot 4.5 software with Mascot search engine and the protein database of National Center for Biotechnology Information (NCBI - February 2015). The sequence query searching was set up using the following parameters: carbamidomethyl (C) as fixed modification, deamidated (NQ), oxidation (HW) and oxidation (M) as variable modifications with one missed cleavage and a *m/z* tolerance of 50 ppm for the precursor ion and a *m/z* tolerance of 0.1 Da for the product ions. Protein identification was based on taxonomic similarities with *M. notabilis* since corresponding proteins in *M. alba* have not yet been sequenced. Only protein Mascot scores greater than 70 are significant ($p < 0.05$) for protein identification.

IgE Measurement

Allergen specific IgE was measured with an ELISA kit (Allercoat™ 6 Microplate ELISA, Euroimmun, Germany). Sera samples were applied to the microplate wells, which were assembled with the rings coated with commercial *M. alba* pollen extract and incubated for 60 minutes at 37°C. After washing the microplate wells with wash buffer, a component of the kit, alkaline phosphatase labelled anti-human IgE antibody was added and incubated for 60 minutes at 37°C. Substrate solution was added into each well and incubated for 30 minutes at 37°C. After washing, bound conjugate was detected with p-nitrophenyl phosphate (PNPP) by incubating for 30 minutes at 37°C. The reaction was stopped with 1 M NaOH and read at 405 nm on an ELISA reader. ELISA for prepared *M.alba* pollen extract could not be performed due to a lack of availability of the relevant allergen ring coated with this extract.

Results

Skin prick tests

Eleven of 23 patients who were sensitized to one or more standardized tree pollens (ALK-Abello, Spain) reacted to *Morus alba* L. pollen extract in different concentrations (1 patient with 1/100 dilution, 3 patients with 1/10 dilution, and 7 patients with the undiluted extract). None of the healthy control subjects reacted to the SPT. The skin prick test results of 11 patients (A-K) with *M. alba* pollen and other standard tree pollens are presented in **Table 1**. The most prevalent tree pollen reactivities were against *Cupressus arizonica* (9 patients) and *Platanus acerifolia* (7 patients).

Table 1. Skin prick test results for *Morus alba* L. pollen extract and some other commercial tree pollen extracts.

Patient	Mora	Commercial pollen extracts									
		Betv	Plaa	Quei	Cupa	Cups	Cora	Alng	Fags	Pins	Quer
A	4 mm	4 mm	4 mm	–	4 mm	5 mm	–	5 mm	5 mm	–	5 mm
B	5 mm	–	–	–	–	–	–	–	–	–	–
C	4 mm	–	–	–	–	–	4 mm	4mm	–	–	–
D	5 mm	4 mm	5 mm	–	5 mm	–	–	–	–	–	–
E	4 mm	–	–	4 mm	4 mm	4 mm	4 mm	4 mm	4 mm	–	–
F	4 mm	4 mm	5 mm	4 mm	4 mm	4 mm	–	–	–	–	+
G	5 mm	–	4 mm	–	5 mm	–	–	–	–	–	–
H	5 mm	–	–	–	5 mm	–	–	–	–	–	–
I	5 mm	–	4 mm	–	6 mm	–	4 mm	–	–	–	–
J	4 mm	–	4 mm	–	4 mm	5 mm	5 mm	4 mm	–	–	–
K	4 mm	4 mm	4 mm	4 mm	4 mm	4 mm	–	4 mm	4 mm	4 mm	5 mm

Mora, *Morus alba*; Betv, *Betula verrucosa*; Plaa, *Platanus acerifolia*; Quei, *Quercus ilex*; Cupa, *Cupressus arizonica*; Cups, *Cupressus sempervirens*; Cora, *Corylus avellana*; Alng, *Alnus glutinosa*; Fags, *Fagus sylvatica*; Pins, *Pinus sylvestris*; Quer, *Quercus robur*; (+) indicates positive response, (–) indicates no response to the pollen sample in patient.

Nasal provocation tests

Nasal provocation with *M. alba* pollen extract was conducted with 23 patients. Seven out of 11 SPT (+) patients (A-G) were also NPT (+) whereas 2 (H and I) were negative and 2 (J and K) were hyperreactive. Five of the remaining 12 SPT (–) patients were hyperreactive while 4 patients reacted to different concentrations of pollen extract in the NPT. Three of the SPT (–) patients were also NPT (–). None of the healthy control subjects reacted to the NPT.

Detection of IgE-binding proteins by 1D-SDS PAGE and immunoblotting

The SDS-PAGE of the *M. alba* pollen extract indicated at least 18 proteins (**Figure 1a**). These proteins were then transferred to a PVDF membrane without staining for the detection of IgE-binding capacity using Western blotting. Each blot was individually incubated with the sera of the 23 seasonal allergic rhinitis patients and 5 healthy control subjects. Specific IgEs against *M.alba* polypeptides were detected in 11 out of 23 patients' sera (**Figure 1b**). The results have been evaluated with both SPT and NPT results. The 1D-immunoblotting profile resulting from interaction between specific IgEs of patient F and *M. alba* pollen proteins is presented in **Figure 1c**. IgE antibodies of the remaining 12 patients and of the control individuals did not react with the pollen proteins.

Immunoblots showed that *M. alba* pollen contained common IgE-binding polypeptides between 55-100 kDa. One protein with a molecular weight of 80 kDa produced a significant reaction in 5 patients (A, C, E, F and K). IgE-binding protein(s) with a molecular weight of < 30 kDa were also detected. These small proteins were predominant in one of the hyperreactive patients (J) whereas the 80 kDa protein was predominant in hyperreactive patient (K). Polypeptide bands corresponding to the identified allergens were excised from the polyacrylamide gel and analyzed by MS/MS analysis.

Identification of potential allergenic proteins

Common IgE-binding protein bands (Band 1-5 from 1D-gel in **Figure 1a**) were excised, digested by trypsin and analyzed using MALDI TOF/TOF mass spectrometer for protein identification. To check the accuracy of this experiment, a 50 kDa marker protein was also analyzed and the protein was identified with a high score (431). Only the proteins identified with significant Mascot scores were summarized in **Table 2**. It should be mentioned that the database research was conducted by similitudes to *M. notabilis*, a recently sequenced *Morus* species as the complete sequences of proteins in *M. alba* are still not well understood.[19,20] Band 1 matched with two isoforms of methionine synthase (5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase) with a Mascot protein scores of 353 and 215. Two isoforms of L-ascorbate oxidase-like protein were also identified from band 1, but with much lower scores (173 and 87). One isoform of L-ascorbate oxidase-like protein was also identified from band 2, which was very close to band 1. The calculated masses from the primary sequences of both L-ascorbate oxidase-like protein isoforms is around 60-62 kDa, a value lower than the observed masses for band 1 and 2 of 82 and 79 kDa, respectively. This difference could be explained by a glycosylation, well described for several ascorbate oxidase enzymes in pollens.[21]

A phosphoglucomutase and the subtilisin-like protease SDD1 were both identified in band 3 (observed mass of 68 kDa). Band 4 and band 5 also gave positive results with the identified proteins hypothetical protein L484_006703, a protein from the glycosyl hydrolase family 9 and hypothetical protein L484_025194 with a conserved domain found in a variety of structurally related metalloproteins like glyoxalase I or dioxygenases. However, these proteins are less significant with mascot scores of 71 and 73, respectively, close to the threshold score of 60 used for the validation of Mascot identifications.

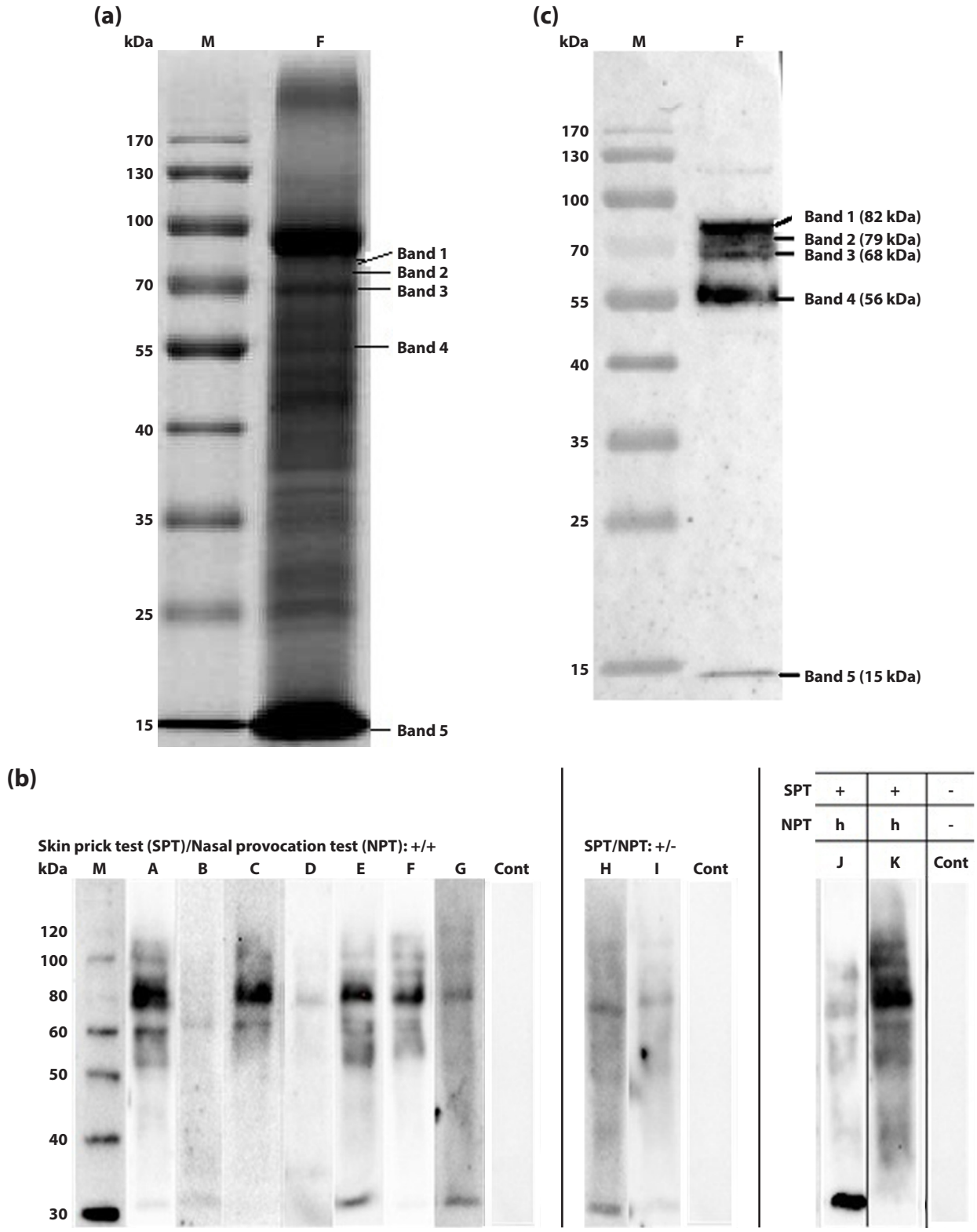

Figure 1. (a) 1-D Coomassie blue stained protein profile of *Morus alba* L. pollen; (b) IgE immunoblotting analysis of the sera of 11 patients (1-11) with *Morus alba* L. pollen extract. Patients were presented in three classes of clinical response; (c) 1D-immunoblotting result from patient F serum. M, molecular weight markers (PageRuler Prestained Protein Ladder (Thermo Scientific) in (a) and MagicMark XP Western Protein Standard (Life Technologies) in (b) and (c); Cont., serum sample from healthy control subjects; h, hypersensitive; Mora: *Morus alba*.

Table 2. The potential allergenic proteins of *Morus alba* L. pollen.

	Protein names	Accession number	Calculated Molecular Mass (Da)	Observed Molecular Mass (Da)	Mascot Score	Matches (# Peptides)	Protein sequence coverage (%)
Band 1	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	XP_010097930	84904	82000	353	19	18
	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	XP_010103482	83389	82000	215	17	17
	L-ascorbate oxidase-like protein	XP_010093150	60499	82000	173	26	23
	L-ascorbate oxidase-like protein	XP_010093149	62333	82000	87	18	21
Band 2	L-ascorbate oxidase-like protein	XP_010093150	60499	79000	103	22	23
Band 3	Subtilisin-like protease SDD1	XP_010108074	85546	68000	115	19	20
	Phosphoglucomutase	XP_010101975	63757	68000	107	22	27
Band 4	hypothetical protein L484_006703	XP_010112623	53747	56000	71	6	9
Band 5	hypothetical protein L484_025194	XP_010106438	16003	15000	73	9	48

ELISA results

Specific IgE antibodies were only detected in two sera samples (patients E and K) with standardized commercial *M. alba* pollen extract possibly due to absence or inadequate concentration of allergenic protein(s) in this extract.

Discussion

In this study, a 1D-immunoproteomics approach provided the first study of the allergenic protein profile of the *M. alba* (white mulberry) pollen extract, which was confirmed as an allergen for 11 patients living in Istanbul, Turkey. It was found that *M. alba* pollen contains many allergenic proteins between 15-100 kDa. The most prominent bands are proteins of approximately 55-100 kDa in the majority of patients.

Until now, only two distinct Ig-E binding proteins around 10- and 18 kDa have been reported for *M. alba* in a case report.[13] The 18 kDa protein was proposed to be in accordance with the Bet v1 allergen and its homologs, however, identification of this protein has not been achieved. Our study revealed that the allergenic proteins of *M. alba* pollen have a molecular weight of 82, 79, 68, 56 and 15 kDa. Thus it can be suggested that *M. alba* pollen contains allergenic proteins with higher molecular weight than known allergenic proteins with low molecular weight as in the other *Morus* species and common tree pollens.[13] In fact, some IgE reactive proteins between 36-98 kDa have also been detected in paper mulberry (*M. papyrifera*) grown in Pakistan, however in this study, the authors focused only on a 10 kDa protein.[10]

After MS/MS analyses of the major protein bands in 1D-gel, methionine synthase (MetE) (Band 1) showed the highest protein score (353) among all identified IgE-binding proteins. This protein with a MW of 85 kDa belongs to the vitamin-B12 independent methionine synthase (MetE) family and catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate (N^5-MeTHF) to homocysteine resulting in methionine formation.[22] Two reports were published on allergenic MetEs among plants, but never in *M. alba*. In the first study Chardin et al. showed that the amino acid sequence of a high molecular weight allergenic protein (approximately 80 kDa) from the oilseed rape (*Brassica napus*) pollen was very similar to that of the cobalamin-independent MetE of *Arabidopsis thaliana* (AtMetE).[23] The authors demonstrated that this 80 kDa protein represented an allergen from the oilseed rape pollen. In 2011, a study from Iran identified the cobalamin-independent MetE as a new allergen of *Salsola kali* pollen.[22] This study showed that *S. kali* MetE shares a high degree of amino acid sequence homology with the MetE from other plants including *Beta vulgaris* (Amaranthaceae) (91%), *Solanum tuberosum* (89%), and *Arabidopsis thaliana* (88%). The new allergen was designated Sal k 3 by the WHO/IUIS Allergen Nomenclature Subcommittee. Our study is the first report on allergenic MetE in *M. alba*. Although we propose MetE as the major allergen of *M. alba* we have no data regarding the cross-reactivity to Amaranthaceae pollens in our patients.

Our results are also partly correlated with the findings of Erler et al. for birch pollen.[24] These researchers evaluated the profile of allergenic and non-allergenic proteins in extracts of birch pollen from different origins by MS-based proteomics, and they detected MetEs with high scores. Thus, *M. alba* pollen might be expected to display cross-reactivity with birch pollen through these proteins. However, we detected skin test positivity for birch pollen in only 4 patients (**Table 1**).

Six other proteins were also detected in MS analyses with lower protein scores. The allergenicity of L-ascorbate oxidase -like protein (Band 1, 2) could be explained by the carbohydrate epitope in the glycan moiety of this protein as in the previous studies with Cupressaceae pollens[21] and olive pollens.[25] Subtilisine-like protease and phosphoglucomutase (Band 3) were already described in other species as potential allergenic proteins.[24,26-29]

Band 4 and band 5 also allowed the identification of potential allergenic proteins: hypothetical protein L484_006703, a protein from the glycosyl hydrolase family 9, and hypothetical protein L484_025194 with a conserved domain found in a

variety of structurally related metalloproteins like glyoxalase I or dioxygenases. There are 10 records related to glycosyl hydrolases in the allergome database (http://www.allergome.org/script/search_step2.php). Two belong to olive tree-derived allergens, Ole e 10 and Ole e 10.0101. A novel allergenic glyoxalase has been demonstrated with rice, and the role of indoleamine 2,3-dioxygenase (IDO), an initiator of tryptophan catabolism, on allergic inflammation has been explored.[30-31]

Although this study provides new data regarding the allergenic proteins in *M. alba* pollen, it contains some limitations because ELISA results did not concur with the immunoproteomic results. The discrepancy might be explained by the failure to detect low antibody levels. Further studies on sIgE detection in patients' sera are needed. In addition, the results should be supported by other diagnostic tests such as a basophil activation test.

In conclusion, IgE-binding proteins detected in our study are relatively different than those reported earlier, probably as a result of the region where the pollen samples were collected as it is well known that the pollen content and the allergenicity are affected by the climate and other environmental conditions.[32] Methionine synthase is a potential allergenic protein in *Morus alba* pollen. Further studies such as 2D-gel electrophoresis and other MS techniques, ELISA testing and extending the clinical data are in progress for a better understanding of the allergy mechanism of mulberries.

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Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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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 (25^{th} - 75^{th})	*P*-value	Polymorphism	Total IgE (IU/ml) Median (25^{th} - 75^{th})	*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)	

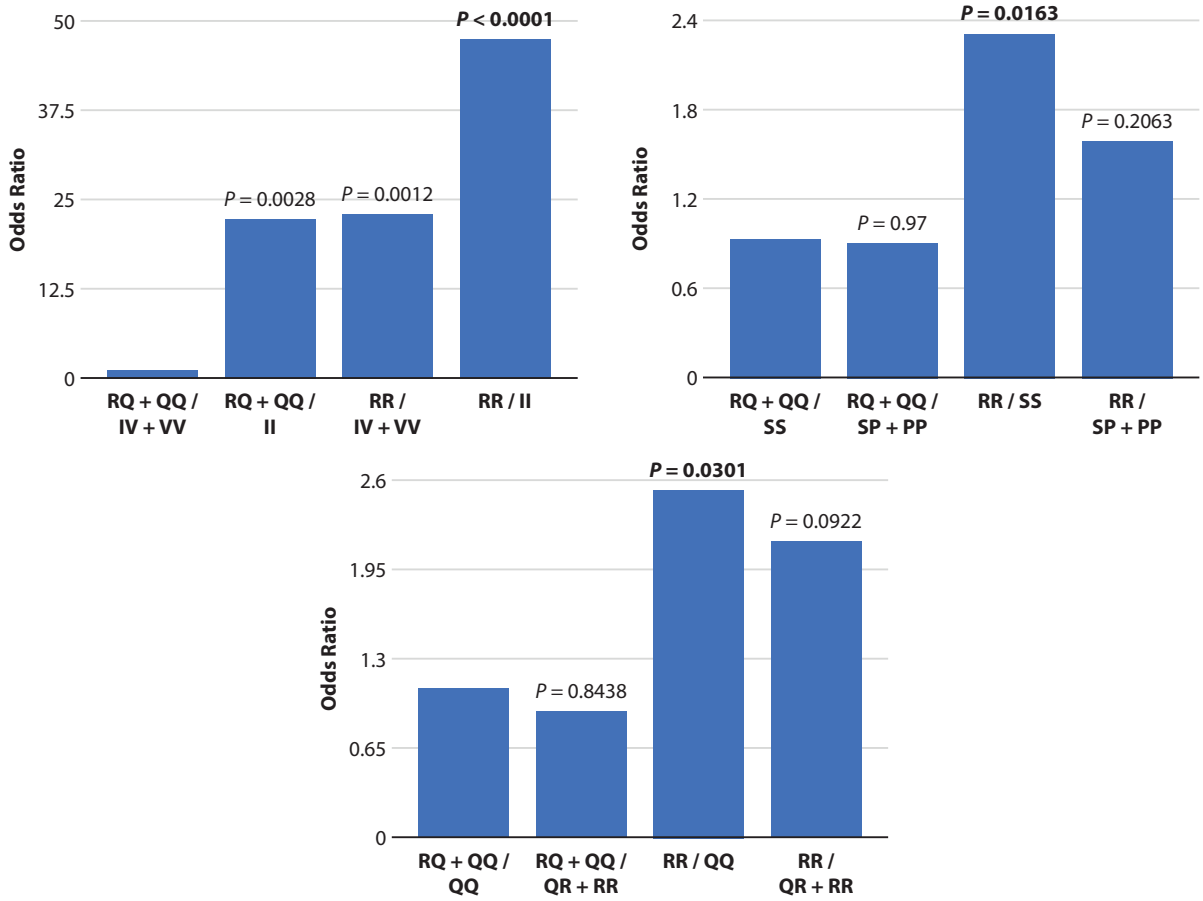

Figure 1. Interaction of IL4RA and IL13 Genotypes.
Bars indicate the odds ratio between the different combinations of genotypes for IL4RA (I50V, S478P, and Q551R) and IL13 R130Q. The non-risk genotype for each gene was used as the reference odds ratio.

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

Acknowledgements

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ORIGINAL ARTICLE

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APJAI

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**.

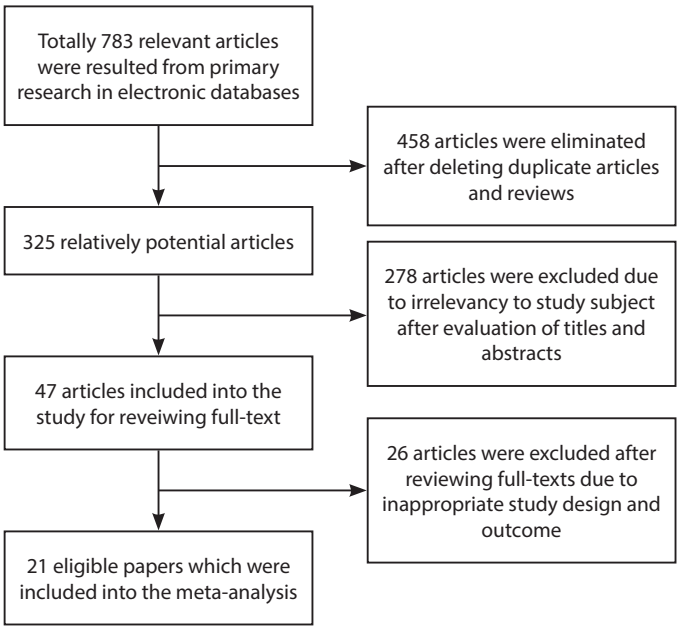

Figure 1. Flowchart for identification of studies selected.

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%

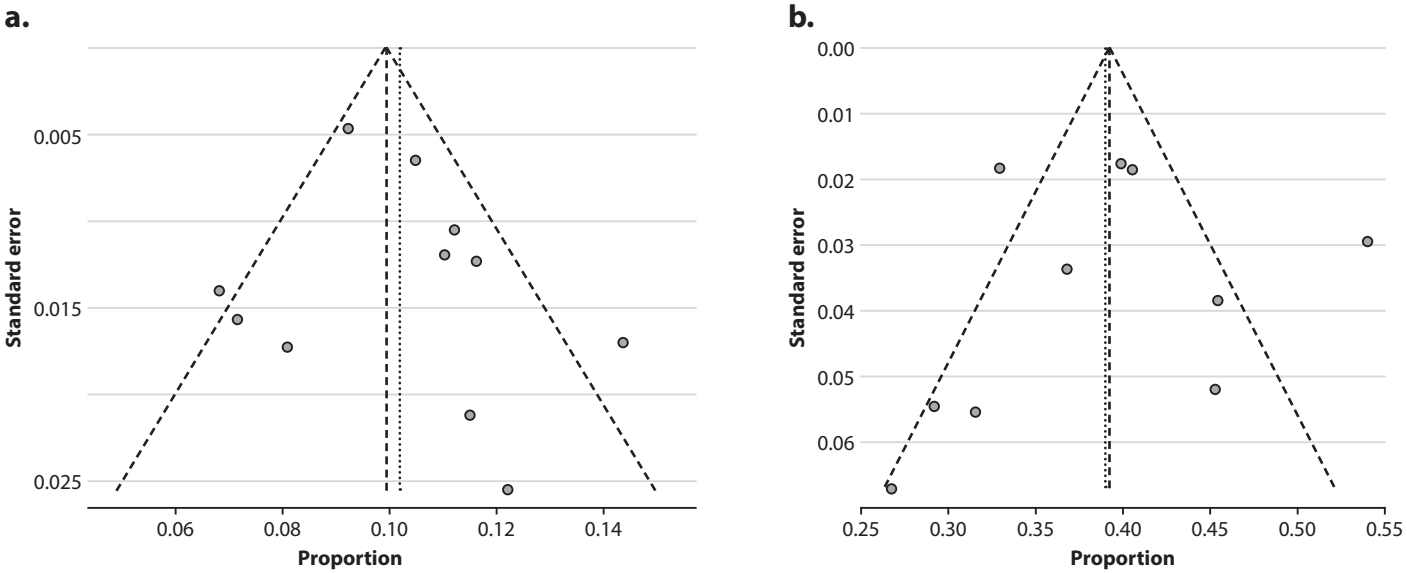

Figure 2. A funnel plot of the overall meta-analysis of metabolic and endocrine comorbidities reflecting publication bias. (a. AR with asthma; b. asthma with AR)

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**)

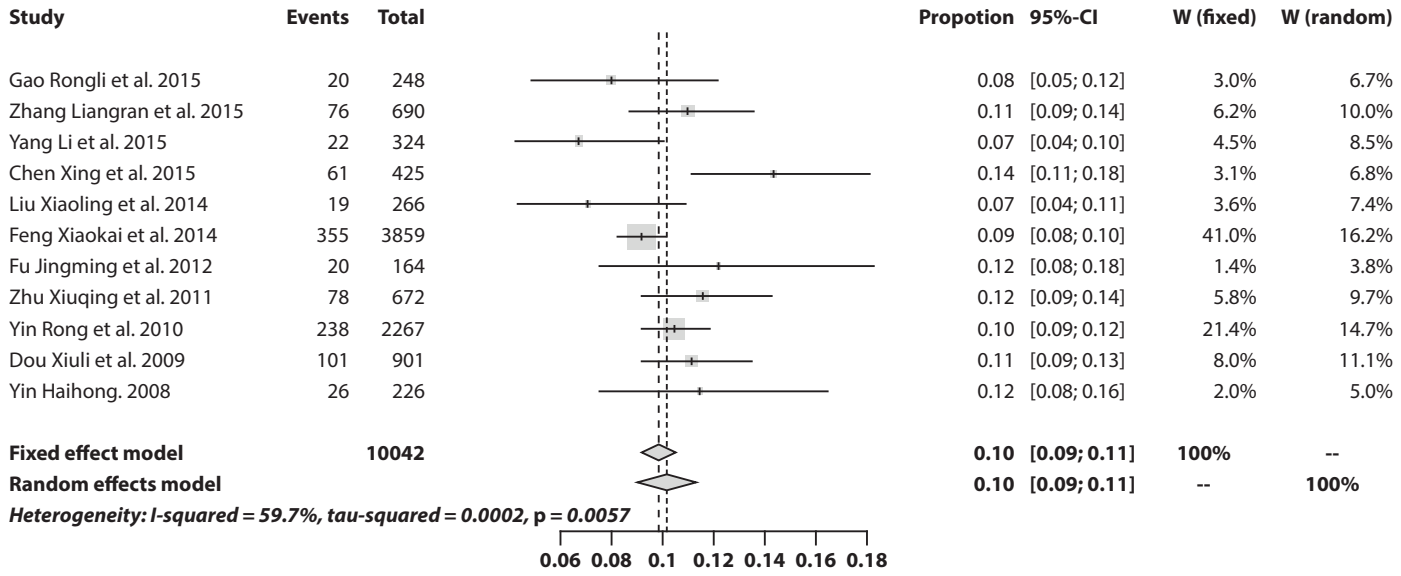

Figure 3. Forest plot of the rate of AR patients with asthma.

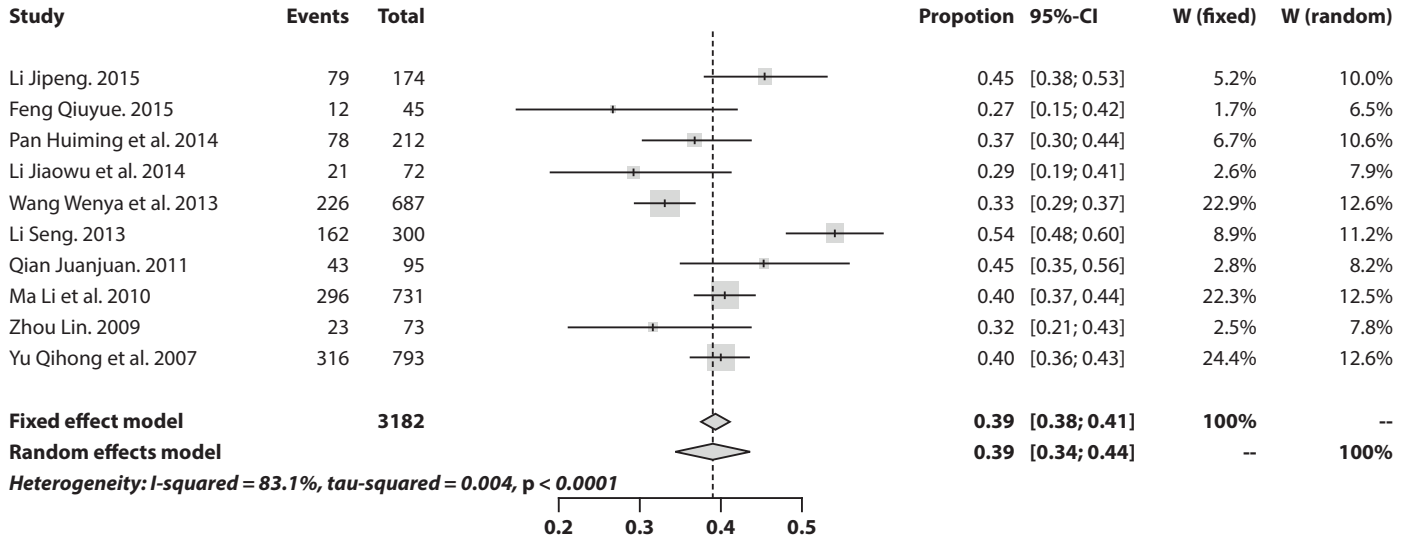

Figure 4. Forest plot of the rate of asthma patients with AR.

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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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, breastfeeding, 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)	

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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

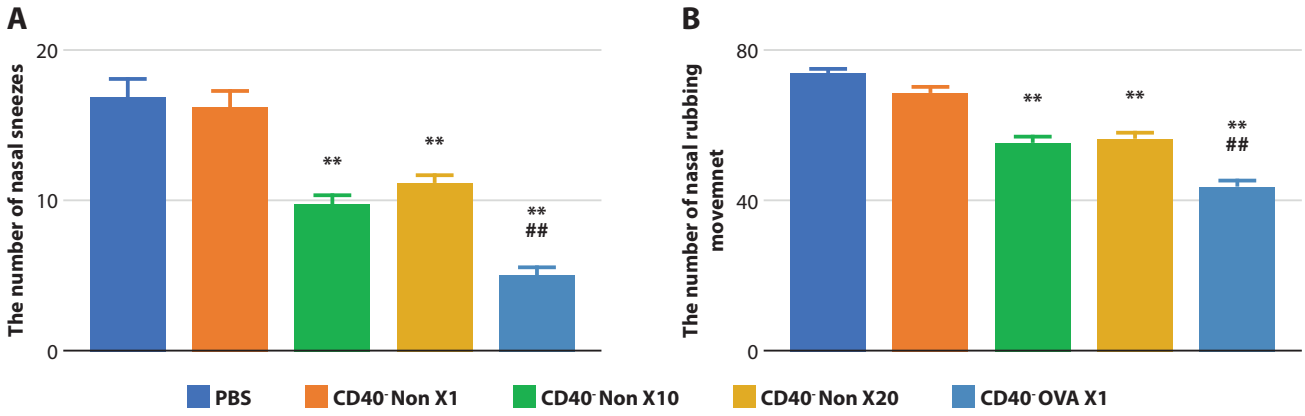

Figure 1. Prevention effects of allergy by $CD4^+CD25^+$ cells induced by CD40-silenced OVA DCs.
Five mice were injected intraperitoneally and challenged intranasally with OVA after treatment of PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells ($CD40^-$ Non, 4×10^5 "X1", 4×10^6 "X10", 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.

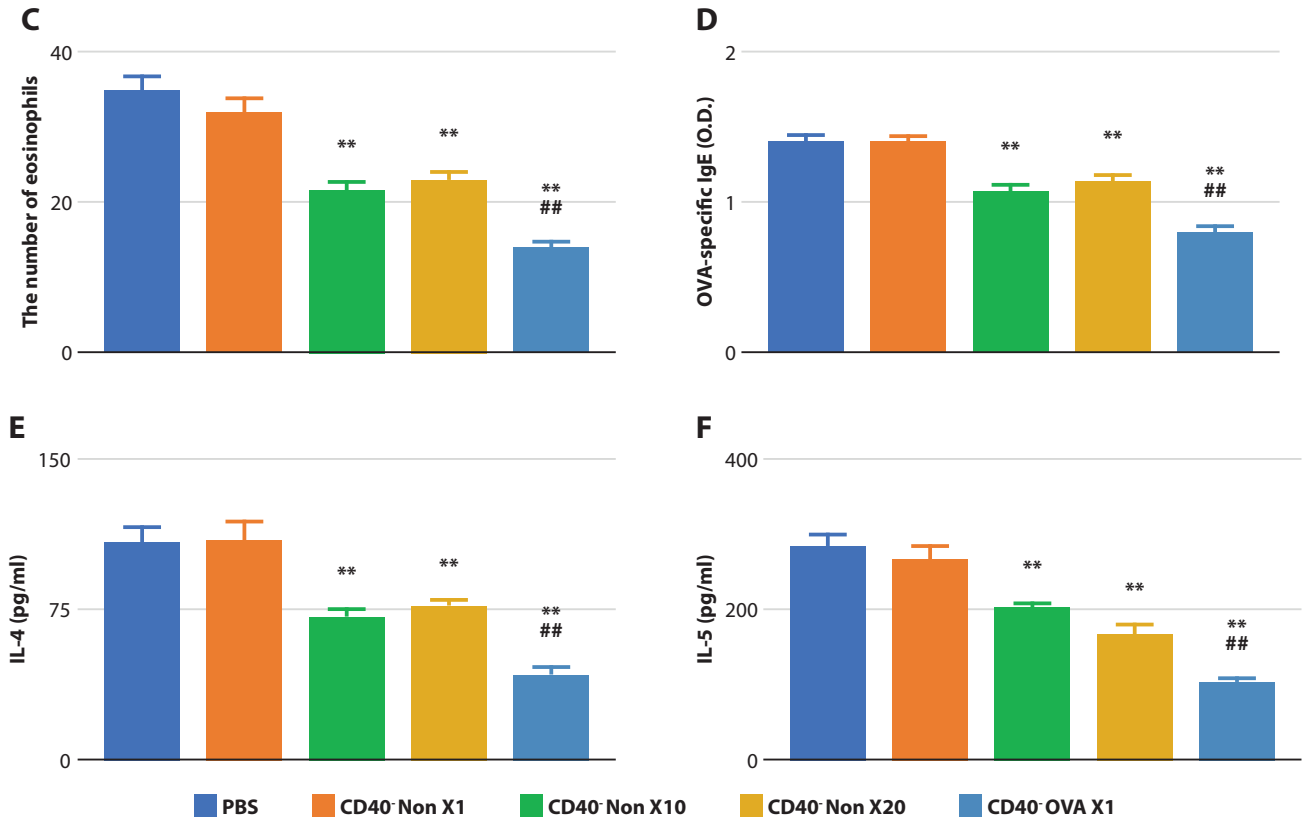

Figure 1. (Continued)
(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. The level of IL-4 (E) and IL-5 (F) production from splenic splenocytes stimulated by OVA was measured by ELISA. ** P < 0.01 versus groups of PBS alone and CD40- Non X1. ##P < 0.01 versus groups of CD40-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

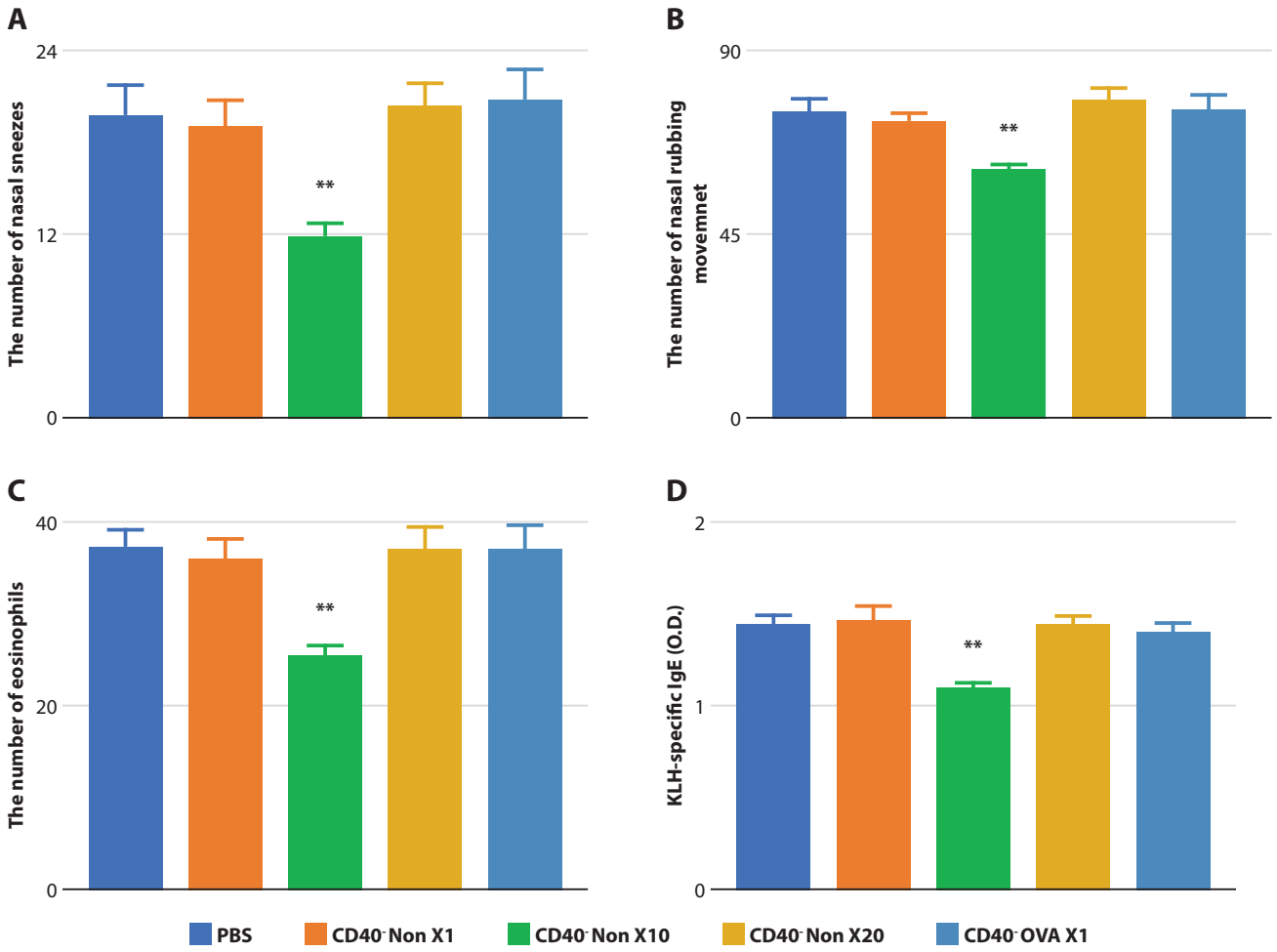

Figure 2. No allergy prevention effect from CD4⁺CD25⁺Tregs induced by CD40-silenced OVA DCs.
Five mice were injected intraperitoneally and challenged intranasally with KLH after treatment with PBS alone, CD40-silenced nonantigen DC-induced CD4⁺CD25⁺ cells (CD40⁻ Non, 4×10^5 "X1" or 4×10^6 "X10" cells/mouse), 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.

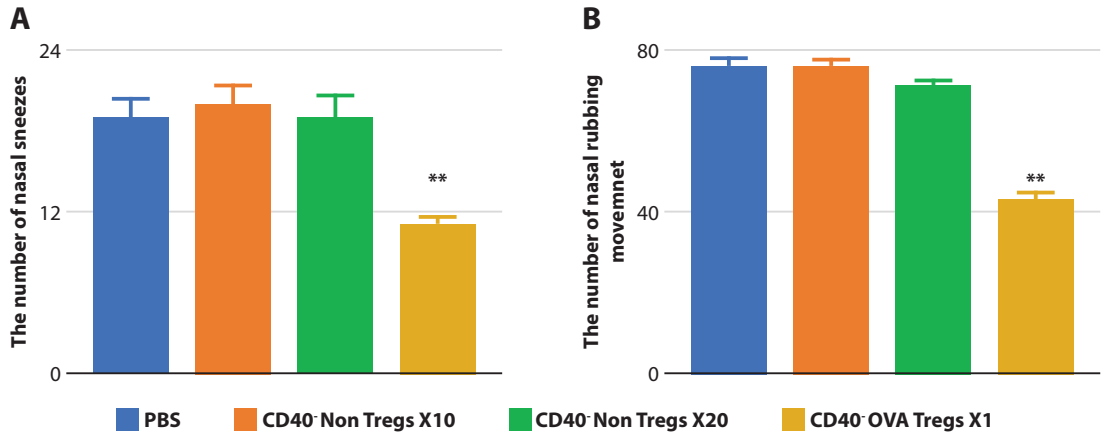

Figure 3. Therapeutic effects of CD4⁺CD25⁺Tregs induced by CD40-silenced OVA DCs in vitro on established allergic rhinitis.
Five mice with OVA-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced CD4⁺CD25⁺ cells (CD40⁻ Non, 4×10^6 "X10" or 8×10^6 "X20" cells/mouse), 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.

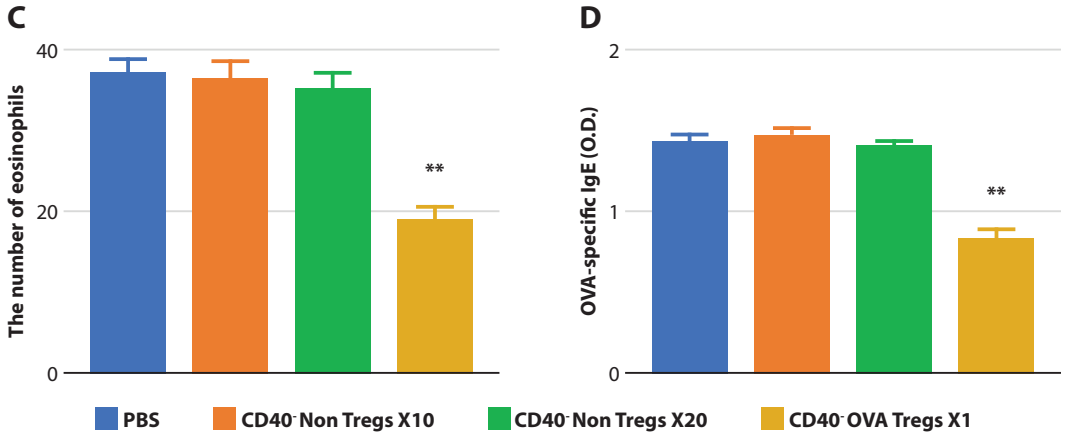

Figure 3. (Continued)
(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. ** P < 0.01 versus group of PBS alone, CD40- Non X10, and CD40- Non X20. Experiments were repeated 3 times with similar result.

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

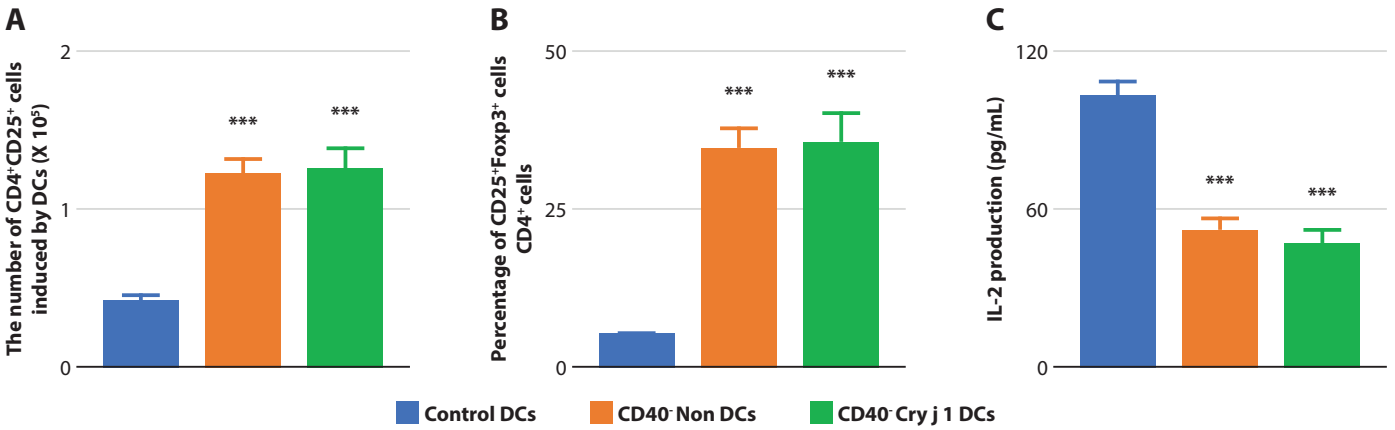

Figure 4. Modulation by CD40 siRNA in vitro. (A) DCs were transfected with Control siRNA (Control DCs) or CD40 siRNA. DCs transfected with CD40 siRNA were pulsed without Cry j 1 (CD40- Non DCs) or with Cry j 1 (CD40- Cry j 1 DCs). The numbers of $CD4^+CD25^+$ cells induced from 3×10^5 naïve $CD4^+$ cells 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

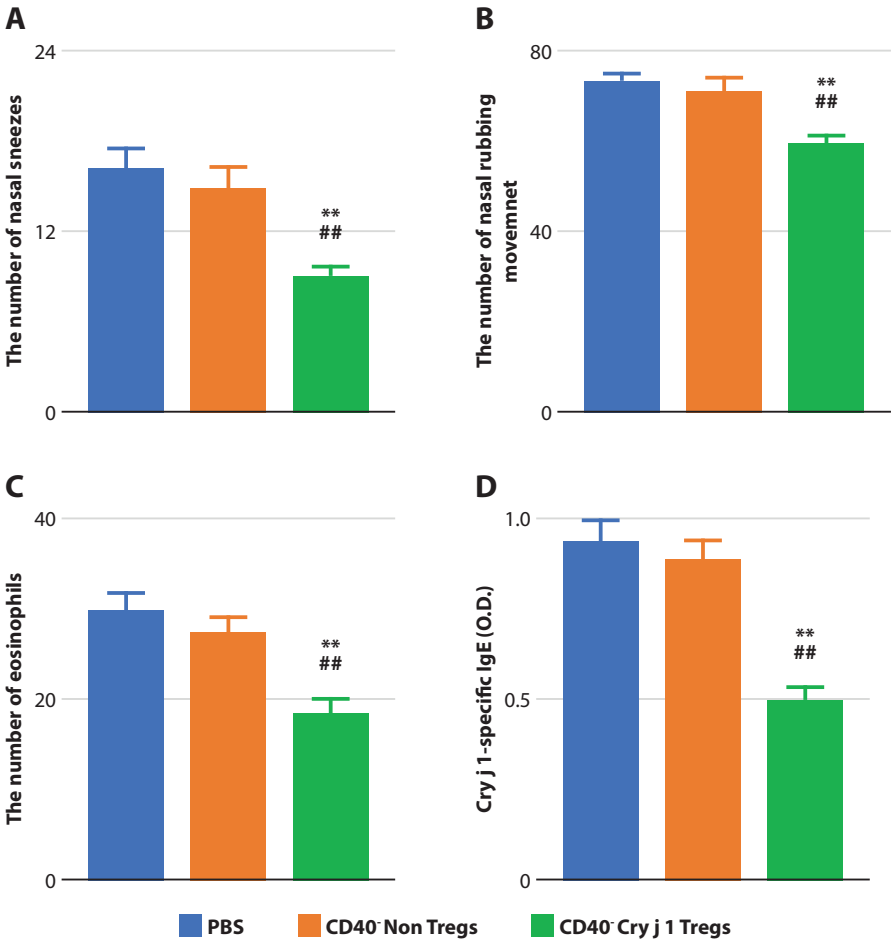

Figure 5. Therapeutic effects of $CD4^+CD25^+$Tregs induced by CD40-silenced Cry j 1 DCs in vitro on established allergic rhinitis. Five mice with Cry j 1-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells (8×10^6 cells/mouse, $CD40^-$ Non Tregs) 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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(effective as of February 1, 2017)

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not more than total of 35 for original research papers
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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.
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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.

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