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

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Abstract

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

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

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

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

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

Key words Asthma, rhinoconjunctivitis, eczema, ISAAC, GAN

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Introduction

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

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

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

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

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

Methods

Study Design

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

Participants

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

GAN Core Questionnaires

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

Sample Size

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

Data Collection and Analysis

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

Results

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

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

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

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

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

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

Current wheeze: wheeze in the past 12 months

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

Strengths and Weaknesses of the Study

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

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

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ORIGINAL ARTICLE

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

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Abstract

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

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

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

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

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

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

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Introduction

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

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

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

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

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

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

Methods

Study Design

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

Participants

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

GAN Core Questionnaires

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

Definitions of AR, Rhinitis, and Hay Fever

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

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

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

Sample Size

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

Data Collection and Analysis

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

Results

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

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

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

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

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

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

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

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

Table 2. (Continued)

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

Table 3. Factor Associate with AR of all children

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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ORIGINAL ARTICLE

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

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Abstract

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

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

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

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

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

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

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Introduction

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

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

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

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

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

Methods

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

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

Generation of Tregs in vitro

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

Immunization and Treatment

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

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

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

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

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

Cry j 1- specific T cell response

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

OVA- specific T cell response

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

Measurement of IL-2 production

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

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

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

Nasal allergic symptoms

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

Pathology

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

Statistical analysis

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

Results

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

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

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

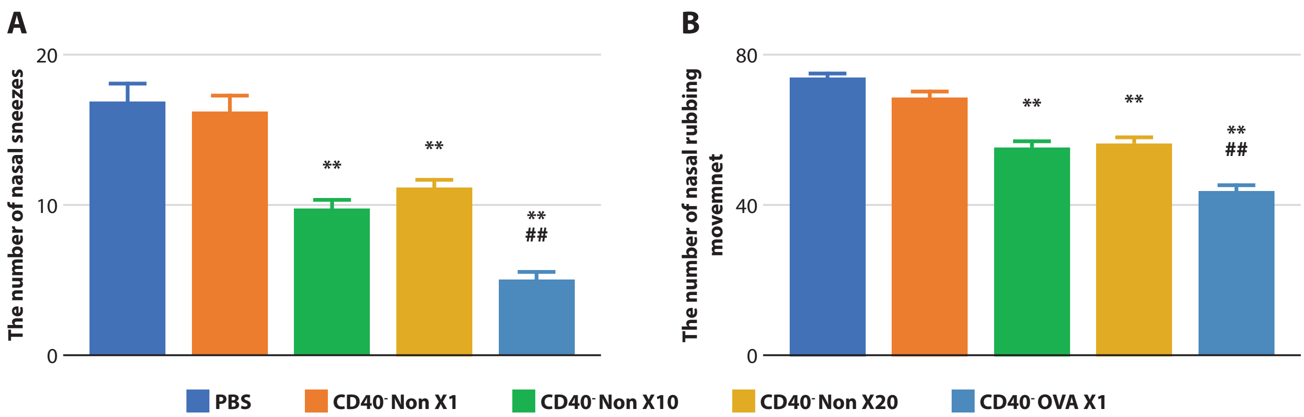

Figure 1. Prevention effects of allergy by $CD4^+CD25^+$ cells induced by CD40-silenced OVA DCs.
Five mice were injected intraperitoneally and challenged intranasally with OVA after treatment of PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells ($CD40^-$ Non, 4 × 10^5 "X1", 4 × 10^6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

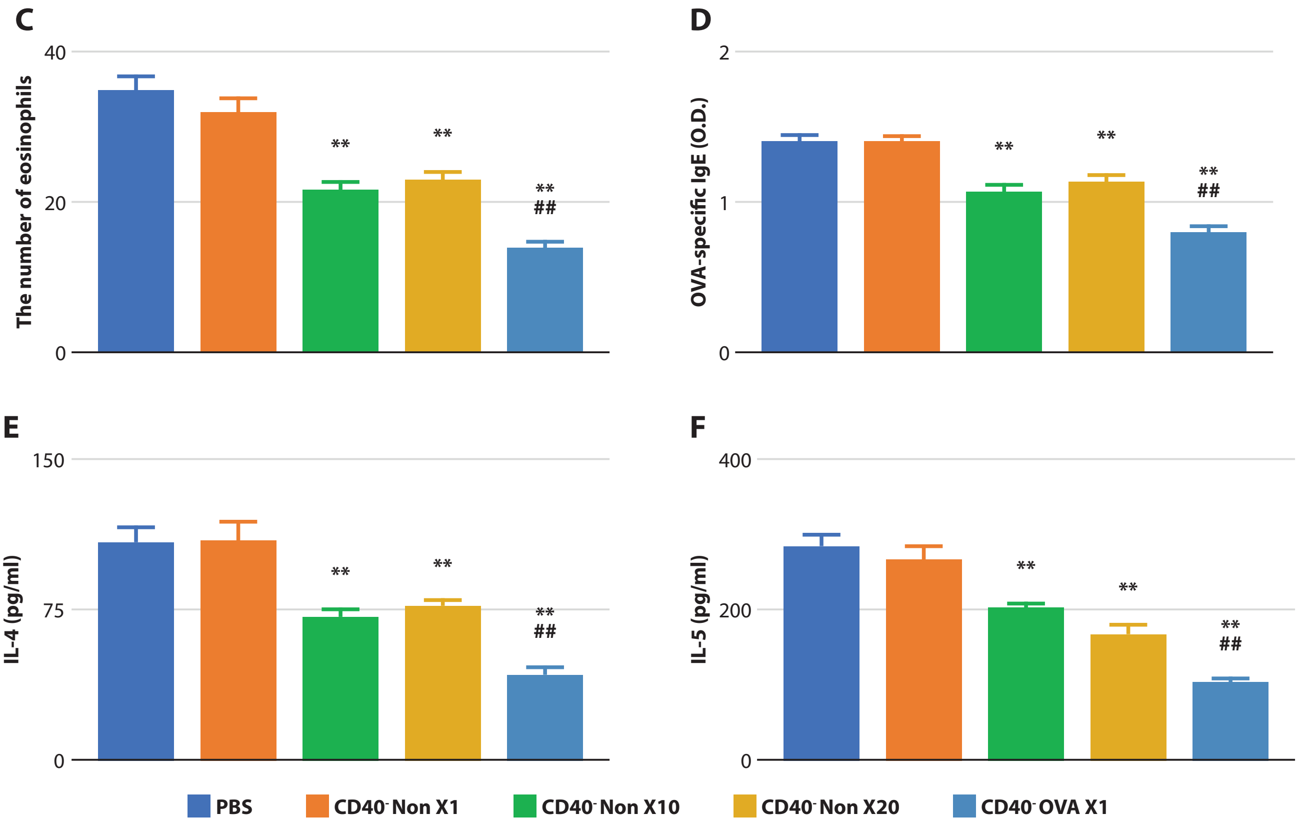

Figure 1. (Continued)
(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. The level of IL-4 (E) and IL-5 (F) production from splenic splenocytes stimulated by OVA was measured by ELISA. ** P < 0.01 versus groups of PBS alone and CD40$^-$ Non X1. ##P < 0.01 versus groups of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

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

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

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

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

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

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

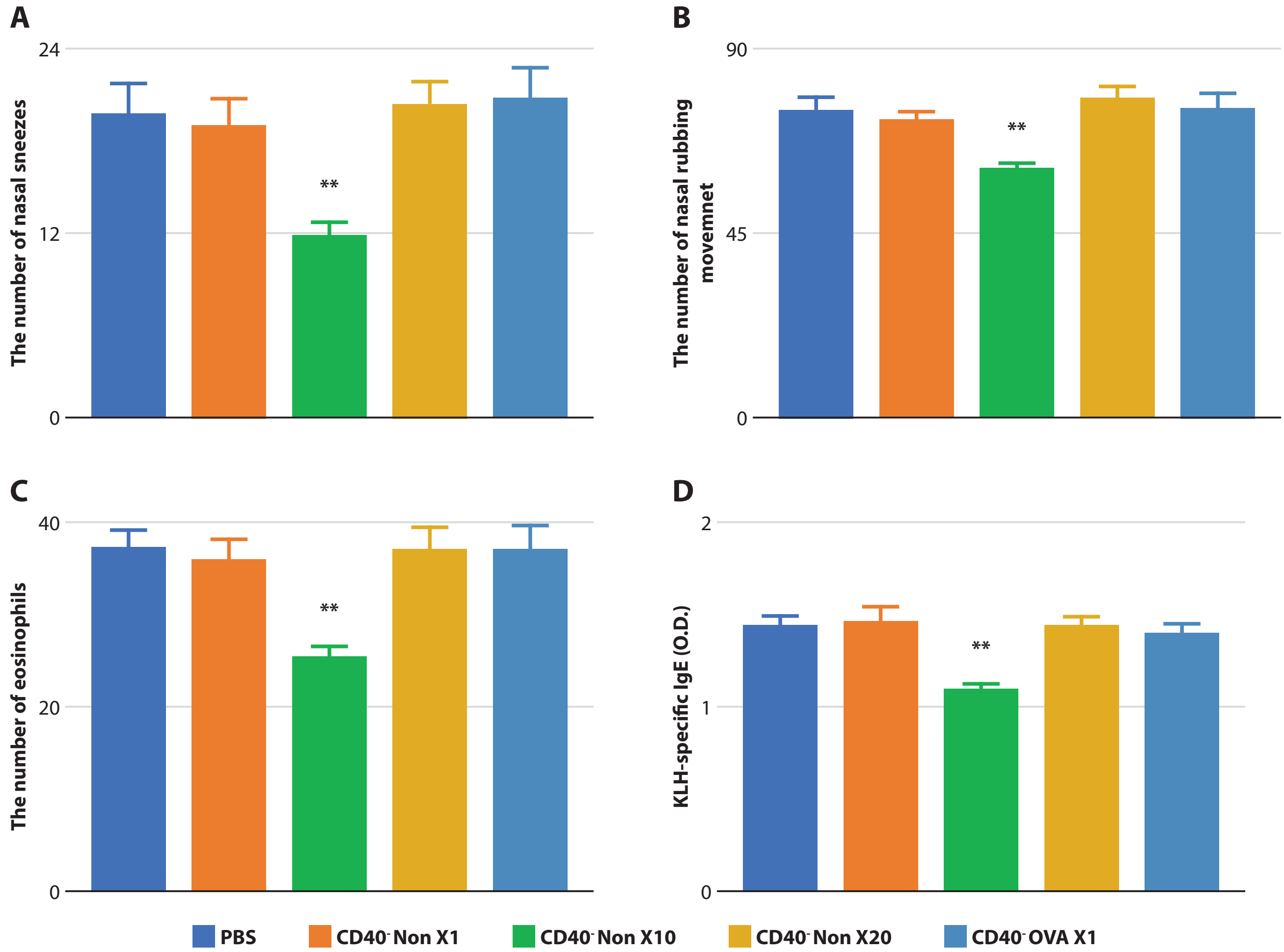

Figure 2. No allergy prevention effect from $CD4^+CD25^+$Tregs induced by CD40-silenced OVA DCs.
Five mice were injected intraperitoneally and challenged intranasally with KLH after treatment with PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells ($CD40^-$ Non, 4×10^5 "X1" or 4×10^6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

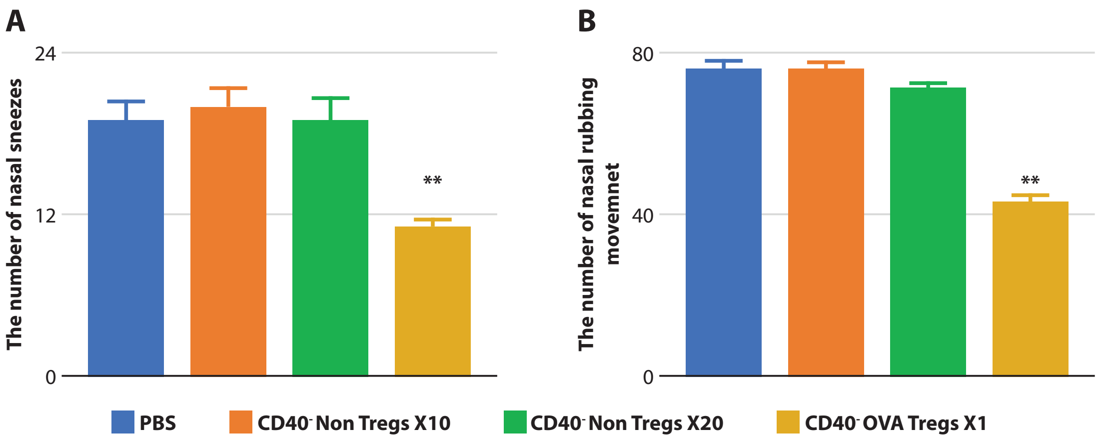

Figure 3. Therapeutic effects of $CD4^+CD25^+$Tregs induced by CD40-silenced OVA DCs in vitro on established allergic rhinitis.
Five mice with OVA-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells ($CD40^-$ Non, 4×10^6 "X10" or 8×10^6 "X20"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

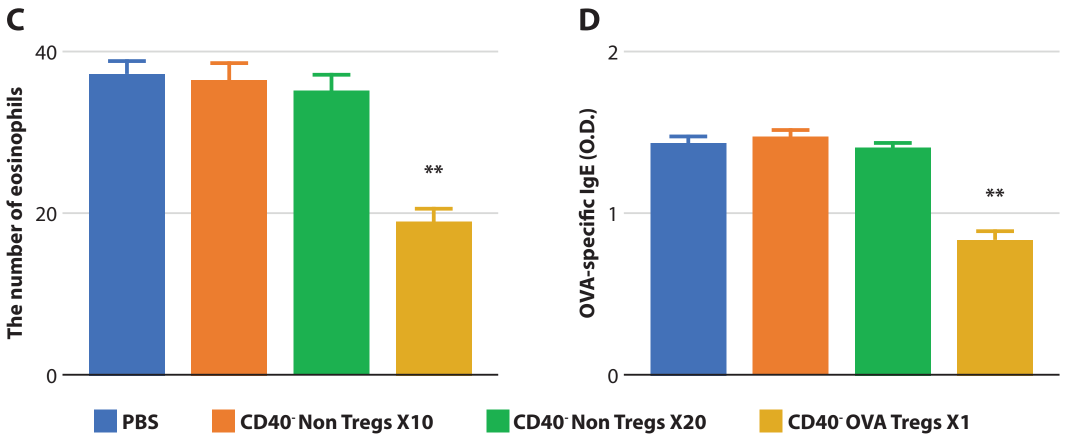

Figure 3. (Continued)
(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. ** P < 0.01 versus group of PBS alone, CD40- Non X10, and CD40- Non X20. Experiments were repeated 3 times with similar result.

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

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

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

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

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

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

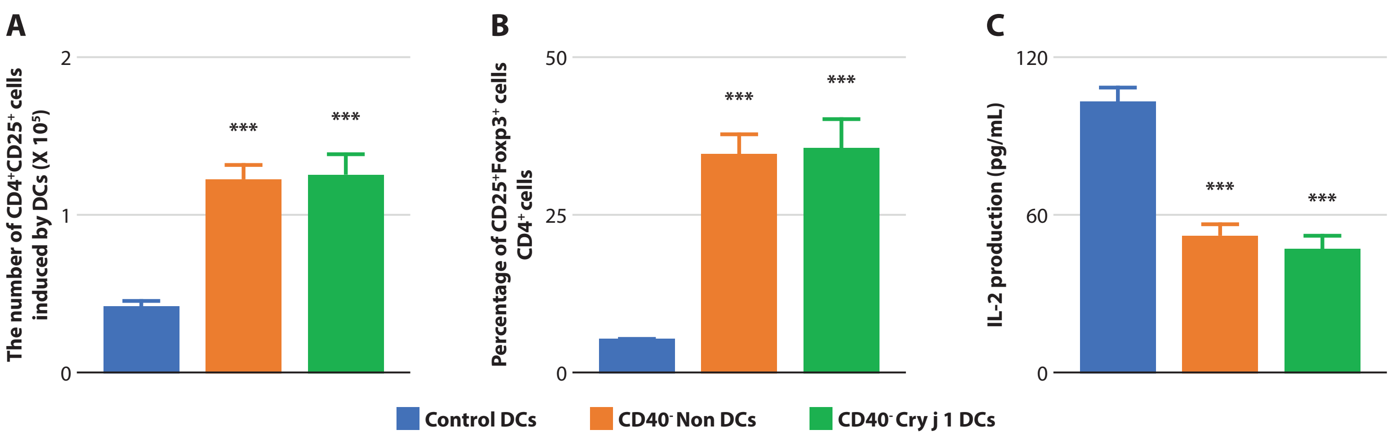

Figure 4. Modulation by CD40 siRNA in vitro. (A) DCs were transfected with Control siRNA (Control DCs) or CD40 siRNA. DCs transfected with CD40 siRNA were pulsed without Cry j 1 (CD40- Non DCs) or with Cry j 1 (CD40- Cry j 1 DCs). The numbers of $CD4^+CD25^+$ cells induced from 3×10^5 naïve $CD4^+$ cells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

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

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

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

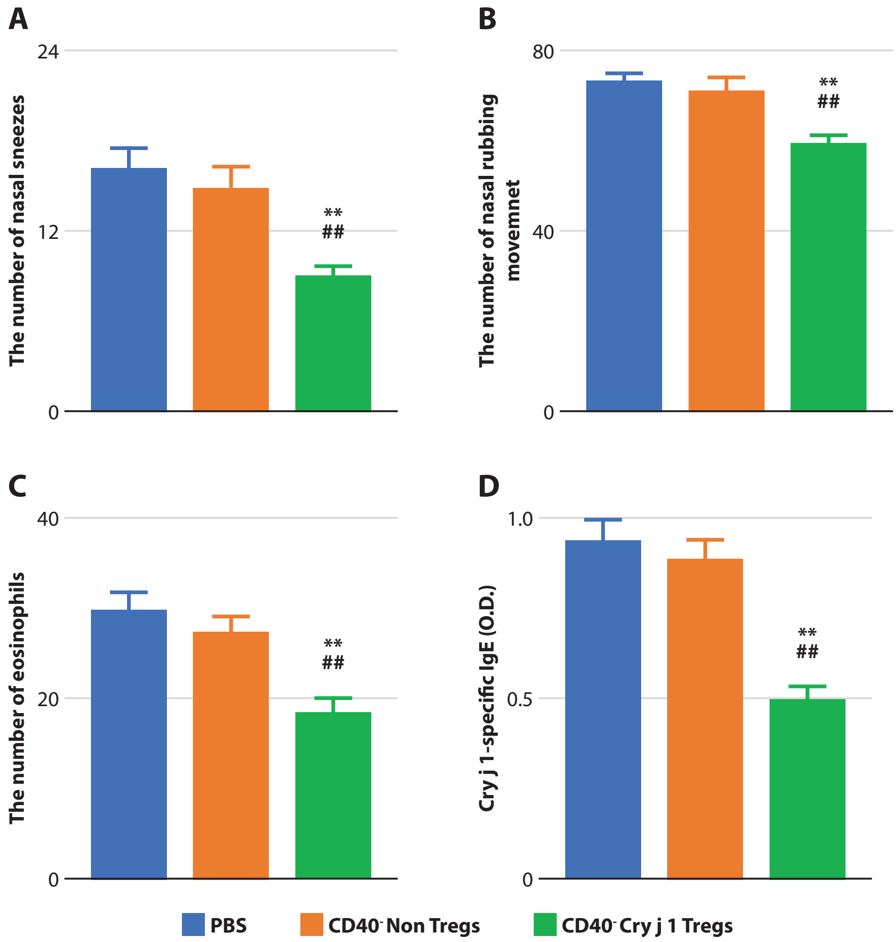

Figure 5. Therapeutic effects of $CD4^+CD25^+$Tregs induced by CD40-silenced Cry j 1 DCs in vitro on established allergic rhinitis. Five mice with Cry j 1-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced $CD4^+CD25^+$ cells (8×10^6 cells/mouse, $CD40^-$ Non Tregs)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

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

Discussion

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

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

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

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

Financial disclosure

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Conflict of interest

None

Authors' contributions

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

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

- **File Description**

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

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

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

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

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

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

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

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

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

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

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

4. **Submit**

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

5. **Submission Fee**

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

6. **Manuscript Format**

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

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

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

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

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

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

   Keywords (at least 5 words or key phrases)

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

6.3 Main text

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

Introduction

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

Methods

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

Results

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

Discussion

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

6.4 Acknowledgements

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

6.5 References

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

Examples

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

Books and other monographs

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

6.6 Figure legends

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

6.7 Tables

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

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

6.8 Figure

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

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

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

Article Types

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

- **Original Research Articles:** The text of original articles should be divided into sections with the following headings in this order: Introduction, Methods, Results, Discussion, and Conclusion. The total text should not exceed 3,500 words (excluding the Abstract, References, and Figure/Table Legends). These should describe fully, but as concisely as possible, the results of original clinical and/or laboratory research. Original articles should have a structured abstract with the following headings: Background, Objective, Methods, Results and Conclusions (maximum 250 words). A minimum of 5 keywords for indexing, and no more than 35 references are required. Text should not exceed 3,500 words. Advice on appropriate sectioning of original articles can be found in the ICMJE's Uniform Requirements. Each original article may be accompanied by a combination of no more than 6 figures and tables. Original article manuscripts that are determined to significantly exceed these limits may be returned to the authors for shortening prior to review. The manuscript should be organized in the following order: title page WITH the names of the authors and affiliations (please see title page requirement mentioned above); abstract and key words; main text; acknowledgements; references; figure legends; tables (each table complete with title and footnotes), and figures. Figures should look sharp and crisp when viewed at 100% magnification. Please note that should your manuscript be accepted, the journal may request for higher resolution TIFF or EPS files.
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