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

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.5 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. 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. 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. 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. 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. Risk factors for AR include paracetamol, antibiotics, truck traffic, breastfeeding, farm animals, cats and dogs, air pollution, tobacco, body mass index (BMI), diet, cooking fuels, birth weight, migration, and siblings. Despite the considerable research efforts, the risk factors of AR remain poorly understood. A family history of atopic diseases seems to be a major risk factor, but various environmental factors and lifestyle are also considered important elements in the evolution of the disease.

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. 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 Bhuminbol 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. 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. 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. 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. 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 questionnaire-based symptoms of rhinitis stratified by age group

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>All (n = 6,291)</th>
<th>6-7 years (n = 3,074)</th>
<th>13-14 years (n = 3,217)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Prevalence 95%CI</td>
<td>N</td>
</tr>
<tr>
<td>Current AR or ARC</td>
<td>1,042</td>
<td>(16.3%, 15.4–17.2%)</td>
<td>462</td>
</tr>
<tr>
<td>Current rhinitis</td>
<td>2,744</td>
<td>(43.6%, 42.4–44.8%)</td>
<td>1,175</td>
</tr>
<tr>
<td>Hay fever (allergic to air)</td>
<td>1,722</td>
<td>(27.4%, 26.3–28.5%)</td>
<td>754</td>
</tr>
<tr>
<td>Severe AR</td>
<td>91</td>
<td>(1.5%, 1.2–1.7%)</td>
<td>30</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total (n = 6,291)</th>
<th>6-7 Years old (n = 3,074)</th>
<th>13-14 Years old (n = 3,217)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n (%)</td>
<td>P-value</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-7</td>
<td>3,074</td>
<td>462 (15.0)</td>
<td>-</td>
</tr>
<tr>
<td>13-14</td>
<td>3,217</td>
<td>562 (17.5)</td>
<td>-</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>3,013</td>
<td>468 (15.6)</td>
<td>1,559</td>
</tr>
<tr>
<td>Male</td>
<td>3,278</td>
<td>555 (16.9)</td>
<td>1,515</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; P85</td>
<td>5,360</td>
<td>857 (16.0)</td>
<td>2,619</td>
</tr>
<tr>
<td>≥ P85</td>
<td>931</td>
<td>167 (17.9)</td>
<td>455</td>
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</table>
Table 2. (Continued)

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total (n = 6,291)</th>
<th>6-7 Years old (n = 3,074)</th>
<th>13-14 Years old (n = 3,217)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n (%)</td>
<td>P-value</td>
</tr>
<tr>
<td>Paternal allergy history</td>
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<td></td>
<td></td>
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<tr>
<td>Asthma</td>
<td>No</td>
<td>6,107</td>
<td>976 (16.0)</td>
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<tr>
<td></td>
<td>Yes</td>
<td>184</td>
<td>48 (26.1)</td>
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<td>AR</td>
<td>No</td>
<td>5,234</td>
<td>775 (14.8)</td>
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<tr>
<td></td>
<td>Yes</td>
<td>1,057</td>
<td>249 (23.6)</td>
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<td>Atopic</td>
<td>No</td>
<td>5,434</td>
<td>811 (14.9)</td>
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<td></td>
<td>Yes</td>
<td>857</td>
<td>213 (24.9)</td>
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<td>Sibling</td>
<td>No</td>
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<td>327 (16.2)</td>
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<td></td>
<td>Yes</td>
<td>4,278</td>
<td>697 (16.3)</td>
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<tr>
<td>Only 6-7 Years old</td>
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<td></td>
</tr>
<tr>
<td>LBW</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Breast Feeding (6 months)</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Antibiotics (first 1 year)</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Paracetamol (first 1 year)</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>-</td>
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<tr>
<td>LRTI (first 1 year)</td>
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<td>-</td>
</tr>
<tr>
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<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Farm animal</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Paracetamol</td>
<td>No</td>
<td>893</td>
<td>99 (11.1)</td>
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<tr>
<td></td>
<td>Yes</td>
<td>5,398</td>
<td>925 (17.1)</td>
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<td>Exercise</td>
<td>No</td>
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<td>558 (13.8)</td>
</tr>
<tr>
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<td>Yes</td>
<td>2,259</td>
<td>466 (20.6)</td>
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<td>Parent Smoke</td>
<td>No</td>
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<td>982 (16.3)</td>
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<tr>
<td></td>
<td>Yes</td>
<td>266</td>
<td>42 (15.8)</td>
</tr>
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<td>Pet</td>
<td>Dog Now</td>
<td>No</td>
<td>4,275</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1,566</td>
<td>283 (18.1)</td>
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<td>Cat Now</td>
<td>No</td>
<td>5,317</td>
<td>813 (15.5)</td>
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<td>974</td>
<td>197 (20.2)</td>
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<td>Truck Traffic</td>
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<td>&lt; 0.001</td>
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<tr>
<td>Never</td>
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<td>3,410</td>
<td>459 (13.5)</td>
</tr>
<tr>
<td>Sometime</td>
<td></td>
<td>2,114</td>
<td>384 (18.2)</td>
</tr>
<tr>
<td>Always</td>
<td></td>
<td>767</td>
<td>181 (23.6)</td>
</tr>
<tr>
<td>Fire Cooking</td>
<td>No</td>
<td>6,036</td>
<td>979 (16.2)</td>
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<td>Yes</td>
<td>255</td>
<td>45 (17.6)</td>
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<td>Env Factors</td>
<td>Cockroach</td>
<td>No</td>
<td>4,273</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2,018</td>
<td>360 (17.8)</td>
</tr>
<tr>
<td>Air Conditioner</td>
<td>No</td>
<td>3,993</td>
<td>619 (15.5)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2,298</td>
<td>405 (17.6)</td>
</tr>
<tr>
<td>Tree or Flower</td>
<td>No</td>
<td>2,238</td>
<td>343 (15.3)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4,053</td>
<td>681 (16.8)</td>
</tr>
<tr>
<td>Perfume</td>
<td>No</td>
<td>3,591</td>
<td>557 (15.5)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2,700</td>
<td>467 (17.3)</td>
</tr>
<tr>
<td>School Type</td>
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<td>0.575</td>
<td>0.763</td>
</tr>
<tr>
<td>Public</td>
<td></td>
<td>4,170</td>
<td>671 (16.1)</td>
</tr>
<tr>
<td>Private</td>
<td></td>
<td>2,121</td>
<td>353 (16.6)</td>
</tr>
</tbody>
</table>

Prevalence and risk factors of allergic rhinitis in children in the Bangkok area
**Table 3. Factor Associate with AR of all children**

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>All</th>
<th>6-7</th>
<th>13-14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude Odds Ratio</td>
<td>Adjusted Odds Ratio</td>
<td>Crude Odds Ratio</td>
</tr>
<tr>
<td></td>
<td>Point (95%CI)</td>
<td>P Value</td>
<td>Point (95%CI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-7</td>
<td>1.20 (1.05,1.37)</td>
<td>0.009</td>
<td>1.11 (0.96,1.29)</td>
</tr>
<tr>
<td>Sex Male</td>
<td>1.11 (0.97,1.26)</td>
<td>0.143</td>
<td></td>
</tr>
<tr>
<td>Paternal allergy history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td>1.86 (1.33,2.60)</td>
<td>&lt; 0.001</td>
<td>1.59 (1.05,2.13)</td>
</tr>
<tr>
<td>Atopic dermatitis</td>
<td>1.77 (1.51,2.08)</td>
<td>0.001</td>
<td>1.43 (1.20,1.71)</td>
</tr>
<tr>
<td>Paternal allergy history</td>
<td>1.89 (1.59,2.24)</td>
<td>0.001</td>
<td>1.54 (1.29,1.86)</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotics (first 1 year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paracetamol (first 1 year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm animal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>1.18 (1.02,1.33)</td>
<td>0.030</td>
<td>1.07 (0.91,1.26)</td>
</tr>
<tr>
<td>Cat</td>
<td>1.38 (1.16,1.64)</td>
<td>&lt; 0.001</td>
<td>1.28 (1.07,1.53)</td>
</tr>
<tr>
<td>Truck Traffic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seldom</td>
<td>1.43 (1.23,1.66)</td>
<td>&lt; 0.001</td>
<td>1.26 (1.01,1.56)</td>
</tr>
<tr>
<td>Always</td>
<td>1.99 (1.64,2.41)</td>
<td>&lt; 0.001</td>
<td>1.72 (1.41,2.11)</td>
</tr>
<tr>
<td>Environmental Factors</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cockroach</td>
<td>1.18 (1.03,1.36)</td>
<td>0.021</td>
<td>1.11 (0.88,1.41)</td>
</tr>
<tr>
<td>Air Conditioner</td>
<td>1.17 (1.02,1.34)</td>
<td>0.028</td>
<td>1.05 (0.83,1.32)</td>
</tr>
<tr>
<td>Perfume</td>
<td>1.14 (1.00,1.30)</td>
<td>0.058</td>
<td>1.07 (0.98,1.23)</td>
</tr>
</tbody>
</table>

* Multivariable logistic regression model

**Ref.**
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. 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.

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. 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. 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. Allergic children showed a significantly higher number of respiratory infections in comparison with the non-allergic group. Epidemiological studies have investigated significant relationships between AR and LRTI.

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. 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. 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.

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. 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 µg/m³ when self-reported traffic was “absent,” 44 µg/m³ when “low,” 48 µg/m³ when “intermediate,” and 52 µg/m³ when “high.” 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. 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.\(^{43}\)

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.

Acknowledgements

The study was completed with significant contributions from the colleagues of the allergy centers, Bhumibol Adulyadej Hospital. The authors wish to thank:

Mr Suthisisak Srisawad
Mr Itti Chinnaratapanisit
Ms Chanut Chinnaratapanisit

The authors would like to thank all the children, parents, and teachers who participated in this study. We also thank those who helped with the field work.

This study was co-supported by grants from the National Research Council of Thailand; the Allergy, Asthma, and Immunology Association of Thailand; the Royal College of Pediatricians of Thailand; and the Pediatric Society of Thailand.

References


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

Motohiko Suzuki, Makoto Yokota, Shinya Ozaki, Yoshinaka Nakamura

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-silenced 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.

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, while the inhibition of this interaction suppresses T cell responses and generates regulatory T cells (Tregs).

RNA interference using small interfering RNA (siRNA) induces specific silencing of gene expression, and is a potent, selective, and easy method. 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.

We previously reported that vector expressing siRNA specific for CD40 (CD40 siRNA) inhibits allergic responses not only as a means of prevention but also as treatment. 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. 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. 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. 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. 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.

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. 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, UUCUCAGCCGAG UGGAAACA) 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. 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.

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 × 10⁹/mL) were co-cultured with 6 × 10⁶/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-β (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).

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 × 10⁶ or 4 × 10⁷ cells/mouse) induced by CD40-silenced nonantigen DCs, or Tregs (4 × 10⁶ 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 × 10⁶ or 4 × 10⁷ cells/mouse) induced by CD40-silenced nonantigen DCs, or Tregs (4 × 10⁶ or 4 × 10⁷ 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 × 10⁶ or 8 × 10⁶ cells/mouse), or Tregs by CD40-silenced OVA DCs (4 × 10⁶ or 8 × 10⁵ 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 × 10⁵ cells/mouse), or Tregs by CD40-silenced Cry j 1 DCs (4 × 10⁵ 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 × 10⁹ cells/mL) and DC (2 × 10⁶ cells/mL) suspensions were cultured for 72 h and stimulated with 10 µg/mL Cry j 1 antigen.
OVA-specific T cell response

Splenetic 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 was stained with Luna staining. The number of eosinophils infiltrating the nasal mucosa in mice in the PBS and CD40-silenced nonantigen DC-induced T cells at levels 10 times greater than (4 × 10^5 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.

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

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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
The number of eosinophils

C

D

OVA-specific IgE (O.D.)

E

F

IL-4 (pg/ml)

IL-5 (pg/ml)

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 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 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, $4 \times 10^5$ “X1” or $8 \times 10^6$ “X10” cells/mouse), or CD40-silenced OVA DC-induced CD4+CD25+ cells (CD40 OVA, $4 \times 10^5$ “X1” or $4 \times 10^6$ “X10” cells/mouse). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge. Experiments were repeated 3 times with similar result.

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, $4 \times 10^5$ “X1” or $4 \times 10^6$ “X10” cells/mouse), or CD40-silenced OVA DC-induced CD4+CD25+ cells (CD40 OVA, $4 \times 10^5$ “X1” or $4 \times 10^6$ “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.
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.16–19 Bone marrow-derived DCs were transfected with CD40 siRNA or Control siRNA (Control DCs). DCs transfected with CD40 siRNA were pulsed with Cry j 1 and were then used to induce Tregs.

**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.

**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 naive 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 induced from 3 × 10^6 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).

Figure 5. 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

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

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 × 10^6 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 pollin.

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-non-specific Tregs. Patients who suffer from sensitization to multiple allergens are increasing.23 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,22-23 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.25 Both blockade of B7-CD28 and CD40-CD40L also activated Foxp3+ regulatory T cells and reduced IL-2 production.26 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).27 And Vogel et al.28 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 infection26,27 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 response30,31 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 administrated (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,27,32 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
This study is partially supported by Grants-in-Aid for Scientific Research C (15K10789) from Japan Society for the Promotion of Science.

Conflict of interest
None

Authors’ contributions
Mototiko Suzuki and Yoshisasa Nakamura designed the study. Mototiko 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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