

## Letter to the Editor

**Genetic variants of inducible costimulator are associated with allergic asthma susceptibility**

To the Editor:

The prevalence of allergic diseases has increased worldwide over the past decades, with 300 million people now affected by asthma and an estimated 400 million more suffering from allergic rhinitis.<sup>1</sup> These allergic phenotypes result from a complex interplay between genetic and environmental risk factors that remain incompletely defined. Previous genetic studies associating inducible costimulator (*ICOS*) promoter polymorphisms with allergic sensitization<sup>2</sup> and IgE<sup>3</sup> were not consistently reproduced.<sup>4,5</sup> Thus, the role of *ICOS* polymorphisms in allergy and asthma risk remained unclear. Here we investigate the association of polymorphisms in the *ICOS* gene with the risk of developing allergic asthma, and report for the first time a significant association between single nucleotide polymorphisms (SNPs) of *ICOS* and asthma. Furthermore, we demonstrate that the minor allele of the tagSNP rs4675374 is associated with increased expression of *ICOS* on T lymphocytes including regulatory T (Treg) cells and lower risk of atopy and asthma.

Our association analysis included an adult Singapore Chinese cohort and an independent Dutch Caucasian cohort.<sup>6</sup> (For more information, see this article's [Methods](#) section and for demographic characteristics, see [Tables E1 and E2](#) in the Online Repository at [www.jacionline.org](http://www.jacionline.org).) We identified the T allele of the tagSNP rs4675374 as having the most significant association with allergic asthma in the Singapore Chinese cohort, with an odds ratio of 0.69 (95% CI, 0.59-0.80) and a *P* value of  $9.77 \times 10^{-7}$  (see [Table E3](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). This SNP was also significantly associated with allergic asthma (*P* = .017) in the Dutch Caucasian cohort, with the same direction of effect (see [Table E4](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). In a meta-analysis using data from the Singapore Chinese and Dutch allergic asthma cohorts, the T allele (minor) was significantly associated with allergic asthma ( $P_{\text{meta}} = 7.16 \times 10^{-8}$  and an odds ratio of 0.72; [Table I](#)). To test whether our findings on *ICOS* variants and asthma risk could be reproduced in an unrelated and independent cohort, we analyzed data from a recently published meta-analysis of asthma in North American populations.<sup>7</sup> We used association summary statistics including risk and alternative alleles, *z* score, and *P* value from the study data to ask whether *ICOS* genetic variants were associated with asthma in this population (see [Table E5](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). We found that the most significantly associated SNP in the Singapore Chinese population, rs4675374, was also the most significantly associated SNP in the North American populations ( $P_{\text{meta}} = .006$ ). In addition, in a Singaporean population of ethnic Chinese from the Singapore Cohort study Of the Risk factors for Myopia study,<sup>8</sup> we observed that the allele frequency of the T genotype of rs4675374 SNP was higher in controls (40%) than in asthmatic cases (36%). In conclusion, the association of the T allele of rs4675374 *ICOS* SNP with lower asthma risk was validated in several independent cohorts of diverse ethnic origin.

IgE levels are a strong indicator of allergic disease and hence we next tested whether rs4675374 was also associated with serum

IgE levels. Analysis of samples from 182 individuals within the adult Singaporean Chinese cohort revealed that the presence of the T allele, which is associated with a lower risk of allergic asthma, was also associated with lower total IgE and house dust mite-specific IgE levels in serum (see [Fig E1, A and B](#), in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). When we tested the same hypothesis in a cohort of Korean asthmatic patients, we found that individuals with the TT allele had significantly lower total IgE levels (mean, 397 U/mL) than did those with the CC or CT genotypes (mean, 305 U/mL), which was consistent with findings in the Singapore Chinese cohort (see [Fig E1, C](#)).

The genetic associations described above for the rs4675374 *ICOS* SNP were indeed significant but it was not clear whether this SNP drove a measurable biological phenotype or function. To address this question, we used a large expression quantitative trait locus meta-analysis data set of European origin that comprised 9 different whole-blood expression data sets with corresponding whole genome SNP information.<sup>9</sup> [Fig 1](#) demonstrates a strong association of rs4675374 with *ICOS* mRNA levels in whole blood in this independent population of unrelated individuals from Europe. Individuals with the T allele had a significantly higher *ICOS* gene expression level than did those with the C allele. The direction of the effect was identical for 7 of the 8 data sets, with a meta *P* value that indicated significance (false-discovery rate, <0.05) with  $P = 1.5 \times 10^{-7}$  (*z* score of 5.25 for all the cohorts combined). Thus, having the low asthma-risk T allele for the tagSNP rs4675374 is associated with higher *ICOS* mRNA levels in an independent population of unrelated individuals of European ethnicity.

To evaluate whether the difference in gene expression translated into a higher *ICOS* protein level, we selected a group of 40 individuals from the Singaporean Chinese adult cohort carrying the CC or TT rs4675374 genotype and determined the expression of *ICOS* protein on peripheral blood T cells. Of the group of 40 individuals, 16 were homozygous for the minor T allele of rs4675374. The expression of *ICOS* on unstimulated CD3<sup>+</sup> T cells from PBMCs was relatively low (see [Fig E2, A](#), in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) but increased after activation with anti-CD3 and anti-CD28 antibodies, as previously described<sup>2</sup> (see [Fig E2, B](#)). Surface expression of *ICOS* in activated total CD3<sup>+</sup> T cells, as well as in the CD4<sup>+</sup> and CD8<sup>+</sup> subsets (see [Fig E2, B, C, and D](#), respectively), was significantly higher in samples from individuals carrying the rs4675374 TT genotype associated with a lower risk for asthma (*P* value .014 for CD3<sup>+</sup> cells, .047 for CD4<sup>+</sup> cells, and .032 for CD8<sup>+</sup> cells).

The effect of the rs4675374 tagSNP genotype on *ICOS* expression level was also assessed in various peripheral blood CD4<sup>+</sup> T helper cell subpopulations (T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17, and T<sub>H</sub>17 and T<sub>H</sub>17) in the absence of stimulation (see [Fig E3](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org); for gating strategy, refer to [Fig E4](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Individuals with the TT genotype associated with lower asthma risk had significantly higher *ICOS* expression on T<sub>H</sub>1 cells (*P* = .04; see [Fig E3, A](#)). The variation in *ICOS* levels on T<sub>H</sub>2 cells followed the same trend but was not significant, perhaps due to the sample size (*P* = .059; [Fig E3, B](#)). The *ICOS* levels in the T<sub>H</sub>17 and T<sub>H</sub>17 cell populations were not significantly linked to the rs4675374 genotype (*P* > .05; [Fig E3, C and D](#)).

**TABLE I.** Association of rs4675374 with allergic asthma phenotype

| CHR                                   | SNP       | BP        | Minor allele | Major allele | MAF  | OR                    | SE                    | L95  | U95   | <i>P</i> <sub>allele</sub> |          |
|---------------------------------------|-----------|-----------|--------------|--------------|------|-----------------------|-----------------------|------|-------|----------------------------|----------|
| A. Singapore Chinese adult population |           |           |              |              |      |                       |                       |      |       |                            |          |
| 2                                     | rs4675374 | 204510823 | T            | C            | 0.40 | 0.68                  | 0.07                  | 0.59 | 0.8   | $9.7 \times 10^{-7}$       |          |
| CHR                                   | SNP       | BP        | Minor allele | Major allele | MAF  | OR                    | SE                    | L95  | U95   | <i>P</i> <sub>allele</sub> |          |
| B. DAG cohort                         |           |           |              |              |      |                       |                       |      |       |                            |          |
| 2                                     | rs4675374 | 204510823 | T            | C            | 0.18 | 0.79                  | 0.097                 | 0.66 | 0.96  | .017                       |          |
| CHR                                   | SNP       | BP        | Minor allele | Major allele | N    | <i>P</i> value        | <i>P</i> (R)          | OR   | OR(R) | <i>Q</i>                   | <i>I</i> |
| C. Meta analysis                      |           |           |              |              |      |                       |                       |      |       |                            |          |
| 2                                     | rs4675374 | 204510823 | T            | C            | 2    | $7.16 \times 10^{-8}$ | $3.45 \times 10^{-6}$ | 0.72 | 0.73  | 0.26                       | 22.19    |

BP, Base pair; CHR, chromosome; DAG, Dutch Asthma GWAS; *I*,  $I^2$  heterogeneity index (0-100); *minor allele*, minor allele for the SNP rs4675374 for which the association statistics have been calculated; *major allele*, major allele for the SNP rs4675374; *MAF*, minor allele frequency; *N*, number of studies for the meta-analysis; *OR*, odds ratio calculated using the fixed-effect model; *OR(R)*, odds ratio calculated using the random-effect model; *P*, *P* value calculated using the fixed-effect model; *P(R)*, probability using the random-effect model; *Q*, *P* value for Cochran's *Q* statistic.

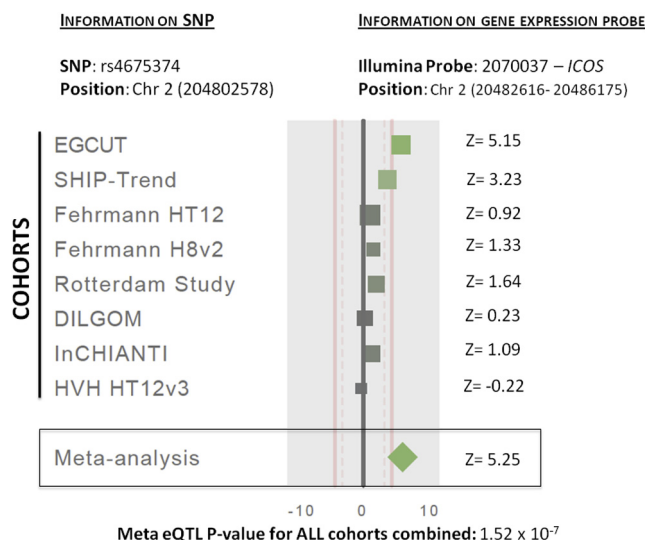


**FIG 1.** Association of rs4675374 tagSNP genotype with *ICOS* mRNA expression level in 8 different data sets from whole blood. The thick red line is the FDR significance threshold (FDR < 0.05), whereas the dotted red line denotes FDR < 0.5. The allele T of rs4675374 is used as the reference allele because it increases the expression of *ICOS*. Green color indicates an increase in gene expression for the T allele considered here. eQTL, Expression quantitative trait locus; FDR, false-discovery rate.

High *ICOS* expression was previously described in the CD45RO<sup>+</sup> population of FOXP3<sup>+</sup> Treg cells.<sup>10</sup> We thus analyzed what proportion of FOXP3<sup>+</sup> CD45RO<sup>+</sup> and CD45RO<sup>−</sup> Treg cells expressed *ICOS* and whether *ICOS* expression was affected by tagSNP rs4675374. We observed a higher percentage of *ICOS*<sup>+</sup>FOXP3<sup>+</sup> Treg cells within the total Treg-cell population in individuals with the TT genotype ( $P = .04$ ; see Fig E5, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org); for gating strategy, refer to Fig E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The surface expression of *ICOS* in the total Treg-cell population of individuals with the TT genotype was also tendentially, albeit not significantly, higher (see Fig E5, B). Finally, both the percentage of *ICOS*<sup>+</sup> cells and the level of *ICOS* expression among CD45RO<sup>+</sup> Treg cells were higher in samples from individuals with the TT genotype than in samples from individuals with the asthma-associated CC genotype ( $P = .02$  and  $.03$ ; see Fig E5, C and D, respectively).

In conclusion, we demonstrated a significant association of rs4675374 with the occurrence of allergic asthma and with IgE levels in independent cohorts. In addition, we linked the specific alleles present at this position to both *ICOS* mRNA level in total blood and *ICOS* protein level in peripheral blood-activated CD4<sup>+</sup> and CD8<sup>+</sup> populations and nonactivated Treg cells and T<sub>H</sub>1 CD4<sup>+</sup> subsets.

*ICOS* is a costimulatory molecule of the CD28 family that is important for effector T<sub>H</sub>2 and T-cell-mediated B-cell responses.<sup>11</sup> However, *ICOS* is also important in tolerance and regulatory T-cell function.<sup>11</sup> Stimulation through *ICOS* induces the expression of IL-10,<sup>12</sup> and it is known that *ICOS*<sup>+</sup> Treg cells suppress through the cytokines IL-10 and TGF- $\beta$ .<sup>10</sup> *ICOS* similarly plays a crucial role in airway tolerance by inducing regulatory cells that secrete IL-10.<sup>13,14</sup> It is thus relevant that the *ICOS* tagSNP rs4675374 genotype is associated with higher *ICOS* expression in CD45RO<sup>+</sup> FOXP3<sup>+</sup> Treg cells, which are known to be highly suppressive.<sup>10</sup> The suppressive activity of Treg cells with the TT genotype may thus be higher than the suppressive activity of those with the disease-associated CC genotype. In addition, higher *ICOS* expression in T<sub>H</sub>1 cells, as well as other effector/memory T cells, may contribute to higher production of the immunosuppressive cytokine IL-10. Expression of *ICOS* in Treg cells and other memory/effector cells such as T<sub>H</sub>1 cells may promote preferential *ICOS*-*ICOSL*-mediated interactions with plasmacytoid dendritic cell, rather than interactions with myeloid dendritic cell, leading to IL-10 production and attenuated responses.<sup>10</sup> Higher *ICOS* expression on Treg cells and T<sub>H</sub>1 cells may also drive interactions with *ICOSL*<sup>+</sup>-activated B cells, resulting in suppression of IgE production. Further functional experiments are needed to elucidate these hypotheses.

In summary, we show here using several independent human cohorts that a genetic *ICOS* variant is associated with higher *ICOS* expression and lower incidence of atopy and allergic asthma. Our findings may be explained by a protective role of higher *ICOS* expression in FOXP3<sup>+</sup> Treg cells and in other populations of effector/memory cells such as T<sub>H</sub>1 cells. This may tilt the balance of the immune response to allergens toward tolerance rather than inflammation, and/or toward the production of nonallergic humoral responses. Our study suggests that a thorough understanding of *ICOS* polymorphisms and their effects on the regulation of *ICOS* expression may provide new avenues for the immunomodulation of inflammatory allergic diseases.

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Anand Kumar Andiappan, PhD<sup>a,b</sup>  
Sriram Narayanan, PhD<sup>a</sup>  
Rachel A. Myers, PhD<sup>c</sup>  
Bernett Lee, PhD<sup>a</sup>  
Maartje A. Nieuwenhuis, PhD<sup>d</sup>  
Alessandra Nardin, PhD<sup>e</sup>  
Choon-Sik Park, MD<sup>f</sup>  
Hyoung Doo Shin, PhD<sup>f</sup>  
Jeong-Hyun Kim, PhD<sup>f</sup>  
Harm-Jan Westra, PhD<sup>g</sup>  
Lude Franke, PhD<sup>g</sup>  
Tonu Esko, PhD<sup>h</sup>  
Andres Metspalu, PhD<sup>h</sup>  
Yik-Ying Teo, PhD<sup>i,j</sup>  
Seang Mei Saw, MD, PhD<sup>j</sup>  
Khor Chiea Chuen, PhD<sup>k</sup>  
Liu Jianjun, PhD<sup>l</sup>  
Gerard H. Koppelman, MD, PhD<sup>m</sup>  
Dirkje S. Postma, MD, PhD<sup>d</sup>  
Michael Poidinger, PhD<sup>n</sup>  
John E. Connolly, PhD<sup>a,n</sup>  
De Yun Wang, MD, PhD<sup>o</sup>  
Olaf Rotzschke, PhD<sup>a</sup>  
Maria A. Curotto de Lafaille, PhD<sup>a</sup>  
Fook Tim Chew, PhD<sup>b</sup>

From <sup>a</sup>the Singapore Immunology Network, Agency for Science, Technology and Research (A\*STAR), Singapore; <sup>b</sup>the Department of Biological Sciences, National University of Singapore, Singapore; <sup>c</sup>the Department of Human Genetics, University of Chicago, Chicago, Ill; <sup>d</sup>the Department of Pulmonology and Tuberculosis, University Medical Center Groningen, GRIAC Research Institute, University of Groningen, Groningen, The Netherlands; <sup>e</sup>the Division of Allergy and Respiratory Medicine, Soonchunhyang University Bucheon Hospital, Bucheon, Korea; <sup>f</sup>the Department of Life Science, Sogang University, Seoul, Korea; <sup>g</sup>the Department of Genetics, University of Groningen, Groningen, The Netherlands; <sup>h</sup>Estonian Genome Center, University of Tartu, Tartu, Estonia; <sup>i</sup>the Department of Statistics and Applied Probability, National University of Singapore, Singapore; <sup>j</sup>Saw Swee Hock School of Public Health, National University of Singapore, Singapore; <sup>k</sup>Infectious Diseases, Genome Institute of Singapore, A\*STAR, Singapore; <sup>l</sup>Human Genetics, Genome Institute of Singapore, A\*STAR, Singapore; <sup>m</sup>the Department of Pediatric Pulmonology and Pediatric Allergy, University Medical Center Groningen, Beatrix Children's Hospital, GRIAC Research Institute, University of Groningen, Groningen, The Netherlands; <sup>n</sup>the Institute of Molecular and Cell Biology, A\*STAR, Singapore; and <sup>o</sup>the Department of Otolaryngology, National University of Singapore, Singapore. E-mail: maria\_lafaille@immunol.a-star.edu.sg. Or: dbseft@nus.edu.sg.

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## METHODS

### Study subjects

**Adult Singaporean Chinese population.** The experimental research described in this article was performed with the approval of the Institutional Review Board (Reference no. NUS07-023) of the National University of Singapore. All procedures complied with the Helsinki declaration. The mouthwash and blood samples used in this study were collected from ethnic Chinese participants after informed consent, as part of an ongoing whole-genome asthma and allergic disease case-control association study in Singapore.<sup>E1,E2</sup> Phenotypic characteristics of the population are described in Table E1. Recruited subjects completed an ARIA/ISAAC-derived<sup>E3,E4</sup> asthma and allergy questionnaire and underwent skin prick tests (SPTs) against a panel of allergens commonly found in Singapore including *Dermatophagoides pteronyssinus*, *Blomia tropicalis*, *Elaeis guineensis*, and *Curvularia lunata*. A SPT result was defined as positive when a wheal diameter of 3 mm or greater was observed after 15 minutes. SPT tests included positive (histamine) and negative (saline) controls. Subjects were classified as asthmatic on the basis of (1) self-reported wheezing symptoms and doctor diagnosis of asthma and (2) positive skin prick reaction to either of the dust mite allergens (*D pteronyssinus* or *B tropicalis*). Nonallergic control subjects were identified by lack of reaction to any of the allergens tested by SPT and by self-reported absence of allergic symptoms.

**IgE cohort.** A subset of 182 individuals was selected from this adult Singapore Chinese cohort on the basis of availability of information on rs4675374 ICOS SNP genotype, total IgE levels, and specific IgE levels to *D pteronyssinus*, with the latter measured using ImmunoCAP. More information on these individuals can be found in Andiappan et al.<sup>E5</sup> The IgE levels were log transformed for further statistical analysis.

**Cohort for functional analysis.** A total of 40 individuals from the described cohort<sup>E5</sup> were selected on the basis of homozygosity of the C and T rs4675374 ICOS SNP alleles. Blood was collected for further analysis of ICOS expression in circulating T cells using flow cytometry. The individuals were classified on the basis of genotype of the rs4675374 ICOS SNP, with 24 having the CC genotype and 16 having the TT genotype. There were no sex- or age-based covariate effects.

**Dutch asthma genome-wide association studies cohort.** The Dutch Asthma genome-wide association studies (GWAS) cohort (DAG cohort)<sup>E6</sup> is a case-control study on asthma. Of the 920 asthma cases with a doctor diagnosis of asthma and presence of bronchial hyperresponsiveness, 575 had a positive skin prick test result and their data were used for this analysis. The 980 controls had no asthma or chronic obstructive pulmonary disease, nor any evidence of significant airway obstruction. All 1555 subjects were of Caucasian descent (see Table E2).

**Pediatric Singaporean Chinese cohort.** The paediatric study subjects were from the Singapore Cohort study Of the Risk factors for Myopia (SCORM). This study is conducted across 3 schools in Singapore<sup>E7</sup> and includes children of ethnic Chinese descent mainly between the ages of 7 and 9 years. Asthma information was obtained by self-reported doctor diagnosis, confirmed by the parents.

**North American cohorts.** The North American cohorts consisted of asthma cases, unaffected controls, asthma case-parent trios, and extended families recruited in clinics across the United States, in Mexico, and in Barbados. Sample characteristics, genotyping, and analysis have been previously described.<sup>E8</sup> Data from a total of 12976 individuals across the 3 cohorts were used in the analysis, each cohort having the following sample sizes: (1) European Americans: 1486 cases, 1539 controls, and 620 case-parent trios; (2) African Americans and African Caribbeans: 1154 cases, 1054 controls, and 413 case-parent trios; and (3) Latino Americans: 606 cases, 792 controls, and 1082 case-parent trios. Thus, there were a total of 3246 asthmatic cases, 3385 controls, and 2115 case-parent trios across the 3 cohorts. Genotypes for the 7 ICOS tagSNPs described in the Singaporean Chinese cohort were extracted from the GWAS data for these individuals along with their corresponding case/control status.

**Cohorts for whole blood gene expression data.** To analyze the potential association of tagSNP genotype with transcript expression, we

used data from a previously described large-scale expression quantitative trait locus (eQTL) study using peripheral blood samples from 5311 individuals.<sup>E9</sup> This whole-genome eQTL meta-analysis was performed over 9 data sets from 7 cohorts: EGCUT14 (n = 891), InCHIANTI (n = 611), Rotterdam study (n = 762), Fehrmann (n = 1240 on the Illumina HT12v3 platform and 229 on the Illumina H8v2 platform), HVH (n = 43 on the Illumina HT12v3 platform and 63 on the Illumina HT12v4 platform), SHIP-TREND (n = 963), and DILGOM (n = 509). Sample preparation, processing, and the eQTL analysis are described in the previous study.<sup>E9</sup> For this study, we used summary statistic eQTL data available from the original meta-analysis for rs4675374 and the ICOS gene (Illumina probe ID, 2070037; position, Chromosome 2 [20482616-20486175]).

**Korean asthmatic cohort for IgE analysis.** A total of 877 asthma patients were recruited from the Genomic Research Center for Allergy and Respiratory Diseases in Soonchunhyang University Hospital, Korea. All subjects provided written informed consent. The study protocols were approved by the Institutional Review Board of the Soonchunhyang University Bucheon Hospital, a member of the National Biobank of Korea (Institutional Review Board no. SCHBC\_IRB\_05\_02). The genotype for ICOS SNP rs4675374 was extracted from the GWAS data previously published<sup>E10</sup> and analyzed in relation to serum IgE levels.

### Selection of tagSNPs

Genotypes from the HapMap Han Chinese from Beijing (CHB) were used to determine tagSNPs for the ICOS gene. The CHB population consists of 45 unrelated individuals from Beijing, China, who were genotyped in the HapMap project<sup>E11</sup> to determine the common haplotypes within the Chinese population. All ICOS genetic variants described in the CHB HapMap<sup>E11</sup> were retrieved and tagged using the de Bakker et al<sup>E12</sup> algorithm. We identified 7 tagSNPs covering the entire ICOS gene at  $r^2 = 0.8$  for association testing.

### Genotyping

For the Singaporean Chinese adult cohort, genomic DNA was extracted from buccal cells obtained from a mouthwash of 0.9% saline solution following a standardized protocol.<sup>E1</sup> DNA samples content was quantified in triplicate on a Nanodrop (ND-1000) before use. Genotyping was performed on purified DNA samples using the Illumina BeadXpress Assay (Illumina, Inc, San Diego, Calif) according to manufacturer recommendations at the University of Utah Genomics Core Facility (Salt Lake City, Utah). For the Singaporean pediatric population, genotypes for the ICOS SNPs were extracted from whole genome genotyping data generated in a previous study.<sup>E13</sup> The genotypes for the Korean population were also extracted from previously reported GWAS data.<sup>E10</sup> Finally, the genotyping of the 182 Singaporean Chinese adults used for IgE correlations was performed using the Sequenom platform and the Mass Array system.

### Statistical analysis

Genotypes of the tagSNPs were then tested for Hardy-Weinberg equilibrium in control subjects by using the  $\chi^2$  test. The primary test of allelic association was performed using the Cochran Armitage trend test, which evaluates the association of the minor allele with the disease phenotype. All *P* values and odds ratios with 95% CIs were estimated using the PLINK program (version 1.06). The meta-analysis was performed using both the fixed effects and random effects using the meta-analysis command. Association summary statistics (risk and alternative alleles, *z* score, and *P* value) were obtained from the meta-analysis of North American populations as previously described.<sup>E8</sup> For association analysis using the functional parameters of ICOS mean fluorescence intensity and percentiles of ICOS<sup>+</sup> cells, a nonparametric Mann Whitney test was used using the PRISM software. Illustrative figures were also generated using the PRISM software.

## PBMC culture and flow cytometric analysis

PBMCs were isolated from blood collected from volunteers of the adult Singaporean Chinese cohort and samples stored frozen over liquid nitrogen. Before analysis, cells were thawed and counted in the presence of a vital stain. For detection of intracellular antigens, cells were first labeled for cell surface markers and then fixed and permeabilized using FoxP3 staining buffer set (from BD Biosciences, San Jose, Calif) according to the manufacturer's protocol before incubation with antibodies recognizing intracellular molecules of interest. PBMCs were labeled with antibodies specific for CD3 (clone UCHT1), CD4 (clone OKT4), CD45RO (clone UCHL1), and CD25 (clone BC96) from eBioscience (San Diego, Calif); FOXP3 (clone 259D/C7), CCR6 (clone 11A9), CCR4 (clone 1G1), CXCR5 (clone RF8B2), CXCR3 (clone 1C6), and ICOS (clone DX29) from BD Biosciences; or ICOS (clone C398.4A) from Biolegend (San Diego, Calif). PBMCs were incubated with antibodies at 4°C for 30 minutes with 2 washes before data acquisition on a 5-laser LSR II flow cytometer (from BD) and data analysis using FlowJo. To measure ICOS expression after T-cell activation, PBMCs were stimulated *in vitro* with Dynabeads Human T-Activator CD3/CD28 beads (Life Technologies, Grand Island, NY) at a concentration of 5 uL stock per  $2 \times 10^5$  cells. After 48 hours of culture, cells were labeled as above with antibodies to CD3, CD4, CD8, and ICOS and analyzed by using flow cytometry.

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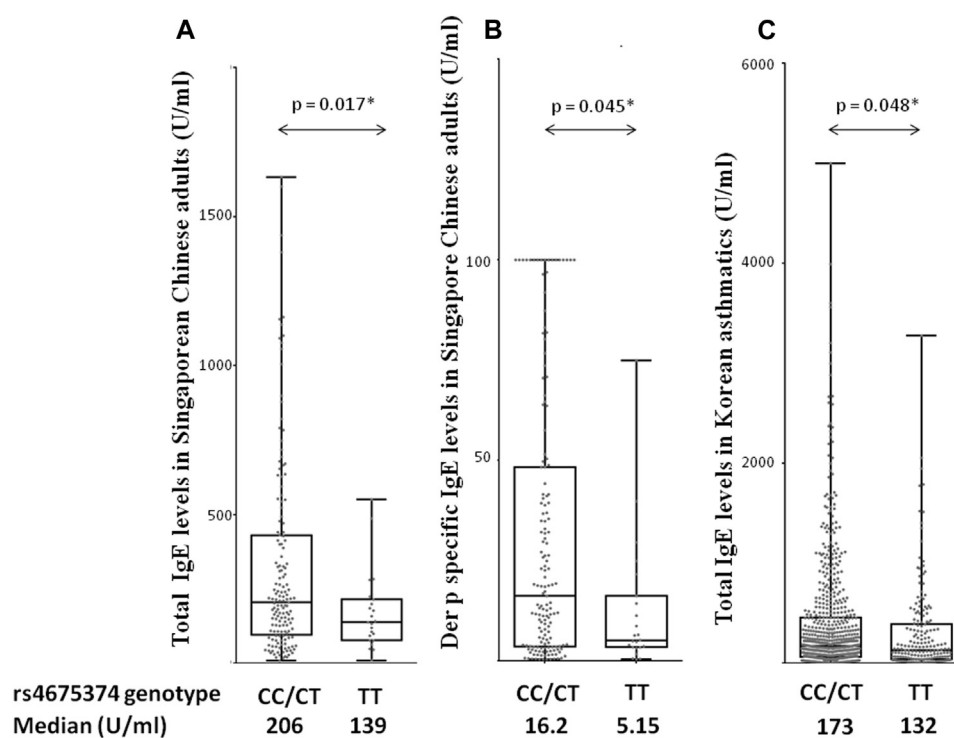


**FIG E1.** Association of the tagSNP rs4675374 genotype with serum IgE levels in the Singaporean Chinese and Korean asthmatic cohorts using nonparametric tests. **A**, Total IgE levels in serum samples from selected individuals within the Singaporean Chinese cohort. *Dermatophagoides pteronyssinus*- (Der p) specific IgE levels (**B**) and total IgE levels (**C**) in serum samples of Korean asthmatic patients stratified by the rs4675374 genotype. \* $P < .05$ . Median IgE levels of the samples analyzed are indicated.

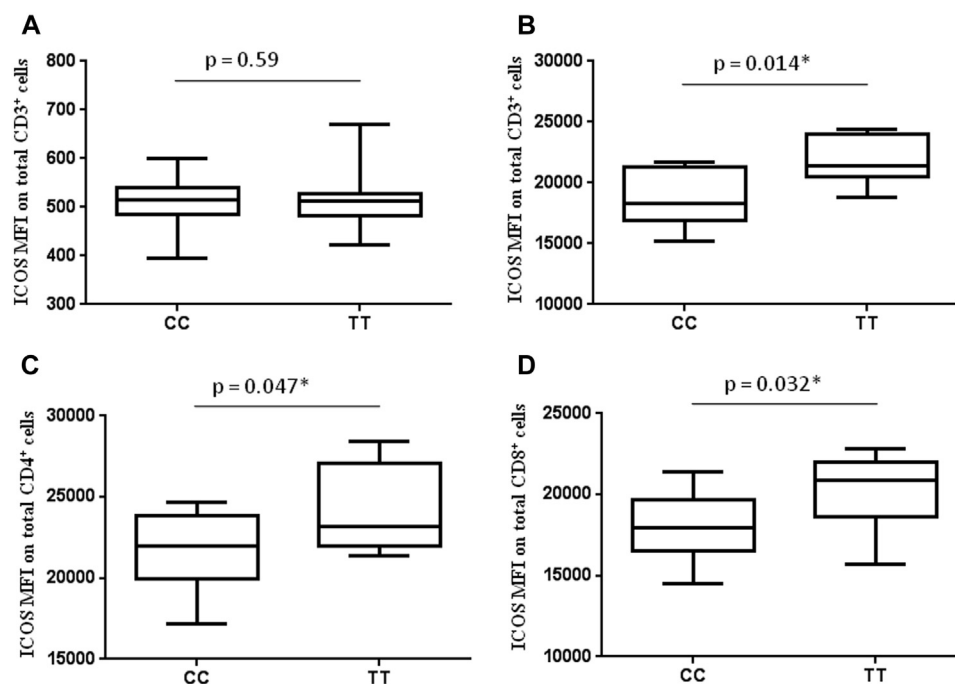


**FIG E2.** ICOS expression level on T cells before and after stimulation *in vitro*. Median fluorescence intensity (MFI) of ICOS labeling on CD3<sup>+</sup> T cells before stimulation (**A**) and after stimulation with anti-CD3 and anti-CD28 *in vitro* (**B**). MFI of ICOS on stimulated CD4<sup>+</sup> (**C**) and CD8<sup>+</sup> (**D**) T cells, stratified by the rs4675374 genotype. Statistical analysis was carried out by using the nonparametric Mann Whitney test. \* $P < .05$  ( $n = 40$ ).

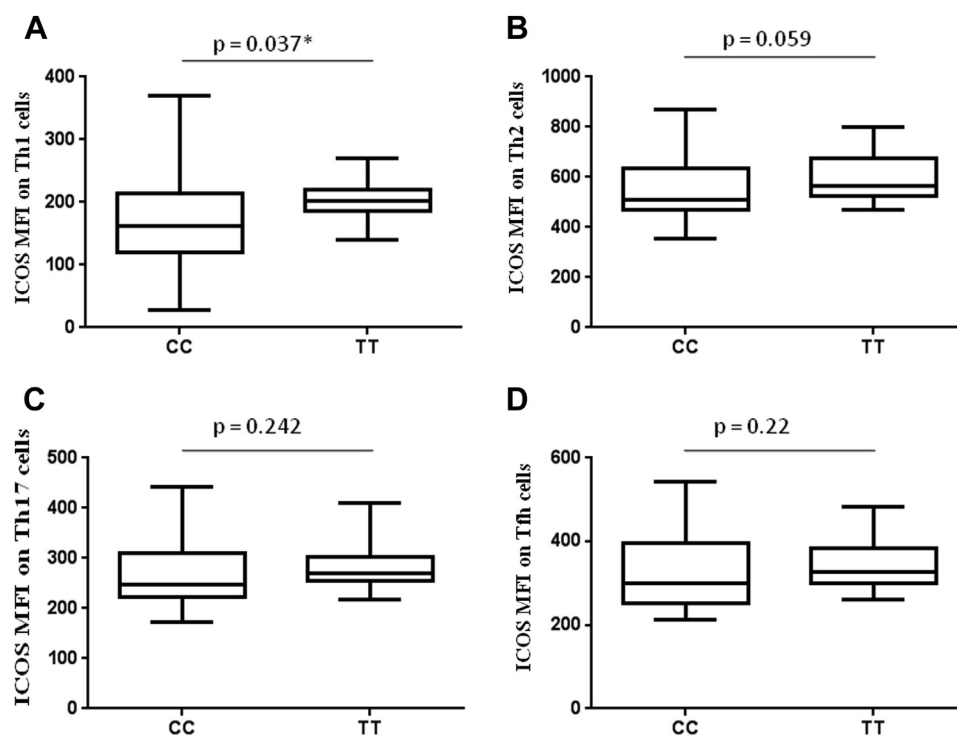


**FIG E3.** ICOS expression level on nonstimulated CD4<sup>+</sup> T helper cell subsets. Median fluorescence intensity (MFI) of ICOS labeling on T<sub>H</sub>1 cells (A), T<sub>H</sub>2 cells (B), T<sub>H</sub>17 cells (C), and Tfh cells (D), stratified by the rs4675374 genotype. \* $P < .05$  ( $n = 40$ ) using nonparametric Mann Whitney test.

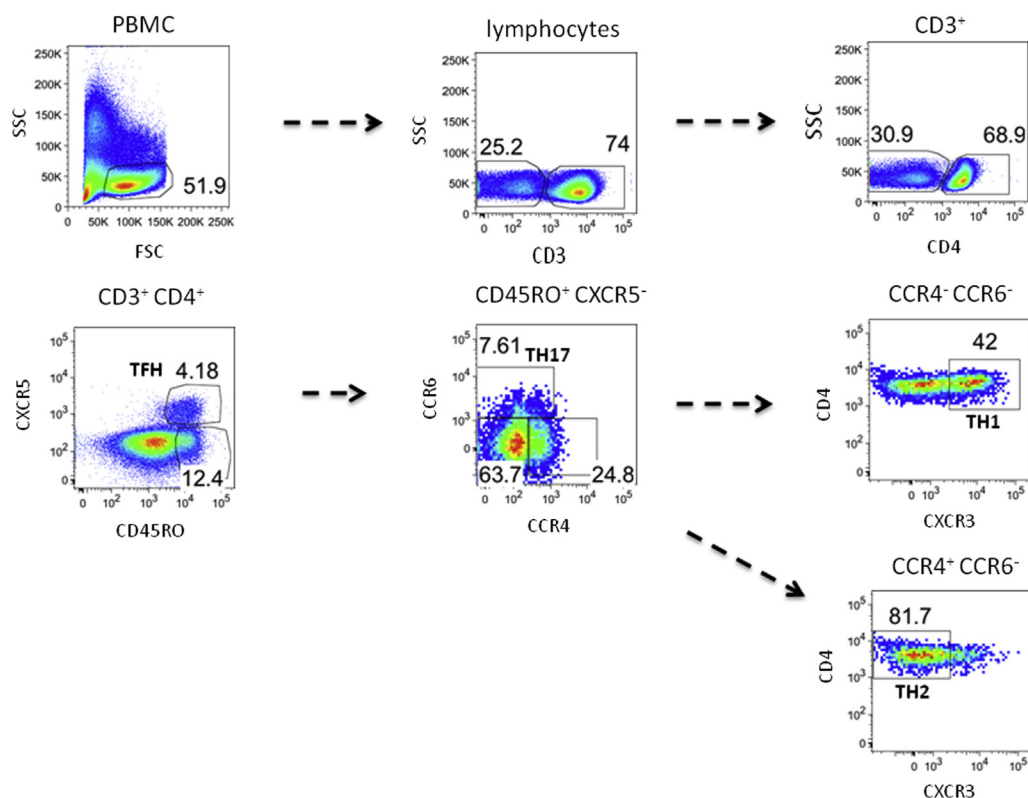


**FIG E4.** Flow cytometry gating strategy for the T helper cell populations T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17, and T<sub>fh</sub>. The numbers in the panels indicate the frequency of the cells within the gates indicated above the panels. *FSC*, Forward scatter; *SSC*, side scatter.

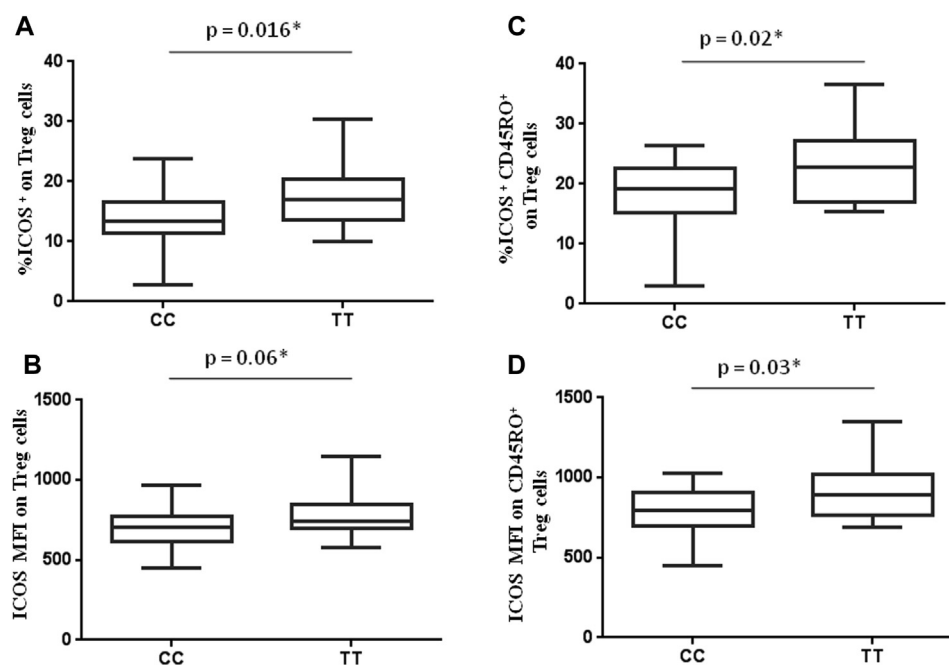


**FIG E5.** ICOS expression in populations of nonstimulated Treg cells. Frequency of ICOS<sup>+</sup> Treg cells (**A**) and median fluorescence intensity (MFI) of ICOS labeling on Treg cells (**B**) stratified by the rs4675374 genotype. Frequency of ICOS<sup>+</sup> cells (**C**) and MFI of ICOS expression (**D**) in CD45RO<sup>+</sup> Treg-cell populations. Statistical analysis was carried out by using a nonparametric Mann-Whitney test. \* $P < .05$  ( $n = 40$ ).

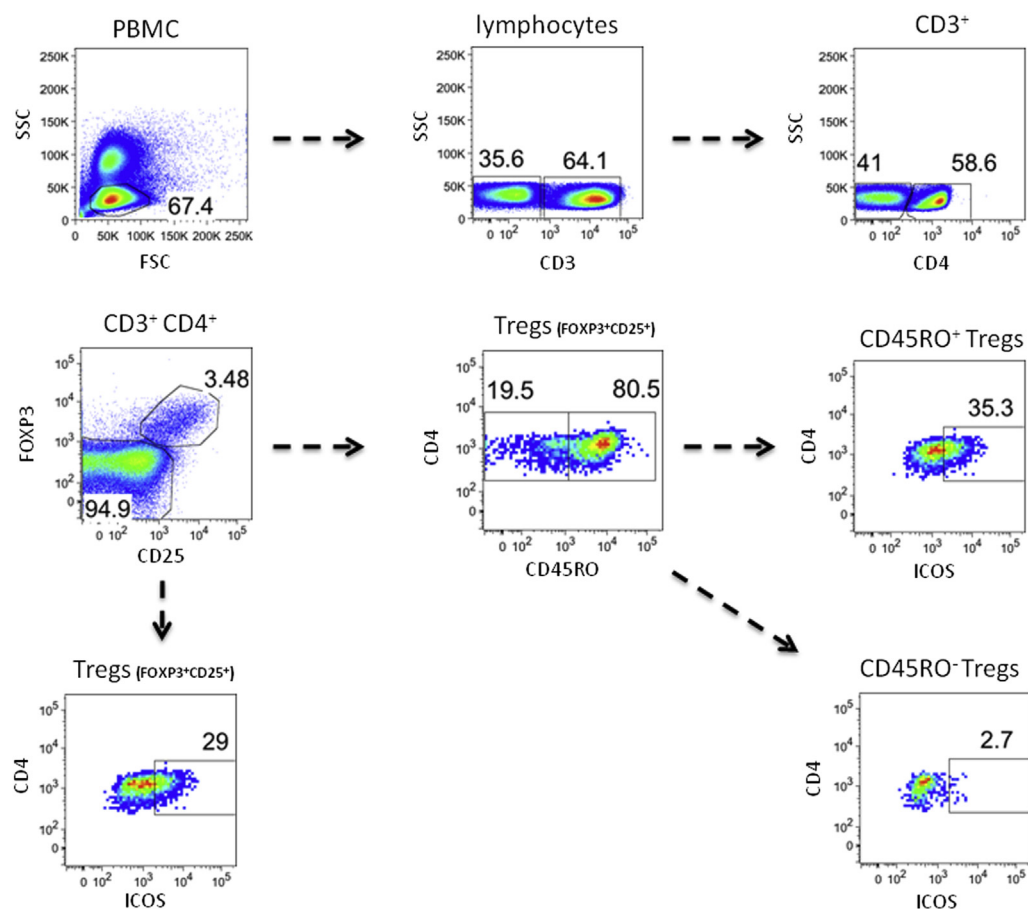


**FIG E6.** Flow cytometry gating strategy for Treg cells. The numbers in the panels indicate the frequency of the cells within the gates indicated on the top of the panels. ICOS expression on total Treg cells and on CD45RO<sup>+</sup> and CD45RO<sup>-</sup> Treg cells is shown. *FSC*, Forward scatter; *SSC*, side scatter.

**TABLE E1.** Demographic characteristics of the adult Singapore Chinese population

| Phenotype                      | Sex    | Count | Age (y), median (min, max) |
|--------------------------------|--------|-------|----------------------------|
| Asthma case*                   | Female | 341   | 20 (18.00, 57.00)          |
|                                | Male   | 377   | 22 (19.00, 34.00)          |
|                                | Total  | 718   | 21 (18.00, 57.00)          |
| Nonatopic nonasthmatic (NANA)† | Female | 520   | 21 (19.00, 59.00)          |
|                                | Male   | 197   | 22 (20.00, 56.00)          |
|                                | Total  | 717   | 21 (18.00, 59.00)          |
| Total                          | Female | 861   | 21 (18.00, 57.00)          |
|                                | Male   | 574   | 22 (18.00, 59.00)          |
|                                | Total  | 2880  | 22 (18.00, 55.00)          |

GINA, The Global Initiative for Asthma; WHO, World Health Organization.

\*Asthma case was classified on the basis of doctor diagnosis of the disease with a positive SPT reaction to 1 of the allergens tested (based on WHO and GINA guidelines for asthma).

†NANA are healthy control individuals classified on the basis of no symptoms and history of allergic disease and a negative SPT reaction.

**TABLE E2.** Demographic characteristics of the Dutch cohort

| Phenotype             | Sex    | Count | Age (y), median<br>(min, max) |
|-----------------------|--------|-------|-------------------------------|
| Allergic asthma case* | Female | 278   | 35 (7, 67)                    |
|                       | Male   | 297   | 39 (6, 75)                    |
|                       | Total  | 575   | 37 (6, 75)                    |
| Controls†             | Female | 74    | 48 (34, 71)                   |
|                       | Male   | 557   | 58 (36, 76)                   |
|                       | Total  | 631   | 57 (34, 76)                   |
| Total                 | Female | 352   | 38 (7, 71)                    |
|                       | Male   | 857   | 54 (6, 76)                    |
|                       | Total  | 1209  | 52 (6, 76)                    |

\*All allergic asthma cases were defined on the basis of asthma symptoms with bronchial hyperresponsiveness, with a doctor diagnosis of asthma and a positive SPT result.

†Individuals classified as controls on the basis of no symptoms or history of asthma or chronic obstructive pulmonary disease. Not all controls have a phenotype; this is due to the pseudo controls. In these controls, the untransmitted alleles from the father and the mother of asthmatic patients are used as the genotype.

**TABLE E3.** Association of *ICOS* tag SNPs with allergic asthma phenotype in the adult Singaporean Chinese population (643 asthmatic patients\* and 717 NANA controls†)

| SNP        | BP        | A1 | A2 | Case* frequency | NANA control† frequency | <i>P</i> <sub>allele</sub> | OR (95% CI)      |
|------------|-----------|----|----|-----------------|-------------------------|----------------------------|------------------|
| rs4675374  | 204802578 | T  | C  | 0.36            | 0.45                    | $7.78 \times 10^{-7}$      | 0.69 (0.59-0.80) |
| rs4675377  | 204814676 | C  | T  | 0.25            | 0.31                    | .0005                      | 0.75 (0.64-0.88) |
| rs4312468  | 204802105 | A  | G  | 0.09            | 0.13                    | .003                       | 0.70 (0.56-0.89) |
| rs4335928  | 204801980 | C  | T  | 0.11            | 0.14                    | .025                       | 0.78 (0.62-0.97) |
| rs10172036 | 204824283 | G  | T  | 0.43            | 0.46                    | .171                       | 0.90 (0.78-1.05) |
| rs11571314 | 204821842 | A  | G  | 0.20            | 0.22                    | .293                       | 0.91 (0.76-1.09) |
| rs4355090  | 204816382 | A  | G  | 0.49            | 0.49                    | .941                       | 1.00 (0.87-1.16) |

A1, Allele 1 for the SNP rs4675374 for which the association statistics have been calculated; A2, allele 2 for the SNP rs4675374; BP, base pair; OR, odds ratio with 95% CI; *P*<sub>allele</sub>, *P* value obtained from the association based on allele for the Cochran Armitage trend test.

\*Asthma cases were classified on the basis of self-reported doctor diagnosis of the disease and a positive SPT reaction to at least 1 of the allergens tested.

†NANAs refers to nonatopic and nonasthmatic healthy controls classified on the basis of absence of symptoms, lack of history of allergic disease, and a negative SPT reaction to the allergens tested.

**TABLE E4.** Association of *ICOS* tag SNPs with allergic asthma phenotype in the Dutch cohort (575 asthmatic patients\* and 980 controls†)

| SNP        | BP        | Minor allele | Major allele | MAF  | OR   | SE   | L95  | U95  | <i>P</i> <sub>allele</sub> |
|------------|-----------|--------------|--------------|------|------|------|------|------|----------------------------|
| rs4675374  | 204510823 | T            | C            | 0.18 | 0.79 | 0.10 | 0.66 | 0.96 | .017                       |
| rs4675377  | 204522921 | C            | T            | 0.19 | 0.81 | 0.10 | 0.67 | 0.98 | .0295                      |
| rs11571312 | 204528303 | T            | C            | 0.14 | 1.04 | 0.11 | 0.85 | 1.28 | .6983                      |
| rs10932037 | 204533591 | T            | C            | 0.13 | 0.87 | 0.11 | 0.70 | 1.08 | .2118                      |
| rs1559931  | 204533974 | A            | G            | 0.27 | 0.94 | 0.08 | 0.80 | 1.11 | .4933                      |

*A1*, Allele 1 for the SNP rs4675374 for which the association statistics have been calculated; *A2*, allele 2 for the SNP rs4675374; *BP*, base pair; *L95* and *U95*, lower and upper levels of 95% CI for the odds ratio estimate; *MAF*, minor allele frequency in the population; *OR*, odds ratio with 95% CI; *P*<sub>allele</sub>, *P* value obtained from the Cochran Armitage trend test; *SE*, standard error for the association analysis.

\*Asthma cases were classified on the basis of self-reported doctor diagnosis of the disease and a positive SPT reaction to at least 1 of the allergens tested.

†Controls refer to nonasthmatic healthy controls classified on the basis of absence of symptoms and lack of history of allergic disease.

**TABLE E5.** Stratified analysis for individual American population and combined meta-analysis for all Americans

| SNP        | Position  | European Americans (EA) |                  | African Americans (AA) |                  | Latinos Americans (LA) |                  | All North American populations |               |
|------------|-----------|-------------------------|------------------|------------------------|------------------|------------------------|------------------|--------------------------------|---------------|
|            |           | EA Meta <i>z</i>        | EA Meta <i>P</i> | AA Meta <i>z</i>       | AA Meta <i>P</i> | LA Meta <i>z</i>       | LA Meta <i>P</i> | Combined <i>z</i> score        | Meta <i>P</i> |
| rs4675374  | 204510823 | 2.410                   | <b>.016</b>      | 1.076                  | .282             | 1.154                  | .249             | 2.108                          | .006*         |
| rs10932034 | 204521238 | −1.950                  | .051             | −1.303                 | .192             | −0.773                 | .440             | −1.094                         | .018*         |
| rs4675377  | 204522921 | −1.839                  | .066             | −0.932                 | .352             | −0.809                 | .419             | −1.186                         | .035*         |
| rs4335928  | 204510225 | −1.704                  | .088             | 0.154                  | .878             | −0.358                 | .720             | −2.738                         | .236          |
| rs4312468  | 204510350 | −1.682                  | .092             | 0.191                  | .848             | −0.255                 | .799             | −2.357                         | .274          |
| rs4355090  | 204524627 | −0.412                  | .680             | −0.371                 | .710             | −0.440                 | .660             | −0.263                         | .480          |
| rs11571314 | 204530087 | −0.223                  | .824             | 0.639                  | .523             | 0.249                  | .804             | 0.345                          | .730          |
| rs10172036 | 204532528 | −0.675                  | .499             | 0.031                  | .975             | 1.182                  | .237             | 0.181                          | .793          |
| rs11895665 | 238217712 | 0.143                   | .887             | −0.693                 | .489             | 0.820                  | .412             | 0.706                          | .856          |

Meta *z*, *z* score obtained by the meta-analysis as described in Torgerson et al<sup>18</sup>; *P*, The *P* value obtained for the meta-analysis; *SNP*, the rs-id of the *ICOS* SNP.

\**P* < .05.