

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

The single nucleotide polymorphism *CRTh2* rs533116 is associated with allergic asthma and increased expression of *CRTh2*

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Abstract

Background: *CRTh2* (chemoattractant-receptor homologous molecule expressed on Th2 cells) is expressed by Th2 cells and other cells involved in allergic inflammation. Single nucleotide polymorphisms (SNPs) in *CRTh2* (rs11571288, rs545659, rs634681) have been associated with various phenotypes of allergy in ethnically distinct populations. Here, we assessed the association between *CRTh2* rs533116 and allergic asthma, expression of *CRTh2* and Th2 cytokine production.

Methods: *CRTh2* rs533116 was genotyped in an ethnically diverse population ($n = 1282$). The proportion of cells expressing *CRTh2* was determined in peripheral blood from subjects with allergic airways disease and controls as well as with *in vitro* differentiated Th2 cells. Receptor function was assessed by stimulating Th2 cells with the *CRTh2*-specific agonist 13,14-dihydro-15-keto-PGD₂ (DK-PGD₂) and measuring IL-4 and IL-13 by intracellular staining and ELISA.

Results: *CRTh2* rs533116 was associated with allergic asthma in White people (2.67 [1.09–6.55], $P < 0.05$), and expression of *CRTh2* was higher in subjects with allergic airways disease compared to controls ($P < 0.05$). Among allergic individuals, the AA genotype was significantly associated with more eosinophils and higher expression of *CRTh2* by both CD4⁺ T cells and eosinophils ($P < 0.05$). *In vitro*, the AA genotype was associated with a higher proportion of *CRTh2*⁺ cells during Th2 differentiation as well as more IL-4 and IL-13 expression following DK-PGD₂ stimulation ($P < 0.05$).

Conclusions: These findings show an association between *CRTh2* rs533116 and allergic asthma and suggest this may be mediated by elevated expression of *CRTh2*, leading to higher numbers of circulating eosinophils and Th2 cytokine production.

CRTh2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) is expressed by human Th2 but not Th1 lymphocytes (1) as well as by other inflammatory cells including eosinophils and basophils (2–5). *CRTh2* is a

G-protein-coupled receptor for prostaglandin D₂ (PGD₂), also referred to as DP2 (6). *In vitro*, PGD₂-*CRTh2* signaling induces chemotaxis (3), expression of Th2 cytokines (7) and inhibits apoptosis, suggesting it may also impede the resolution of allergic inflammation (8).

Wild-type mice treated with *CRTh2* antagonists and *crth2*^{−/−} mice both exhibit blunted responses in models of allergic asthma, dermatitis and rhinitis (9–11). In humans, there are increased numbers of *CRTh2*-expressing cells in the nasal mucosa of subjects with allergic rhinitis (5, 12)

Abbreviations

CRTh2, chemoattractant receptor homologous molecule expressed on Th2 cells; SNP, single nucleotide polymorphism; PGD₂, prostaglandin D₂; DK-PGD₂, 13,14-dihydro-15-keto-PGD₂; COX-2, cyclo-oxygenase-2.

and skin lesions of those with atopic dermatitis (4, 13, 14). There are also higher numbers of lung T cells expressing C_{CRTh2} in human asthmatics (15) and elevated PGD₂ levels following allergen challenge (16), which has been associated with disease severity (17). A recent clinical trial of a C_{CRTh2} antagonist in asthma has shown promise for this approach (18). Together, these studies indicate the C_{CRTh2} pathway is likely involved in allergic responses within tissues, but whether there are increases in circulating C_{CRTh2}-expressing cells in allergic airways disease has not yet been well studied.

We and others have shown that the C_{CRTh2} single nucleotide polymorphisms (SNPs) rs11571288, rs545659 and rs634681 are associated with asthma and allergic sensitization in ethnically distinct populations (19–22). Our previous report also suggested association between C_{CRTh2} rs533116 and allergic asthma, but significance was lost after correcting for multiple comparisons (21). However, C_{CRTh2} rs533116 may still represent an independent risk factor because it was not in linkage disequilibrium with the other associated SNPs (21). While reporter assays indicate C_{CRTh2} SNPs may influence mRNA stability and/or transcription (19, 22), no study to date has examined their association with C_{CRTh2} expression. Here, our further investigation of C_{CRTh2} rs533116 shows it is associated with allergic asthma as well as more circulating eosinophils and higher C_{CRTh2} expression and function.

Materials and methods

Subjects

Participants in the present study were from two independent populations from Toronto and Edmonton, Canada. In the Toronto Nutrigenomics and Health (TNH) study (23), allergic asthmatic subjects were identified by questionnaire. In the Edmonton population, subjects were White people and atopic status was assessed by skin testing and allergic airways disease by physician diagnosis. Controls had negative skin tests and no evidence of airway disease (Appendix S1). Demographics are presented in Table 1.

Genotyping

Genotyping of C_{CRTh2} rs533116 (g6391G>A, upstream of the ATG) was performed with TaqMan[®] allelic discrimination, Sequenom iPLEX Technology or sequencing (Appendix S1).

Flow cytometry and ELISA

The proportion of circulating cells expressing C_{CRTh2} was determined by staining of whole blood cells and cell surface markers: CCR3 (eosinophils), CD203c (basophils), CD4 or CD8 (T lymphocytes), and CD14 (monocytes). Intracellular

Table 1 Subject characteristics

University of Toronto						
Characteristic	Allergic asthma			Control		
Gender (% male)	29.8%			30.9%		
Age (mean, SD)	22.8 ± 2.5			22.6 ± 2.5		
BMI (mean, SD)	24.0 ± 4.3			22.8 ± 3.5		
	Allergic asthma			Control		
	Genotype, <i>n</i> (%)					
	GG	GA	AA	GG	GA	AA
Ethnicity						
Whites	11 (31.4)	14 (40.0)	10 (28.6)	237 (40.7)	266 (45.6)	80 (13.7)
Asians	8 (100)	0	0	349 (81.5)	77 (18.0)	2 (0.5)
South Asians	1 (100)	0	0	79 (58.5)	48 (35.6)	8 (5.9)
Other	3 (100)	0	0	59 (66.3)	24 (27.0)	6 (6.7)
University of Alberta						
Characteristic	Allergic			Control		
Gender (m/f)	8/6			3/5		
Age (mean, SD)	31.9 ± 6.7			27.2 ± 4.4		
Positive skin prick	14			0		
Allergic rhinitis	11			0		
Asthma	6			0		
Inhaled CS	3			0		
Oral CS	0			0		

SD, standard deviation; CS, corticosteroid.

staining for IL-13 and IL-4 was performed following stimulation (4 h). IL-4 and IL-13 levels were assayed by ELISA (Appendix S1).

Th2 differentiation

Human CD4⁺ T cells were cultured in Th2-polarizing conditions [recombinant human (rh) IL-4, anti-IFN- γ , anti-IL-12] on cycles of priming [3 days; anti-CD3/CD28, recombinant human (rh) IL-2] and rest (4 days; rhIL-2). For CRTh2-enriched Th2 cell lines, CRTh2⁺ cells were isolated on day 7 of Th2 differentiation and maintained on cycles of priming and rest. Experiments were performed after the rest phase (Appendix S1).

Quantitative RT-PCR

CRTh2 mRNA was detected with a predesigned Taqman Gene Expression Assay and abundance calculated relative to GAPDH (Appendix S1).

Statistical analyses

Differences in genotypic frequencies were examined using chi-square tests or Fisher's exact test. Unconditional logistic regression models were used to estimate the odds ratio (OR) and 95% confidence intervals (CI) associated with CRTh2 genotypes and allergic asthma in White people. Models were adjusted for age (years), sex, BMI and smoking status. We created terms to evaluate the genetic effects under additive, recessive and dominant models as well as to examine genotypic effects. CRTh2 and cytokine data are represented as mean $\pm$ standard error of the mean (SE). Differences in CRTh2 expression by circulating cells were determined by Mann-Whitney rank sum test. *In vitro* experiments were paired and therefore analyzed with a one-sample Student's *t*-test (Appendix S1).

Results

CRTh2 rs533116 is associated with allergic asthma among White people

The frequency of rs533116 genotypes in the TNH study differed between ethnic groups, with the AA genotype most frequent in White people (14.6%) compared to South Asians (5.9%) and Asians (0.5%; Table 1). To avoid population stratification and because allergic asthma was rare in the other ethnicities, we analyzed data from White people only. A significant association between the AA genotype and allergic asthma was observed (OR, 2.67; 95% CI, 1.09–6.55; $P = 0.02$). We also observed a significant association with the additive (OR, 1.61; 95% CI, 0.99–2.61; $P = 0.05$) and recessive models (OR, 2.57; 95% CI, 1.18–5.57; $P = 0.02$), but not the dominant model (OR, 1.45; 95% CI, 0.7–3.04; $P = 0.32$; Table 2). These data indicate CRTh2 rs533116 is associated with development of allergic asthma among White people.

Table 2 Association of the CRTh2 rs533116 with allergic asthma among White people

Genotype	Cases N (%)	Controls N (%)	OR* (95% CI)	P-values
GG	11 (31.4)	237 (40.7)	1 (ref)	
GA	14 (40.0)	266 (45.6)	1.08 (0.48–2.45)	0.2540
AA	10 (28.6)	80 (13.7)	2.67 (1.09–6.55)	0.0173
Additive			1.61 (0.99–2.61)	0.0534
Recessive (AA vs GA and GG)			2.57 (1.18–5.57)	0.0175
Dominant (AA and GA vs GG)			1.45 (0.70–3.04)	0.3200

*Adjusted for age, sex, BMI and smoking status.

Expression of CRTh2 is increased in allergic airways disease

To characterize the profile of circulating cells expressing CRTh2, White subjects were recruited at the University of Alberta. Figure 1A shows two major cell populations expressing CRTh2, denoted as gates R1 (granulocytes) and R2 (mononuclear cells). Virtually all cells in R1 were also CCR3⁺ (96.8% $\pm$ 0.52, Fig. 1A), indicating they are eosinophils. Similarly, analyzing CCR3⁺ cells of high side scatter (eosinophils) showed 97.6% $\pm$ 0.40 were CRTh2⁺ (data not shown). R2 contained CRTh2-expressing basophils, CD4⁺ T cells and monocytes. Analysis of these populations revealed that 66.5% $\pm$ 3.30 of basophils, 5.1% $\pm$ 0.43 of CD4⁺ T cells and 3.9% $\pm$ 0.36 of monocytes were CRTh2⁺ ($n = 22$, all donors), and no differences in the proportion of CRTh2⁺ cells were observed between allergic and control subjects (Fig. 1B). However, when examined as a percentage of total cells, there were more circulating eosinophils, basophils and CD4⁺ T cells expressing CRTh2 in allergic compared to nonallergic control subjects ($P < 0.05$; Fig. 1C). Mean fluorescence intensity (MFI) of CRTh2 staining was also significantly higher on all three cell types from allergic subjects compared to nonallergic controls ($P < 0.01$, Fig. 1D), indicating CRTh2 is more highly expressed. The percentages of total CRTh2⁺ cells were increased in subjects with allergic asthma and allergic rhinitis compared to controls, indicating elevated CRTh2 expression is a general characteristic of allergic airway disease (Table S1).

CRTh2 rs533116 is associated with an increase in the percentage of circulating eosinophils

We next examined the frequency of eosinophils, calculated as a percentage of total cells, segregated by rs533116 genotype. Figure 2A shows that allergic subjects with the AA genotype had significantly more eosinophils than those with the GG genotype (2.3-fold, $P < 0.05$), while a significant increase was not observed between the GA and GG genotype (1.5-fold, $P = 0.31$). These data indicate that in allergic subjects, the AA genotype is associated with a higher percentage of circulating eosinophils.

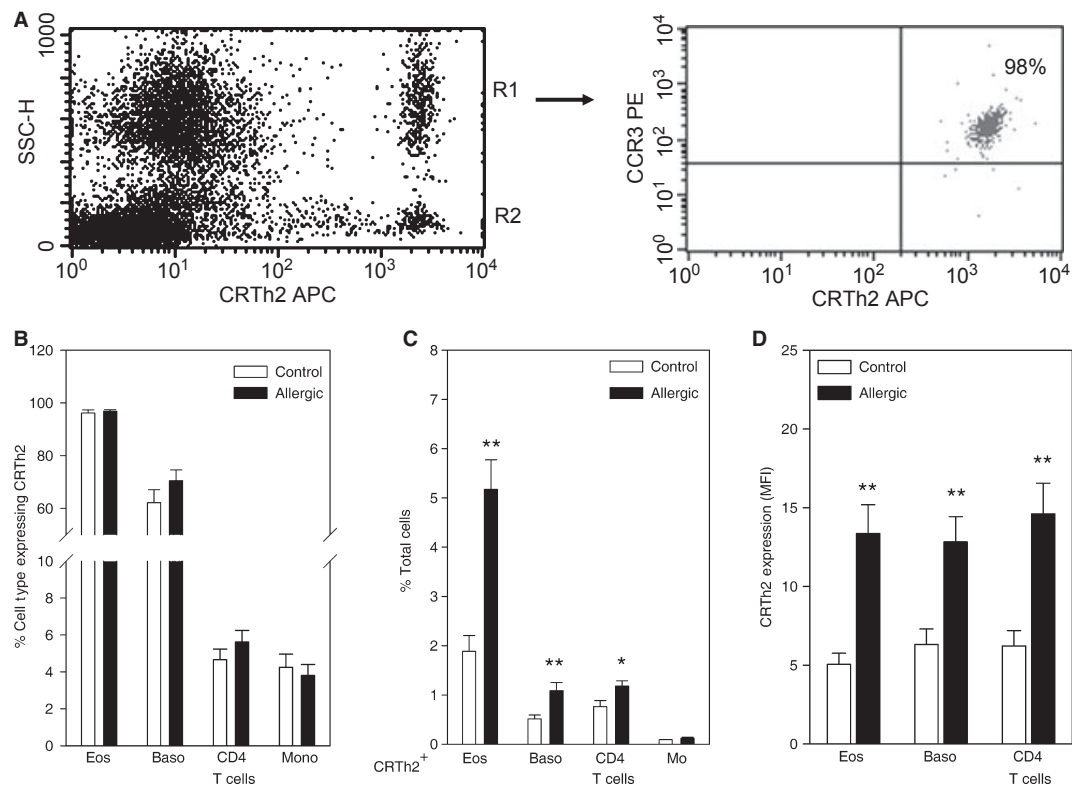


Figure 1 Profile of CRTh2 expression in circulating white blood cells. Representative whole blood staining for CRTh2 plotted against side scatter revealed a granulocyte (R1) and mononuclear cell (R2) population. The R1 gate is virtually all eosinophils, identified by CCR3 co-staining (A). The identity of mononuclear cells expressing CRTh2 was determined by co-staining with cell type-specific markers for basophils (CD203c⁺), helper T cells (CD4⁺) and

monocytes (CD14⁺). The data are presented as the mean $\pm$ SE of the percentage of each cell type expressing CRTh2 in allergic ($n = 14$) and nonallergic controls ($n = 8$, B) and then calculated as a proportion of total cells (C). Mean fluorescence intensity (MFI) of CRTh2 staining was also assessed for each cell type (D). Statistical significance was assessed by Mann–Whitney rank sum test, * $P < 0.05$, ** $P < 0.01$.

CRTh2 rs533116 is associated with an increase in CRTh2 expression

Eosinophils from allergic individuals with the AA genotype also showed more CRTh2 expression (higher intensity staining) compared to the GG genotype (3.2-fold, $P < 0.05$; Fig. 2B). The AA genotype was also associated with significantly higher expression of CRTh2 on CD4⁺ T cells, compared to cells from carriers of the GG genotype (2.4-fold, $P < 0.05$; Fig. 2B). A trend for higher expression of CRTh2 on eosinophils with the GA vs GG genotype (2-fold, $P = 0.09$) was observed, though not for CD4⁺ T cells (1.3-fold, $P = 0.51$). These data indicate that on both eosinophils and CD4⁺ T cells from allergic subjects, the AA genotype is associated with higher CRTh2 expression.

CRTh2 rs533116 is associated with enhanced Th2 differentiation

To investigate the influence of rs533116 on Th2 cell differentiation, CD4⁺ T cells from allergic subjects with the AA or GG genotype were isolated and cultured under Th2 polarizing conditions. To reduce variability, each experiment

was performed with a pair of AA and GG donors. In every case, the AA genotype was associated with a higher percentage of CRTh2-expressing cells compared to the GG genotype, with a mean difference of 1.7-fold ($P < 0.02$; Fig. 3A). One set of subjects was re-called and again showed the AA genotype to be associated with a higher percentage of CRTh2⁺ cells (day 6: AA, 21% vs GG, 15%; day 12: AA, 31% vs GG, 24%). CRTh2 mRNA in differentiating Th2 cells was 9.8-fold higher in cells with the AA compared to GG genotype, although this did not reach significance (Fig. 3B). These data suggest the AA genotype influences the propensity of CD4⁺ T cells to undergo Th2 differentiation.

CRTh2 rs533116 is associated with enhanced Th2 cytokine production

To study whether rs533116 was associated with enhanced receptor function, CRTh2⁺ Th2 cells with the AA or GG genotype were stimulated with the CRTh2-specific agonist, DK-PGD₂. Cells with the AA genotype demonstrated ~2-fold higher percentage of cells expressing IL-4 and IL-13 compared to cells with the GG genotype ($P < 0.05$, Fig. 3C). Th2 cells with the AA genotype also showed higher secretion of IL-13,

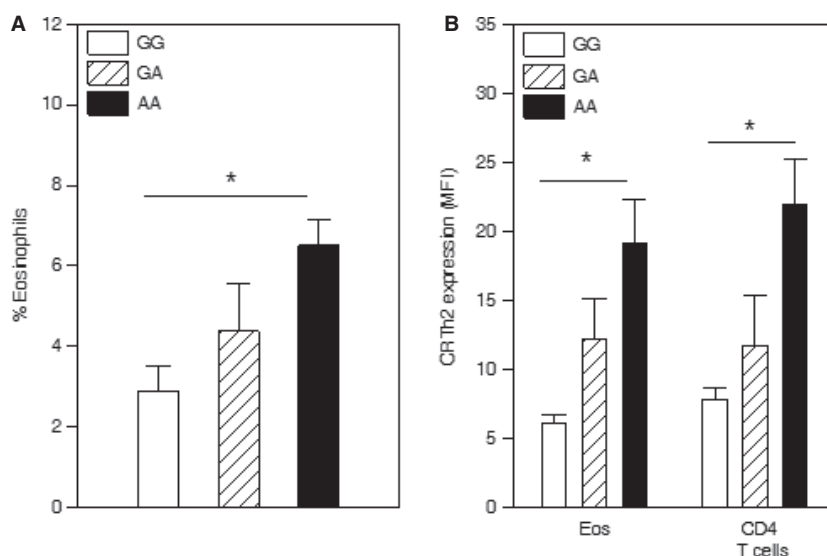


Figure 2 *CRTh2* rs533116 is associated with a higher percentage of circulating eosinophils and expression of CRTh2. Data represent mean $\pm$ SE of the percentage of eosinophils (A) and mean fluorescence intensity (MFI) of CRTh2 staining on eosinophils and CD4⁺ T

cells (B) in allergic subjects segregated by rs533116 genotype (GG, $n = 4$; GA, $n = 4$; AA, $n = 4$). Statistical significance was assessed by one-way ANOVA, * $P < 0.05$.

both at baseline and in response to DK-PGD₂ (Fig. 3D, $P < 0.05$), while the level of IL-4 was below detection (data not shown). These data indicate that the AA genotype is associated with heightened expression of Th2 cytokines.

Discussion

CRTh2 plays an important role in allergic inflammation, mediating expression of Th2 cytokines and chemotaxis (3, 7). The present study demonstrates that *CRTh2* rs533116 is associated with both allergic asthma and expression of CRTh2. It also provides the first comprehensive characterization of the proportion of circulating cells expressing CRTh2, demonstrating increases in CRTh2-expressing eosinophils, basophils and CD4⁺ T cells. Furthermore, we observed that virtually all CCR3⁺ eosinophils express CRTh2, which may reflect synergy between these chemotactic pathways. PGD₂ has been shown to enhance eotaxin-induced eosinophil release from the bone marrow and to maintain activity in blood, suggesting a role in mediating tissue infiltration (24, 25). The higher percentage of CRTh2⁺ CD4⁺ T cells is in line with a recent study showing that allergen-specific CD4⁺ T cells expressing CRTh2 are increased in blood of allergic compared to nonallergic subjects (26). In contrast, Mutalithas et al. showed no increase in blood CD3⁺ CRTh2⁺ cells in asthmatics compared to non-asthmatics, although they did not control for atopy (15). Collectively, these studies indicate that the increases in CRTh2-expressing cells observed within tissues of allergic subjects may be due, at least in part, to infiltration of circulating cells.

This study shows a significant association between *CRTh2* rs533116 and allergic asthma among White adults, those demonstrating the highest frequency of the AA genotype. This finding supports our previous report with German children in

which significance for the association between rs533116 and allergic asthma was lost following correction for multiple testing (21). The weaker association in children could be due to population differences, because children were from Munich, Dresden and Leipzig. Alternatively, as PGD₂ is metabolized from essential omega-6 fatty acid, complex gene-diet interactions could also explain the discrepancies. For instance, the influence of *CRTh2* rs533116 may rely on exceeding a threshold level of omega-6 fatty acid intake/PGD₂ production over time and be more obvious in adults than children.

In our study, the AA genotype was rare in controls, with only 1 subject in the first 20 investigated. As expression of CRTh2 is regulated by mediators driving allergic inflammation, including IL-4 and the transcription factor GATA-3 (27, 28), we limited analysis to cells from allergic individuals in an effort to increase our ability to detect a genotype-specific effect on CRTh2 expression. Subjects with the AA genotype exhibited a higher percentage of circulating eosinophils and expression of CRTh2 per cell compared to those with the GG genotype. As elevated eosinophil levels in the blood and/or sputum are considered predictors of uncontrolled asthma and exacerbations (29), subjects with the AA genotype may be at risk of more severe asthma phenotypes. Further studies with clinically characterized asthmatics are needed to address this possibility.

The AA genotype was also associated with increased surface expression of CRTh2 on circulating CD4⁺ T cells, a greater propensity for Th2 differentiation and more Th2 cytokine production. A higher percentage of IL-13-expressing cells and secreted IL-13 were observed for the AA compared to GG genotype. There was also a higher percentage of IL-4-expressing cells, indicating the AA genotype is associated with heightened IL-4 responsiveness. The inability to detect secreted IL-4

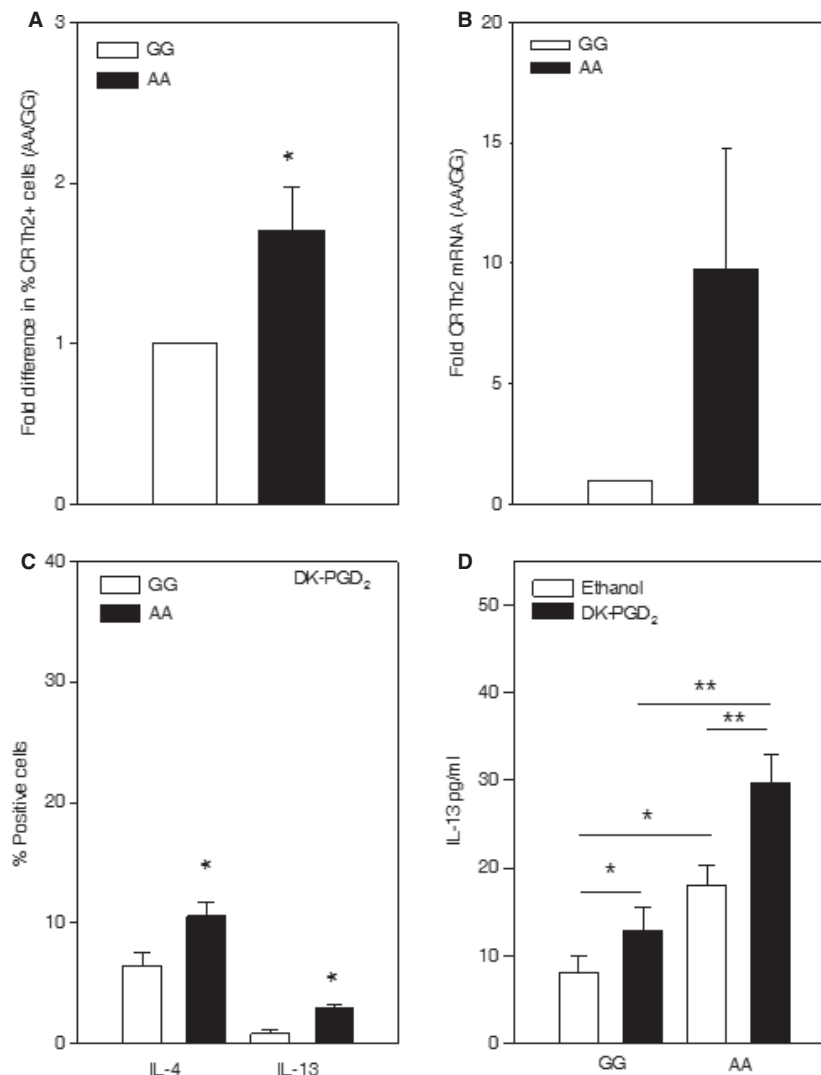


Figure 3 *CRTh2* rs533116 influences C $RT\theta$ 2 expression during Th2 differentiation. CD4⁺ T cells isolated from subjects with the AA or GG genotype were cultured under Th2-polarizing conditions. Fold difference for the percentage of C $RT\theta$ 2⁺ cells (AA over GG, $n = 11$, A). Data represent mean $\pm$ SE of four paired experiments. Influence of rs533116 on *CRTh2* mRNA levels in cells undergoing Th2 differentia-

tion ($n = 4$, B). C $RT\theta$ 2⁺ Th2 cells from subjects with the AA or GG genotype were stained for intracellular IL-4 ($n = 9$) and IL-13 ($n = 6$) following stimulation (4 h) with DK-PGD₂ (C). Th2 cell secretion of IL-13 was assessed by ELISA ($n = 6$, D). Data represent mean $\pm$ SE of the percentage of positive cells or pg/ml. Statistical significance was assessed by Student's *t*-test, * $P < 0.05$, ** $P < 0.01$.

may be due to the binding of soluble IL-4 receptor, an issue previously reported to hamper detection of IL-4 by ELISA (30). Collectively, these data suggest rs533116 is associated with increased expression of C $RT\theta$ 2 and Th2 cytokines.

Our data suggest the influence of *CRTh2* rs533116 may be through a recessive effect. We detected association with allergic asthma using the recessive model and significantly higher expression of C $RT\theta$ 2 was observed for the comparison of carriers of the AA vs GG, but not the GA genotype. A recessive effect would be similar to our previous finding for the effect of *IL13* rs1800925 on IL-13 production (31). However, association was also observed with the additive model and a trend for higher C $RT\theta$ 2 expression on eosinophils of GA vs

GG genotype was observed (2-fold, $P = 0.09$). Although CD4⁺ T cells did not show a trend (1.3-fold, $P = 0.51$), this was likely due to the challenge of detecting differences from the much lower percentage of CD4⁺ T cells expressing C $RT\theta$ 2 (~6%), compared to eosinophils (~98%). Therefore, we cannot rule out the possibility that a dose-response/additive effect may exist.

This is the first study to examine whether *CRTh2* SNPs coincide with altered C $RT\theta$ 2 expression and as rs533116 is located upstream of the transcription start site, our results indicate it may influence transcription from the *CRTh2* promoter. While the AA genotype was associated with significantly more surface C $RT\theta$ 2, the increase in *CRTh2* mRNA (9.8-fold) was not

statistically significant. This is likely due to examining time points optimized for surface expression of CRTh2 rather than transcript levels that are reported to be influenced by stability (19). Although the rs533116 SNP is not in linkage disequilibrium with SNPs in other allergy-associated genes, such as FcεR₁ and CC10, or other *CRTh2* SNPs associated with allergic phenotypes (21), it is co-inherited with a number of currently unstudied *CRTh2* SNPs within the intronic and 3' regions (21). Therefore, experiments to determine whether rs533116 is a *bona fide* gain-of-function polymorphism are necessary and currently underway.

Elevated circulating and/or sputum eosinophils are considered a marker of asthma exacerbation (29, 32), while Th2 cytokine expression has been suggested to mediate day-to-day symptoms (33). Our findings indicate that both circulating eosinophil and CD4⁺ T cell expression of CRTh2 are increased in subjects with allergic airways disease, with the highest levels observed in subjects with the rs533116AA genotype. Further increase in susceptibility may occur through gene–gene interactions such as co-inheritance of *CRTh2* rs533116AA with rs20417CC in cyclo-oxygenase 2, associated with higher release of PGD₂ (34). In conclusion, this work suggests *CRTh2* rs533116 represents an important genetic determinant for susceptibility to allergic airway disease through an increase in CRTh2 expression by both eosinophils and Th2 cells.

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Author contributions

Eduardo Campos Alberto performed whole blood staining and data analysis; Emily MacLean generated the genotyped Th2 cell lines, the enriched CRTh2⁺ Th2 cells and performed flow cytometry and qRT-PCR analysis; Courtney Davidson genotyped the population recruited from the University of Alberta and performed qRT-PCR; Nami Shrestha Palikhe

performed ELISA experiments; Jessica Storie performed Th2 cell cultures and intracellular cytokine staining; Christopher Tse performed some Th2 differentiations and flow cytometry analysis; Darren Brenner performed all statistical genetic analysis; Irvin Mayers and Harissios Vliagoftis facilitated recruitment and characterization of subjects; Harissios Vliagoftis also participated in critical review of the manuscript; Ahmed El-Soheemy provided the association data for the University of Toronto population and critical manuscript preparation and review; Lisa Cameron designed the study, supervised all experiments, collection of data, data analysis and wrote the final version of the manuscript.

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Conflict of interest

All Authors declare that they have no competing financial or personal interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Methods.

Table S1. CRTh2 expression in allergic asthma and rhinitis.

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Supplementary Material

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Methods*Subjects*

The Toronto Nutrigenomics and Health (TNH) study is a population of University of Toronto students, a diverse ethno-cultural population representative of Toronto, being studied for biomarkers of chronic disease (1). Allergic asthmatic subjects were identified by answering “yes” to both: “Have you ever had a doctor diagnosis of allergy” and “Have you ever had a doctor diagnosis of asthma”. The study protocol was approved by the Research Ethics Board at the University of Toronto and informed consent was obtained from all subjects. Exclusion criteria were symptoms of infectious diseases. The University of Alberta study was approved by the Human Ethics Committee of the

University of Alberta and informed consent was obtained from all subjects. Subjects were recruited at the University Asthma and Allergy Clinic. Atopy was determined by a positive skin test to 1 of 12 common aeroallergens. Genotyping was performed on 30 subjects, while only 22 of 30 were available for further study of CRTh2 expression.

Genotyping

Genomic DNA was isolated from peripheral blood mononuclear cells or whole blood using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to manufacturer instructions. Genotyping of *CRTh2* rs533116 in the Toronto population was performed using a TaqMan® allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) on an ABI 7000 Sequence Detection System. Plates (96-well) that contained controls and a 10% random replication of samples were genotyped consecutively. Genotyping the Edmonton population was performed either by Sequenom iPLEX Technology (Quebec Genome Centre, Montreal, Canada) or sequencing. All subjects were successfully genotyped.

Flow cytometry

Antibodies Flow cytometric analysis of CRTh2 was performed using biotinylated anti-CRTh2 (clone BM16; Miltenyi Biotec, Auburn, CA, USA) and streptavidin-APC (eBioscience, San Diego, CA, USA) or anti-CRTh2 directly labeled with APC (clone 301108, R&D Systems, Minneapolis, MN, USA). Antibodies for cell type markers were anti-CD4 (Serotec, Oxford, UK); anti-CD8 and anti-CCR3 (BD, Mississauga, ON,

Canada); anti-CD203c (Coulter Immunotech, Prague, CR); anti-CD14 and anti-CD16 (eBioscience). Respective isotype controls were used.

Surface staining Staining was performed with 100 µl of well-mixed anti-coagulated whole blood with all steps performed at room temperature. Non-specific binding was blocked (30 min) using Fc blocker (Miltenyi Biotec) and mouse and rat IgG (Invitrogen, Burlington, ON, Canada). Cells were then incubated with biotinylated anti-CRTh2 (30 min). Lysis buffer (BD) was added (15 min), cells washed twice and APC-conjugated streptavidin and the directly labeled primary antibodies for cell type identification were added (30 min). Cells were washed and resuspended in PBS/paraformaldehyde (2%, 30 min). Expression was assessed using a FACSCalibur flow cytometer and gates set in accordance with the profiles of the isotype control.

Cell type analysis Cell types were identified using side scatter and expression of cell surface markers: CCR3 (eosinophils, gate R1, Fig. 1) (2), CD203c (basophils) (3), CD4 or CD8 (T lymphocytes), CD14 (monocytes) (4) and CD16 (neutrophils) (5). Mean fluorescence intensity data was based on 30,000 total events and calculated for each sample using fold increase over isotype control (Mean CRTh2 staining – Mean Control Antibody)/Mean Control Antibody.

Th2 cell staining CRTh2 staining of *in vitro* differentiated Th2 cells was performed as above (but on ice) with biotinylated anti-CRTh2 and streptavidin-APC. Intracellular cytokine staining was performed on Th2 cells following 4 hours stimulation with either mitogenic stimulation with PMA (20ng/ml) and ionomycin (1µM) or DK-PGD₂ (0.5 or

1 μ M; Cayman, Chemicals, Ann Arbor, MI, USA) in the presence of brefeldin A (10 μ g/ml; Sigma Aldrich, Oakville, ON, Canada) for intracellular sequestration of cytokines. Cells were fixed with paraformaldehyde (4%; Sigma) and permeabilized with saponin (0.4%; Sigma). IL-13-PE and IL-4-Alexa 488 antibodies (BD) and isotypes were incubated on ice (30 min), washed and fluorescence read immediately by FACSCalibur.

Th2 cell differentiation

For human Th2 cell differentiation peripheral blood mononuclear cells were isolated by density centrifugation over Ficoll-Hypaque (Pharmacia Biotech/GE, Baie d'Urf, PQ, Canada). CD4⁺ T cells were isolated by negative selection with the CD4⁺ T cell Isolation Kit II (Miltenyi Biotec). Purity was typically > 96%. All antibodies and recombinant cytokines were from R&D Systems, unless otherwise specified. CD4⁺ T cells (2 x 10⁶/ml) were cultured in X-VIVO-15 medium (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA) on priming (3 days) and rest (4 days). Priming was with antibodies against CD3 and CD28 (plate bound, 1 μ g/ml) under Th2 polarizing conditions: recombinant human (rh) IL-2, 2.5 ng/ml; rhIL-4, 10 ng/ml; neutralizing antibodies against IFN- γ , 1 μ g/ml; IL-12, 1 μ g/ml (eBiosciences). Cells were then rested (4 days) in rhIL-2 (5 ng/ml), IL-4 (10 ng/ml), anti-IFN- γ and anti-IL-12 (1 μ g/ml). Experiments with differentiating Th2 cells were conducted after one and two weeks of culture. CRTh2 enriched Th2 cell lines were generated using the Th2 differentiation method described above and on day 7 CRTh2⁺ cells were isolated using the CRTh2 isolation kit and MACS columns (Miltenyi). CRTh2⁺ cells (2 x 10⁶/ml) were maintained on cycles of priming (3 days, anti-CD3, anti-

CD28, rhIL-2) and rest (4 days, rhIL-2) as above. Experiments with CRTh2⁺ Th2 cells were performed one and two weeks after CRTh2 isolation.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit and QIAshredders (Qiagen, Valencia, CA, USA). Reverse transcription (RT) reactions were done using oligo-dT primers and Superscript II Reverse Transcriptase (Invitrogen). *CRTh2* mRNA was quantified using a pre-designed Taqman Gene Expression Assay (Applied Biosystems, Carlsbad, CA, USA). *GAPDH* mRNA was used as an internal control and was quantified using a custom 6FAM-labeled TAMRA probe (5'-AAA TCC CAT CAC CAT CTT CCA GGA GCG A-3') (Applied Biosystems) and the following primers *GAPDH*-F (5'-CAA GGCT GAG AAC GGG AAG-3') and *GAPDH*-R (5'-GCA AAT GAG CCC CAG CCT T-3'). The PCR protocol consisted of 10 minutes at 95°C followed by 40 cycles of 30 seconds at 95°C and 60 seconds at 60°C. All samples were run in triplicate. The *CRTh2* and *GAPDH* PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen), quantified and serial dilutions of linearized plasmid were used as standard curves of known copy number. Data were calculated as copy of *CRTh2* per copy of *GAPDH* and represented as the fold difference in *CRTh2* mRNA from *CRTh2* rs533116AA compared to *CRTh2* rs533116GG homozygotes

ELISA

Th2 cells (2 x 10⁶/ml) were incubated with DK-PGD₂ (0.5 or 1μM, 4hrs). IL-4 and IL-13 levels were assayed using a commercially available ELISA kit (Diacclone Research, France, detection limits 1.1 pg/ml and 3.12 pg/ml, respectively).

Statistical analyses

Differences in genotypic frequencies were examined using χ^2 tests or a Fisher's exact test, where cell sizes were small. Statistical analyses were performed using Graphpad Prism version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SAS version 9.1 (SAS Institute, Cary, NC, USA). Models examining the TNH data were adjusted for age, sex, body mass index (BMI) and smoking status. The ORs for the multiple genotype-specific effects were created using CLASS statements in SAS in adjusted logistic regression models to create indicator variables for each of the genotype groups to show the effects across the groups. This allows for the estimation of individual effect of genotype groups in a polytomous independent variable. *In vitro* experiments examining genotyped Th2 cells were paired, with cells of each genotype differentiated and tested on the same day, and therefore analyzed with a one-sample Student's *t* test.

Results

CRTh2 staining CRTh2 staining was further tested in experiments using 3% human serum and 0.5% human serum albumin as well as an independently generated anti-CRTh2 antibody. In all cases, the % of CRTh2 expressing cells remained similar.

Although monocytes may also express low CD4, we detected only $0.12\% \pm 0.03$ of CD14⁺ cells (as a proportion of total cells) expressing CRTh2 and therefore considered CD4⁺CRTh2⁺ cells as T helper cells. CRTh2 expression by CD8⁺ T cells, B cells, NK cells, $\gamma\delta$ T cells and neutrophils was low (< 1%, data not shown).

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Table S1

CRTh2 Expression in Allergic Asthma and Rhinitis

Number of cells/Total cells (%)	Allergic Asthma n = 6	Allergic Rhinitis n = 7	Controls n = 8
CRTh2+	9.03 ± 0.87* [‡]	5.84 ± 0.74*	3.76 ± 0.28
CRTh2+ Eosinophils	6.25 ± 0.93*	4.39 ± 0.76	1.88 ± 0.31
CRTh2+ Basophils	1.37 ± 0.31*	0.77 ± 0.09	0.51 ± 0.07
CRTh2+ CD4 T cells	1.33 ± 0.23*	1.03 ± 0.76	0.76 ± 0.12
CRTh2+ Monocytes	0.16 ± 0.03	0.10 ± 0.02	0.09 ± 0.01
MFI of CRTh2			
CRTh2+ Eosinophils	17.5 ± 3.22*	10.6 ± 2.00	5.05 ± 0.70
CRTh2+ Basophils	16.7 ± 2.84*	10.2 ± 1.69	6.31 ± 0.99
CRTh2+ CD4 T cells	18.4 ± 3.12*	12.4 ± 2.77	6.22 ± 0.98

*P-values determined by one-way ANOVA, * $p < 0.05$ allergic asthma or allergic rhinitis compared to control, $p < 0.05$ allergic asthma compared to allergic rhinitis*