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Title: Elevated Levels of Circulating CD4⁺CRTh2⁺ T cells Characterize Severe Asthma

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ABSTRACT

Background: Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2) is a receptor for PGD₂ and expressed by T cells, eosinophils, basophils and ILC2 cells. CRTh2 expression by CD4⁺ T cells identifies the Th2 subset and these cells have been characterized as allergen-specific central memory Th2 cells. Recently, activation of the PGD₂-CRTh2 pathway in the lungs was associated with severe asthma.

Objective: To assess circulating levels of Th2 cells and related mediators in severe asthma and those who experience asthma exacerbations.

Methods: Peripheral blood cells expressing CRTh2 were characterized by flow cytometry and qRT-PCR. Serum IL-13 and PGD₂ were measured by ELISA and compared with asthma severity and tendency to exacerbate.

Results: Severe asthmatics had more circulating CD4⁺CRTh2⁺ T cells, CRTh2 and GATA3 mRNA and a higher level of serum IL-13 compared to mild/moderate asthmatics. The proportion of CD4⁺CRTh2⁺ T cells was associated with lower lung function and was highest in severe asthmatics that exacerbated in the last year. Circulating CD4⁺CRTh2⁺ T cells, unlike eosinophils, were positively correlated with inhaled steroid dose.

Conclusions and Clinical Relevance: Elevated levels of circulating CD4⁺CRTh2⁺ T cells are a feature of severe asthma, despite high dose corticosteroids. Tracking the systemic level of these cells may help identify type-2 severe asthmatics at risk of exacerbation.

Keywords: CRTh2, CD4⁺CRTh2⁺ T cells, Th2 cells, type-2 immunity, PGD₂, severe asthma, exacerbation, glucocorticosteroid responsiveness, biomarker

ABBREVIATIONS:

ATS: American Thoracic Society

AUC: Area under curve

BAL: Bronchial alveolar lavage

CRTh2: Chemoattractant receptor-homologous molecule expressed on Th2 cells

ERV: Emergency Room Visit

FEV₁: Forced expiratory volume in 1 sec

ICS: Inhaled corticosteroid

ILC2: Innate lymphoid cells, group 2

OCS: Oral corticosteroid

pWBC: Peripheral white blood cells

PGD₂: Prostaglandin D₂

PGDS: Prostaglandin D synthase

q-RT-PCR: Quantitative reverse transcriptase PCR

ROC: Receiver operating characteristics

INTRODUCTION

Asthma is a heterogeneous condition and many efforts have been made to identify asthma phenotypes (reviewed in [1]). Recently, two distinct molecular phenotypes of asthma, Th2-high and Th2-low, were identified. Compared to Th2-low, Th2-high asthmatics had increased expression of IL-5 and IL-13 in bronchial biopsies, elevated eosinophil levels in the blood and bronchial alveolar lavage (BAL) and were corticosteroid responsive [2]. However, some patients with more severe forms of asthma showing little improvement from steroid treatment also have elevated blood and sputum eosinophils [3, 4], a cell type dependent on IL-5 [5]. In light of this, the Th2-high asthma phenotype has now been proposed to encompass both mild/moderate corticosteroid responsive as well as severe corticosteroid-refractory asthmatics [1]. New treatment strategies have been developed based on careful patient selection and blocking specific Th2 cytokine pathways. For instance, in patients with high sputum or blood eosinophils blocking IL-5 improves FEV₁ and symptoms in those with an exacerbating phenotype [3, 4]. Similarly, administering anti-IL-13 to patients with high serum periostin, a marker of IL-13 activity [2], was moderately effective in improving FEV₁ and reducing exacerbations [6], though not day-to-day symptoms [7]. These studies are helping to link the relative importance and non-redundant roles of these Th2 molecules to the clinical features of asthma, but suggest that strategies blocking Th2 immunity upstream of these cytokines may be a useful approach.

Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2) is a receptor for PGD₂, and so is formally named prostaglandin D receptor 2 (PTGDR2, OMIM 604837). CRTh2 is considered a marker of human Th2 cells, within the CD4⁺ T cell subset [8, 9], but is also expressed by eosinophils, basophils [10] and group 2 innate lymphoid cells (ILC2) [11]. Though a major source of PGD₂ is mast cells following allergen cross-linking of IgE [12], it is

also produced by other inflammatory cells activated by bacterial [13] and viral [14] products. *In vitro*, PGD₂-CRTh2 activation mediates production of IL-4, IL-5 and IL-13 from both Th2 cells [15] and ILC2 [16] and so these mediators are now referred to as type-2 cytokines. PGD₂ ligation of CRTh2 activates eosinophils, causing shape change [17], and mediates chemotaxis of both eosinophils [18] and Th2 cells [19]. PGD₂ also inhibits Th2 cell apoptosis and is therefore considered to interfere with resolution of inflammation [20].

In vivo studies using CRTh2^{-/-} mice or CRTh2 antagonists showed that this receptor mediates cellular and physiologic responses in models of allergic disease [21-23]. In humans, CRTh2⁺CD3⁺ T cells are increased in allergic tissues [24, 25] and we and others showed that blood levels of CRTh2⁺CD4⁺ T cells are higher in subjects with allergic airways disease compared with non-allergic controls [26, 27]. Furthermore, the level of CRTh2 has been reported to be upregulated on eosinophils in eosinophilic esophagitis [28]. Evidence also suggests this pathway is involved in disease severity as Fajt *et. al.* observed a higher proportion of CRTh2-expressing cells and levels of PGD₂ in the lungs of severe compared to mild/moderate asthmatics and that they associate with poor asthma control [29].

Studies have shown that CRTh2⁺CD4⁺ T cells are allergen specific Th2 cells [27, 30, 31] and of a central memory phenotype [32]. These are long-lived T cells that circulate between the lymph nodes, blood and site of exposure, providing the immunological memory that mediates rapid recall responses and chronicity of allergic inflammation [33-35]. As such, we hypothesized that the circulating level of Th2 cells would be higher in patients designated as having severe asthma based on persistence of asthma symptoms. We observed that the proportion of blood CD4⁺CRTh2⁺ T cells, but not eosinophils, were higher in severe compared to mild/moderate asthmatics, were associated with lower lung function and positively correlated with daily dose of

inhaled corticosteroid. In severe asthmatics, CD4⁺CRTh2⁺ T cells were also higher in those that exacerbated in the last year. Collectively, our findings suggest that CD4⁺CRTh2⁺ T cells may mediate persistence of symptoms and that tracking their level could help identify severe asthmatics at risk of exacerbation.

METHODS

Additional information on study design and experimental detail is provided in the article's Online Supplement.

Subjects

This study was approved by the institutional review board (approval number PRO1784) and all subjects gave informed consent. Severe asthma was defined by the American Thoracic Society (ATS) criteria [36] which included being on high dose inhaled corticosteroids (ICS, ≥ 1000 μ g/day) and/or use of oral corticosteroid (OCS) therapy for 50% or more of the previous year. In addition, patients with severe asthma also exhibited ≥ 2 of 7 criteria indicative of persistent symptoms. All other asthmatics were classified as the mild/moderate group. Atopy was determined by skin testing. (See Supplement for further detail). Patients were recruited from the tertiary care Asthma Clinic at the University of Alberta. Smokers with asthma were not excluded.

Profiling peripheral blood cells by asthma phenotype

Peripheral blood was stained with antibodies against cell type-specific surface markers as well as CRTh2. Positivity was assessed by flow cytometry. Figure 1 shows the gating strategy for CRTh2-expressing CD4⁺ T cells and eosinophils. Helper T cells were identified by low side scatter (SSC^{low}) and high CD4^(high). Eosinophils were identified by high SSC^(high) and CCR3 positivity⁽⁺⁾. Mean fluorescence intensity (based on 30,000 events) was calculated for each sample using fold increase over isotype control (Mean CRTh2 staining – Mean Control Antibody)/Mean Control Antibody.

Quantitative real time PCR (qRT-PCR)

Gene expression was assessed with RNA isolated from whole blood. *CRTh2* and *GATA3* were amplified with TaqMan assays (Applied Biosystems, Carlsbad, CA, USA) and expressed relative to *GAPDH* mRNA.

Measurement of serum IL-13 and PGD₂

IL-13 was measured by enzyme-linked immunosorbent assay (ELISA, detection > 3.1 pg/ml) in serum or cell culture supernatants of peripheral blood mononuclear cells (PBMC) activated (24 hours) with PMA (20 ng/ml) and ionomycin (1 μM). PGD₂ was measured using an enzyme immunoassay (EIA) that detects PGD₂ converted to PGD₂-MOX (> 3 pg/ml). The assay has 100% specificity for PGD₂-MOX and does not detect PGE₂, PGF₂ or Thromboxane B₂ (< 0.01%).

Statistical analysis

Median values of phenotypic clinical characteristics between study subjects were compared using the Mann-Whitney test for continuous variables. Fisher exact test was used for categorical variables. For receiving operating characteristics (ROC) curves and area-under-curve (AUC), 95% confidence intervals (CI) were used. Statistical analyses were performed using SPSS (version 21.1, Chicago, IL, USA), except optimum cut offs were mathematically determined using R software (version 3.1.1, The R Foundation for Statistical Computing). *P* values of less than .05 were considered significant.

RESULTS

Demographic and clinical characteristics

From March 2012 - November 2013, 66 patients consented to join the study and gave blood. As expected, FEV₁ (% predicted) was significantly lower in severe compared to mild/moderate asthmatics ($P = 0.027$), while there were no differences in age, sex, BMI, atopy, IgE, blood eosinophils or history of smoking (Table 1). The severe asthma group also had more patients with FEV₁ < 80% predicted ($P = 0.046$), who used oral corticosteroid ($P = 0.002$) and who had been hospitalized for asthma in the last year ($P = 0.024$).

Circulating Th2 cells are increased in severe asthma

Figure 1 (A-B) shows the gating strategies used to characterize the profile of CRTh2-expressing cells within whole blood. We found no significant differences in the percentage of circulating CD4⁺ T cells or eosinophils between the two groups (Fig. S1). However, analysis of CRTh2 expression by immune cells showed a higher proportion of the CD4⁺ T cell population was expressing CRTh2 in severe compared to mild/moderate asthmatics ($P = 0.008$; Fig. 1C). Furthermore, as a percentage of total peripheral white blood cells, severe asthmatics had 50% more CD4⁺CRTh2⁺ T cells than patients with milder disease ($P = 0.02$; Fig. 1D). The proportion of these cells was also higher than healthy control subjects (Table S1). Despite this, the level of CRTh2 (MFI) on CD4⁺CRTh2⁺ T cells was similar in mild/moderate (95.72 [58.70 - 159.27]) and severe asthmatics (91.09 [40.47 - 164.58], $P = 0.594$). There were no differences in the proportion of CRTh2⁺ eosinophils (SSc^{high}CCR3⁺, Fig. 1E) or CRTh2-expressing eosinophils as a proportion of white blood cells between the two asthma groups (Fig. 1F). The level of CRTh2 (MFI) on eosinophils also did not differ (mild/moderate, 65.75 [45.60 - 93.57] vs severe, 73.59 [44.0 - 112.70], $P = 0.570$).

Increased expression of CRTh2 mRNA and type-2 mediators in severe asthma

Whole blood preparations from severe asthmatics had more mRNA for CRTh2 ($P = 0.024$) and the Th2 transcription factor GATA3 ($P = 0.025$) than mild/moderate asthmatics (Fig. 2A). CRTh2 mRNA correlated with the level of CRTh2 (MFI) on CD4⁺CRTh2⁺ T cells in severe ($r_s = 0.43$, $P = 0.03$) but not mild/moderate asthmatics ($r_s = -0.06$, $P = 0.78$) and did not correlate with CRTh2 MFI on eosinophils (severe, $r_s = 0.32$, $P = 0.117$; mild/moderate, $r_s = 0.144$, $P = 0.48$).

Severe asthmatics with a high level of CRTh2 mRNA had significantly more surface CRTh2 on CD4⁺ T cells ($P = 0.005$) compared to those with a low level of CRTh2 mRNA (Table S2). These data suggest the increased level of CRTh2 mRNA in severe asthmatics is driven by the CD4⁺ T cell population, though does not discount contribution from other cell types.

Serum IL-13 was below detection in 51% of samples, which were assigned 3.1 pg/ml (lower limit). Despite this, more severe asthmatics had detectable serum IL-13 (63%) than mild/moderate asthmatics (34%, $P = 0.026$). Furthermore, the median level of IL-13 was also significantly higher in the severe group ($P = 0.015$; Fig. 2B). All serum samples had detectable levels of PGD₂, but we found no difference in the level of this lipid mediator between the asthma groups (Fig. 2C).

The PGD₂-CD4⁺CRTh2⁺ T cell pathway is not influenced by smoking

Most asthma studies exclude patients with more than 10 pack years of smoking, to avoid influence from chronic obstructive pulmonary disease (COPD) [37]. In an effort to conduct a ‘real world’ study, we included smoking asthmatics. While some also had a COPD co-diagnosis (n=10, see online supplement for details), removing this subset from the analysis showed that the severe asthma group still had a prominent Th2 phenotype (Table S3). We also segregated patients based on smoking status and found that those with a history of ≥ 10 years of smoking and/or current smokers compared to those that never smoked or did so for < 10 years showed no significant differences in most mediators of the PGD₂- CD4⁺CRTh2⁺ T cell pathway. Interestingly, however, the group with a history of ≥ 10 years of smoking and/or current smokers

had fewer eosinophils ($P = 0.03$, Table S4).

Circulating levels of CD4⁺CRTh2⁺ T cells and clinical characteristics

We also examined whether the proportion of CD4⁺CRTh2⁺ T cells was associated with clinical characteristics of asthma, independent of phenotype categorization. We found more CD4⁺CRTh2⁺ T cells in patients with lower lung function ($FEV_1 < 80\%$, $P = 0.026$; Fig. 3A), though not more serum IL-13, PGD₂ or blood eosinophils (Fig. S2). We also examined the proportion of CD4⁺CRTh2⁺ T cells in patients segregated based on whether or not they had an exacerbation requiring an emergency room visit in the last year. There were significantly more CD4⁺CRTh2⁺ T cells in severe asthmatics that exacerbated compared to those that did not ($P = 0.041$). Amongst exacerbators, severe asthmatics had 2.4-fold more CD4⁺CRTh2⁺ T cells than mild/moderate patients ($P = 0.008$; Fig. 3B). There was only a trend for more IL-13 in those that did vs did not exacerbate ($P = 0.062$). This was perhaps attributed to the fact that in non-exacerbating patients, the level of IL-13 was significantly higher in severe asthmatics than those with mild/moderate disease ($P = 0.008$; Fig. 3C). The level of PGD₂ was significantly higher in those that exacerbated in the total asthma population ($P = 0.011$) as well as the mild/moderate group ($P = 0.025$; Fig. 3D). Blood eosinophil levels were not different between those that did and did not exacerbate in the last year (Fig. 3E).

CD4⁺CRTh2⁺ T cells are positively correlated with dose of inhaled corticosteroid

The total amount of inhaled corticosteroid was positively correlated with the proportion of CD4⁺CRTh2⁺ T cells ($r_s = 0.447$, $P < 0.0001$), but not with the level of blood eosinophils (Table

S5). This relationship was similar in mild/moderate ($r_s = 0.365$, $P < 0.022$) and severe asthmatics ($r_s = 0.330$, $P < 0.093$), though only a trend was observed in the severe group. These data indicate that the correlation with inhaled steroid dose may be related to CD4⁺CRTh2⁺ T cells *per se*, rather than a lack of steroid responsiveness of severe asthmatics.

Level of circulating CD4⁺CRTh2⁺ T cells as a classifier of type-2 severe asthma

We next asked whether these cells could be used to identify patients with type-2 severe asthma. We stratified patients based on the median level of CD4⁺CRTh2⁺ T cells or CRTh2 mRNA as well as detectable serum IL-13 or blood eosinophils, both previously used to identify difficult-to-treat asthmatics [3, 7]. Table 2 shows there were more severe asthmatics in the CD4⁺CRTh2⁺ T cell high group ($P = 0.044$) as well as those with high CRTh2 mRNA ($P = 0.024$) or IL-13 ($P = 0.030$), but not high blood eosinophils. Patients with high CD4⁺CRTh2⁺ T cells also had more inflammatory cells and IL-13 production following *ex vivo* activation (n=10, Table 3), though stratifying based on high serum IL-13 or blood eosinophils did not coincide with elevated levels of inflammatory cells or IL-13 production (Table S6). Receiver operator characteristics (ROC) curve analyses showed that while using the proportion of CD4⁺CRTh2⁺ T cells (AUC = 0.67, $P = 0.022$), level of CRTh2 mRNA (AUC = 0.70, $P = 0.024$) or serum IL-13 (AUC = 0.66, $P = 0.024$), was somewhat able to classify severe from mild/moderate asthma, the sensitivity was low (56%, Table S7, Fig. S3).

Level of CD4⁺CRTh2⁺ T cells, PGD₂ and IL-13 identifies those with a history of exacerbation

Severe asthmatics that exacerbated had the highest proportion of CD4⁺CRTh2⁺ T cells (Fig. 3B) and those with high CD4⁺CRTh2⁺ T cells were more likely to have exacerbated in the last year (47%) compared to those with low CD4⁺CRTh2⁺ T cells (8%, $P = 0.043$; Table 4). Therefore, we examined the ability of these cells to identify exacerbators. In severe asthmatics, the level of circulating CD4⁺CRTh2⁺ T cells ($> 0.26\%$, optimum cut off) was a sensitive indicator of exacerbation in the last year (100%, AUC = 0.75, $P = 0.041$; Table 3, Fig. S4), though it was not specific (58%). When we examined patients with high CD4⁺CRTh2⁺ T cells and high PGD₂ (> 346.1 pg/ml) or IL-13 (> 3.12 pg/ml), those that did or did not exacerbate differed substantially in the level of PGD₂ ($P = 0.055$), though not IL-13 ($P = 0.774$; Table 4) and ROC analysis using these criteria showed this method could discriminate exacerbators (AUC 0.889, sensitivity and specificity $> 80\%$, $P = 0.025$; Table 4, Fig. S5).

DISCUSSION

This study shows that blood levels of CD4⁺CRTh2⁺ T cells were higher in severe compared to mild/moderate asthmatics, in patients with lower lung function and were positively correlated with daily dose of inhaled corticosteroid. Severe asthmatics with a high level of CD4⁺CRTh2⁺ T cells were also more likely to have exacerbated in the last year. Collectively, these data show that the level of circulating CD4⁺CRTh2⁺ T cells associates with type-2 severe asthma and suggest they participate in persistence and exacerbation of symptoms.

A key feature of allergic disease is the adaptive immune response and development of memory to an allergen [35]. After acute exposure, the effector memory T cell population contracts, while the central memory population persists as long-lived cells [33, 35]. Upon re-exposure to the sensitizing allergen, central memory T cells are activated in the draining lymph nodes and migrate through the blood to infiltrate the airways [33]. Circulating $CD4^+CRTh2^+$ T cells are allergen-specific central memory T cells [27, 30-32]. Our finding of higher levels of these cells in the blood of severe compared to mild/moderate asthmatics could suggest either that these patients experience more frequent exposure to their sensitizing allergen or that an allergen reservoir is continually stimulating these cells [38]. Genetic variation within CD83, a surface receptor expressed by mature dendritic cells, was recently associated with severe asthma in a genome-wide association study [39] and a mutation in CD83 was shown to mediate Th2 cell differentiation [40]. Taken together, these studies suggest severe asthmatics have heightened Th2 cell differentiation which may involve gene-environment interactions.

In addition to comparing asthma groups, we also assessed the ability of $CD4^+CRTh2^+$ T cells to serve as a biomarker of severe asthma. We did find that asthmatics with high blood $CD4^+CRTh2^+$ T cells were more likely to have severe asthma, more blood eosinophils and IL-13 production following activation. However, the level of circulating $CD4^+CRTh2^+$ T cells was not a sensitive biomarker for predicting severe asthma, though promising (AUC = 0.666, $P = 0.022$). Recently, subpopulations of Th2 cells have been identified. $CRTh2^+CD4^+PGDS^+CD161^{high}$ T cells were shown to express significantly more Th2 cytokines and to be more strongly correlated with blood eosinophils in patients with eosinophilic gastrointestinal disease and atopic dermatitis than the entire Th2 population ($CRTh2^+CD4^+$ T cells) [31]. Additionally, Lam *et. al.* showed that polyp-derived $CD4^+IL-17RB^+$ cells exhibited a Th2 cytokine profile, which could be further

induced by stimulation with IL-25 and IL-33 [41]. In light of this, further work may show that this (or another) Th2 cell subpopulation correlates more closely with asthma severity.

Once Th2 cells migrate into the lung, the majority will become effector memory T cells and respond to activation by producing large amounts of Th2 cytokines [34]. However, dual Th2-Th17 cells were recently reported in BAL and shown to correspond with asthma severity and steroid resistance [41]. Therefore, within the lung some Th2 cells may alter phenotype due to local inflammation and contribute other mediators such as IL-17 [42]. Our analysis suggests the higher level of CRTh2 mRNA in severe compared to mild/moderate asthmatics may be attributed primarily to Th2 cells. While we did not assess ILC2, which also express CRTh2 [11], their abundance has been shown to be orders of magnitude lower than blood Th2 cells [42] and so they likely contribute minimally to the overall level of CRTh2 mRNA we measured.

Nonetheless, increasing evidence suggests a role for ILC2 in asthma. They are elevated in the blood of asthmatics compared to healthy controls [43], BAL of asthmatics compared to disease matched controls [44, 45] and were recently shown to track with severity, being higher in severe compared to mild asthmatics despite corticosteroid use [42]. Indeed, ILC2 are considered to provide important links between innate and adaptive type 2 responses within tissues [46], though whether they primarily migrate to or develop within the tissues is not well understood.

Steroids are known to mediate anti-inflammatory effects through many mechanisms [47], including inhibition of cytokine production [48] and induction of apoptosis [49]. However, a defining feature of severe asthma is persistence of symptoms despite high dose ICS [36]. Indeed, we found that the proportion of circulating CD4⁺CRTh2⁺ T cells, but not eosinophils, was positively correlated with dose of inhaled steroid. This may be related to the fact that memory T cells, unlike eosinophils [50, 51], have been shown to be resistant to glucocorticoid-induced cell

death [52] and 100-fold less sensitive to steroid in terms of inhibition of proliferation and cytokine production [53]. Furthermore, T cells are known to become steroid resistant when treated with IL-2 and IL-4 [54] and when activated through the T cell receptor [55]. Interestingly, these are the conditions that drive Th2 differentiation and the memory T cell phenotype. Therefore, persistence of symptoms may ultimately be associated with abundance of Th2 cells and their inherent steroid insensitivity.

Severe asthmatics that exacerbated had the highest levels of blood $CD4^+CRTh2^+$ T cells and more severe events, with 15% requiring hospitalization. Respiratory viruses are a major trigger of asthma exacerbation and are considered to be mediated by epithelial IL-33 and IL-25 induction of type-2 cytokines, from both Th2 cells and ILC2 [56]. PGD_2 is also released by inflammatory cells activated by bacterial [13] and viral components [14]. Since PGD_2 activation of CRTh2 inhibits Th2 cell apoptosis [20], innate stimuli may also contribute to type-2 immunity by sustaining Th2 cells. In contrast to a recent paper by Fajt *et. al.* showing increased PGD_2 in BAL of severe compared to mild/moderate asthmatics [29], we observed no difference in blood PGD_2 levels based on asthma severity. Together these findings indicate that assessment of PGD_2 in the airways may be more sensitive, likely due to the source being from local mast cells [12] and its short lived nature in the circulation [57]. However, we did observe an increase in the serum level of PGD_2 in those that exacerbated in the last year, supporting a role for this mediator in worsening symptoms and that its detection in blood can be informative. Indeed, when we included an individual's serum level of PGD_2 or IL-13 along with % $CD4^+CRTh2^+$ T cells, this combined estimate of pathway activation discriminated those that did or did not exacerbate in the last year quite well (AUC = 0.889, $P = 0.025$). Further study is needed to test whether tracking these mediators can be used to diagnose imminent exacerbation.

One caveat of these data is that we did not assess compliance. So, it is possible that increased CD4⁺CRTh2⁺ T cells in patients categorized as severe asthmatics may be due to the actual amount of steroid taken by the patients (*vs* the amount prescribed) being too low. However, the fact there was a positive correlation between CD4⁺CRTh2⁺ T cells and the prescribed dose of inhaled steroid, rather than a lack of correlation, makes that possibility seem less likely. However, it cannot be ruled out and needs to be studied.

In summary, we observed that having a high proportion of circulating CD4⁺CRTh2⁺ T cells is a feature of the type-2 severe asthma phenotype, though the underlying mechanism(s) remains to be determined. Clinical trials with CRTh2-selective antagonists have shown variable efficacy in treating asthma [58, 59], as improved lung function was observed in steroid naïve asthmatics taking a CRTh2-specific antagonist [58], but not when a DP1/CRTh2 dual antagonist was given to moderate/severe asthmatics taking corticosteroids [59]. The effectiveness of a therapeutic approach that targets CRTh2 would rely not only on the activity and selectivity of the anti-CRTh2 molecule, but also on determining the responsive asthma phenotype. Our results suggest that the level of blood CD4⁺CRTh2⁺ T cells may identify those with susceptibility for developing severe asthma, but that assessing measures of disease activity and/or Th2 cell subpopulations [31, 41] is necessary to accurately discriminate severe asthma and those at risk of exacerbation.

Conflict of Interest: Authors have no conflicts of interest to declare.

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Figure Legends

Figure 1. The proportion of circulating CD4⁺CRTh2⁺ T cells is increased in severe asthma. (A-B) Gating strategies used to characterize the profile of CRTh2-expressing cells within whole blood in severe (n=27) compared to mild/moderate asthma (n=39). (C) CRTh2⁺ cells as a proportion of the CD4⁺ T cell population, (D) CD4⁺CRTh2⁺ T cells as a proportion of total peripheral white blood cells. (E) CRTh2⁺ eosinophils (SSc^{high}CCR3⁺ cells) and (F) CRTh2⁺ eosinophils as a proportion of total peripheral white blood cells. pWBC, peripheral white blood cells.

Figure 2. Type-2 mediators are increased in severe asthma. (A) CRTh2 and GATA3 mRNA (n=55), serum level of (B) IL-13 (n=65) and (C) PGD₂ (n=61) in severe compared to mild/moderate asthmatics.

Figure 3. CD4⁺CRTh2⁺ T cells are associated with lung function and exacerbation. (A) The proportion of CD4⁺CRTh2⁺ T cells in patients segregated based on having FEV₁ < or ≥ 80% predicted (n=66). (B) Proportion of CD4⁺CRTh2⁺ T cells, (C) IL-13, (D) PGD₂ levels and (E) blood eosinophils segregated based on having ≥ 1 emergency room visits for asthma in the last year (n=7 mild/moderate, n= 8 severe). pWBC, peripheral white blood cells; ERV, emergency room visit

Table 1. Demographic and clinical characteristics

	Mild/Moderate (n=39)	Severe (n=27)	P
Age (range)*	39 (31-57)	47 (31-59)	0.57
Sex (% female)	22 (56.4%)	15 (55.6%)	1
BMI (kg/m ² ,	30.4 (23.4-36.8)	27.7 (24.6-28.7)	0.315
Atopy (%)**	30 (88.2%)	17 (65.4%)	0.056
Serum IgE (kU/L)*	109.0(25-237.5)	87.0(21-233.5)	0.627
Eos (cells/μl)*	200 (100-500)	200 (100-300)	0.238
FEV ₁ (% predicted)*	87.0 (76.0-91.0)	73.0(53.0-91.0)	0.027
FEV ₁ /FVC*	72.0 (64.0-79.0)	61.0(49.0-68.0)	0.001
History of smoking (%)	16 (41%)	13 (48.1%)	0.62
Pack years*	0(0.0-7.5)	0(0.0-15.0)	0.703
Current smoking (%)	5 (12.8%)	1 (3.7%)	0.388
Total dose of inhaled corticosteroid	500 (139-586)	1000(1000-1173)	<0.0001
Daily 2 nd line controller	28 (72%)	24 (89%)	0.13
FEV ₁ < 80%	13 (33%)	16 (59.3%)	0.046
Use of short acting β-agonist	9 (23.1%)	12 (44.4)%	0.106
1 or more OCS burst /last year	9 (23.1)%	17 (63%)	0.002
3 or more OCS burst /last year	1 (3%)	7/27 (26%)	0.006
1 or more emergency room visit/last year	7 (18%)	8 (30%)	0.371
# hospitalization for asthma/last year	0 (0%)	4 (15%)	0.024
Nasal polyposis ^F	8 (20.5%)	8 (29.6%)	0.560

*Data were presented as median (Interquartile range) and analyzed by using Mann-Whitney U test for continuous variables. All other categorical variables were presented as n (%) and were analyzed by Fisher exact test; ** Atopy was determined as the presence of at least one positive skin test to a panel of 12 aeroallergens. Data was for mild-

moderate (n=34) and severe asthmatics (n= 26); [‡]Nasal polyposis represents those with a history of sinus disease and/or sinus surgery; *BMI*, body mass index; *OCS*, oral corticosteroid

Table 2. Frequency of severe asthma based on level of type-2 inflammatory markers

% CD4 ⁺ CRTh2 ⁺ T cells *			
	Low (n=33)	High (n=33)	<i>P</i>
		> 0.24%	
No of SA (%) [‡]	9 (27.3)	18 (54.5)	0.044
CRTh2 mRNA*			
	Low (n=27)	High (n=28)	<i>P</i>
		> 3.1	
No of SA (%) [‡]	7 (26)	15 (60)	0.024
IL-13*			
	Low (n=35)	High (n=30)	<i>P</i>
		> 3.12	
No of SA (%) [‡]	10 (28.6)	17 (56.7)	0.03
Eos (cells/μl)*			
	Low (n=37)	High (n=27)	<i>P</i>
		> 300	
No of SA (%) [‡]	17 (45.9)	10 (37)	0.61

* % CD4⁺CRTh2⁺ T cells/pWBC (median = 0.24%);

CRTh2 mRNA in whole blood calculated

as copies of CRTh2/1000 copies GAPDH (median = 3.1);

Serum IL-13 (high is above detection > 3.12 pg/ml);

Eos (eosinophils/ μ l blood; high is > 300 cells);

[†]SA, severe asthma; pWBC, peripheral white blood cells

Table 3. Level of CD4⁺CRTh2⁺ T cells coincides with other mediators of type-2 inflammation

	% CD4 ⁺ CRTh2 ⁺ T cells *		
	Low (n=33)	High (n=33) > 0.24%	P
CD4 ⁺ lymph	4.36 (3.3-6.7)	9.13 (6.4-13.05)	<0.0001
%CD4 ⁺ CRTh2 ⁺ T cells	0.16 (0.11-0.20)	0.41 (0.28-0.53)	<0.0001
[†] CRTh2 ⁺ Eos	0.94 (0.64-1.42)	2.09 (1.02-2.96)	0.001
Serum PGD ₂	369.5 (118.9-885.03)	339.43 (104.6-540.1)	0.254
Serum IL-13	3.1 (3.1-30.07)	3.1 (3.1-21.7)	0.627
Eos/ μ l *	300 (100-500)	200 (100-350)	0.278
**IL-13 after ex-vivo activation	(n=10) 278.2 (127.5-426.6)	(n=10) 641.8 (294.1-861.3)	0.049

* % CD4⁺CRTh2⁺ T cells/pWBC (median = 0.24%);

[†]CRTh2⁺CCR3⁺/peripheral blood;

Serum IL-13 (high is above detection > 3.12 pg/ml);

Eos (eosinophils/ μ l blood; high is > 300 cells);

**IL-13 was measured by ELISA following overnight stimulation of peripheral blood mononuclear cells from asthma patients (n=20) with PMA (20ng/ml) and Ionomycin (1μM); pWBC, peripheral white blood cells

Table 4. CD4⁺CRTh2⁺ T cells and exacerbation in severe asthmatics

% CD4 ⁺ CRTh2 ⁺ T cells *					
	Low (n=12)	**High (n=15)		P	
ERV, n (%)	1 (8.3%)	7 (46.7%)		0.043	
ROC for Exacerbation in Severe Asthmatics					
	^{\$} Optimum cut off	Sensitivity (%)	Specificity (%)	AUC	
% CD4 ⁺ CRTh2 ⁺ T cells *	0.26% (E=8)	100	57.9	0.753	0.041
**High CD4 ⁺ CRTh2 ⁺ T cells and ^{&} High PGD ₂ or IL-13					
	No ERV (n=7)	ERV (n=6)			
IL-13	20.73 (9.71-30.65)	22.93 (1-75.18)		0.774	
PGD ₂	259.61 (63.30-535.79)	868.26 (337.62-1576.02)		0.055	
ROC for Exacerbation in Severe Asthmatics with High CD4 ⁺ CRTh2 ⁺ T cells					
	^{\$} Optimum cut off	Sensitivity (%)	Specificity (%)	AUC	
^{&} High PGD ₂	[†] 0.4633	83	100	0.889	0.025

or IL-13[&] (E=6)

*% CD4⁺CRTh2⁺ T cells/pWBC; ** High CD4⁺CRTh2⁺ T cells (> 0. 26%)

ROC analysis for predicting exacerbation was performed in the severe asthma population (n=27) and severe asthmatics with **high % CD4⁺CRTh2⁺ T cells (n=15);

[§]Optimum cut off determined mathematically using R software; [‡]Combined optimum cutoff;

[&]High PGD₂ (> 346.1 pg/ml) or IL-13 (> 3.12 pg/ml);

pWBC, peripheral white blood cells; ERV, emergency room visit; ROC, receiver operating characteristic; AUC, area under the curve; E, exacerbators













