

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Th2 cell markers in peripheral blood increase during an acute asthma exacerbation

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Abstract

Background: Allergic asthma is characterized by type 2 inflammation. We have shown the presence of increased type 2 inflammation in patients with severe asthma and those with frequent exacerbations. However, it is not known whether increased type 2 inflammation drives asthma exacerbations. This study aims to determine Th2 immune parameters in patients presenting to the emergency department (ED) with an acute asthma exacerbation and correlate these parameters with clinical and physiological measures of asthma.

Methods: Sixteen adults presenting to the ED with acute asthma exacerbations were recruited after giving informed consent. Ten patients returned 2 weeks later for follow-up. Physiological parameters, asthma control (ACQ6), asthma quality of life (AQLQ) questionnaires, and venous blood were collected during both visits. An immune cell profiling was performed by whole blood flow cytometry: CD4⁺ T cells, Th2 cells (CD4⁺CRTh2⁺ T cells and % of CD4⁺ T cells expressing CRTh2), eosinophils and innate lymphoid cells (ILC2).

Results: During exacerbation, peripheral blood Th2 cell numbers correlated with ACQ6 and AQLQ scores, while ILC2 and eosinophil numbers did not. Subjects had higher % of CD4⁺ T cells expressing CRTh2 and worse FEV₁ during exacerbation compared with the follow-up. The decrease in the % of CD4⁺ T cells expressing CRTh2 seen during the follow-up visit correlated with the improvement in lung function.

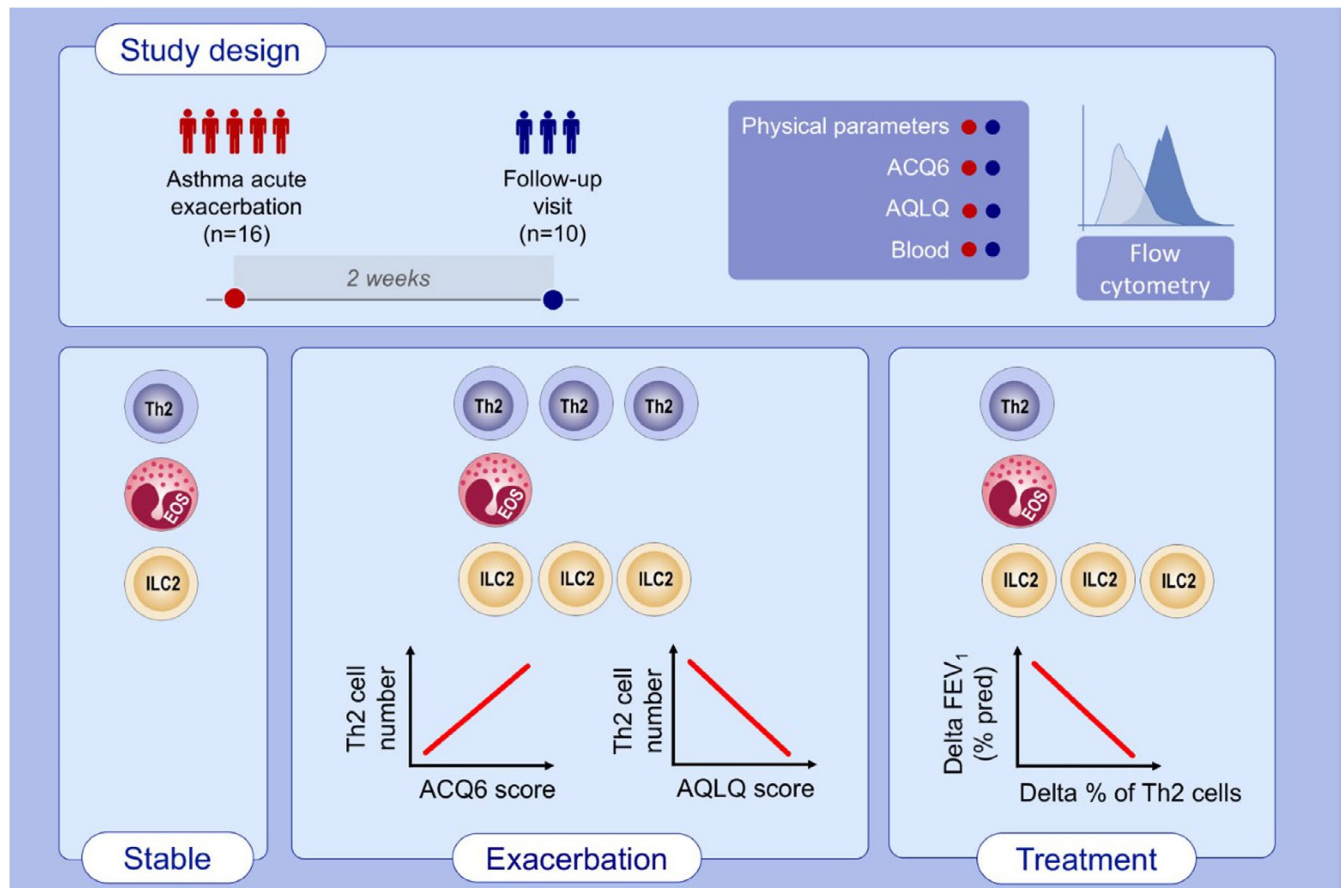
Conclusions: These data suggest that Th2 cells in peripheral blood may be a sensitive measure of increasing symptoms in patients with asthma exacerbations and may serve as a biomarker of an asthma exacerbation.

KEYWORDS

asthma, biomarkers, flow cytometry, lymphocytes, T cells

Abbreviations: ACQ6, Asthma control questionnaire; AQLQ, Asthma quality of life questionnaire; BMI, Body mass index; CRTh2, Chemoattractant receptor-homologous molecule expressed on T helper type 2 cells; ED, Emergency department; ELISA, Enzyme-linked immunosorbent assay; FEV₁, Forced expiratory volume in 1 second; FVC, Forced vital capacity; IL-13, Interleukin-13; ILC2, Innate lymphoid type-2 cells; PGD2, Prostaglandin D2; UAH, University of Alberta Hospital.

Harissios Vliagoftis and Lisa Cameron contributed equally to this work



GRAPHICAL ABSTRACT

Th2 cell number in peripheral blood correlates with asthma control and quality of life during asthma exacerbations. Patients with asthma exacerbations have a higher % of Th2 cells (CD4⁺ T cells expressing CRTh2) in peripheral blood compared to patients with stable asthma, and this parameter decreases after treatment. Eosinophils and ILC2 cells in peripheral blood do not change during asthma exacerbations. The decrease in the % of Th2 cells seen during the follow-up visit correlates with the improvement in lung function.

1 | INTRODUCTION

Asthma exacerbations, defined by Global Initiative for Asthma (GINA) as progressive increase in symptoms or decrease in lung function, requiring a change in treatment,¹ are a driver for increased healthcare costs.² Decreasing the frequency of exacerbations, especially in patients with severe asthma, is one of the main targets of asthma therapeutics and has been the primary outcome for most of the studies testing the efficacy of biologics.³ Despite the availability of effective therapeutic options, acute asthma exacerbations remain a frequent cause of emergency department (ED) and hospital admissions.⁴

One of the reasons for the persistence of asthma exacerbations as a driver for morbidity and healthcare cost in asthma is the lack of reliable biomarkers that could be easily obtained in everyday practice to predict acute asthma exacerbations. With better understanding of the pathophysiology of asthma, evaluation of biomarkers during and following exacerbation are needed so we can better predict severe exacerbations and understand which biomarkers correspond to exacerbation recovery. Symptom and quality of life

scores, airway physiology and a number of blood, sputum and/or urine biomarkers have been proposed, but lack of predictive value and various other problems have prevented widespread adoption.^{5,6} Immune parameters in induced sputum, primarily increased sputum eosinophil counts can predict asthma exacerbations, and follow-up of patients with regularly performed induced sputum is effective in preventing exacerbations.⁷⁻⁹ However, induced sputum is an expensive and time-consuming test, available only in specialized laboratories in urban tertiary centers. Identifying peripheral blood biomarkers would be an easier approach to achieve this objective.⁷

Type-2 inflammation is associated with asthma symptoms.¹⁰ Type-2 biomarkers such as peripheral blood eosinophils, fractional exhaled nitric oxide (FeNO), serum periostin and serum total IgE have different time courses and magnitude of change during and following treatment for a severe exacerbation of asthma.¹¹

Chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTh2), also called DP2, is a receptor for prostaglandin D₂ (PGD₂). In humans, CRTh2 expression by CD4⁺ T cells is considered a marker of Th2 cells,¹² but is also expressed by eosinophils and basophils¹³ as well as innate lymphoid type 2 cells

(ILC2).¹⁴ CRTh2 ligation by PGD₂ mediates Th2 cell activation leading to the chemotaxis, proliferation, and release of pro-inflammatory cytokines.^{15,16}

We have previously shown that blood CD4⁺CRTh2⁺ T-cell numbers are increased in patients with severe asthma compared to those with mild/moderate disease and that increased numbers are also associated with a phenotype with frequent asthma exacerbations.¹⁷ However, there are no data to indicate whether these cells present in the peripheral blood are directly associated within the pathophysiology of an asthma exacerbation or whether they could be used as a biomarker to predict impending asthma exacerbations.

In this study, we evaluated whether CD4⁺CRTh2⁺ T cells in the peripheral blood increase during a severe asthma exacerbation requiring emergency department (ED) visit and compared the changes in the presence of these cells with other markers of type-2 inflammation.

2 | METHODS

2.1 | Subjects

Patients over 18 years of age presenting to the ED of University of Alberta Hospital (UAH) with an acute asthma exacerbation, as per ED attending physician diagnosis, were recruited (March 2016-Dec 2018) during the visit. The study was approved by the institutional review board (approval number Pro00052055) and all subjects provided written informed consent. Patients received treatment as per ED guidelines without the involvement of the study investigators; all patients were given a 5-day course of oral corticosteroids and were instructed to adhere to their prescribed inhaler medications after discharge from ED. Eighteen patients were recruited, but data from sixteen were used for analyses; two were excluded because immune parameter analysis was not successful due to poor red blood cell lysis. After recruitment during the ED visit (visit 1), the patients were asked to return for a clinic visit 2 weeks later (visit 2 – follow-up visit). Ten patients attended the follow-up visit and their data were used in comparisons between the two visits.

Age, sex, body mass index (BMI), current smoking (%), history of >10 pack-years smoking (%), and oral corticosteroid use in the past year (%) were recorded from the ED chart at the time of recruitment. Percent predicted forced expiratory volume in 1 second (FEV₁, % predicted), ratio of FEV₁ to force vital capacity (FVC; FEV₁/FVC ratio), and the asthma control questionnaire (ACQ6)¹⁸ and asthma quality of life questionnaire (AQLQ)¹⁹ were collected at the time of the ED visit and during the follow-up visit. Venous blood was drawn during both visits. Immune parameters were studied in whole blood by flow cytometry and in serum by ELISA.

To study whether immune parameters during acute asthma exacerbations are different than during the stable phase of the disease, we compared data from the ED visits with those from a cohort of stable asthmatics we reported previously²⁰; all values used for comparison were taken from the fourth visit of the previous study.

2.2 | Profiling of peripheral blood immune cells

Blood was collected in sodium heparin tubes (BD Bioscience) and whole blood staining for flow cytometry was performed at room temperature. We used antibody panels for the identification of Th2 cells (CD4⁺CRTh2⁺ T cells [cells/μL] and % of CD4⁺ T cell expressing CRTh2), eosinophils (SSC^{high}CCR3⁺) and ILC2 (Lin⁻CD127⁺CRTh2⁺) by flow cytometry. Flow cytometry data were collected on BD LSR Fortessa (BD) using FACS Diva software and gates set in accordance with the profiles of the isotype control and/or negative control beads. Results were analyzed using FlowJo (TreeStar). Using flow cytometry (gating strategy described in Appendix S1 and shown in Figure S1), we calculated absolute numbers of CD4⁺ and CD4⁺CRTh2⁺ T cells in peripheral blood (cells/μL) as well as the % of CD4⁺ T cells expressing CRTh2. We also calculated absolute numbers of eosinophils (cells/μL) and ILC2 (cells/mL) in peripheral blood. Additional information on flow cytometry and gating strategy is provided in the article's Appendix S1.

2.3 | Enzyme-linked immunosorbent assay (ELISA)

Blood was collected in BD Vacutainer serum tubes, left to clot at room temperature (30 minutes) and then centrifuged at 300 g (10 minutes). Serum was aspirated, aliquoted, and stored at -20°C until use. Commercially available ELISA kits for IL-13 (Interleukin-13, Diaclone Research; detection limit 3.12 pg/mL) and PGD₂ (Prostaglandin D₂-MOX ELISA Kit, Cayman; detection limit 3 pg/mL) were used.

2.4 | Statistical analysis

Median values of clinical and immune parameters were calculated. Correlations between immune parameters, ACQ6 scores, and FEV₁ were analyzed using Spearman's Rank correlation coefficient. These values were further compared between those patients having two visits vs one visit and acute exacerbation vs stable asthma using the Mann-Whitney test for continuous variables and Fisher's exact test was used for categorical variables. Median values for clinical and immune parameters during exacerbation and after treatment were analyzed by nonparametric Wilcoxon rank test. Statistical analyses were performed using SPSS (version 21.1, Chicago, IL, USA). The *P* values of <.05 were considered significant.

3 | RESULTS

3.1 | Demographics and clinical characteristics

Demographics of the 16 patients included in our study are shown in Table 1. The median age of study patients was 30 years, BMI 29.4 and 50% were female. Although 37.5% of patients were current smokers, only 12.5% had a history of smoking >10 pack years (median pack

years 4.9). Figure S2 shows boxplots of all the physiological and peripheral blood immune parameters evaluated during ED visit.

3.2 | CD4⁺CRTh2⁺ T cells in peripheral blood correlate with asthma control and asthma quality of life during an acute asthma exacerbation

Associations between blood immune parameters and physiological parameters during ED visit were studied using Spearman's Rank correlation coefficient (Table 2). The number of CD4⁺CRTh2⁺ T cells

TABLE 1 Demographics and clinical characteristics of study subjects

Parameters	(N = 16)
Age (y)	30 (25-40)
Female patients	8 (50%)
BMI (kg/m ²) [†]	29.4 (23.8-32.2)
Smoking > 10 pack years	2 (12.5%)
Number of pack years	4.9 (0.6-10)
Current smoking	6 (37.5%)
Oral corticosteroids in the past year	9 (56%)
FEV ₁ (% predicted) [‡]	67.2 (42.7-77.7)
FEV ₁ /FVC (%) [§]	63.3 (52.6-71.6)
ACQ6	2.9 (2.2-4.4)
AQLQ	4.0 (3.3-5.1)

Note: Data were calculated as median (interquartile range) for continuous variables and n (%) for categorical variables. Because of missing data point, "n" for the categories labeled, [†]N = 14; [‡]N = 13; [§]N = 13.

Abbreviations: ACQ6: Asthma control questionnaire; AQLQ: Asthma quality of life questionnaire; BMI: Body mass index; FEV₁: Forced expiratory volume in 1 s; FVC: Forced Vital Capacity.

in peripheral blood was positively correlated with ACQ6 ($R = .553$, $P = .026$) scores and negatively correlated with overall AQLQ ($R = -.568$, $P = .022$) among the 16 patients included in this study. The number of CD4⁺CRTh2⁺ T cells in peripheral blood was also negatively correlated with the emotional function ($R = -.661$, $P = .005$) and for the activity limitation ($R = -.519$, $P = .039$) of AQLQ while there was a trend for correlation for symptoms ($R = -.456$, $P = .076$) domains (Table S1). In contrast, the number of total CD4⁺ T cells, eosinophils, and ILC2 in peripheral blood did not correlate with ACQ6 or AQLQ. Number of ILC2 in this study subjects were similar with previously published data.²¹ Further, there was a trend toward correlation for the number of CD4⁺CRTh2⁺ T cells with measures of obstruction, such as FEV₁, % predicted ($R = -.500$, $P = .082$), and FEV₁/FVC ($R = -.538$, $P = .058$). All the correlation coefficients between immune and physiological parameters and their P -values are shown in Table 2.

We then studied whether there were associations between the various immune parameters measured (Table 3). The number of CD4⁺ T cells was correlated with both the number of CD4⁺CRTh2⁺ T cells ($R = .709$, $P = .002$) and eosinophils ($R = .697$, $P = .003$) in peripheral blood. The % of CD4⁺ T cells expressing CRTh2 was negatively correlated with the number of CD4⁺ T cells ($R = -.634$, $P = .008$) and eosinophil cells ($R = -.555$, $P = .026$). The number of CD4⁺ T cells and eosinophil cells, but not CD4⁺CRTh2⁺ T cells, was positively correlated with the concentration of serum IL-13 ($R = .564$, $P = .036$ and $R = .708$, $P = .005$, respectively). Finally, the number of CD4⁺CRTh2⁺ T cells was negatively correlated with PGD₂ ($R = -.560$, $P = .037$).

3.3 | Patients with acute asthma exacerbations have a higher % of CD4⁺ T cells expressing CRTh2 than patients with stable asthma

Since we did not recruit control patients for this study, we then compared the subjects we recruited here with a previous cohort

TABLE 2 Correlation Coefficients between immune and physiological parameters in subjects during an acute asthma exacerbation (N = 16)

	CD4 ⁺ T cells in PB (cells/ μ L)	CD4 ⁺ CRTh2 ⁺ T cells in PB (cells/ μ L)	% CD4 ⁺ T cells expressing CRTh2	Eos in PB (cells/ μ L)	ILC2 in PB (cells/mL)	IL-13 (pg/mL)	PGD ₂ (pg/mL)
ACQ6	$R = .408$ $P = .117$	$R = .553$ $P = .026$	$R = -.049$ $P = .858$	$R = .366$ $P = .163$	$R = .215$ $P = .424$	$R = .402$ $P = .154$	$R = -.257$ $P = .375$
AQLQ	$R = -.409$ $P = .115$	$R = -.568$ $P = .022$	$R = .116$ $P = .670$	$R = -.325$ $P = .219$	$R = -.077$ $P = .778$	$R = -.318$ $P = .268$	$R = .192$ $P = .512$
FEV ₁ (% predicted) [‡]	$R = .198$ $P = .517$	$R = -.500$ $P = .082$	$R = -.681$ $P = .010$	$R = .701$ $P = .817$	$R = -.038$ $P = .901$	$R = .131$ $P = .686$	$R = .301$ $P = .342$
FEV ₁ /FVC (%) [§]	$R = -.099$ $P = .748$	$R = -.538$ $P = .058$	$R = -.401$ $P = .174$	$R = -.280$ $P = .354$	$R = -.165$ $P = .590$	$R = -.145$ $P = .653$	$R = -.168$ $P = .602$

Note: P -value was calculated by Spearman's rank correlation coefficient. Because of missing data point, "n" for the categories labeled, [‡]N = 13; [§]N = 13.

Bold indicates a statistically significant difference with a P -value less than 0.05.

Abbreviations: ACQ6, Asthma control questionnaire; AQLQ, Asthma quality of life questionnaire; FEV₁, Forced expiratory volume in 1 s; FVC, Forced Vital Capacity; PB, Peripheral blood.

TABLE 3 Correlation coefficients between immune parameter in subjects during an acute asthma exacerbation (N = 16)

	CD4 ⁺ T cells in PB (cells/ μ L)	CD4 ⁺ CRTh2 ⁺ T cells in PB (cells/ μ L)	% of CD4 ⁺ T cells expressing CRTh2	Eos in PB (cells/ μ L)	ILC2 in PB (cells/mL)	IL-13 (pg/mL)	PGD ₂ (pg/mL)
CD4 ⁺ T cells in PB (cells/ μ L)	---						
CD4 ⁺ CRTh2 ⁺ T cells in PB (cells/ μ L)	<i>R</i> = .709 <i>P</i> = .002	---					
% of CD4 ⁺ T cells expressing CRTh2	<i>R</i> = -.634 <i>P</i> = .008	<i>R</i> = -.001 <i>P</i> = .996	---				
Eos in PB (cells/ μ L)	<i>R</i> = .697 <i>P</i> = .003	<i>R</i> = .450 <i>P</i> = .080	<i>R</i> = -.555 <i>P</i> = .026	---			
ILC2 in PB (cells/mL)	<i>R</i> = -.097 <i>P</i> = .721	<i>R</i> = .135 <i>P</i> = .617	<i>R</i> = .440 <i>P</i> = .088	<i>R</i> = .044 <i>P</i> = .871	---		
IL-13 (pg/mL)	<i>R</i> = .564 <i>P</i> = .036	<i>R</i> = .434 <i>P</i> = .121	<i>R</i> = -.297 <i>P</i> = .302	<i>R</i> = .708 <i>P</i> = .005	<i>R</i> = .245 <i>P</i> = .399	---	
PGD ₂ (pg/mL)	<i>R</i> = -.218 <i>P</i> = .455	<i>R</i> = -.560 <i>P</i> = .037	<i>R</i> = -.156 <i>P</i> = .594	<i>R</i> = -.007 <i>P</i> = .982	<i>R</i> = -.059 <i>P</i> = .840	<i>R</i> = .025 <i>P</i> = .933	---

Note: *P*-value was calculated by Spearman's rank correlation coefficient.

Bold indicates a statistically significant difference with a *P*-value less than 0.05.

Abbreviation: PB, Peripheral blood.

TABLE 4 Comparison of demographics and clinical characteristics between patients with an asthma exacerbation and stable asthmatics

Parameters	Exacerbation (N = 16)	Stable (N = 18)	<i>P</i>
Age (y)	30 (25-40)	46 (30-56)	.004
Female patients	8 (50%)	11 (61.1%)	.721
BMI (kg/m ²) [†]	29.4 (23.8-32.2)	30.1 (23.1-38.8)	.879
Current Smoking	6 (37.5%)	0 (0%)	.003
FEV ₁ (% predicted) [‡]	67.2 (42.7-77.7)	79.5 (70.2-91.5)	.037
FEV ₁ /FVC (%) [§]	63.3 (52.7-71.6)	80.5 (65.5-84.5)	.025
ACQ6	2.9 (2.2-4.4)	0.8 (0.3-2.0)	<.0001
Eos in PB (cells/ μ L)	94 (31-493)	138 (73-243)	.971
ILC2 in PB (cells/mL)	1924 (1499-2378)	1366 (893-2197)	.049
CD4 ⁺ T cells (cells/ μ L)	492 (152-664)	904 (524-1346)	.016
CD4 ⁺ CRTh2 ⁺ T cells in PB (cells/ μ L)	25(12-38)	29 (14-46)	.605
% of CD4 ⁺ T cells expressing CRTh2	5.96 (3.7-7.6)	3.2 (2.3-3.9)	.003

Note: Data were calculated as median (interquartile range) for continuous variable and n (%) for categorical variables. *P*-value was calculated by Mann-Whitney U test. Because of missing data point, "n" for the categories labeled, [†]N = 14; [‡]N = 13; [§]N = 13 for exacerbation.

Bold indicates a statistically significant difference with a *P*-value less than 0.05.

Abbreviations: ACQ6, Asthma control questionnaire; BMI, Body mass index; FEV₁, Forced expiratory volume in 1 s; FVC, Forced Vital Capacity; PB, Peripheral blood.

consisting of patients with stable asthma.²⁰ Demographics, clinical characteristics as well as physiological and immunological parameters for patients with an acute exacerbation and those with stable asthma are shown in Table 4. There was a significant difference in the median age between the two groups (*P* = .004), though biological sex and median BMI were similar (*P* > .05). A significant difference was observed in the number of current smokers between

acute asthma exacerbation (37%) vs those with stable asthma (0%, *P* = .003). Patients with acute asthma exacerbations were more likely to have poor asthma control compared to those with stable asthma (2.9 vs 0.8, *P* < .0001) and had a lower FEV₁/FVC ratio (*P* = .025) and FEV₁ % predicted (*P* = .037). Compared to patients with stable asthma, those with acute asthma exacerbation had significantly lower numbers of CD4⁺ T cells (492 vs 904, *P* = .016), while those

with acute asthma had a higher % of CD4⁺ T cells expressing CRTh2 compared to stable asthma (5.96 vs 3.2, $P = .003$, Table 4).

Since we had a difference in age and smoking status between the two cohorts, we performed two separate sub-group analyses. We first limited both cohorts only to subjects 20-50 years old and we also analyzed only the nonsmoking subjects of the cohorts. In both cases, these sub-groups analyses showed the same results as the full dataset, specifically a lower number of CD4⁺ T cells and a higher % of CD4⁺ T cells expressing CRTh2 in the patients with acute exacerbations compared to patients with stable asthma (Tables S3 and S4). In addition, the differences in pulmonary lung functions, asthma control, and quality of life measures still held in these sub-group analyses.

3.4 | Changes in % CD4⁺ T cells expressing CRTh2 between exacerbation and follow-up visits

Among the 16 patients recruited, 10 returned for the follow-up visit. We compared the returning group with those that did not return (Table S2) to understand if there were differences between those returning and those that did not and whether these differences could affect the interpretation of the data. No significant differences were observed in age, BMI, ACQ6, AQLQ, use of oral corticosteroid in the past year or any of the immune parameters studied between the two groups. Patients who did not return for the follow-up visit had higher rates of current smoking (83.3% vs 10.0%, $P = .008$) and better lung function (FEV₁% predicted, 76.5% vs 59.8%, $P = .045$).

We compared asthma control and quality of life as well as pulmonary function changes between the ED and follow-up visits (Table 5).

Overall, FEV₁ (% predicted) increased and ACQ6 decreased significantly during the follow-up visit (FEV₁, 59.8% vs 82.7%, $P = .008$, Figure 1B; ACQ6, 3.2 vs 2.3, $P = .041$) indicating that treatment improved both lung function and asthma symptoms. We also compared immune parameters between the two visits. Asthmatic patients during ED visit had a lower number of CD4⁺ T cells compared with the follow-up visit (470 vs 761, $P = .022$). During exacerbation, asthmatic patients had a higher % of CD4⁺ T cells expressing CRTh2 compared with the follow-up visit (6.8 vs 3.0, $P = .005$, Figure 1A and C). It is also interesting that % of CD4⁺ T cells expressing CRTh2 during the second visit decreased to similar levels with % of CD4⁺ T cells expressing CRTh2 in stable asthmatics (Figure 1A). In contrast, total number of CD4⁺CRTh2⁺ T cells in peripheral blood did not change between the two visits. Therefore, changes in % of CD4⁺ T cells expressing CRTh2, may reflect the change on the total number of % of CD4⁺ T cells between the exacerbation and follow-up visits. There was no change in the number of ILC2 and eosinophils in peripheral blood or serum level of IL-13 and PGD₂ between the two visits (Table 5).

Since asthmatic patients had lower lung function (Figure 1B) and a higher % of CD4⁺ T cells expressing CRTh2 during exacerbation compared with follow-up (Figure 1C), we calculated the difference in % CD4⁺ T cells expressing CRTh2 (delta % CD4⁺ T cells expressing CRTh2 = exacerbation-treatment) and the difference in FEV₁ (delta FEV₁ = exacerbation-treatment) and found a significant negative correlation between the change in FEV₁ and % CD4⁺ T cells expressing CRTh2 ($R = -.783$, $P = .013$; Figure 1D). This analysis indicates a drop in the proportion of CD4⁺ T cells expressing CRTh2 coincides with improvement in lung function in asthmatics following treatment for an acute exacerbation.

	Exacerbation	Treatment	P
FEV ₁ (% predicted) [‡]	59.8 (39.3-72.9)	82.7 (76.8-89.7)	.008
FEV ₁ /FVC (%) [§]	61.7 (49.9-71.6)	70.1 (68.0-80.8)	.038
ACQ6	3.2 (1.7-4.5)	2.3 (1.4-3.4)	.041
AQLQ	4.3 (3.2-5.8)	5.4 (2.8-5.8)	.445
Eos in PB (cells/μL)	72.0 (31-258)	196 (77-467)	.169
ILC2 in PB (cells/mL)	1640 (1321-2364)	1855 (1024-2611)	.508
CD4 ⁺ T cells (cells/μL)	470 (189-577)	761 (560-1851)	.022
CD4 ⁺ CRTh2 ⁺ T cells in PB (cells/μL)	22 (14-43)	26 (17-44)	.646
% of CD4 ⁺ T cells expressing CRTh2	6.8 (3.6-10.2)	3.02 (2.6-3.4)	.005
IL-13 (pg/mL)	3.8 (1-22.1)	9.6 (2.1-72.3)	.31
PGD ₂ (pg/mL)	263.6 (76.4-774.1)	426.9 (201.2-603.3)	.575

Note: Data were calculated as median (interquartile range) for continuous variable and n (%) for categorical variables. P -value was calculated by Wilcoxon-rank test. Because of missing data point, "n" for the categories labeled, [‡]N = 9 and [§]N = 9 for exacerbation and treatment.

Bold indicates a statistically significant difference with a P -value less than 0.05.

Abbreviations: ACQ6, Asthma control questionnaire; AQLQ, Asthma quality of life questionnaire; FEV₁, Forced expiratory volume in 1 s; FVC, Forced Vital Capacity; PB, Peripheral blood.

TABLE 5 Comparison of immune and physiological parameters between visit 1 and visit 2 (N = 10)

4 | DISCUSSION

In this study, we recruited patients with an acute asthma exacerbation upon presentation to the ED and studied physiological and immunological parameters to identify parameters that may characterize an asthma exacerbation. Previous studies have identified that therapeutic approaches targeting increased sputum eosinophilia can prevent asthma exacerbations.^{22,23} Sputum-based molecular biomarkers are, however, not widely available in clinical practice, and other valid and reliable biomarkers are urgently needed. In our attempt to identify such biomarkers, we have performed immune cell profiling of peripheral blood by flow cytometry; even though flow cytometric assays will still require a specialized laboratory, the source material is easily obtainable from patients. Parameters studied here and shown to be increased during an asthma exacerbation could be further tested to evaluate their usefulness as biomarkers of impending exacerbations.

Our study shows that the number of Th2 cells ($CD4^+CRTh2^+$ T cells) in peripheral blood during an asthma exacerbation correlates with asthma control and asthma quality of life measures, but not with pulmonary function parameters. This finding is in contrast to our previous

report in a population of stable asthmatics that showed the number of Th2 cells in peripheral blood did not correlate with ACQ6 scores.²⁰ It is possible to speculate that the increased numbers of Th2 cells and possibly their activation status during an asthma exacerbation affect asthma control and subsequently quality of life in these subjects; especially if a previously reported²⁴ pathogenic phenotype of Th2 cells is overrepresented in patients during acute asthma exacerbations. On the other hand, this may not be the case for patients with stable disease and lower numbers of Th2 cells. This observation also suggests that the increase in Th2 cells during an asthma exacerbation may have a pathogenic role in asthma exacerbations. Further studies are needed to delineate these possibilities. Although we have previously reported that Th2 cells correlate with asthma severity and with a phenotype of frequent asthma exacerbations in patients with severe asthma, this is the first report showing that Th2 cells are increased during an acute asthma exacerbation, compared to the stable phase of the disease, and that they decrease rapidly following treatment.

It is also interesting that the numbers of eosinophils and ILC2 in peripheral blood, cells that have been previously associated with asthma pathophysiology,²⁵ do not correlate with any of the clinical and

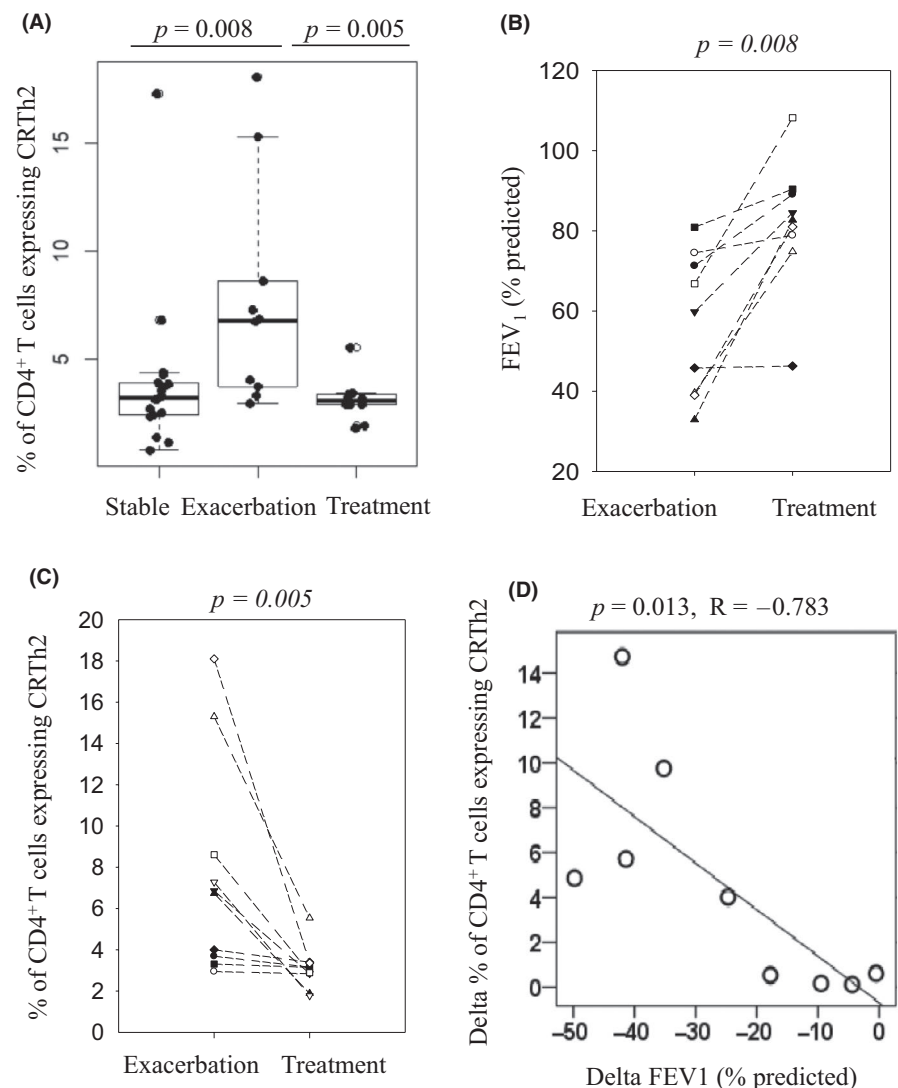


FIGURE 1 A, % of $CD4^+$ T cells expressing CRTh2 in patients with stable asthma (stable) and patients with an asthma exacerbation during the first (exacerbation) and the second (treatment) visits. B, FEV₁ (% predicted) (C) and % of $CD4^+$ T cells expressing CRTh2 during exacerbation and treatment visits for the patients included in the study (D) Correlation of changes in FEV₁ and % of $CD4^+$ T cells expressing CRTh2 between exacerbation and treatment visits ($n = 10$ in all cases)

physiological parameters in our patient population. In a model of allergic airway inflammation, ILC2 cell numbers decreased in blood and increased in lung tissue after inhalation allergen challenge²⁶ and they also decreased in blood after nasal challenge.²⁷ Similarly, eosinophils in induced sputum increase during exacerbations²³ or after an inhalation allergen challenge.²⁸ These observations along with our data suggest that for eosinophils and ILC2, in contrast to Th2 cells, their presence in lung tissue and/or their activation status in the lungs/airways, but not their numbers in peripheral blood, may be associated with asthma pathophysiology and predictive of asthma control and/or quality of life.

A second interesting observation was that compared to patients with stable asthma, those with an acute asthma exacerbation had significantly lower numbers of CD4⁺ T cells in peripheral blood. Similar decrease in CD4⁺ lymphocytes has been shown in children with asthma exacerbations.²⁹ The reason for this result is not clear. On the other hand, presence of viral infections leading to asthma exacerbations may also in part responsible for this effect since viral infections can cause lymphopenia.^{30,31} We have no direct data supporting the presence of viral infections in our study population. However, plasmacytoid dendritic cells (pDCs), which are the main producers of IFN α in response to RNA or DNA viruses³² and are decreased in the peripheral blood of children during RSV-induced asthma exacerbations,³³ are also significantly lower in our population during the ED visit compared with the treatment visit (manuscript in preparation), suggesting a potential viral etiology for the exacerbations. Alternatively, this decrease in lymphocyte numbers may be the result of lymphocytes being recruited to the airways, since there is evidence that induced sputum lymphocyte counts increase prior to and during an asthma exacerbation.³⁴ We also observed that the number of CD4⁺ T cells increase during the follow-up visit, supporting the hypothesis that decreased lymphocyte numbers are associated with the pathophysiology of asthma exacerbations. However, we have no direct data to support one or the other of these hypotheses.

We found that IL-13 correlates with eosinophil and CD4⁺ T-cell numbers, but not with the number of ILC2 cells. Unfortunately, we were not able to detect consistently IL-4 or IL-5 in the serum samples from the patients we report here (data not shown). The correlation between eosinophils and IL-13 may suggest that eosinophils, that have been shown to produce IL-13,³⁵ are a major source of IL-13 in peripheral blood in this population. On the other hand, the finding that the number of CD4⁺ T cells in peripheral blood was positively correlated with serum IL-13, though the number of Th2 cells did not, may suggest that CD4⁺ T cells other than CRTh2⁺ cells, release IL-13 during an asthma exacerbation. This could also mean, however, that serum IL-13, possibly released from eosinophils, affects the number of CD4⁺ cells through indirect mechanisms. Finally, it is interesting that ILC2 cell numbers do not correlate with IL-13 levels, even though ILC2 cells are considered as one of the major cell types producing IL-13,³⁶ a belief based primarily on their ability to release high amount of cytokines *ex vivo*. Their relative contributions *in vivo*, especially considering their small numbers, are not as clear. For example, in our experiments, we identified approximately 13 times more Th2 cells and 48 times more eosinophils than ILC2, indicating that cumulatively these other cells may have higher effects on cytokine levels.

Our observation that the proportion of Th2 cells within the CD4 population (% CD4⁺ T cells expressing CRTh2) was high during the ED visit and decreased during the follow-up visit to levels similar to those of stable asthmatics suggests that Th2 cells may be a biomarker of imminent exacerbations. We have previously shown a similar increase in frequency of Th2 cells in the peripheral blood of patients with severe asthma compared to mild/moderate disease and to patients prone to frequent exacerbations.³⁷ It is interesting that in our study we also show a decrease in total lymphocyte counts during the acute exacerbation, which may have precipitated the relative change in Th2 cell frequency compared with other T-cell subsets, since the number of Th2 cells/ μ L of peripheral blood did not change (Table 5). Previous studies have also shown that the frequency of CD4⁺ T cells producing type-2 cytokines is increased during an asthma exacerbation,³⁸ similar to what we have shown in our study. However, previous studies did not report total cell numbers, so it was unclear whether their finding was an increase in the number of these cells or just a relative change in their frequency compared to other T-cell subsets. Independently of the reason for the change in Th2 frequency, our observation indicates a shift in the balance of Th subsets 2 weeks after an exacerbation, although we do not have data for numbers of Th1 or Th17 cells. Whether the observed alteration in Th2 cell balance could be ascribed to the use of oral corticosteroids, or other reasons that happen after 15 days of treatment of an acute asthma exacerbation, is not clear at this point. Further studies will be needed to clarify this issue.

Recent data have generated a lot of interest in the use of CRTh2 antagonists as a therapeutic strategy for asthma.^{39,40} If an asthma exacerbation is characterized by increased presence of CRTh2⁺ lymphocytes within the lymphocyte pool as our data suggest, and since these cells are expected to have a pathogenic role in type 2 inflammation,²⁴ treatment with CRTh2 antagonists during an exacerbation may prove beneficial either as a more directed approach than oral corticosteroids, or at least as an add-on to corticosteroid-based therapies. To understand the dynamics of CRTh2⁺ lymphocytes and their increase in numbers during exacerbation, further time-dependent longitudinal studies are needed to determine the potential utility of such an approach for the treatment of asthma exacerbations. Alternatively, a study following stable asthmatics on monthly basis until they reach the point of exacerbation could help clarify the role of changes in Th2 cells in stable compared to acute exacerbating asthma.

Other Th2 immune parameters, such as eosinophil and ILC2 numbers, did not change between the ED and the follow-up visits. This is interesting since eosinophils are sensitive to corticosteroids and a decrease in their numbers after a 5-day course of oral corticosteroids would be expected. Although oral prednisone has been shown to decrease sputum and blood eosinophils in patients with an acute exacerbation of asthma,²² a previous report has shown that the blood numbers are increased again 10 days after discontinuation of oral corticosteroid. This published observation is in line with our finding of no difference in eosinophil numbers between the exacerbation and follow-up visits, since the follow-up visit occurred approximately 10 days after the end of the 5-day course of oral corticosteroids prescribed during the acute exacerbation visit.

Further, the pathway involved in increased PGD₂/CRTh2 activation needs to explore in this setting. Exacerbation can be triggered by allergen-, bacteria-, and virus-induced release of PGD₂, which initiates a signaling cascade through CRTh2 on Th2 cells with the release of Th2 cytokines, which promote IgE class-switching in B lymphocytes. However, PGD₂ activation of CRTh2 also inhibits Th2 cells apoptosis⁴¹ and so is also considered to contribute to type 2 immunity by sustaining Th2 cells.

This study has several limitations that must be discussed. First, clinical data to validate the diagnosis of asthma in our recruited patients are lacking. The diagnosis of asthma was assumed from the ED diagnosis of "asthma exacerbation" following history, physical examination, and response to therapy. One concern would be whether patients with COPD exacerbations may have been included in our cohort. The fact that our subjects were young (median age 32.6) with a fairly short smoking history (37.5% current smokers but median smoking of 4.9 pack years and none of the patients had more than 12 pack-years history of smoking) decrease the likelihood of contamination of our cohort with patients with COPD.

A limitation of our study was lack of data from our cohort before the development of the asthma exacerbation, so we cannot conclude that Th2 cells increased due to the exacerbation. To address this issue, we presented data (Table 4) comparing our ED cohort with a cohort of patients with stable asthma we had previously recruited. Since, the acute exacerbation cohort was younger and included more smokers, we have performed a sub-analysis by excluding smokers from the acute exacerbation cohort which showed similar results, similar to the main dataset as we expected based on our previous paper.¹⁷ We also performed an aged-matched analysis between these two cohorts that also retained the finding of differences in % of CD4⁺ T cells expressing CRTh2. Finally, the sample size was small due largely to decreasing numbers of patients presenting to the UAH ED with an acute asthma exacerbation. A larger study, possibly in association with sputum induction during the two visits, may be needed to validate these results.

In conclusion, this study shows Th2 cells are high during an asthma exacerbation and return to levels similar to those from a cohort of stable asthmatics following aggressive treatment with systemic corticosteroids. Therefore, determination of the representation of Th2 cells in the CD4⁺ T-cell compartment may be biomarkers of an impending asthma exacerbation. Larger longitudinal prospective studies on changes in markers of Th2 immunity before an exacerbation and during recovery will improve our understanding of their role in asthma exacerbations.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

NSP participated in study design and the development of documents required for ethics approval, performed flow cytometry experiments, and data analysis and authored for the first draft of the manuscript. YW, EK, and VDG performed flow cytometry and participated in blood sample collection process. BR recruited patients from Emergency Department, University of Alberta, participated in the study design and supervised clinical data collection and reviewed the draft of the manuscript. HV was responsible for study design, supervised data collection, and authored the final draft of the manuscript. LC was responsible for study design and supervised data collection and reviewed the final draft of the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Appendix S1

Online Repository

Th2 cell markers in peripheral blood increase during an acute asthma exacerbation

Methods

Flow cytometry

To block non-specific binding of antibodies Fc blocker (Miltenyi Biotec, Auburn, CA, USA), mouse IgG (50µg; Invitrogen, Burlington, ON, Canada) and rat IgG (50µg; Invitrogen) were added to whole blood and incubated for 20 min at room temperature (RT). Whole blood was then incubated with biotinylated anti-CRTh2 (clone BM16; Miltenyi Biotec) or isotype control antibody for CRTh2 (rat IgG2a; AbD Serotech, Raleigh, NC, USA) for 30 min at RT. Whole blood was then lysed by red blood cell lysis buffer (BD Bioscience) for 15 min with vortexing (10sec) before centrifugation. Cells were centrifuged at 300xg (5 min) and washed with 2 ml of FACS buffer (PBS, 0.5% BSA, 0.1% NaN₃, 3% FBS). For detection of CRTh2⁺ cells streptavidin-APC (200ng/µl) was added (eBioscience, San Deigo, CA, USA). Multi-colour flow cytometry was performed using the following antibodies or isotype control for cell identification: anti-CD4 (FITC, Clone 1F6; Serotec, Oxford, UK), anti-CCR3 (PE, Clone 5E8; BD Bioscience), anti-CD3 (APC, Clone HIT3a; BD Bioscience), Lin 1 (FITC, anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, anti-CD56 - all from BD Bioscience), anti-CD127 (BV421, Clone HIL-7R-M21; BD Bioscience), anti-FcεR1 (FITC, Clone AER-37 [CRA1]; eBioscience), anti-CD11c (FITC, Clone B-ly6; BD Bioscience), anti-CD123 (FITC, Clone 6H6; eBioscience).

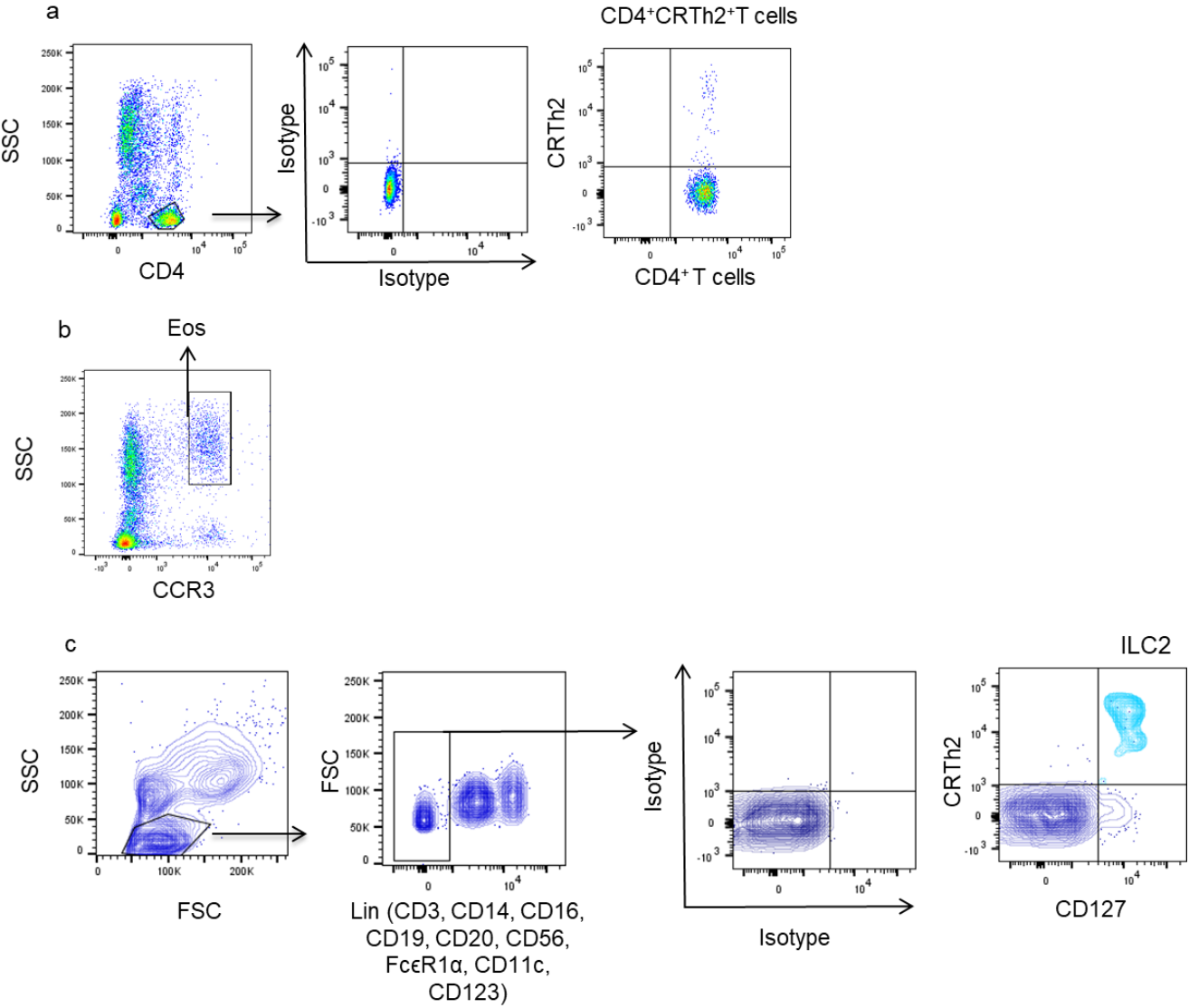
BD CompBeads Plus (BD CompBeads are beads that have been coupled to an antibody specific for the kappa light chain of immunoglobulin (Ig) from mouse, anti-mouse Ig, κ; BD Bioscience) were used to establish compensation corrections for spectral overlap for any combination of

fluorochrome-labeled antibodies. Cells and compensation bead tubes were incubated in the dark (30 min), washed with 2ml of PBS, centrifuged (300xg, 5min) and resuspended in 250 µl of 2% paraformaldehyde (Sigma Aldrich).

Flow cytometry data were collected on BD LSR Fortessa (BD Bioscience, CA, USA) using FACS Diva software and gates set in accordance with the profiles of the isotype control and/or negative control beads. Results were analyzed using FLOWJO (TreeStar, Ashland OR, USA).

Gating strategy for flow cytometry analysis: Figure S1A shows the gating strategy for CD4 T cells identified as low SSC CD4 expressing cells ($SSC^{low}CD4^{+}$). This method of identifying $CD4^{+}$ T cells was confirmed with double staining with CD3, which showed the selected population to be over 99% positive for CD3.¹ The % of $CD4^{+}CRTh2^{+}$ T cells was calculated with reference to CRTh2 isotype control. Figure S1B shows the gating strategy for eosinophils that were identified as high side scatter (SSC) and CCR3 expressing cells ($SSC^{high}CCR3^{+}$), as we have also done previously.² This gating strategy excludes other CCR3-expressing cells, such as peripheral blood fibrocytes²⁻⁴, Th2 cells⁵ and basophils⁶ as these cells have lower side scatter and are found in the lymphocyte and monocyte gates of forward and side scatter plots, gates that are not included in our analysis of eosinophils. Group 2 innate lymphoid cells type 2 (ILC2) were identified within the lymphocyte region of an SSC/FSC scatter plot as $Lin^{negative}$ (CD3, CD14, CD16, CD19, CD20, CD56, FcεR1α, CD11c, CD123) cells expressing CRTh2 and CD127 (Figure S1C).

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Fig S1

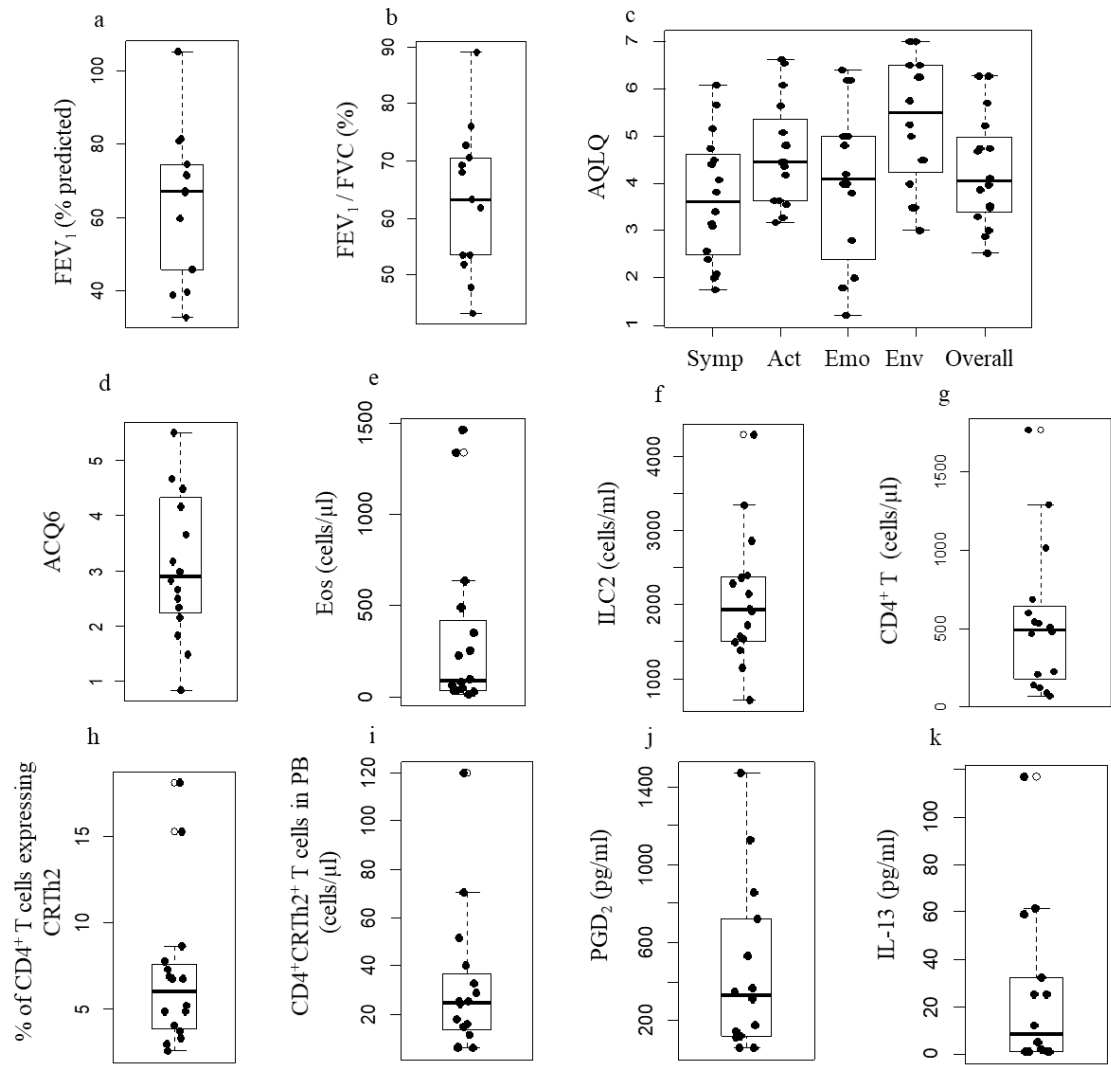


Fig S2

45 Figure legend

46 S1. Gating strategy for flow cytometry analysis of peripheral blood immune parameters, a. Th2
47 cells; b. eosinophils; c. ILC2.

48 S2. Boxplots of a. FEV₁ (% predicted); b. FEV₁/FVC (%); c. AQLQ scores; d. ACQ6 scores
49 [Symp: Symptom, Act: Activity limitation, Emo: Emotional function, Env: Environmental
50 stimuli]; e. Eos (cells/ μ l); f. ILC2 (cells/ml); g. CD4⁺ T (cells/ μ l); h. % of CD4⁺ T cells expressing
51 CRTh2; i. CD4⁺CRTh2⁺ T cells (cells/ μ l); j. PGD2 (pg/ml); and k. IL-13 (pg/ml) (n=16).

Table S1. Correlation coefficients between AQLQ domains and immune parameter during an acute asthma exacerbation (N=16)

AQLQ	CD4 ⁺ T cells in PB (cells/ μ l)	CD4 ⁺ CRTh2 ⁺ T cells in PB (cells/ μ l)	% of CD4 ⁺ T cells expressing CRTh2	Eos in PB (cells/ μ l)	ILC2 in PB (cells/ml)	IL-13 (pg/ml)	PGD ₂ (pg/ml)
Symptoms	R = -0.256 <i>p</i> = 0.339	R = -0.456 <i>p</i> = 0.076	R = -0.004 <i>p</i> = 0.987	R = -0.250 <i>p</i> = 0.350	R = -0.168 <i>p</i> = 0.535	R = -0.236 <i>p</i> = 0.417	R = 0.213 <i>p</i> = 0.464
Activity limitation	R = -0.372 <i>p</i> = 0.156	R = -0.519 <i>p</i> = 0.039	R = 0.113 <i>p</i> = 0.678	R = -0.274 <i>p</i> = 0.305	R = 0.106 <i>p</i> = 0.696	R = 0.234 <i>p</i> = 0.421	R = 0.147 <i>p</i> = 0.615
Emotional function	R = -0.491 <i>p</i> = 0.054	R = -0.661 <i>p</i> = 0.005	R = 0.082 <i>p</i> = 0.762	R = -0.390 <i>p</i> = 0.135	R = -0.098 <i>p</i> = 0.719	R = -0.468 <i>p</i> = 0.091	R = 0.283 <i>p</i> = 0.327
Environmental stimulation	R = -0.543 <i>p</i> = 0.030	R = -0.290 <i>p</i> = 0.276	R = 0.442 <i>p</i> = 0.087	R = -0.349 <i>p</i> = 0.185	R = 0.135 <i>p</i> = 0.619	R = -0.408 <i>p</i> = 0.148	R = -0.100 <i>p</i> = 0.735

AQLQ is a disease-specific health-related quality of life questionnaire includes 32 questions grouped into 4 domains: symptoms (12 items), activity limitations (11 items), emotional function (5 items), and environmental stimuli (4 items). Each question is scored on a 7-point Likert scale, which ranges from 7 (no impairment) to 1 (severe impairment). The overall score is calculated as the mean of all questions, and the 4 domain scores are the means of the scores for the questions in the respective domains.

Table S2. Comparison between demographics and clinical characteristics between patients that returned for a second visit and those that did not

<i>Clinical Parameters</i>			
	Both visits (N=10)	One visit (N=6)	<i>p</i>
Age (y)	31 (26-41)	28 (23-43)	0.786
Female patients	5 (50%)	3 (50%)	1
BMI (kg/m²)[†]	29.4 (23.5-32.2)	28.0 (23.5-62.1)	0.888
Current smoking	1 (10%)	5 (83.3%)	0.008
Oral corticosteroids in the past year	6 (60%)	3 (50%)	1
ACQ6	3.2 (1.7-4.5)	2.8 (2.4-3.7)	0.828
AQLQ	4.3 (3.2-5.8)	4.0 (3.4-4.7)	0.745
FEV₁ (% predicted)[‡]	59.8 (39.3-72.9)	76.5 (68.3-99.2)	0.045
FEV₁/FVC[§]	61.7 (49.9-71.6)	66.3 (55.9-84.0)	0.355
<i>Blood Immune Parameters</i>			
Eos in PB (cells/μl)	72.0 (31-258)	374 (41-1376)	0.193
ILC2 in PB (cells/ml)	1640 (1321-2364)	2213 (1819-3757)	0.129
CD4⁺ T cells (cells/μl)	470 (189-577)	550 (107-1205)	0.588
CD4⁺ CRTh2⁺ T cells in PB (cells/μl)	22 (14-43)	25 (6-51)	0.914
% of CD4⁺ T cells expressing CRTh2	6.8 (3.6-10.2)	5.0 (4.3-7.0)	0.515
IL-13 (pg/ml)	3.8 (1-22.1)	29 (1-59)	0.428
PGD₂ (pg/ml)	263.6 (76.4-774.1)	338.5 (137.3-825.6)	0.699

ACQ6: Asthma control questionnaire; AQLQ: Asthma quality of life questionnaire; BMI: Body mass index; FEV₁: Forced expiratory volume in 1 sec; FVC: Forced Vital Capacity; PB: Peripheral blood; Data was calculated as median (interquartile range) for continuous variable and n (%) for categorical variables. *p*-value was calculated by Mann-Whitney U test. Because of missing data point, “n” for the categories labeled, [†]N=10 vs 4, [‡]N=9 vs 4 for both visits vs one visit respectively.

Table S3. Comparison of demographics and clinical characteristics between patients with an asthma exacerbation and stable asthmatics after excluding current smokers

Parameters	Exacerbation (N=10)	Stable (N=18)	<i>p</i>
Age (y)	31 (26-49)	46 (30-56)	0.031
Female patients	5 (50%)	11 (61.1%)	0.698
BMI (kg/m ²)*	28.8 (22.9-31.9)	30.1 (23.1-38.8)	0.471
Current Smoking	0 (0%)	0 (0%)	1
FEV ₁ (% predicted) **	52.8 (39.1-70.2)	79.5 (70.2-91.5)	0.003
FEV ₁ /FVC (%)**	57.6 (48.9-69.9)	80.5 (65.5-84.5)	0.009
ACQ6	3.2 (1.7-4.5)	0.8 (0.3-2.0)	0.001
Eos in PB (cells/μl)	62.0 (31-490)	138 (73-243)	0.504
ILC2 in PB (cells/ml)	1749 (1321-2364)	1366 (893-2197)	0.250
CD4 ⁺ T cells (cells/μl)	470 (189-618)	904 (524-1346)	0.013
CD4 ⁺ CRTh2 ⁺ T cells in PB (cells/μl)	27(14-43)	29 (14-46)	0.886
% of CD4 ⁺ T cells expressing CRTh2	6.8 (3.9-10.3)	3.2 (2.3-3.9)	0.003

ACQ6: Asthma control questionnaire; BMI: Body mass index; FEV₁: Forced expiratory volume in 1 sec; FVC: Forced Vital Capacity; PB: Peripheral blood. Data was calculated as median (interquartile range) for continuous variable and n (%) for categorical variables. *p*-value was calculated by Mann-Whitney U test. Because of missing data point, “n” for the categories labeled, *N=9; **N=8 for exacerbation.

Table S4. Comparison of demographics and clinical characteristics between patients with an asthma exacerbation and stable asthmatics after aged 20-50 years old

Parameters	Exacerbation (N=15)	Stable (N=13)	<i>p</i>
Age (y)	31 (25-41)	41 (30-49)	0.092
Female patients	8 (53.3%)	7 (53.8%)	1.000
BMI (kg/m ²)*	30.0 (23-32)	30 (22.6-33.3)	0.663
Current Smoking	5 (33.3%)	0 (0%)	0.044
FEV ₁ (% predicted) **	67 (41.2-79.3)	80 (70.0-88.5)	0.050
FEV ₁ /FVC (%)**	65.6 (52.3-72.1)	82 (70.0-85.0)	0.017
ACQ6	3.0 (2.1-4.5)	0.8 (0.24-2.0)	<0.001
Eos in PB (cells/μl)	78.0 (31-384)	117 (72-254)	0.627
ILC2 in PB (cells/ml)	1937 (1486-2386)	1428 (706-2231)	0.102
CD4 ⁺ T cells (cells/μl)	477 (134-687)	987 (604-1375)	0.012
CD4 ⁺ CRTh2 ⁺ T cells in PB (cells/μl)	25(11-40)	29 (14-39)	0.662
% of CD4 ⁺ T cells expressing CRTh2	6.7 (3.7-7.7)	3.1 (2.3-3.9)	0.001

ACQ6: Asthma control questionnaire; BMI: Body mass index; FEV₁: Forced expiratory volume in 1 sec; FVC: Forced Vital Capacity; PB: Peripheral blood. Data was calculated as median (interquartile range) for continuous variable and n (%) for categorical variables. *p*-value was calculated by Mann-Whitney U test. Because of missing data point, “n” for the categories labeled, *N=13; **N=12 for exacerbation.

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