

LETTER

Increased circulating CRTH2⁺Tregs are associated with asthma control and exacerbation

To the editor,

Asthma is a chronic airway inflammatory disease consisting of several variable clinical presentations (phenotypes) and distinct pathophysiological mechanisms (endotypes).¹ Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) is a type 2 receptor for prostaglandin D2 (PGD2), expressed on Th2 and other various cell types including regulatory T cells (Tregs).² Tregs can suppress proliferation and activation of effector type 2 inflammatory cells. On the other hand, dysregulated Tregs have been implicated in developing chronic allergic diseases.³ Among patients with allergic asthma, the *FOXP3* gene expression and Treg suppressive function decrease compared with healthy controls.⁴ GATA3 expression in Tregs may contribute to their Th2-like responses by switching Tregs to Th2 cells under the type 2 milieu.⁵ Dysregulated Tregs represent a Th2-like phenotype. That may be due to overexpression of GATA3 and producing high amounts of interleukin (IL)-4 after local exposure to PGD2.⁶ In this present study, we assessed the presence of CRTH2⁺Tregs in peripheral blood of patients with different inflammatory phenotypes of asthma. We found that the frequency of CRTH2⁺Tregs was increased in patients with asthma, particularly in those with concomitant allergy, and this increase correlated negatively with asthma control. Furthermore, we discovered that the frequency of CRTH2⁺Tregs increased during an acute asthma exacerbation. Finally, we demonstrated that Tregs of allergic patients with asthma exhibited Th2-biased character.

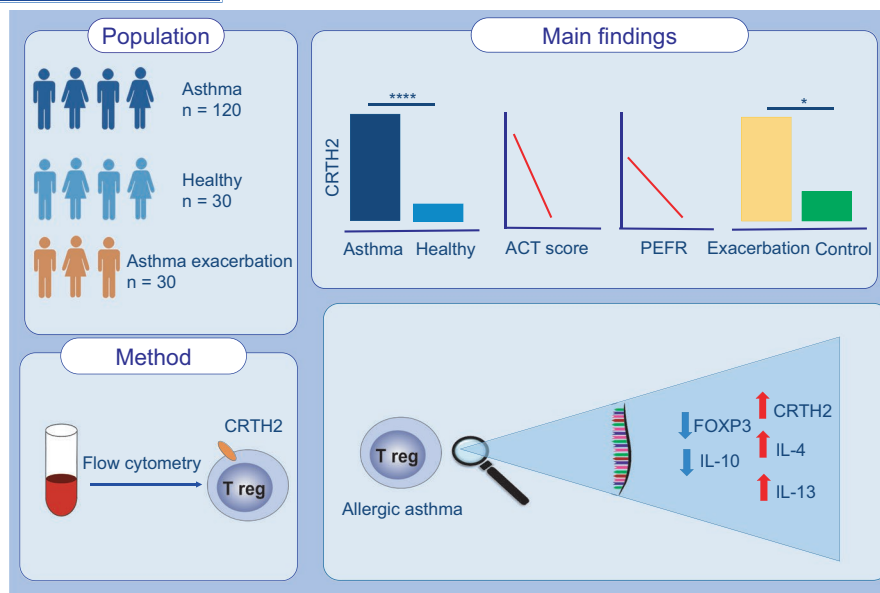
We first studied the frequency of CRTH2⁺ and CRTH2⁻Tregs (CD3⁺CD4⁺CD25⁺CD127⁻) and Th2 (CD3⁺CD4⁺CD25⁻CRTH2⁺) in 120 patients with asthma compared with 30 healthy controls. For a detailed description of the method, see the methods section in this article's supplementary data. The gating strategy is shown in Figure S1A. The frequency of CRTH2⁺Tregs in the asthma group, 0.09% [0.06–0.18], was significantly higher as compared to the control group, 0.04% [0.03–0.06], $p < .0001$ (Figure 1A). The frequencies of CRTH2⁻Tregs in patients with asthma were significantly lower (Figure 1A), whereas Th2 cells did not differ between controls and asthmatics (Figure S2A). Next, we compared the frequency of T-cell subsets in patients with different asthma phenotypes: eosinophilic allergic (EA), eosinophilic non-allergic (EN), non-eosinophilic allergic

(NA), and non-eosinophilic non-allergic (NN) patients and controls. The median of CRTH2⁺Tregs in EA, NA, and NN was significantly higher than in controls (Figure 1B and Figure S1B). No significant difference was observed in the median of CRTH2⁻Tregs and Th2 cells between asthma phenotypes and control (Figure S2B). Patients with allergic asthma had higher frequencies of CRTH2⁺Tregs than those with non-allergic asthma (Figure 1C). Also, patients with controlled asthma exhibited lower CRTH2⁺Tregs than those with uncontrolled asthma (Figure 1C). In this study, well-controlled asthma was determined by a high asthma control test (ACT) score which correlated with a low number of CRTH2⁺Tregs (Figure 1D). Besides, percentage of predicted peak expiratory flow rate (PEFR) negatively correlated with CRTH2⁺Tregs levels (Figure 1D). We also observed that the frequency of CRTH2⁺Tregs was associated with absolute eosinophil counts and Th2 cells (Figure S2C).

To explore the role of CRTH2⁺Tregs in an asthma attack, we examined 30 asthma donors during exacerbation in the absence of infection and 15 controls (Figure S3A). The level of CRTH2⁺Tregs was significantly higher among the patients with asthma exacerbation, 0.13% [0.1–0.22] than that of patients with controlled asthma, 0.09% [0.08–0.11], $p = .007$ (Figure 1E and Figure S3B). After three weeks of follow-up, the frequency of CRTH2⁺Tregs in those patients during asthma control decreased significantly compared with periodic asthma exacerbation, median difference 0.03%, $p = .01$ (Figure 1E), whereas the differences were not significant among patients with stable, controlled asthma.

Next, we analyzed the heterogeneity of T-cell phenotypes using t-distributed stochastic neighbor embedding (tSNE) algorithm. T-cell populations could be classified based on clustering results, except CRTH2⁺Tregs which grouped with Th2 cells (Figure 2A). Of note, the numbers of CRTH2⁺Tregs were higher among patients with allergic asthma than those with non-allergic phenotypes and healthy individuals. To examine the relative expression of Th2 genes in Tregs, we determined mRNA levels from purified Tregs of patients with asthma and healthy controls. We observed statistically higher expression of *PTGDR2* mRNA in patients with allergic phenotype compared with those with the non-allergic phenotypes and healthy controls (Figure 2B). Patients with asthma showed also significantly

Abbreviations: ACT, asthma control test; ALI, air-liquid interface; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; HBEC, human bronchial epithelial cell; PEFR, peak expiratory flow rate; PGD2, prostaglandin D2; TER, transepithelial electrical resistance; Th2, T helper 2 cells; TJ, tight junction; Tregs, regulatory T cells; tSNE, t-distributed stochastic neighbor embedding.



GRAPHICAL ABSTRACT

The frequency of CRTH2⁺Tregs in patients with asthma, especially allergic phenotype is higher compared to healthy controls and shows a negative correlation with ACT scores and PEFR. CRTH2⁺Tregs increase during exacerbation and decrease after asthma control. In vitro investigations demonstrate that Tregs of allergic patients with asthma display Th2-biased character. Abbreviation: CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; Tregs, regulatory T cells; ACT, asthma control test; PEFR, peak expiratory flow rate.

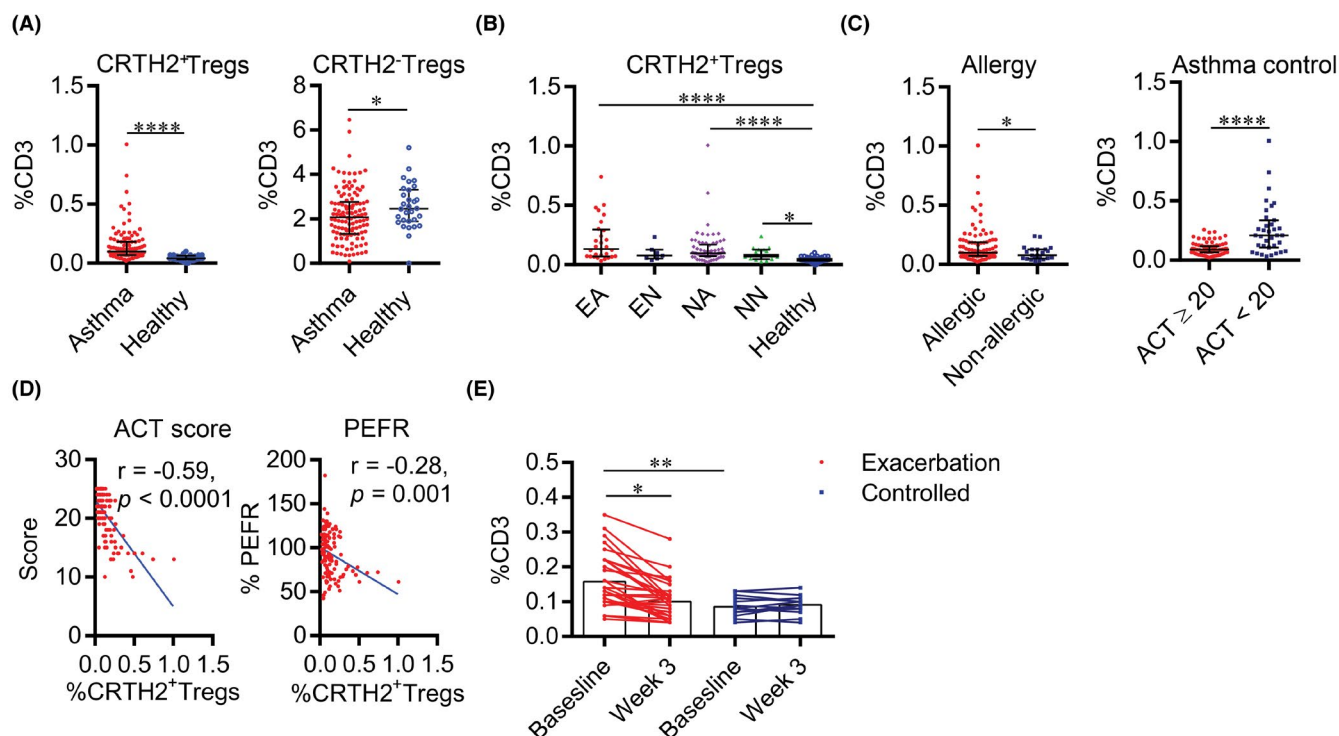


FIGURE 1 (A) Frequencies of CRTH2⁺ and CRTH2⁻Tregs in patients with asthma ($n = 120$) and healthy controls ($n = 30$). (B) Frequency of CRTH2⁺Tregs in patients with eosinophilic allergic (EA, $n = 30$), eosinophilic non-allergic (EN, $n = 7$), non-eosinophilic allergic (NA, $n = 69$), non-eosinophilic non-allergic (NN, $n = 14$), and healthy control (HC, $n = 30$). (C) Frequency of CRTH2⁺Tregs compared between allergic and uncontrolled asthma (asthma control test (ACT) < 20). (D) Correlation between the frequency of CRTH2⁺Tregs and ACT and % predicted peak expiratory flow rate (PEFR) in all asthmatic patients. (E) Frequency of CRTH2⁺Tregs in patients with asthma exacerbation ($n = 30$) and controlled ($n = 15$) compared between baseline and after 3 weeks. * $p < .05$, ** $p < .01$, **** $p < .0001$

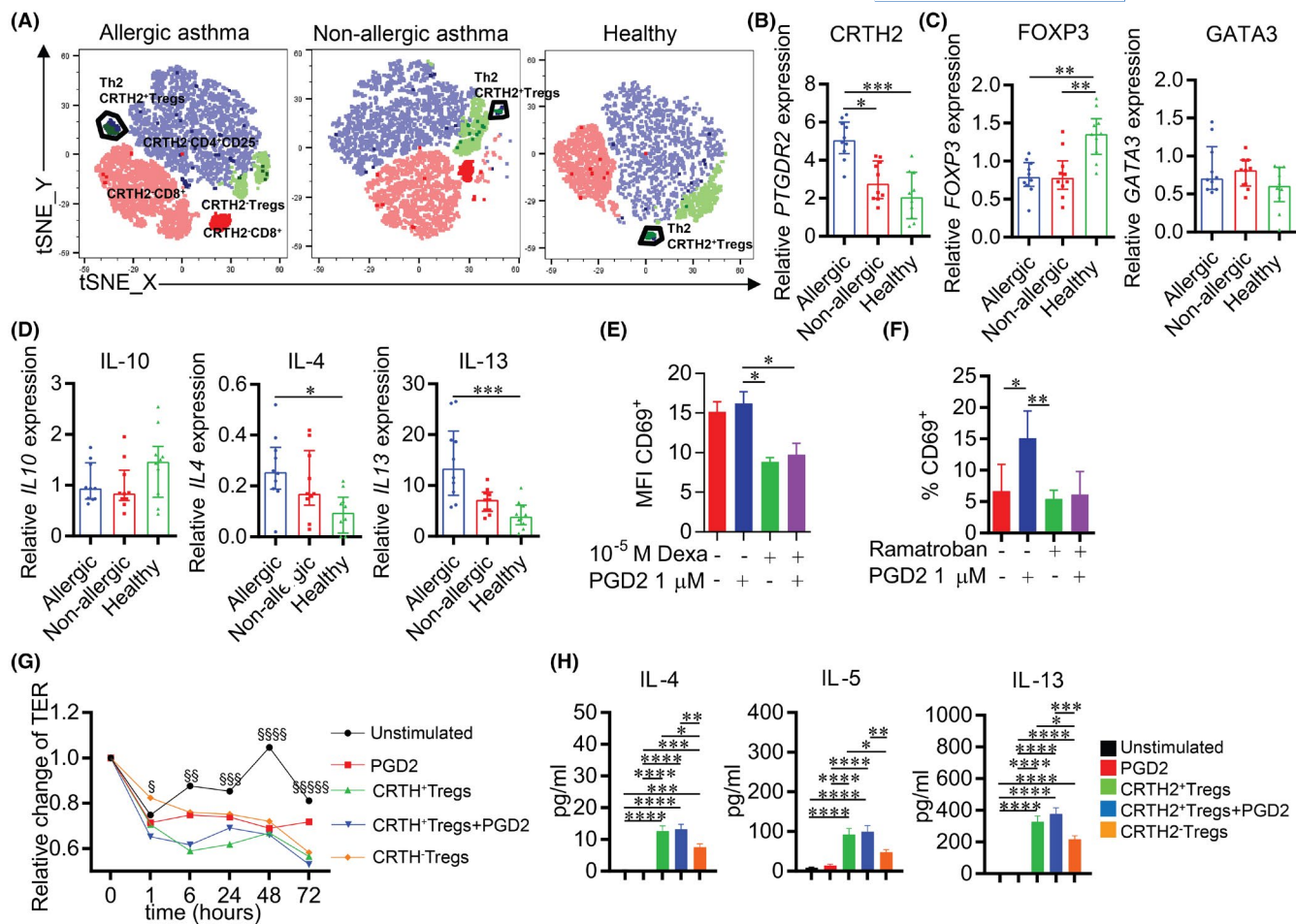


FIGURE 2 (A) Representative tSNE plot clustered 6 T cells population, CRTH2⁺CD4⁺CD25⁻ (dark blue), CRTH2⁺CD8⁺ (dark red), CRTH2⁺Tregs (dark green), CRTH2⁻CD4⁺CD25⁻ (light blue), CRTH2⁻CD8⁺ (light red) and CRTH2⁻Tregs (light green) of patient with allergic, non-allergic asthma and healthy control. Relative *PTGDR2* (B), transcription factor genes (*FOXP3* and *GATA3*) (C), cytokine genes (*IL-10*, *IL-4*, and *IL-13*) (D) expression in Tregs of patients with allergic (n = 10), non-allergic asthma (n = 10), and healthy controls (n = 10). (E) *In vitro* experiment, after 3 days of CD4⁺ T cells culture, 10⁻⁵ M dexamethasone (Dexa) decreased CD69 expression on CRTH2⁺Tregs in condition with/without PGD2 stimulation of asthmatic patients (n = 10). (F) Fresh whole blood of asthmatic patients (n = 10) were pretreated with 1 μM ramatroban (DP2 antagonist) and then stimulated with PGD2. The numbers of CD69⁺CRTH2⁺Tregs were investigated 1 h after PGD2 administration. (G) Tight junction of bronchial epithelial cells of asthmatic patients (n = 10) compared between unstimulated, PGD2, CRTH2⁺Tregs with or without PGD2 and CRTH2⁻Tregs. Transepithelial electrical resistance (TER) measurements were examined at 0, 1, 6, 24, 48, and 72 h. H, Levels of IL-4, IL-5, and IL-13 in the supernatant of bronchial epithelial culture. [§]CRTH2⁺Tregs+PGD2 vs. CRTH2⁻Tregs *, ^{§§}Unstimulated vs. CRTH2⁺Tregs ****, Unstimulated vs. CRTH2⁺Tregs+PGD2 ****, CRTH2⁺Tregs vs. CRTH2⁻Tregs *, CRTH2⁺Tregs+PGD2 vs. CRTH2⁻Tregs *, ^{§§§}Unstimulated vs. CRTH2⁺Tregs **, Unstimulated vs. CRTH2⁺Tregs+PGD2 *, ^{§§§§}Unstimulated vs. CRTH2⁺Tregs ****, Unstimulated vs. CRTH2⁺Tregs+PGD2 ****, Unstimulated vs. CRTH2⁻Tregs ****, ^{§§§§§}Unstimulated vs. CRTH2⁺Tregs ****, Unstimulated vs. CRTH2⁺Tregs+PGD2 ****, Unstimulated vs. CRTH2⁻Tregs **. *p < .05, **p < .01, ***p < .001, ****p < .0001

reduced *FOXP3* and a trend to higher expression of *GATA3* compared with controls (Figure 2C). Accordingly, we observed higher expression of *IL-4* and *IL-13* in Tregs of patients with allergic asthma as compared to healthy controls, but *IL-10* showed no significant difference between the groups (Figure 2D). These data indicate that type 2 gene expression was elevated, whereas regulatory signature was decreased in patients with asthma, particularly in those with allergic phenotypes.

All patients during asthma exacerbation received systemic corticosteroid treatment. We hypothesized that corticosteroids might affect the frequency of the CRTH2⁺Tregs. To assess the efficacy of dexamethasone and DP2 antagonist (ramatroban) in response to

PGD2 engagement, we examined activated (CD69⁺) CRTH2⁺Tregs. Reduced CD69 expression was observed in the presence of dexamethasone with and without PGD2 compared with only PGD2 stimulation (Figure 2E and Figure S4A). Under DP2 antagonist treatment, the frequency of activated CRTH2⁺Tregs decreased compared with the condition with only PGD2 stimulation (Figure 2F and Figure S4B). These data indicate that dexamethasone and DP2 antagonists could alleviate CRTH2⁺Tregs activation.

Disordered epithelial regulation and disruption of bronchial epithelial tight junction (TJ) are crucial for asthma pathogenesis. Recent studies have been reported that IL-4 and IL-13 can disrupt bronchial epithelial TJ.^{7,8} We determined whether IL-4- and IL-13-producing

CRTH2⁺Tregs might influence the bronchial epithelial barrier. After the first hour of the coculture, transepithelial electrical resistance (TER) was significantly lower in the presence of CRTH2⁺Tregs with PGD₂ than CRTH2⁺Tregs (Figure 2G). Noticeably, TER in the presence of CRTH2⁺Tregs with or without PGD₂ significantly decreased after 6, 24, 48, and 72 h compared with the control values (Figure 2G). As shown in Figure S5, TJ protein was markedly disrupted in the HBEC-CRTH2⁺Tregs cocultures compared with the absence of CRTH2⁺Tregs. In this study, CRTH2⁺Tregs stimulated with PGD₂ led to slightly increased levels of IL-4, IL-5, and IL-13 compared with CRTH2⁺Tregs without of PGD₂ but without a statistically significant difference between both groups (Figure 2H). Moreover, type 2 cytokines were produced by CRTH2⁺Tregs more than CRTH2⁺Tregs (Figure 2H).

In the current study, we investigated CRTH2⁺Tregs in a sufficient sample size. The limitation was the cross-sectional study and when we analyzed phenotypes of asthma, the number of participants in each group was imbalance. However, the main limitation though is the lack of the broader and very detailed investigation of the differences between CRTH2⁺Tregs, CRTH2⁺Tregs, and Th2 cells. In addition, there were no remaining cell samples that could be used to measure intra-cellular FOXP3. Further studies will determine other surface markers, transcription factors, and cytokines which may lead to deeper understanding of the importance of these populations in the steady state and in allergic disease.

Here, we suggested that CRTH2⁺Tregs might be implicated in the immunopathology of allergic asthma. However, CRTH2⁺Tregs are not very frequent and the suppressive effect of CRTH2⁺Tregs may overwhelm these dysfunctional Tregs. Currently, many novel antagonists for PGD₂ receptors have entered clinical trials. Information on whether CRTH2⁺Tregs might be a target for this new class of drugs seems to be of particular interest. Additional validation of the CRTH2⁺Tregs approach as a biomarker will be needed to support clinical implementations.

KEYWORDS

ALI culture, allergic, asthma, asthma control test score, asthma exacerbation, CRTH2, epithelial tight junction, PGD₂, Treg cells, Tregs dysfunction

FUNDING INFORMATION

Phramongkutklao Hospital and Phramongkutklao College of Medicine foundation Scholarship; Christine Kühne-Center for Allergy Research and Education (CK-CARE)

ACKNOWLEDGMENTS

This study was supported by Phramongkutklao Hospital and Phramongkutklao College of Medicine foundation Scholarship and Christine Kühne-Center for Allergy Research and Education (CK-CARE), Davos. The authors wish to acknowledge Mrs. Jitta Tubkate for help in contacting the patients and Asst. Prof. Nattapon Jaisupa

for advice in the laboratory work. We thank Dr. David Groeger for proofreading our manuscript.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest in relation to this work.

AUTHOR CONTRIBUTIONS

T.C., S.S., C.C., and T.B. carried out the experiment. T.B., S.J., M.A., M.S., and C.A. conceived and planned the experiments. T.B. and M.S. wrote the manuscript with support from A.S., M.A., and C.A. T.B., T.C., S.S., C.C., P.P., and W.M. recruited the participants. T.C., S.S., C.C., and T.B. prepared the samples. M.W. prepared the HBEC samples and ALI culture. T.B., A.S., and C.A. supervised the study.

Teerapol Chantveerawong¹
Sasipa Sangkangjanavanich^{1,2}
Chirawat Chiewchalermisri^{1,3} 
Panitan Pradubpongsa¹ 
Wat Mitthamsiri¹ 
Sarawat Jindarat⁴
Ming Wang⁵ 
Mübecce Akdis⁶ 
Milena Sokolowska⁶ 
Cezmi A. Akdis⁶
Atik Sangasapaviliya¹ 
Tadech Boonpiyathad^{1,6} 

¹Division of Allergy and Clinical Immunology, Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand

²Division of Allergy, Immunology and Rheumatology, Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

³Department of Medicine, Panyanantaphikkhu Chonprathan Medical Center, Srinakharinwirot University, Nonthaburi, Thailand

⁴Department of Pharmacology, Phramongkutklao College of Medicine, Bangkok, Thailand

⁵Department of Otolaryngology, Head and Neck Surgery, Beijing TongRen Hospital, Capital Medical University, and the Beijing Key Laboratory of Nasal Diseases, Beijing Institute of Otolaryngology, Beijing, China

⁶Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, and the Christine Kühne-Center for Allergy Research and Education (CK-CARE), Davos, Switzerland

Correspondence

Tadech Boonpiyathad, Division of Allergy and Clinical Immunology, Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand.
Email: tadechb@pmk.ac.th

ORCID

Chirawat Chiewchalemsri  <https://orcid.org/0000-0002-4437-3146>

Panitan Pradubpongsa  <https://orcid.org/0000-0001-6323-4910>

Wat Mitthamsiri  <https://orcid.org/0000-0003-4740-6107>

Ming Wang  <https://orcid.org/0000-0002-8560-3964>

Mübeccel Akdis  <https://orcid.org/0000-0003-0554-9943>

Milena Sokolowska  <https://orcid.org/0000-0001-9710-6685>

Atik Sangasapaviliya  <https://orcid.org/0000-0002-7117-2868>

Tadech Boonpiyathad  <https://orcid.org/0000-0001-8690-7647>

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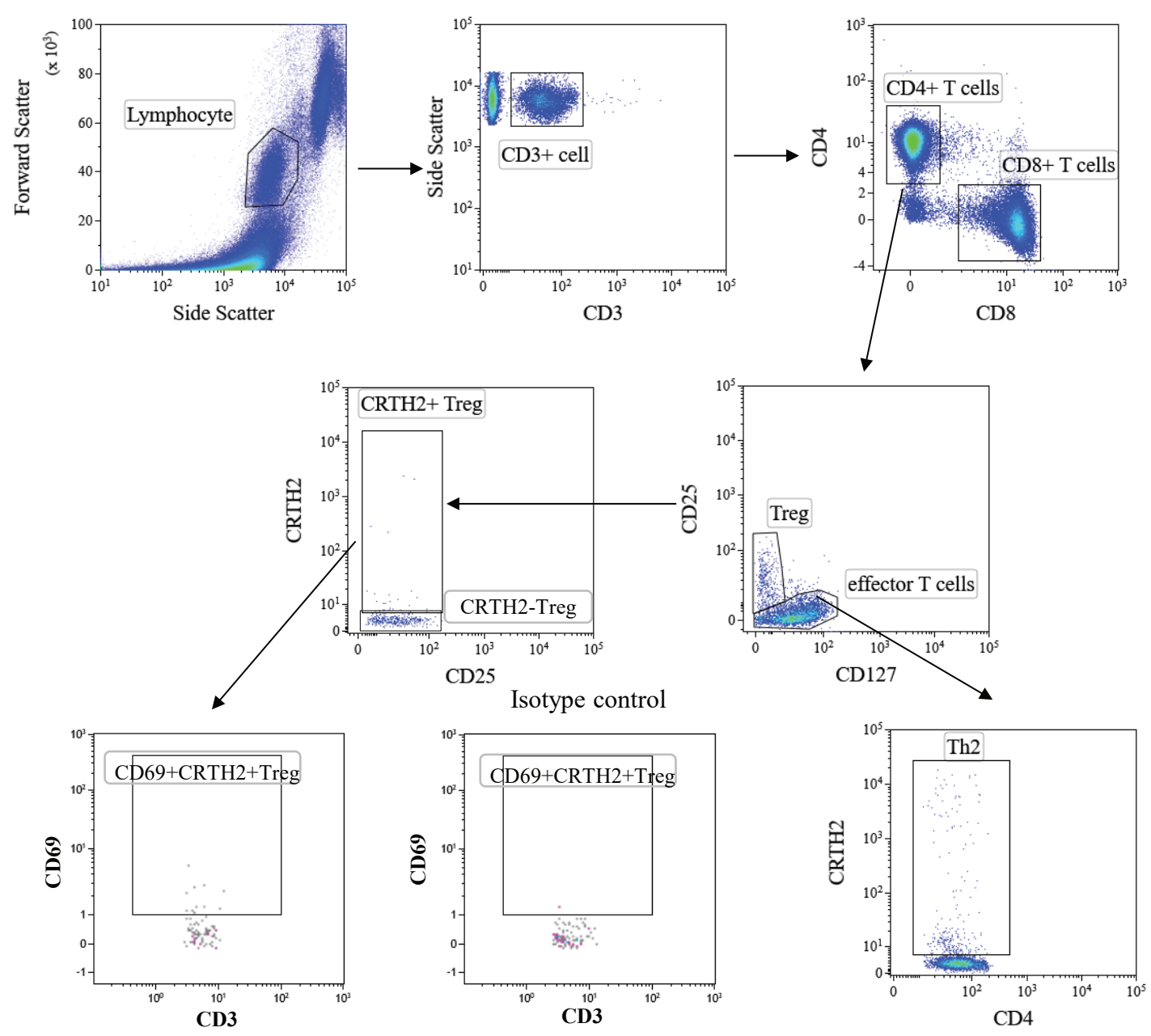
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SUPPORTING INFORMATION

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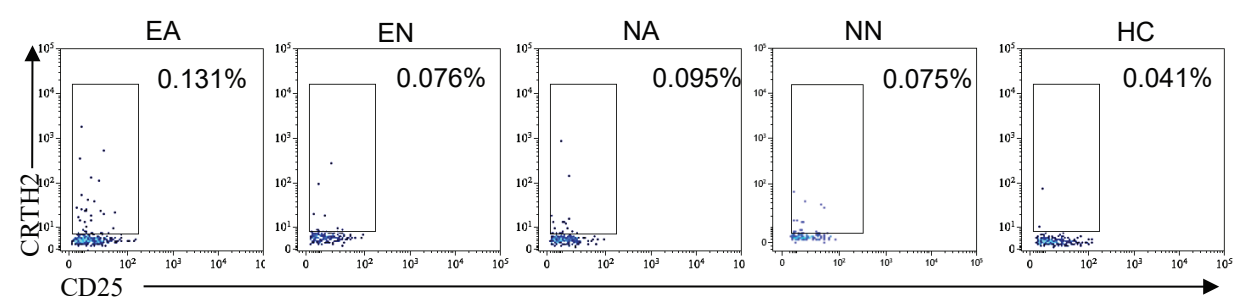


Figure S1_Chantveerawong et al.

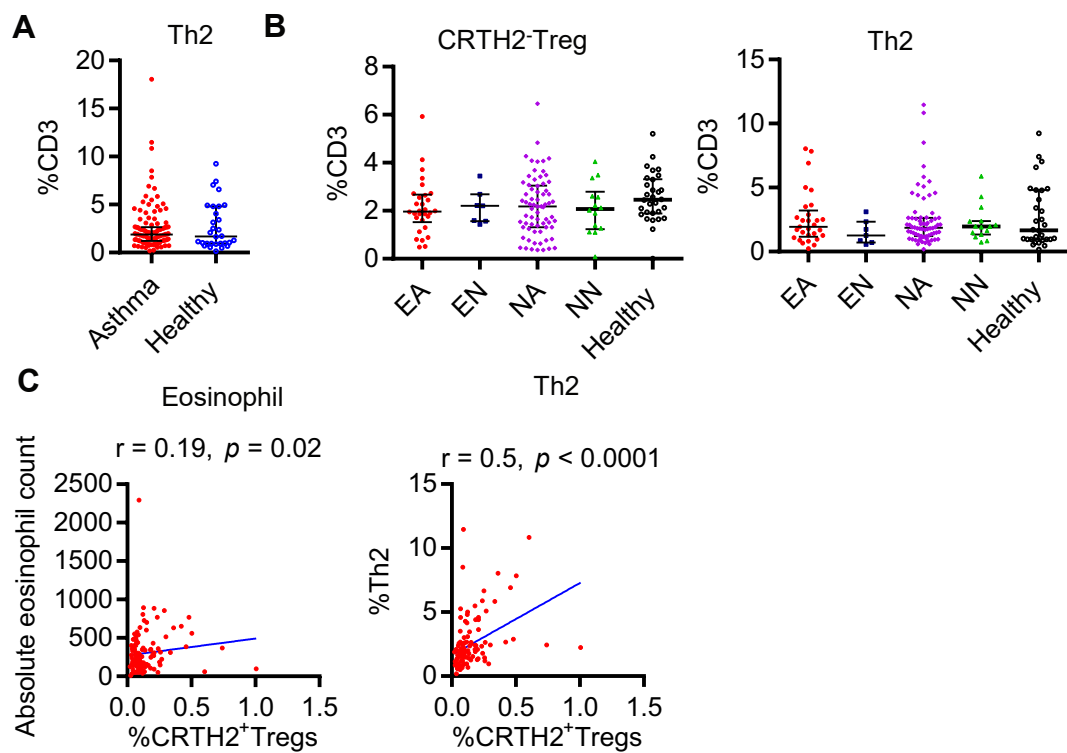
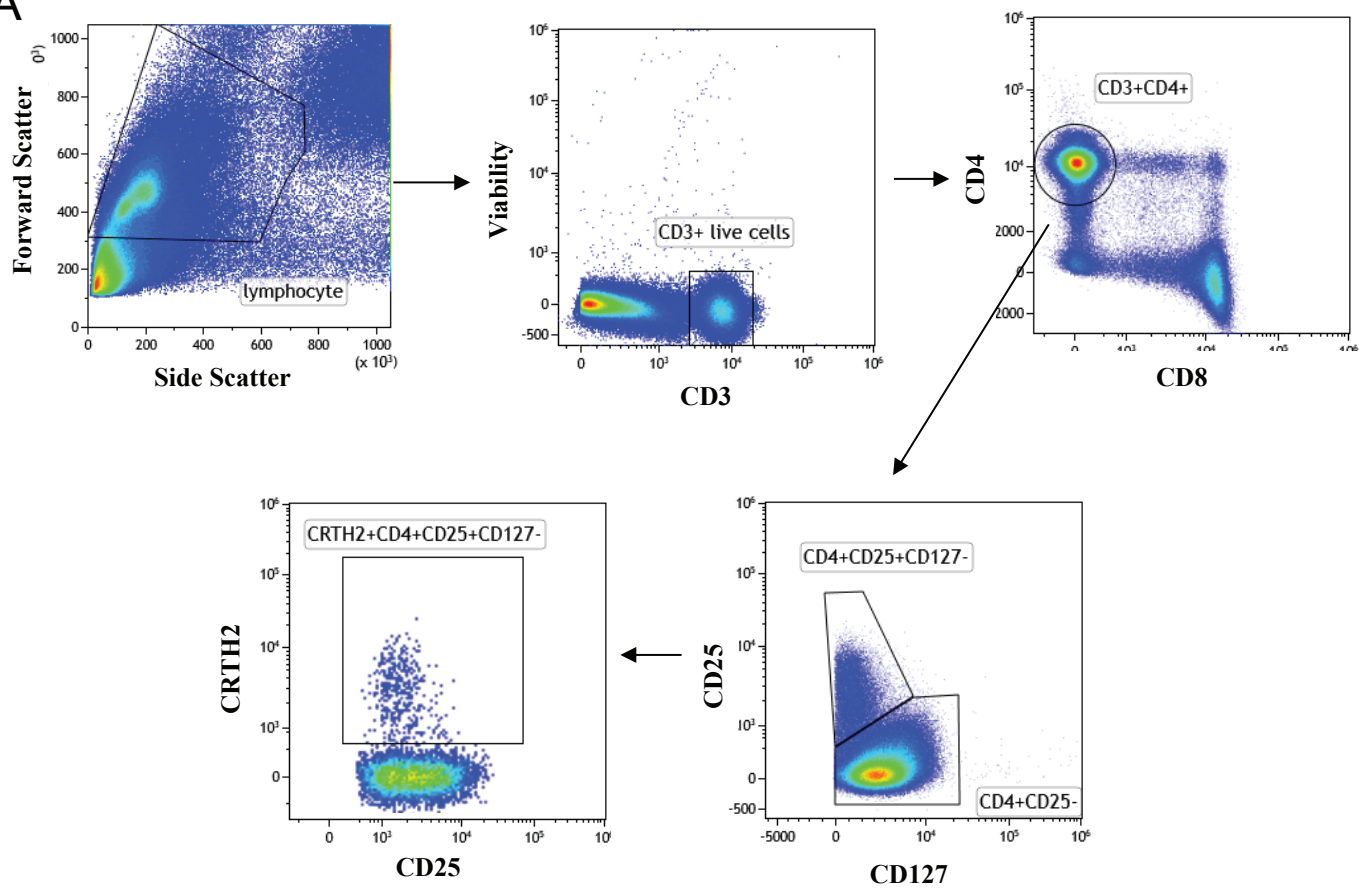


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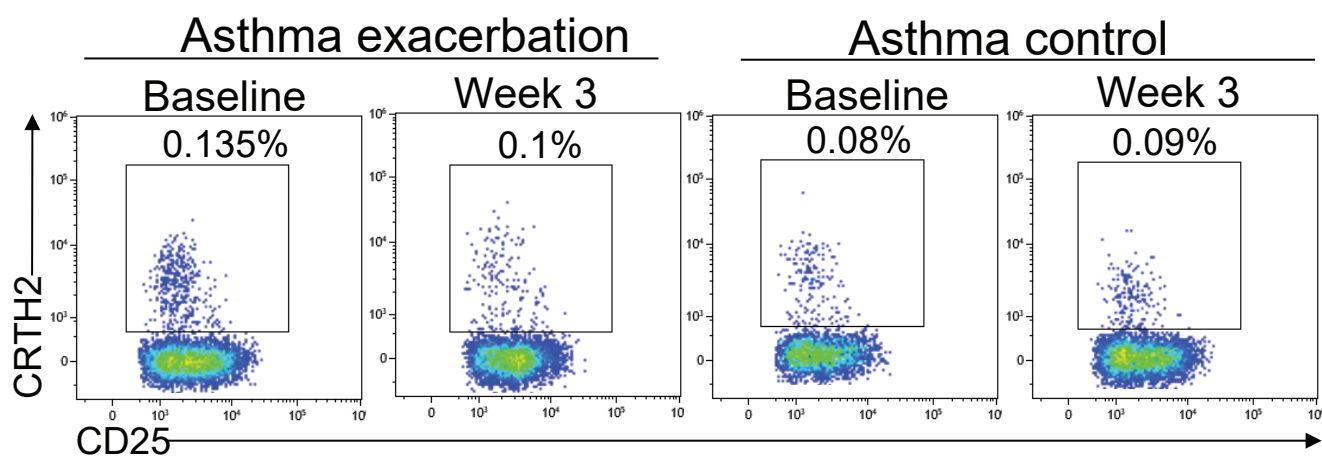


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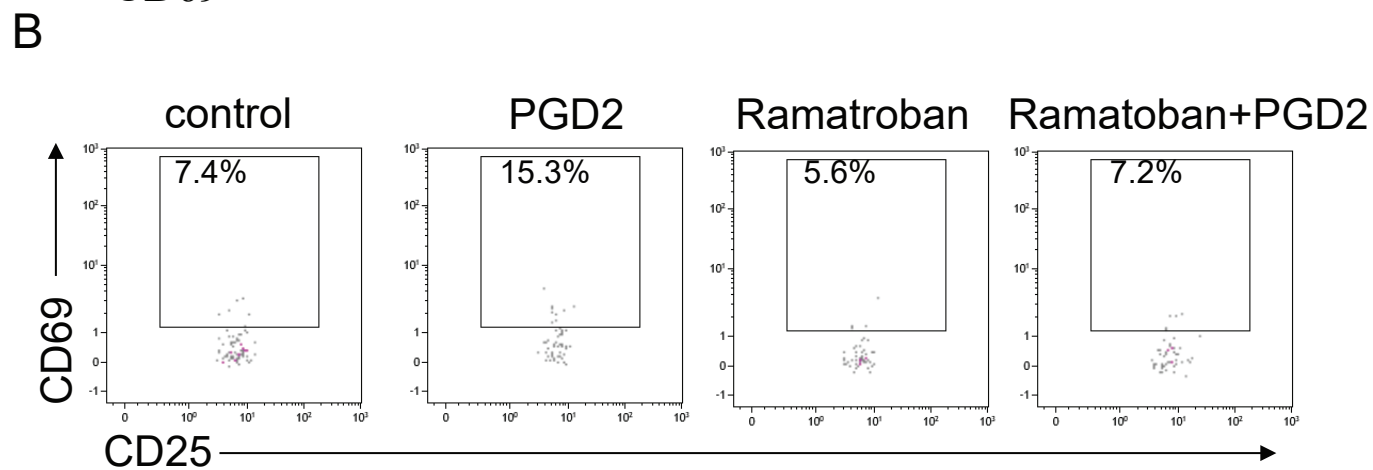
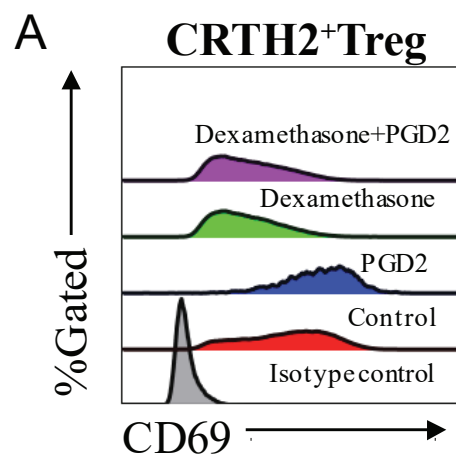


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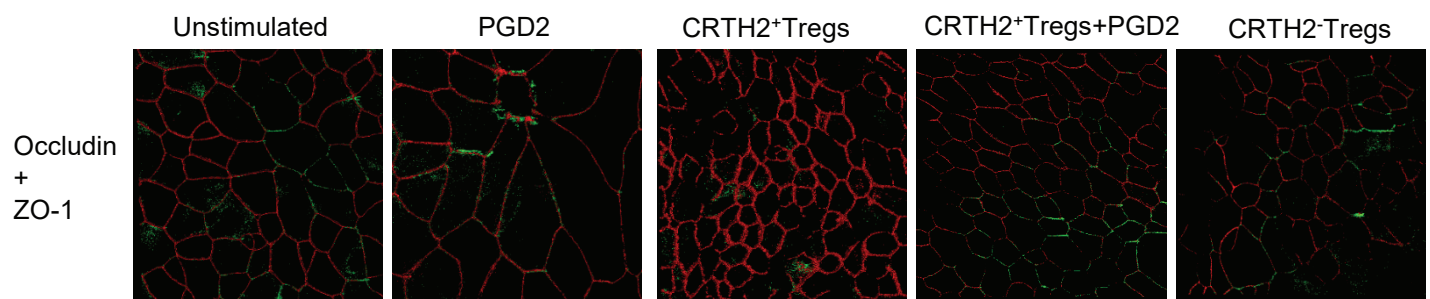


Figure S5_Chantveerawong et al.

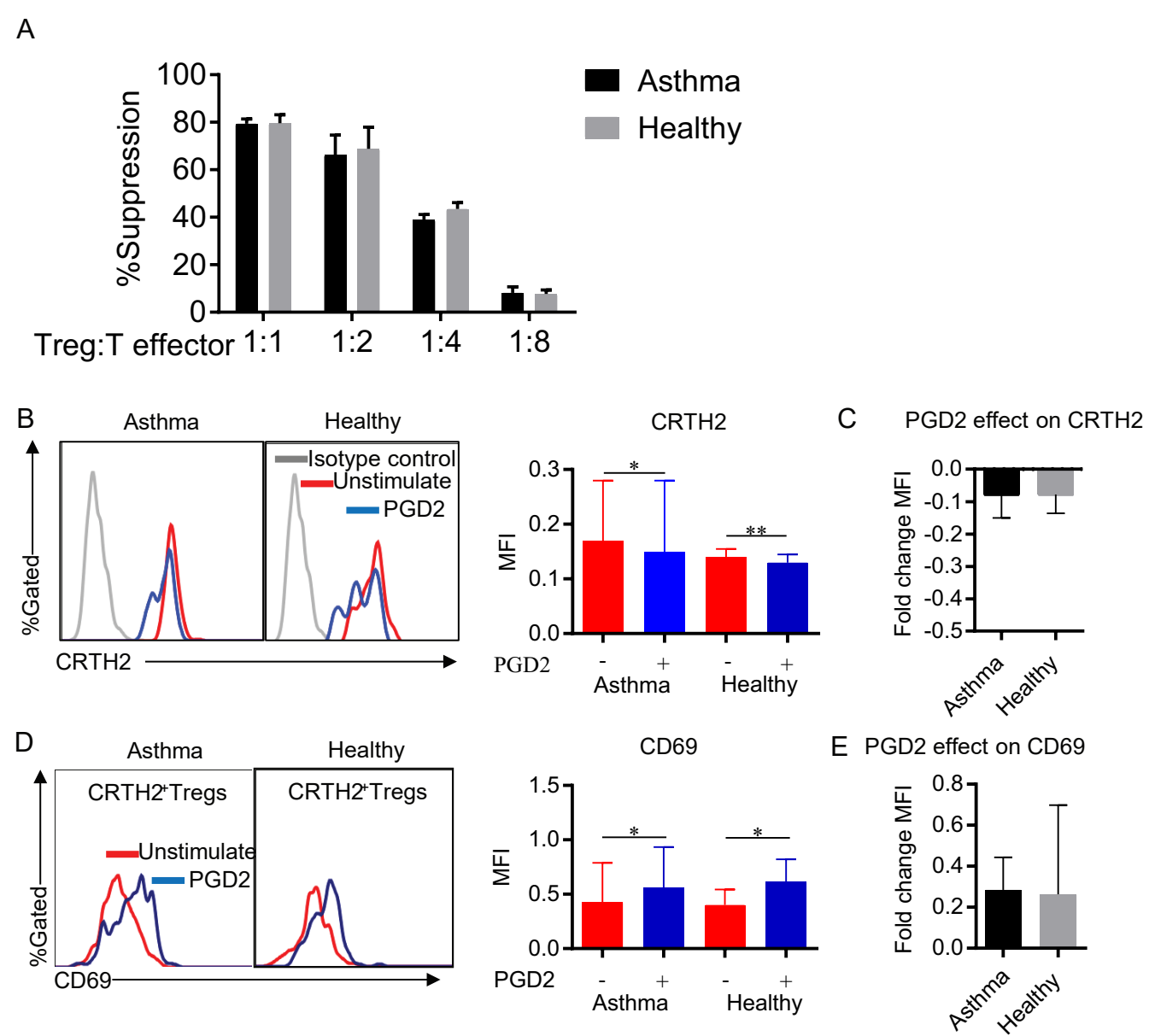


Figure S6_Chantveerawong et al.

Online repository

Increased circulating CRTH2⁺Tregs are associated with asthma control and exacerbation

Short running title: Increased circulating CRTH2⁺Tregs in asthmatic patients

Teerapol Chantveerawong^a, Sasipa Sangkangjanavanich^{a,b}, Chirawat Chiewchalernsri^{a,c}, Panitan Pradubpong^a, Wat Mitthamsiri^a, Sarawut Jindarat^d, Ming Wang^e, Mübeccel Akdis^f, Milena Sokolowska^f, Cezmi A. Akdis^f, Atik Sangasapaviliya^a, Tadech Boonpiyathad^{a,f}

^aDivision of Allergy and Clinical Immunology, Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand

^bDivision of Allergy, Immunology and Rheumatology, Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

^cDepartment of Medicine, Panyanantaphikkhu Chonprathan Medical Center, Srinakharinwirot University, Nonthaburi, Thailand

^eDepartment of Pharmacology, Phramongkutklao College of Medicine, Bangkok, Thailand

^dDepartment of Otolaryngology, Head and Neck Surgery, Beijing TongRen Hospital, Capital Medical University, and the Beijing Key Laboratory of Nasal Diseases, Beijing Institute of Otolaryngology, Beijing, China

^fSwiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, and the Christine Kühne-Center for Allergy Research and Education (CK-CARE), Davos, Switzerland

Corresponding author:

Tadech Boonpiyathad

Division of Allergy and Clinical Immunology, Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand.

tadechb@pmk.ac.th

Tel: +66-2354-7614

Fax: +66-2763-3278

Methods

Subjects

One hundred and twenty adult asthmatic patients were recruited at the Allergy Clinic, Phramongkutklo Hospital, Bangkok, Thailand. The patients were diagnosed with asthma by the patient's history and pulmonary function test that showed a positive response to a bronchodilator (forced expiratory volume at 1 sec $\geq 12\%$ and ≥ 200 mL as an absolute value compared with a baseline). The allergic phenotype was defined based on skin prick test reactivity to the allergen (wheal diameter ≥ 6 mm higher than negative control to classify as allergic). In this study, all the patients with allergic asthma had positive skin prick test with *D. pteronyssinus* extract (Alk-Abello, Round Rock, TX, USA). Also, the patients with allergic asthma had symptoms year-round and a history of respiratory symptoms become worse when there was exposure to domestic activity that stirs up house dust such as vacuuming, dusting, sweeping, making the bed, or shaking out blankets. Absolute total eosinophil count > 350 cells/ μ l defines eosinophilic phenotype. For 30 healthy controls, the people without a history of asthma and allergic disease were enrolled in the study. All subjects were donated 10 ml peripheral blood in a heparin tube. The baseline patient characteristics are shown in **Table S1**. All asthma patients were evaluated with an asthma control test score (ACT) and peak expiratory flow rate (PEFR) % predicted. The asthma control test (ACT) score ranges from 5 (poor control of asthma) to 25 (complete control of asthma). An ACT score ≥ 20 indicates controlled asthma.¹ Peak expiratory flow rate (PEFR) was measured and compared with standard value to calculate the percent predicted.²

In an experiment of 30 patients with asthma exacerbation, asthma exacerbation without infection is defined by acute episodes of progressively worsening shortness of breath, coughing, wheezing, chest tightness, and no fever. Patient with asthma exacerbation requires treatment with nebulized short-acting beta-2 agonist, intravenous dexamethasone, and oral corticosteroid added to daily asthma medications for 7 days. At least three weeks after the exacerbation, the patients during the period in the control condition (control asthma without inhaled short-acting beta-2 agonist) were recruited to give peripheral blood at the clinic. Venipuncture was taken on the day of asthma exacerbation and follow-up at the outpatient clinic. Fifteen patients with stable control asthma were recruited when patients reported ACT score ≥ 20 and used reliever medication less than twice a week (**Table S2**). The study protocol was approved by the Institutional Review Board of the Royal Thai Army's Ethics Committee. All patients provided written informed consent before participating in the study.

Flow cytometry

For flow cytometry analysis, first, 200 μ l of heparin anticoagulated peripheral blood were added with fluorescein isothiocyanate (FITC)-anti-CD3 (clone OKT3, Biolegend, San Diego, CA, USA), phycoerythrin (PE)-anti-CD25 (clone BC96, Biolegend), PerCP-Cy5.5-anti-CD8 (clone SK1, Biolegend), Alexa Fluor 647 (AF647)-anti-CRTH2 (clone BM16, BD biosciences, Franklin Lakes, NJ, USA), PE-Cy7-CD127 (clone A019D5, Biolegend) and APC-Cy7-anti-CD4 (clone OKT4, Biolegend) for 15 min in the dark at room temperature. After staining, 2 ml (x10 dilution with distilled water) BD FACSTTM lysing solution (BD Biosciences) was added for 10 min at room temperature. Next, cells were washed 2 times with phosphate buffer saline (PBS), pH 7.2, 0.5% bovine serum albumin and 2 nM EDTA and centrifuge at 300xg for 5 minutes at room temperature and then added with a suitable amount of buffer for analysis by flow

cytometry. Matched isotype controls were used at the same protein concentration as the antibodies description.

The patients with asthma exacerbation donated 10 ml peripheral blood to collect peripheral blood mononuclear cells (PBMCs). PBMCs were isolated by the Ficoll Hypaque centrifugation method and froze at -80 °C until analysis. The frozen PBMCs were studied after 45 participants donated peripheral blood completed 2 visits. The cryopreserved PBMCs, following thawing, were treated with 100 U recombinant DNase I (Sigma-Aldrich, St Louis, MO, USA). Afterward, cells were stained using fixable viability dye eFlour 450 (eBioscience, San Diego, CA, USA) at 4°C for 30 min in the dark, and then cells were stained with surface antibodies as previously described. A Guava easyCyte (Merck, Darmstadt, Germany) was used for flow cytometry analysis, and data were analyzed with Kaluza software (Beckman Coulter, Indianapolis, IN, USA). The t-distributed stochastic neighbor embedding (tSNE) algorithm was analyzed by FlowJo 10.1 (TreeStar, Ashland, OR, USA).

RT-PCR detection of mRNA levels

Tregs (CD3⁺CD4⁺CD25⁺CD127⁺) were purified from fresh 1 x 10⁷ PBMCs from patients with allergic asthma, non-allergic asthma, and healthy donors using FACSARIA II (Beckton Dickinson, Franklin Lakes, NJ, USA). Sorted cells were lysed in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA by using a Superscript II Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. The prepared cDNAs were amplified using the SYBR-PCR master mix (Thermo Fisher Scientific) according to the recommendations of the manufacturer in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequences of primers for detecting *IL4*, *IL10*, *IL13*, *PTGDR2*, *FOXP3* and *GATA3* expression are shown in **Table S3**. Results were normalized to endogenous control *EF1α* (ΔC_t values). The relative quantification and calculation of the range of confidence were performed using the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) method (relative gene expression). The RNA samples were prepared at least in duplicate.

In vitro experiment

Fresh whole blood stimulation with 1 μM PGD2 (Sigma-Aldrich) was performed to evaluate the activation of CRTH2⁺Tregs in patients with allergic asthma and healthy controls. PGD2 upregulates IL-4, IL-5, and IL-13 expression in Th2 cells and induces Th2 cell migration.^{1,2} Increased levels of PGD2 in the airways of patients with asthma and after allergen challenge has been repeatedly reported.^{3,4} Furthermore, severe asthma is associated with higher numbers of mast cells in airway submucosa and increased level of PGD2.⁵ The PGD2 stimulation method has been described in detail elsewhere.⁴ Then, whole blood was stained with surface mAbs against cell surface molecules as previously described above and PE/Dazzle 594-anti-CD69 (clone FN50, Biolegend). Ramatroban (Sigma-Aldrich) is an antagonist of the thromboxane receptor and also an antagonist of the DP2 receptor.⁴ It is powerfully potent at the DP2 4-5 fold than the thromboxane receptor.^{5,6} 1 μM ramatroban was added to the whole blood before PGD2 stimulation to evaluate the effect of blocking the DP2 receptor in CRTH2⁺Tregs activation.

For the dexamethasone experiment, CD4⁺ T cells from patients with allergic asthma were isolated by CD4⁺ T cells isolation kit (Miltenyl Biotec, Bergisch Gladbach, Germany). The CD3⁺CD4⁺ cells were selected as the final negative fraction.⁴ Cells were cultured in complete RPMI (cRPMI) 1640 medium (Gibco, Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, anti-CD3/anti-CD28 coated bead and IL-2 (Sigma-Aldrich) at 37 °C in 5% CO₂. Cells were also cultured with 1 μM PGD2 and/or 10⁻⁵ M dexamethasone (Sigma-Aldrich) in different conditions. After 3 days, cells were harvested and stained with surface mAbs as previously described.

For Treg suppression assay, PBMCs from allergic asthmatic patients and healthy donors were purified CRTH2⁺CD4⁺CD25⁺CD127⁻ (CRTH2⁺ Treg cells) and CD4⁺CD25⁻ T cells (T effector cells) by using FACSaria II. T effector cells were stained with CellTrace violet cells proliferation kit (ThermoFisher, Waltham, MA, USA), according to the manufacturer's instructions. Then Treg, T effector, and irradiated T cells were cultured with anti-CD3/anti-CD28 coated bead and IL-2 in 96 U bottom well plate for 5 days. Treg cell and T effector cells were co-cultured in different Treg: T effector cell ratios as 0:1, 1:1, 1:2, 1:4, 1:8 and 0:1 without any stimulation. After 5 days, cells were harvested and washed 2 times with PBS. Cells were stained with 0.5 μl viability dye eFluor 780 (eBioscience) for 30 min at 4°C, and then cells were washed 2 times with PBS and 2 nM EDTA. Cells were stained with anti-CD3-FITC (clone UCHT1, Biolegend), anti-CD4-AF700 (clone RPA-T4, Biolegend) and anti-CD25-PE (clone BC96, Biolegend) for 15 min at room temperature. Afterward, cells were washed 2 times and analyzed by flow cytometry. We found that percentage of CRTH2⁺Treg suppression did not show a significant difference between allergic asthmatic patients and healthy donors (**Fig S6A**).

We investigated the response of CRTH2⁺Tregs to PGD2 stimulation in fresh whole blood samples. Whole blood was stimulated with PGD2 and mean fluorescence intensity (MFI) of CRTH2 and CD69 (a marker of lymphocyte activation) was measured. We found that CRTH2 expression on CRTH2⁺Tregs at baseline was not significantly different between asthmatic patients and healthy individuals (Fig S6B). When CRTH2 responses were examined, PGD2 reduced the significant MFI of CRTH2 on Tregs in both groups (Fig S6B). There was no significant difference in fold change of CRTH2 MFI between asthmatic patients and healthy controls (Fig S6C). In unstimulated conditions, we could not detect any significant difference in CD69 expression on CRTH2⁺ and CRTH2⁻Tregs (Fig S6D). After PGD2 stimulation, increasing CD69 levels on CRTH2⁺Tregs of asthmatic patients and healthy controls was observed. In addition, we could not see the difference of fold change of CD69 MFI between asthmatic patients and healthy controls (Fig S6E). Thus, our data demonstrated that CRTH2⁺Tregs in asthmatic patients and healthy donors have similar responses to PGD2 stimulation.

Human bronchial epithelial cells (HBEC) air-liquid interphase (ALI) culture

The method to expand CRTH2⁺Tregs, CRTH2⁻Tregs, HBEC and ALI-cultured have been described in detail elsewhere.^{6,7,8} HBECs and Tregs originated from different asthmatic donors. HBECs were seeded at a density of 150,000 cells in a 6.5-mm diameter polyester membrane with a pore size of 0.4 μm (Corning, Corning, New York) in the medium for the ALI culture. To expand CRTH2⁺ Tregs, PBMCs were isolated by CD4⁺ T cells isolation kit (Miltenyl Biotec,

Bergisch Gladbach, Germany). The CD3⁺CD4⁺ cells were selected as the final negative fraction, and the final positive fraction was irradiated (25 Gy) and used as feeder cells. CD3⁺CD4⁺ cells were cultured in complete RPMI medium (Sigma-Aldrich) with anti-CD3/anti-CD28 coated beads and IL-2 (all from Sigma-Aldrich) 37 C, 5% CO₂ for 2 weeks. Cells were stained with the viability dye eFluor 780 (eBioscience), anti-CD3-FITC (clone UCHT1, Biolegend), anti-CD4-AF700 (clone RPA-T4, Biolegend), anti-CD25-PE (clone BC96, Biolegend), anti-CD127-PE/Cy7 (clone A019D5, Biolegend) and anti-CRTH2-AF 647 (clone BM16, BD Biosciences) to purify CRTH2⁺CD4⁺CD25⁺CD127⁻ (CRTH2⁺ Tregs), CRTH2⁻CD4⁺CD25⁺CD127⁻ (CRTH2⁻ Tregs) and CD4⁺CD25⁻ T cells (T effector cells) by using a FACSaria III (Beckton Dickinson, Franklin Lakes, NJ, USA). HBECs and 50 × 10³ CRTH2⁺ Tregs were cultured together in the presence of transwell inserts, in which HBECs and CRTH2⁺ Tregs were placed in the upper and lower compartments, respectively. IL-2 (100 U/mL) with/without 1 μM PGD2 were added in bronchial epithelial basal medium (Lonza, Basel, Switzerland) supplemented and cRPMI medium (at ratio 1:1). Transepithelial electrical resistance (TER) measurements were done at 0, 1, 6, 24, 48, and 72 hours after PGD2 exposure by using a Millicell-ERS Volt Ohm Meter (Millipore, Temecula, CA, USA). Samples are measured in triplicates in multiple-well systems. Immunofluorescence staining with anti-occludin and anti-zonula occludens 1 (ZO-1) mAbs (both from Invitrogen, Carlsbad, CA, USA) were described in detail elsewhere.⁸ Cytokine levels in cultured supernatant were determined by using the Bioplex human cytokine immune assay panel (Bio-RAD, Hercules, CA, USA) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7.0 Software (GraphPad Software, La Jolla, CA, USA). Data were expressed as a median with [interquartile range (IQR)]. For unmatched groups, differences were evaluated by using the Mann-Whitney. ANOVA and Dunn's multiple comparison tests were used to compare the data of more than two groups. The frequency of CRTH2⁺ Tregs in patients with asthma exacerbation and relative change of TER were analyzed by repeated ANOVA. The Spearman rank test was used for analysis correlation. A *p*-value of less than 0.05 was considered statistically significant.

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Legend to Supplementary-Figures

Figure S1. A, Representative gating strategy of CRTH2⁺Tregs in fresh whole blood. Identified cells were first gated on lymphocytes and CD3⁺ cells. CD4⁺T cells were gated after gating out CD8⁺T cells. CRTH2⁺Tregs were identified by the expression of CD4⁺CD25⁺CD127⁻ and then gated on CRTH2. Activated CRTH2⁺Tregs was gated on CD69⁺CRTH2⁺ CD4⁺CD25⁺CD127⁻ T cells. Th2 cells were gated on CRTH2⁺CD4⁺CD25⁻ T cells. **B,** Dot plot of CRTH2⁺Tregs in patients with eosinophilic allergic (EA), eosinophilic non-allergic (EN), non-eosinophilic allergic (NA), non-eosinophilic non-allergic (NN) and healthy control (HC). Number represented as median.

Figure S2. A. Frequency of Th2 cells (CRTH2⁺CD4⁺CD25⁻) in patients with asthma (n = 120) and healthy controls (n =30). **B,** Frequency of CRTH2⁺Tregs and Th2 in patients with eosinophilic allergic (EA, n =30), eosinophilic non-allergic (EN, n = 7), non-eosinophilic allergic (NA, n =69), non-eosinophilic non-allergic (NN, n = 14) and healthy control (HC, n = 30). **C,** Correlation between the frequency of CRTH2⁺Tregs and circulating eosinophil levels and the frequency of Th2 cells in all asthmatic patients.

Figure S3. A, Flow cytometry gating strategy of CRTH2⁺Tregs. T lymphocytes were selected based on lymphocyte and CD3⁺ live cells. The T cells population was further gated into CD4⁺ and CD8⁺ T cells. The CD4⁺ T cells population was gated into CD25⁺CD127⁻ (Tregs) and CRTH2⁺Tregs. **B,** Dot plot of CRTH2⁺Tregs in patients with asthma exacerbation and controlled compared between baseline and after 3 weeks. Number represented as median.

Figure S4. A, *In vitro* experiment, after 3 days of CD4⁺ T cells culture, 10⁻⁵ M dexamethasone (Dexa) decreased CD69 expression on CRTH2⁺Tregs in condition with/without PGD2 stimulation of asthmatic patients. **B,** Flow cytometry dot plot demonstrate CD69 expression on CRTH2⁺Tregs in various conditions of ramatoban *in vitro* experiment. Number represented as median.

Figure S5. Immunocytochemistry revealed staining of occludin (green) and ZO-1 (red) after 72 hours between unstimulated, PGD2, CRTH2⁺ Tregs, CRTH2⁺ Tregs+PGD2 and CRTH2⁻ Tregs.

Figure S6. CRTH2⁺Tregs suppressive function and responses to PGD2 stimulation were similar in asthmatic patients and healthy controls. **A**, Percentage of Treg suppression using CRTH2⁺ Treg isolated from allergic asthmatic patients (n = 6) and healthy controls (n =6). **B**, PGD2 decreased CRTH2 expression in CRTH2⁺Tregs of patients with asthma (n = 10) and healthy controls (n = 10). **C**, Fold change of CRTH2 response after PGD2 stimulation. **D**, PGD2 increased CD69 expression on CRTH2⁺Tregs of patients with asthma and healthy controls. **E**, Fold change of CD69 response after PGD2 stimulation. Data were analyzed by ANOVA and Mann-Whitney test. Data Represented as median with IQR. **p* < 0.05, ***p* < 0.01.

Table S1. Baseline characteristics of asthma patients and healthy subjects

Characteristics	Asthma (N =120)				Healthy (n= 30)
	EA (n = 30)	EN (n = 7)	NA (n = 69)	NN (n = 14)	
Gender, female (%)	20 (66.7)	6 (85.7)	47 (68.1)	10 (71.4)	15 (50)
Age (years±SD)	56.1±10.7	65.8±6.4	56.6±15.2	69.2±9.7	48.7±15.3
Body mass index ±SD	25.5±4.5	27.6±6.5	25.4±5.4	26.4±3.9	25.3±5.6
Co-morbidity, Rhinitis, n (%)	26 (86.7)	7 (100)	61 (88.4)	10 (71.4)	
Duration of asthma (years, ±SD)	20.6±16.3	18.2±9.9	22.3±13.8	19.1±10.7	
Patients with ≥ 1 exacerbation in previous 12 mo, n (%)	4 (13.3)	1 (14.3)	12 (17.4)	2 (14.3)	
Smoking status, n (%)					
Never smoke	27 (90)	5 (71.4)	50 (72.5)	12 (85.7)	
Ex-smoke	2 (6.7)	2 (28.6)	17 (24.6)	2 (14.3)	
Current smoke	1 (3.3)	0 (0)	2 (2.9)	0 (0)	
PEFR (L/min)	290.9±90.7	295.5±85.9	335.3±98.1	301±78.5	
PEFR % predicted±SD	83.6±24.2	98.6±23.3	97.6±25.4	86.7±19.2	
FEV1 % predicted±SD last time	75.3±15.9	78.4±22.4	85.5±18.1	70.9±18.7	
FVC % predicted±SD last time	94.5±16.1	95.2±20.5	99.4±18.2	90.8±18.8	
Frequency of asthma symptoms in past month, n (%)					
Never	14 (46.7)	2 (28.6)	23 (33.3)	4 (28.6)	
≤2 times/wk	3 (10)	1 (14.3)	15 (21.7)	4 (28.6)	
>2 times/wk	12 (40)	4 (57.1)	23 (33.3)	5 (35.7)	
Everyday	1 (3.3)	0 (0)	8 (11.6)	1 (7.1)	
ICS dose#, n (%)					

High dose	6 (20)	2 (28.6)	13 (18.8)	3 (21.4)	
Moderate dose	14 (46.7)	3 (42.9)	30 (43.5)	8 (57.1)	
ACT±SD	18.4±4.4	21.0±3.9	21.7±3.3	19.9±3.3	
AQLQ±SD	5.6±0.9	4.9±0.8	5.1±1.0	3.9±0.9	
Absolute eosinophil count (cells/μl ±SD)	585.2±360.2	599.2±187.7	177.3±88.9	175.7±100.3	103.5±58.2

EA, Eosinophilic allergic asthma; EN, Eosinophilic non-allergic asthma; NA, Non-eosinophilic allergic asthma; NN, Non-eosinophilic non-allergic asthma; PEFR, peak expiratory flow rate; ACT, Asthma Control Test; AQLQ, Asthma Quality of Life Questionnaire; ICS, inhaled corticosteroids; #, budesonide or equivalent dose, high dose ICS > 800 μg, moderate dose ICS 400 – 800 μg

*No significant different between the group in patient baseline characteristics were analyzed by the ANOVA and chi-square test except absolute eosinophil count.

Table S2. Baseline characteristics of asthma exacerbation and controlled patients

Characteristics	Asthma exacerbation (n = 30)	Asthma controlled (n = 15)
Gender, female (%)	23 (76.7)	11 (73.3%)
Age (years±SD)	58.6±12.5	54.3 ±10.4
Body mass index ±SD	24.6±5.8	25.9±3.2
Allergic (%)	28 (93.3)	12 (80)
Co-morbidity, Rhinitis (%)	28 (93.3)	13 (86.6)
Duration of asthma (years, ±SD)	21.4±15.7	25.5±18.2
PEFR % predicted±SD	81.2±15.4	86.3±22.7
FEV1 % predicted±SD last time	77.5±17.3	75.2±20.2
FVC % predicted±SD last time	89.5±21.5	92.2±19.6
Frequency of asthma symptoms in past month, n (%)		
Never	2 (6.7)	9 (60)
≤2 times/wk	8 (26.7)	6 (40)
>2 times/wk	17 (56.7)	0 (0)
Everyday	3 (10)	0 (0)
ICS dose#, n (%)		
High dose	14 (46.7)	4 (26.7)
Moderate dose	12 (40)	8 (53.3)
ACT ±SD	15.5±3.2	22.2±1.2
AQLQ±SD	3.8±0.6	5.6±1.1

Absolute eosinophil count (cells/ μ l $\pm$ SD) 395.6 $\pm$ 240.8 285.9 $\pm$ 169.8

mRNA	Primer	Sequences (5'-3 ')
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Asthma phenotype (%)

Eosinophilic allergic (EA)	3 (10)	2 (13.3)
Eosinophilic non-allergic (EN)	1 (3.3)	0 (0)
Non-eosinophilic allergic asthma (NA)	21 (70)	11 (73.3)
Non-eosinophilic non-allergic (NN)	5 (16.7)	2 (13.3)

PEFR, peak expiratory flow rate; ACT, Asthma Control Test; AQLQ, Asthma Quality of Life Questionnaire; ICS, inhaled corticosteroids; #, budesonide or equivalent dose, high dose ICS > 800 μ g, moderate dose ICS 400 – 800 μ g

Table E3. Sequences of primers designed

IL4	Forward	TCTGTGCACCGAGTTGACCG
	Reverse	GCAGCCCTGCAGAAGGTTTC
IL13	Forward	TGAGGAGCTGGTCAACATCA
	Reverse	CAGGTTGATGCTCCATACCAT
IL10	Forward	GGGCACCCAGTCTGAGAACAG
	Reverse	TGGCAACCCAGGTAACCCTT
PTGDR2	Forward	ATCCTGTGCTCCCTCTGTGC
	Reverse	GGATGCTGGTGTGCTGTGG
FOXP3	Forward	GAACCTTCCAGGGCCGAGAT
	Reverse	GAGTGCCTGTAAGTGGGGCA
GATA3	Forward	CTTCGGATGCAAGTCCAGGC
	Reverse	CGAGGTTGCCCCACAGTTCA
EF1 α	Forward	CTGAACCATCCAGGCCAAAT
	Reverse	GCCGTGTGGCAATCCAAT

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