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Impact of High Altitude Therapy on Type-2 Immune Responses in Asthma Patients

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Short title

Immune response of asthma treatment in high altitude

Abstract

Background: Asthma patients present with distinct immunological profiles, with a predominance of type 2 endotype. The aim of this study was to investigate the impact of high altitude treatment on the clinical and immunological response in asthma.

Methods: Twenty-six hospitalized asthma patients (9 eosinophilic allergic; EA, 9 non-eosinophilic allergic; NEA and 8 non-eosinophilic non-allergic; NN) and 9 healthy controls in high-altitude for 21 days were enrolled in the study. We assessed eosinophils, T cells, Tregs, and innate lymphoid cells (ILC) from peripheral blood using flow cytometry.

Results: The number of eosinophils (both resting and activated) and CRTH2-expressing $CD4^+$ and $CD8^+$ T cells decreased significantly in EA patients after altitude treatment. The frequency of $CRTH2^+$ Tregs as decreased significantly in all the asthma phenotypes as well as the frequency of ILC2 was significantly reduced in EA after altitude treatment. After 21 days of altitude therapy, CRTH2-expressing ILC2, $CD4^+$ and $CD8^+$ T cells and Treg cells showed attenuated responses to exogenous PGD2. Furthermore, PGD2 signaling via CRTH2 was found to diminish the suppressive function of $CRTH2^+$ Tregs which partially normalized during high altitude treatment. Improved asthma control was particularly evident in allergic asthma patients and correlated with decreased frequencies of $CRTH2^+$ Treg cells in EA patients. Serum IL-5 and IL-13 decreased during climate treatment in asthma patients with high baseline levels.

Conclusions: Asthma treatment in high altitude reduced the type 2 immune response, corrected the increased CRTH2 expression and its dysregulated functions.

Keywords: Eosinophils, T cells, CRTH2, asthma phenotype, high altitude.

Abbreviations

CRTH2: Chemoattractant receptor homolog expressed on Th2 cells

EA: Eosinophilic allergic asthma

NEA: Non-eosinophilic allergic asthma

NN: Non-eosinophilic non-allergic asthma

PGD2: Prostaglandin D2

Th2: T helper 2

Tc: T cytotoxic

Tregs: Regulatory T cells

ILC: Innate lymphoid cells

Introduction

Subtypes of asthma defined by the type of inflammation and complex immune-regulatory networks open the opportunity for new pathway precision diagnosis and targeted-treatments (1, 2). Asthma management can be individualized not only depending on the severity of the disease, but more importantly on the phenotypic and endotypic characteristics of the patient. An asthma endotype is defined by a distinct pathophysiological mechanism, such as the type 2 endotype (3, 4). Allergic asthma is the most common asthma phenotype with an earlier onset (5-7). Non-allergic or intrinsic asthma includes a subset of patients with asthma without allergic sensitization (8, 9). These patients show quite variable response to standard therapy (5). Eosinophilic asthma is defined as blood eosinophilia (>350 cells/µl), associated with tissue and sputum eosinophilia, thickening of the basement membrane zone and often by good response to inhaled corticosteroid therapy (10, 11). Improvements in the blood and tissue eosinophilia are associated with fewer exacerbations and lower health care costs (6). Particularly, CD69 expression on eosinophils is an indicator for eosinophil activation (12). It is expressed at a low level on unstimulated blood eosinophils and upregulated by interleukin (IL)-5 (7).

Chemoattractant receptor homolog expressed on Th2 cells (CRTH2) represents one of two functional prostaglandin D2 (PGD2) receptors. CRTH2 is expressed on various types of cells; eosinophils, Th2 cells, ILC2, basophils, $CD8^+$ T cells, mast cells, macrophages, monocytes and dendritic cells (13, 14). The PGD2/CRTH2 axis has emerged into a potential pathophysiologic factor for allergy and asthma (15). In allergic asthma patients, the production of PGD2 is increased by allergen exposure (16). After an allergen challenge, the concentration of PGD2 is increased in bronchoalveolar lavage fluid, and its 9α, 11β-PGF2 metabolite is increased in plasma (17). PGD2 effects are mediated partially by prostaglandin D2 receptor 1 (DP1) which causes bronchoconstriction, vasodilation, increases in capillary permeability and mucus production (18). CRTH2 on immune cells is involved in the migration and activation of Th2 cells, eosinophils, basophils and ILC2, and with production of type 2 cytokines IL-4, IL-5 and IL-13 (19). Elevated levels of peripheral $CD4^+CRTH2^+$ T cells is a feature of severe allergic asthma (20).

ILC2s and Th2 cells are a significant source of type 2 cytokines and play a role in eosinophilic inflammatory response, allergy and remodeling in asthma (21, 22). Increased circulating and sputum IL-5 and IL-13-producing ILC2s were detected in severe asthma compared to mild asthma patients (15). Furthermore, increased numbers of $IL\text{-}5^+$ and $IL\text{-}13^+$ ILC2s were found in sputum after allergen challenge in asthma patients (23). Moreover, increased frequency of IL-13-producing ILC2 were enhanced by PGD2 that was co-stimulated with IL-2, IL-25 and IL-33 in asthma patients. (24, 25). IL-13-expressing ILC2 and Th2 cells are also responsible for bronchial epithelial tight junction barrier leakiness in asthma patients (26, 27).

Several studies have demonstrated the efficacy of altitude therapy in treating asthma patients. The hypothesis the effect of high-altitude treatment combines anti-inflammatory treatment as allergen avoidance, lower pressure, less stress, less particle exposure and exposure to sunshine stimulate vitamin D photosynthesis. High altitude treatment showed significantly decreased monocyte activation and CRTH2 expression on $CD4^+CD25^+$ T cell in asthmatic patients (28). In children with atopic dermatitis, high altitude treatment showed significant reductions in memory Tregs, transitional B cells and plasmablasts. Concomitant, increases in memory B cells, effector memory $CD8^+$ T cells, central memory $CD4^+$ T cells and $CCR7^+$ Th2 cells were reported (29). However, detailed changes to the immunological landscape after altitude therapy has not been described in asthma patient subgroups. The present

study asesses the kinetics of immunological changes in different asthma phenotypes in response to treatment in high altitude with specific focus on the CRTH2 receptor and its functions on ILCs, CD4 and CD8 T cell subsets, Tregs and eosinophils during the treatment.

Methods

Subjects

This observational study enrolled 29 adult inpatients (14 males, 15 females and average aged 53.3 years) with asthma at the Pulmonary Clinic, Hochgebirgsklinik, Davos, Switzerland (1560m above sea level). Patients had an average stay of 21 days in Davos. All patients came from low altitude locations and chose their 21 days stay in Davos in to receive high altitude treatment. Nine healthy control subjects (5 males and 4 females) who visited Davos for the first time were recruited in the study (Table S1). All patients were treated with short-acting inhaled beta-agonists for quick relief asthma symptoms and long-acting inhaled beta-agonists plus inhaled corticosteroid (Table S2). No systemic corticosteroid treatment was used in the study. All patients showed good compliance with their treatment and proper inhalation technique of inhaled medication.

Medical history, physical examination, pulmonary function test, exhaled nitric oxide (FeNO2) measurement were performed during the first days of admission to the hospital. The patients received questionnaires on asthma control test (ACT) score (30). Lithium heparin-coagulated blood were collected from patients with asthma and healthy control subjects at baseline and 21 days. Total eosinophil counts > 350 cells/µl to classify as eosinophilia was assessed as well as medical history skin pricking test or allergen-specific IgE for allergy diagnosis. All subjects gave informed consent before participation in the study. The study was approved by the Kanton Zurich Ethical Committee.

Flow cytometry staining

Whole blood was collected into lithium heparin tubes and stored at room temperature until analyses. First, 500 µl whole blood were stained with 0.5 µl viability dye eFluor 780 (eBioscience, San Diego, CA, USA) for 30 min 4°C, protect from light. After that, whole blood was lysed with 500 µl of red blood cell soft lysis buffer for 10 min at room temperature, then washed twice with phosphate buffer saline (PBS). Cells were then stained with anti-CRTH2-Alexa Flour 647 (AF 647) (BD Biosciences San Jose, CA, USA) for 15 min at room temperature and washed with PBS. Cells were stained extracellularly for markers associated with eosinophils, T cells, and innate lymphoid cells. Flow cytometry was performed using a Gallios (Beckman Coulter, Indianapolis, IN, USA). Data and figures were analysed with Kaluza Software (Beckman Coulter). For a detailed description of reagents and procedures, see the Method section in this article's Supplements.

Stimulation of the cells with PGD2

Fresh whole blood stimulation with PGD2 was performed to evaluate the activation of CRTH2 expressing ILCs and T cell subsets. DK-PGD2 (Caymanchem, Ann Arbor, MI, USA) from a stock solution (10 mM) was diluted to a 200 µM solution and then 500 µl whole blood was stimulated with 2.5 µl of PGD2 of 200 µM to a final concentration of 1 µM for 1 hour. Then, whole blood was stained with viability dye, lysis of red blood cells and surface mAbs against cell surface molecules as previously described above.

Suppression assay for Tregs

Peripheral blood mononuclear cells were purified $CRTH2^{+}CD4^{+}CD25^{+}CD127^{-}$ ($CRTH2^{+}$ Tregs), $CRTH2^{-}CD4^{+}CD25^{+}CD127^{-}$ ($CRTH2^{-}$ Tregs) and $CD4^{+}CD25^{-}$ T cells (T effector cells) by using FACSAria III (Beckton Dickinson, Franklin Lakes, NJ, USA). 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. Cells were also stimulated with PGD2 on day 0 to compare the suppressive function between with and without PGD2 stimulation. The vehicle in PGD2 stimulation was 10% ethanol and diluted as

the same PGD2 dilution. For a detailed description of reagents and procedures, see the Method section in this article's Supplements.

Detection of cytokines in serum and supernatant

Serum IL-13 levels were measured using the Erenna Instrument (Merck KGaA, Darmstadt, Germany). Serum IL-5 levels were measured on the Ella system (ProteinSimple, San Jose, CA, USA). Serum Eosinophil-derived neurotoxin (EDN) levels were analyzed using an IVD ELISA from Immundiagnostik AG, Bensheim, Germany.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Baseline subject characteristics data were analysed using either the Chi-square test or 2-way ANOVA. Nonparametric Wilcoxon-matched pair test was used to calculate differences between time points. Repeated ANOVA and Tukey's multiple comparisons were used to calculate differences between the groups. Data were presented as mean ± standard error mean (SEM). *P* values of less than .05 were considered significant.

Results

Patients characteristics

Nine eosinophilic allergic asthma (EA), 9 non-eosinophilic allergic asthma (NEA), 8 non-eosinophilic non-allergic (NN) patients and 9 healthy controls (HC) completed the study. Three asthmatic patients dropped out of the study since they stayed shorter than 21 days in the hospital but not because of therapy responses. The baseline subject characteristics are shown in Table S1. No significant differences in the average age, gender proportion, body mass index, family history of asthma, history of past smoking, total IgE levels, the lung function test, the severity of asthma (as graded by pulmonary function) and asthma control were observed between the asthma groups. NEA patients had longer durations of asthma compared to EA and NN patients.

High altitude decreased circulating eosinophils and activated CRTH2$^+$ eosinophils after treatment

Total numbers of eosinophils were significantly decreased in the patients with EA after 21 days of therapy (Fig. 1A). Activation markers of these eosinophils were then assayed by flow cytometry (Fig. S1A). A significant decrease compared to baseline was observed in the number of activated CRTH2$^+$ eosinophils (CD11b$^+$CD16$^-$Siglec8$^+$CCR3$^+$CRTH2$^+$CD69$^+$) in EA patients (Fig. 1B and Fig. S1B). Activated eosinophils (CD11b$^+$CD16$^-$Siglec8$^+$ CD69$^+$) and activated CRTH2$^-$ eosinophils (CD11b$^+$CD16$^-$Siglec8$^+$CCR3$^-$CRTH2$^-$CD69$^+$) were higher in EA compared with other groups at baseline and decreased after treatment (Fig. S2A and B). There was no statistically significant change in activated CRTH2$^-$ eosinophils. The IL-5, IL-13 and EDN levels in EA patients' sera at baseline were higher compared to other groups (Fig. 1C). Serum IL-5 and IL-13 levels decreased in patients with high baseline levels (Fig. 1C). After 21-day treatment, the mean serum IL-5, IL-13 and EDN levels were still higher in EA patients than other asthma subgroups. Moreover, serum IL-5 and IL-13 in EA patients correlated with the absolute total number of eosinophils in EA patients (Fig. 1D). EDN levels correlated with the absolute total number of eosinophils in EA and NEA patients (Fig. 1E).

High altitude decreased CRTH2 expression on T cell subsets after treatment

To determine the changes in T cell subsets occurring after 21 days of therapy, we analysed CD4$^+$, CD8$^+$ and CD4$^+$CD25$^+$CD127$^-$ (Treg) populations (Fig. S3). The absolute numbers of lymphocytes were significantly decreased in EA and NN patients after 21 days (Fig. S4A). The total numbers of CD4$^+$ T cells also decreased in EA after treatment but no significant changes in Treg cells were observed (Fig. S4B and C). The absolute numbers of CD8$^+$ T cells at baseline were higher in NN patients than EA patients (Fig. S4D).

Next, we analysed CRTH2 expression on T cell subsets. Patients with EA and NEA had significantly more absolute counts and percentage of CRTH2$^+$ T-helper (Th) (CD3$^+$CD4$^+$CD25$^-$CRTH2$^+$) cells compared to healthy controls at baseline (Fig. 2A and Fig. S5A). After altitude therapy, EA and NEA patients showed a reduction in CRTH2$^+$ Th cells (Fig. 2A). The frequency and absolute numbers of CRTH2$^+$ Tregs

($CD3^+CD4^+CD25^+CD127^-CRTH2^+$) were higher at baseline in EA and NEA patients than healthy controls (Fig. 2B). Interestingly, we observed significantly reduced $CRTH2^+$ Treg cells among all asthma phenotypes after 21 days of treatment in high altitude (Fig. 2B). Before treatment, the frequency and absolute numbers of $CRTH2^+$ Tc ($CD3^+CD8^+CRTH2^+$) cells revealed also increased in EA and NEA patients compared with healthy controls (Fig. 2C). During the 21 days of treatment, $CD8^+CRTH2^+$ T cells showed a decrease in EA and NEA patients (Fig. 2C). The results of the CRTH2 expression on T cell subsets are shown in Fig. S5A - C. Furthermore, we analysed how CRTH2 expression on T cell subsets correlates with serum IL-5 and IL-13 levels. The frequency and absolute numbers of $CRTH2^+$ Th cells showed a negative correlation with serum IL-13 levels in EA patients (Fig. 2D). This analysis demonstrates that 21 days of high altitude treatment reduces frequencies and cellularity of CRTH2-expressing T cell subsets.

High altitude reduced CRTH2 expression on ILCs after treatment

We analysed total ILCs ($CD45^+Lin^-CD127^+CD161^+$) and group 1, 2 and 3 ILC subsets in whole blood (Fig. S6). The circulating numbers and percentages of ILC2s ($CD45^+Lin^-CD127^+CD161^+CRTH2^+$) showed no significant difference between asthma patients and healthy controls at baseline. Notably, the frequency of ILC2s significantly in relation to other identifiable ILC populations decreased after 21 days in EA patients (Fig 3A and B). The frequency of ILC1s and ILC3s NCR^+ and NCR^- also showed no significant difference between asthma patients and healthy controls at baseline (Fig. S7 A - C). The frequency of ILC1s was increased ($CD45^+Lin^-CD127^+CD161^+c\text{-}kit^-CRTH2$) in NN patients after treatment (Fig. S7A). We did not find any correlation between circulating ILC2s and serum cytokine levels (Fig. 3C).

High altitude decreased response to PGD2 stimulation in CRTH2-expressing ILCs and T cell

To determine the effect of treatment on the functional PGD2 response in ILCs and T cell subsets, we stimulated whole blood with PGD2 and measured the frequency of activated ($CD69^+$) cells. In unstimulated whole blood, we could not detect any significant difference on activated cell numbers between baseline and after 21 days of treatment in high altitude in asthma patients (Fig. 4A – D). In PGD2-stimulated whole blood, $CD69^+$ ILC2s as a proportion of ILC2s were significantly decreased in asthma patients after 21 days in Davos, while there was no change in healthy controls (Fig. 4A and Fig. S8A). Interestingly, activated $CD4^+CD25^-CRTH2^+$, $CD4^+CD25^+CD127^-CRTH2^+$, and $CD8^+CRTH2^+$ T cells were strikingly reduced after 21 days of treatment in PGD2-stimulated peripheral blood of asthma patients (Fig. 4B - D and Fig. S8B - D). We also found the frequency of $CD69^+CD8^+CRTH2^+$ T cells in asthma patients was higher at baseline in PGD2 stimulated whole blood compared to healthy controls (Fig. 4D).

Decreased suppressive function in CRTH2 expressing Tregs

We next assessed functional properties of Tregs with and without CRTH2 expression. There was a relatively weak suppressor function of CRTH2 positive Tregs of asthma patients, which was partially reconstituted after 21 days of treatment (Fig. 5A). In PGD2 stimulated cultures, the frequency of T effector cell proliferation was also higher when cultured with $CRTH2^+$ Tregs compared to $CRTH2^-$ Tregs (Fig. 5A). Moreover, $CRTH2^+$ Tregs showed less suppressive function in the PGD2-stimulated cultures, whereas PGD2 stimulation showed no effect with $CRTH2^-$ Tregs. We also analysed the supernatants in the cells cultured and found IL-4 levels to be significantly higher in $CRTH2^+$ Tregs with PGD2 stimulation (Fig. 5B). Thus, we demonstrated that $CRTH2^+$ Tregs had defective suppressive function after PGD2 stimulation, which partially recovered after 21 days of treatment.

Asthma control, FEV1 and exhaled nitric oxide correlated with immune responses

Overall, altitude treatment significantly improved asthma control, lung function and significantly decrease lung inflammation in asthma patients (Fig. S9A). FeNO levels had a positive correlation with the number of eosinophils, activated eosinophils and serum IL-5 in asthma patients, and correlated negatively with the number of $CRTH2^+$ Tc cells (Fig. S9B). Improved lung function correlated with reduced numbers of lymphocytes, $CRTH2^+$ Th cells, and eosinophils (Fig. S9B).

In the asthma phenotype analysis, patients with EA and NEA showed significantly increased asthma control after treatment (Fig. 6A). Improved asthma control that determined by increased ACT score in EA patients showed a strong correlation with decreased number and frequency of $CRTH2^+$ Treg cells (Fig. 6B). Furthermore, FeNO levels had a negative correlation with the number and frequency of $CRTH2^+$ Tc cells (Fig. 6C). In NEA patients, improved lung function correlated with decreased lymphocytes and FeNO levels and had a negative correlation with the number of $CRTH2^+$ Tc cells (Fig. 6C). In NN patients, ACT scores had a positive correlation with the number and frequency of $CRTH2^+$ Th cells. Also, FEV1 levels correlated with FeNO levels, serum EDN levels and lymphocytes (Fig. 6C). Furthermore, FeNO levels showed a negative correlation with lymphocytes in NN patients (Fig. 6C). The network analyses of immune markers in the different phenotype of asthma showed differences in correlation between eosinophils, T cell subsets, ILC2s and clinical responses (Fig. S9C). A strong correlation network was observed in eosinophilic and allergic asthma, whereas there was a weak correlation in non-eosinophilic non-allergic asthma. Heat map correlation and correlation graphs between asthma phenotypes are shown in Fig. S10A. The duration of asthma in non-eosinophilic patients had negative correlation with the frequency of $CRTH2^+$Th and $CRTH2^+$Treg cells (Fig. S10B).

Discussion

Asthma treatment in high altitude has been shown to improve asthma control, quality of life, pulmonary function, decrease lung inflammation (decreased exhaled nitric oxide) and reduce oral corticosteroids use in asthma patients (28, 31). High-altitude climates have low concentrations of house dust mite (HDM) due to increased altitude and decreased humidity (32). In addition, exposure to fungal spores, molds and pollen are shorter in duration and lower in concentration at high altitude (33), resulting in reduced bronchial hyperresponsiveness, total blood eosinophils, eosinophilic cationic protein and HDM-IgE level (34). This study enrolled the patients during the period of early winter to end summer, therefore controlling for the avoidance of the relevant allergen (grass and tree pollen) during the season. High altitude may have a direct physiological benefit because of the lower oxygen concentration and viscosity of the air that promoted the full expansion of the lungs and decreased lung resistance (35). Moreover, moving away from psychological stress is an add-on which may also affect neuroimmune mediators with reduced airway inflammation and triggers of asthma exacerbation (36, 37). Other factors related to the exposome (e.g. pollution, traffic and healthier diet while in hospital, etc.) might account for the changes observed.

This study brings together several novel approach to detail the functional immune response during 21 days of treatment in high altitude, while showing that asthma treatment in high altitude simultaneously improved asthma control, FEV1 and FeNO, while modifying the immunological characteristics of effector cells known to contribute to eosinophilic and allergic inflammation. This study had certain limitations, such as another group of asthma patients for comparison in a similar clinical setting at low altitude. In addition, sampling of bronchial samples were lacking, such as induced sputum and bronchoalveolar lavage.

In eosinophilic and allergic asthma, type 2 immune response, is a significant driver of asthma pathology (38, 39). CRTH2 is the hallmark of both Th2 and ILC2 and regarded as a potent inducer of type 2 cytokine secretion. We show that serum IL-5, IL-13 and EDN are elevated in the eosinophilic allergic phenotype (40). Asthma treatment in high altitude reduces peripheral blood and sputum eosinophil levels (34, 41). In patients with allergic asthma, numbers of eosinophils increase after exposure to specific allergens (42). The CD69 expression on eosinophils is a marker of eosinophil activation by various cytokines and after specific allergen-challenge in

asthma patients (43, 44). Our results describe elevated CD69 expression on $CRTH2^+$ and $CRTH2^-$ eosinophils in EA patients compared to other groups at baseline, but only activated $CRTH2^+$ eosinophils decreased significantly after therapy.

The decreased peripheral blood lymphocytes showed a correlation with improved lung function and decreased lung inflammation in NN patients. Many of the immunopathologic features of non-allergic asthma are similar to allergic asthma but higher expression of RANTES and GM-CSF receptor alpha in mucosa and bronchoalveolar lavage were described in non-allergic asthma (45). Th17 response appears to play a crucial role in airway neutrophilia (46). Allergen and other environmental stimuli have been shown to trigger Th17-mediated airway inflammation . In this study, we did not measure cytokine-producing T cells that are related to neutrophilic inflammation. Thus we could not conclude that asthma treatment in high altitude altered systemic neutrophilia inducing immunologic responses in the group of NN patients. The present study demonstrated that most of the patients without allergy and eosinophil (NN) had a benefit after 21 days of the treatment.

CRTH2 expression on T cells in the bronchoalveolar lavage of asthma patients is higher than healthy controls (47, 48). In allergic asthma, increased type 2 cytokines are seen after allergen-challenged (49). CRTH2 has been shown to be a highly selective hallmark for Th2 cells and ILC2s in human cells (50). Our results showed CRTH2 expression on $CD4^+$ Th, Treg and $CD8^+$ Tc cells were higher in allergic asthma patients compared to healthy controls and reduced after the treatment. Interestingly, higher CRTH2 expression on Tregs was a visible marker in allergic asthma patients together with dysfunction in suppressor capacity of $CRTH2^+$ Tregs. Dysregulation of $CRTH2^+$ Tregs produced IL-10 and type 2 cytokines together. There was partial reconstitution after 21 days of high altitude therapy.

PGD2 activates Th2 cells and ILC2s and is associated with increased expression of CD69 (51-53). We demonstrated that, at baseline, PGD2-activated T cell subsets and ILC2s were not different between asthma patients and healthy controls except for $CRTH2^+CD8^+$ T cells. PGD2 and LTE4 regulate diverse genes in $CRTH2^+CD8^+$ T cells inducing type2 cytokines and many other pro-inflammatory cytokines and chemokines, which could contribute to eosinophilia (54). Asthma treatment in high altitude reduced sensitivity of ILC2s and CRTH2 T cell subsets in response to PGD2. Interestingly, CRTH2 antagonists have shown efficacy in patients

with mild to moderate asthma with improvement of FEV1 and reduced sputum eosinophils (55-58). Our data collectively suggest that altitude therapy may result in resolution of allergic inflammation via similar mechanisms to drugs targeting the CRTH2 and PGD2 interaction *in vivo*, by reducing effector immune cell response to PGD2, and enhancing suppressive function of CRTH2+ Tregs.

In summary, we demonstrated that the clinical improvement of high altitude treatment is dependent of the asthma phenotypes. Molecular and cellular analysis of the patient endotype revealed a decrease in type 2 immune response, mostly evident in allergic, eosinophilic asthma patients. Furthermore, desensitization of the CRTH2 system was shown in all asthma phenotypes, indicating a potential implication of the CRTH2 pathway not only in eosinophilic asthma patients.

Conflict of Interest

Andrew L. Croxford, Herve Farine, Peter M.A. Groenen, Martine Clozel and Daniel S. Strasser are Idorsia employees and have received shares and options.

Authors' Contribution

G.C., HW.D., EDR and C.A. planed the study. T.B., N.L. and P.S. carried out the experiment. T.B., A.L.C., H.F., D.S.S., M.A. and C.A. conceived and planned the experiments. T.B. wrote the manuscript with support from D.S.S., M.S. and C.A.. G.C., HW.D., J.S., EDR., P. K.. recruited the participants. A.D., B.R. and D.M. prepared the samples. G.T. was analysis of statistics. P. MA. G., EDR, M.C. and C.A. supervised the study.

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Figure legend

Figure 1. Type 2 endotype is predominant in EA patients and there is reduction of type 2 endotype after treatment in high altitude. Absolute total number of peripheral blood eosinophils (**A**), numbers and frequency of activated $CRTH2^+$ eosinophils ($CD11b^+CD16^-Siglec8^+CCR3^+CRTH2^+CD69^+$) (**B**) and serum IL-5, IL-13 and EDN levels (**C**) of eosinophilic allergic asthma (EA), n = 9, non-eosinophilic allergic asthma (NA), n = 9, non-eosinophilic non-allergic asthma (NN), n = 8, and healthy control (HC), n = 9 compared between baseline (red dot) and 21 days (blue dot) of the treatment. **D**, Serum IL-5, IL-13 and EDN levels correlated with absolute eosinophils. **E**, Heat map Spearman's correlation between serum cytokine levels and eosinophils and activated eosinophils in different asthma phenotypes. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Figure 2. $CRTH2^+$ cells decrease over 21 days in all asthma subgroups after treatment in high altitude. **A - C**, Numbers and frequency of CRTH2 expression on Th ($CD4^+CD25^-$), Treg ($CD4^+CD25^+CD127^-$) and Tc ($CD8^+$) of eosinophilic allergic asthma (EA), n = 9, non-eosinophilic allergic asthma (NA), n = 9, non-eosinophilic non-allergic asthma, n = 8, and healthy control (HC), n = 9 compared between baseline (red dot) and 21 days (blue dot) of the treatment. **D**, Heat map Spearman's correlation between serum IL-5 and IL-13 levels and T cell subsets in different asthma phenotypes. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Figure 3. Decreased type 2 innate lymphoid cells (ILC2s) in type 2 endotype after treatment in high altitude. **A,** Representative flow cytometry dot plot of ILCs. Expression of CRTH2 defined type 2 innate lymphoid cells (ILC2s). Data are shown as means. **B**, Numbers and frequency of ILC2s of eosinophilic allergic asthma (EA), n = 9, non-eosinophilic allergic asthma (NA), n = 9, non-eosinophilic non-allergic asthma (NN), n = 8, and healthy control (HC), n = 9 compared between baseline (red dot) and 21 days (blue dot) of the treatment. **C**, Heat map Spearman's correlation between serum IL-5 and IL-13 levels and ILC2s in different asthma phenotypes. **P* < 0.05, ***P* < 0.01.

Figure 4. Reduced sensitization of $CRTH2^+$ cells after treatment in high altitude **A - D,** Frequency of activated ($CD69^+$) $CRTH2^+$ ILCs, $CRTH2^+CD4^+CD25^-$, $CRTH2^+CD4^+CD25^+CD127^-$ and $CRTH2^+CD8^+$ T cells compared between before

and after 1 hour PGD2 stimulation of 8 asthma patients (2 EA, 4 NA and 2 NN) and 4 healthy controls. *$P < 0.05$, **$P < 0.01$.

Figure 5. A, Treg suppression assay using $CRTH2^+$ and $CRTH^-$ Tregs isolated from allergic asthma patients (n = 6). Percentage of T effector cells proliferation following 5 days of culture with $CRTH2^+$ and $CRTH^-$ Tregs and compared between the vehicle and PGD2 stimulation (*Left*). Histogram of T effector cells proliferation in different Treg: T effector proportion of different conditions and numbers of each histogram indicated mean the percentage of proliferating cells (*Right*). **B,** Levels of cytokines IL-4, IL-13 and IL-10 in cell culture supernatants of Treg suppression assay, Treg: T effector proportion (1:1), n = 6. Data are shown as means ± SEM.*$P < 0.05$, **$P < 0.01$.

Figure 6. A, Asthma control test (ACT) score, force expiratory volume in 1 s (FEV1) and exhaled nitric oxide (FeNO) in patients with eosinophilic allergic asthma (EA), n = 9, non-eosinophilic allergic asthma (NA), n = 9 and non-eosinophilic non-allergic asthma (NN), n = 8 compared baseline (red dot) and 21 days (blue dot) of the treatment. **B**, Correlation between ACT score and $CRTH2^+$ Tregs in EA patients. **C,** Heat map Spearman's correlation between clinical responses and immunologic responses in different asthma phenotypes. *$P < 0.05$, **$P < 0.01$**.**

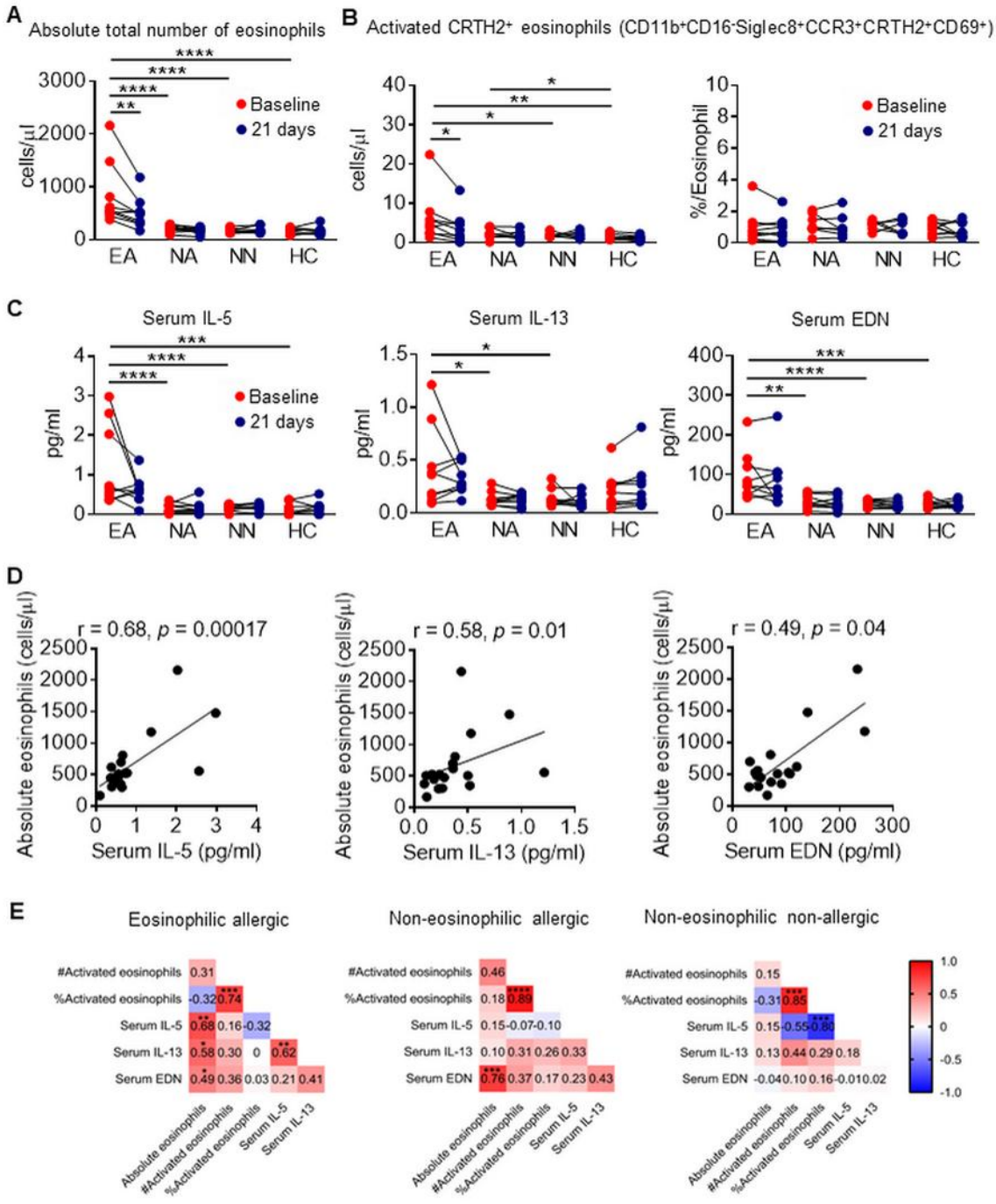


Figure 1 Boonpiyathad et al.

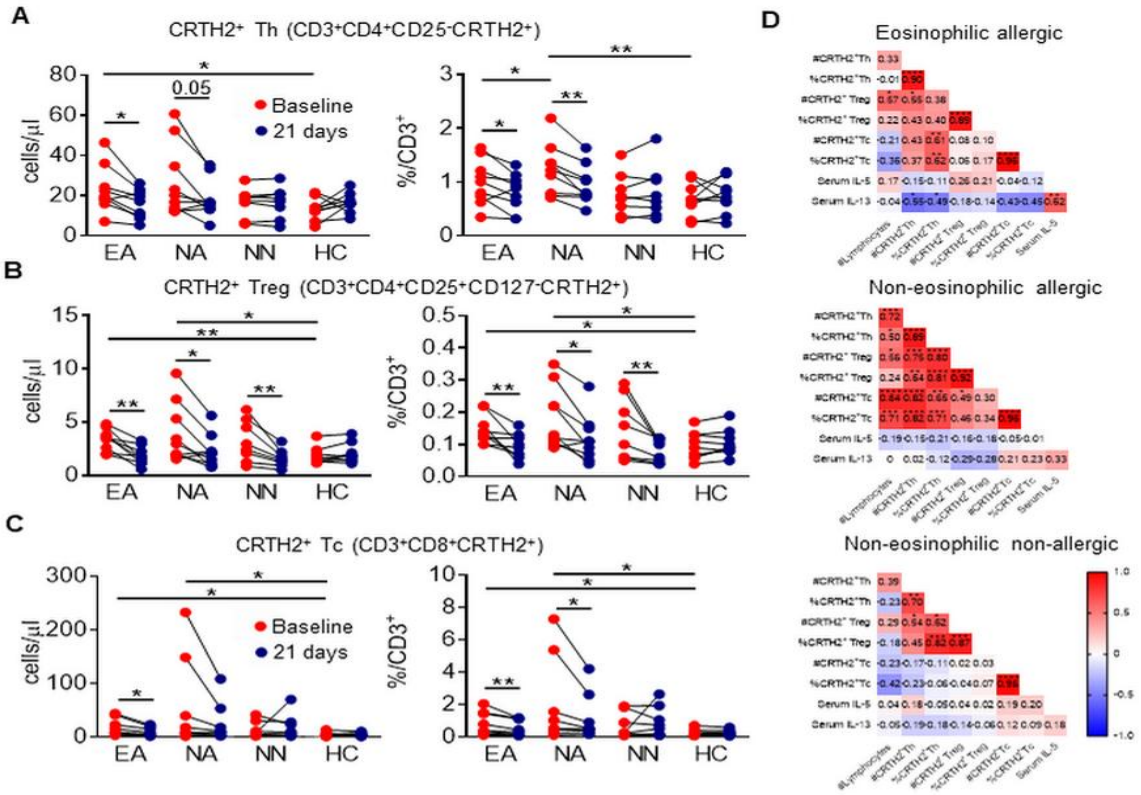
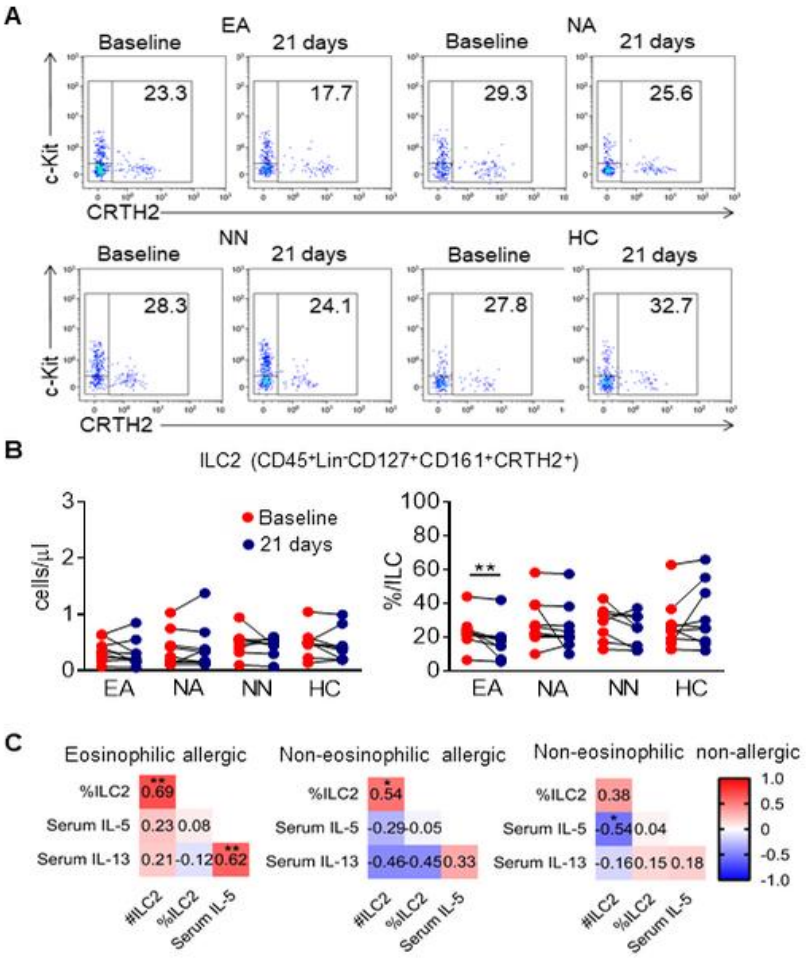


Figure 2 Boonpiyathad et al.



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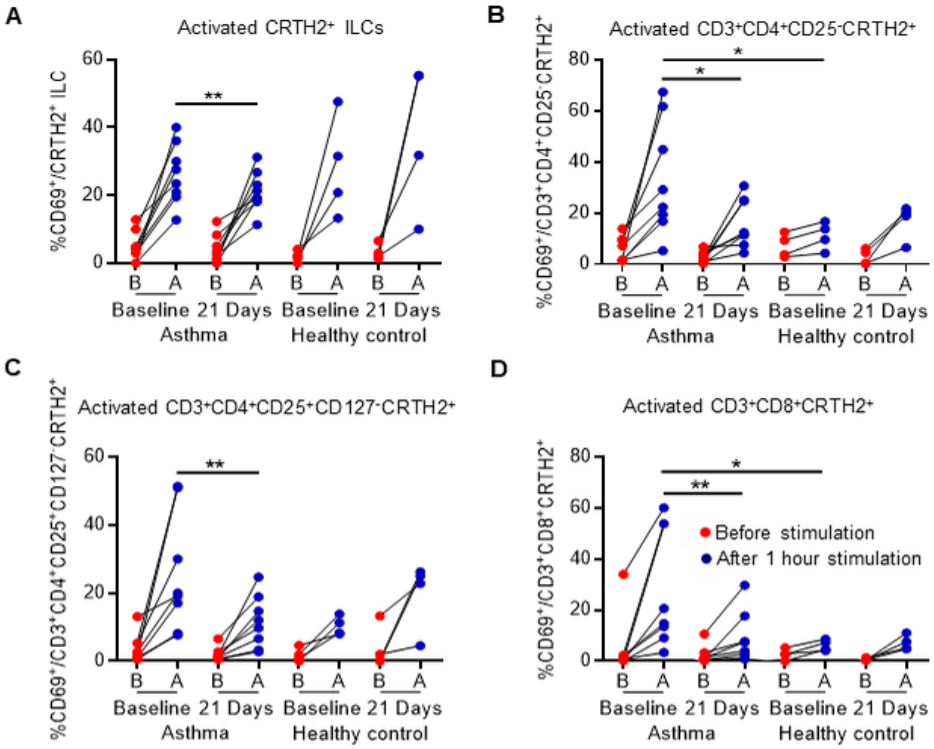

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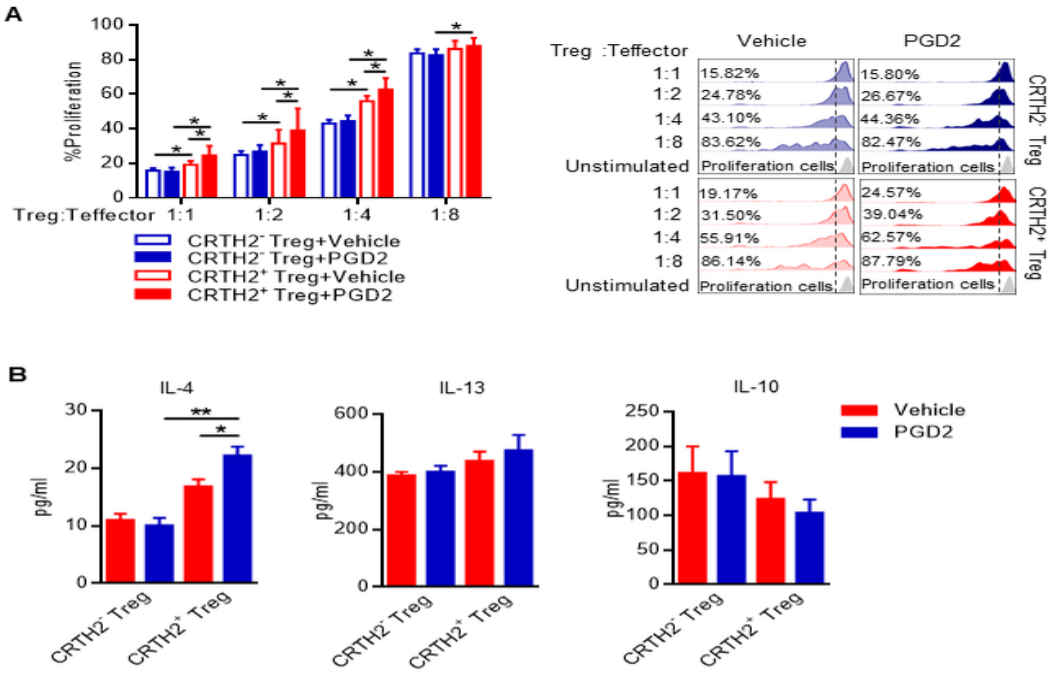

Figure 5 Boonpiyathad et al.

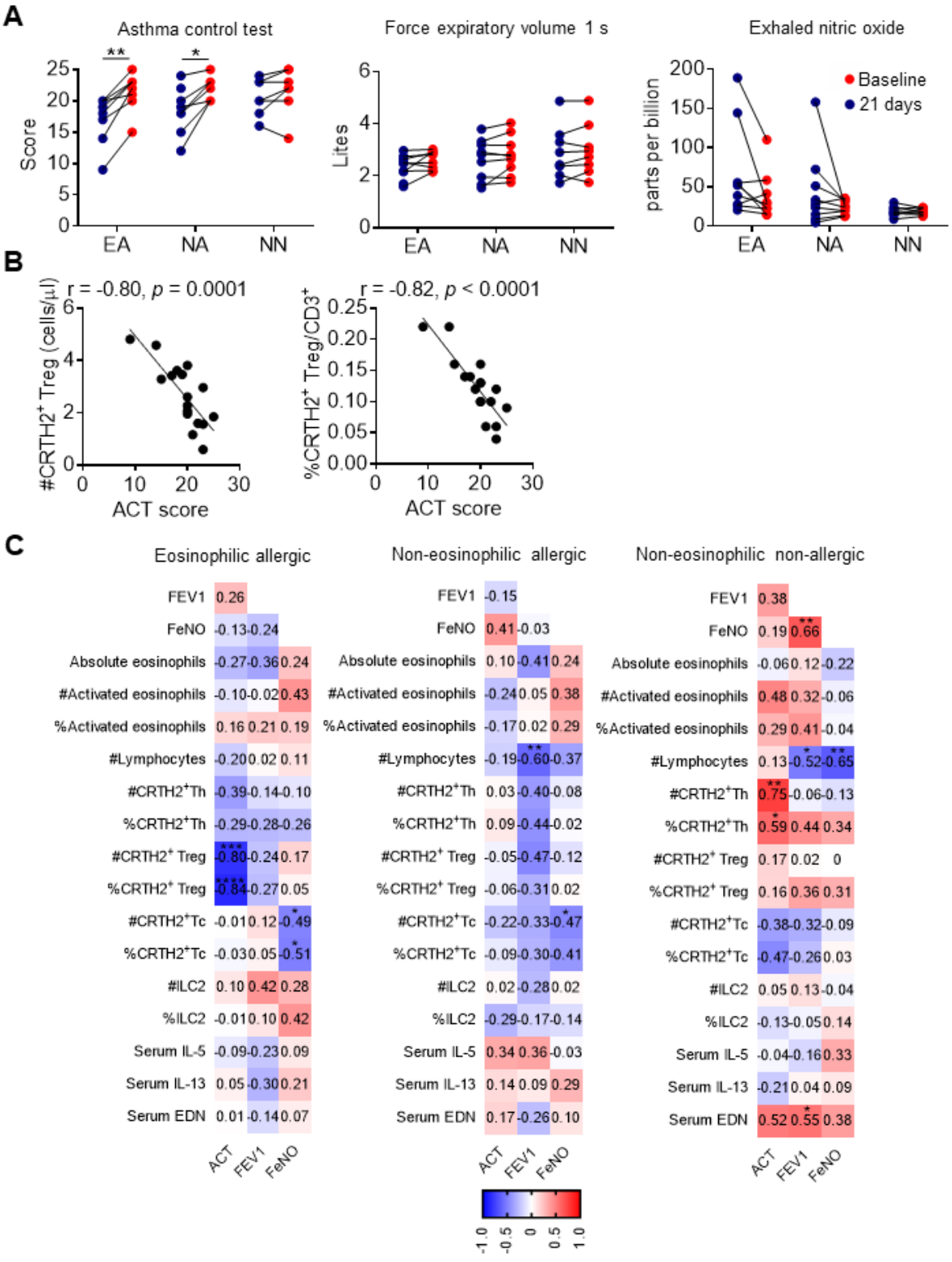

Figure 6 Boonpiyathad et al.