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Circulating CXCR5⁺CD4⁺ T cells participate in the IgE accumulation in allergic asthma

Running head: Circulating CXCR5⁺CD4⁺ T cells in asthma

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Highlights

- IL-4⁺-, IL-21⁺- CXCR5⁺CD4⁺ T cells was increased in allergic asthma patients.
- Circulating CXCR5⁺CD4⁺ T cells boosted IgE production.
- The IgE production could be inhibited by IL-4 or IL-21blockage.
- IL-4⁺-, IL-21⁺-CXCR5⁺CD4⁺ T cells positively correlated with total IgE level.

Abstract

The pathogenesis of allergic asthma is primarily characterized by abnormality in immunoglobulin(Ig)E pathway, suggesting a possible role for follicular helper T cells (Tfh) in the genesis of excessive IgE accumulation. The blood chemokine (C-X-C motif) receptor 5 (CXCR)5⁺CD4⁺ T cells, known as “circulating” Tfh, share common functional characteristics with Tfh cells from germinal centers. The aim of this study was to determine the phenotypes and functions of circulating CXCR5⁺CD4⁺ T cells in allergic asthmatics. Here we found the frequency of the circulating CXCR5⁺CD4⁺ T cells was raised in allergic asthma compared with healthy control (HC). Phenotypic assays showed that activated circulating CXCR5⁺CD4⁺ T cells display the key features of Tfh cells, including invariably coexpressed programmed

cell death (PD)-1 and inducible costimulator (ICOS). The frequency of interleukin IL-4⁺, IL-21⁺-producing CXCR5⁺CD4⁺ T cells was increased in allergic asthma patients compared with HC. Furthermore, sorted circulating CXCR5⁺CD4⁺ T cells from allergic asthma patients boosted IgE production in coculture assay which could be inhibited by IL-4 or IL-21 blockage. Interestingly, IL-4⁺, IL-21⁺-CXCR5⁺CD4⁺ T cells positively correlated with total IgE in the blood. Our data indicated that circulating CXCR5⁺CD4⁺ T cells may have a significant role in facilitating IgE production in allergic asthma patients.

KEY WORDS Follicular helper T; CXCR5; Allergic asthma; IL-21; IL-4.

1. Introduction

Asthma is an inflammatory disorder of the conducting airways leading to variable airflow obstruction in association with airway hyperresponsiveness[1,2]. Abnormality in immunoglobulin (Ig)E pathway has commonly been considered a cardinal feature in allergic asthma, associating not only with the inception of asthma but also with its acute deterioration[1,2].

While T cell help for the pathologic abundance of IgE has long been considered as a helper T cell (Th)2 property[3], recent studies have identified follicular helper T (Tfh) cells as the specialized subset indispensable for providing cognate help to B cells in germinal centers (GCs) [4-10]. Tfh cells distinguishable from other Th cells by the expression of the B cell zone-homing chemokine receptor CXCR5, programmed cell death (PD)-1, inducible costimulator (ICOS), CD40L and the transcriptional repressor B cell lymphoma 6 (BCL-6). Tfh cells perform their functions via interleukin (IL)-21, their hallmark cytokine [6-10]. Through a combination of cytokine secretion and highly regulated cell-cell interactions, Tfh cells provide essential help for B-cell affinity maturation, class switch recombination, and somatic hypermutation within GC[4-10]. The fundamental role of Tfh cells in humoral immunity has boosted many studies in animal models with antibody accumulation or deficiencies. However, it is extremely challenging to obtain lymphoid tissue from patients and healthy controls for research purposes.

Recently, human blood CXCR5⁺CD4⁺ T cells sharing similar functional properties with bona fide GC Tfh cells have been defined[11]. Alteration of CXCR5⁺CD4⁺ T cells has been described in, for example, human autoimmune diseases[12-18] and infections[19,20]. Our previous study also found that peripheral CXCR5⁺CD4⁺ T cells expanded in acute asthma patients and positively correlated with total IgE in the

blood[21,22]. However, the function of these cells was not addressed, and their relevance as B-cell helpers in asthma is not fully understood.

To determine whether circulating CXCR5⁺CD4⁺ T cells might be relevant to the pathogenesis of allergic asthma, we examined the phenotypes and functions of circulating CXCR5⁺CD4⁺ T cell population in patients with allergic asthma. In addition, the T-B cell coculture assay system was employed to investigate the capacity of circulating CXCR5⁺CD4⁺ T subset in promoting pathogenic IgE production.

2. Materials and methods

2.1 Study population

Characteristics of patients with allergic asthma (n = 25) and healthy controls (n = 25) are summarized in **Table 1**. Subjects with allergic asthma were diagnosed on the standard of Global Initiative for Asthma (GINA) [23]. The criteria for inclusion were as follows: (1) a history of variable respiratory symptoms such as wheeze, shortness of breath, chest tightness and/or cough; (2) reversible expiratory airflow limitation characterized by an increase in forced expiratory volume in 1 s (FEV1) by more than 12% and 200 mL after inhaling a bronchodilator; (3) no treatment with systemic glucocorticoids or other immune-suppressive agents within 1 month before entry, but allowing to receive inhaled steroids to control an asthma exacerbation. Atopy was defined by a positive clinical history and skin prick testing to at least one of 11 common aeroallergens (ALK-Abello, Horsholm, Denmark), or a history of severe allergy or anaphylaxis. Patients with acute infections within 1 week of recruitment and those with other chronic diseases were excluded. Other exclusion criteria included pregnant, current smokers, or ex-smokers with 10 or more pack-years. Twenty five age and sex-matched healthy controls were enrolled from our hospital staff. All of the healthy controls were nonatopic, nonasthmatic, and had a normal lung function and no history of respiratory diseases. Written informed consents were obtained from all subjects, and the study was approved by Medical Ethics Committee of The Third Hospital Affiliated to Nantong University. All procedures were carried out in accordance with the approved guidelines and all experimental protocols were approved by the above authority.

2.2 Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were collected into tubes containing sodium heparin. PBMC were isolated by density centrifugation using Ficoll-HypaqueTM (AXIS-SHIELD PoCAs, Oslo, Norway).

2.3 Surface and intracellular staining and flow cytometry

Human PBMCs at 10^6 /tube were stained at room temperature for 30 minutes in the dark with the following antibodies or isotype matched controls: CD4-APC-CY7 (RPA-T4), CCR6-PE-CY7 (11A9), CXCR5-PerCP Cy5.5 (TG2), CXCR3-APC (G025H7), CD95-PE (DX2), PD1-FITC (MIH4), ICOS-PE (DX29), CD69-PE (FN50), HLA-DR-FITC (TU36). All monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA). After being washed with PBS, the cells were processed on a FACSCanto II cytometer (BD Biosciences) and data were analyzed with FlowJo software (Tree Star Inc., Oregon, USA). The cells were gated on the forward scatter of living cells. The numbers of CXCR5⁺CD4⁺ T cells were determined by flow cytometric analysis. Intracellular staining was performed with Leukocyte Activation Cocktail with BD GolgiPlug™ (BD PharMingen, San Jose, CA, USA), Fix & Perm cell permeabilization kit (Nordic-MUBio, Susteren, The Netherlands) and IFN- γ -APC (B27), IL-4-PE (8D4-8), IL-17A-PE (SCPL1362), IL-21-Alexa647 (3A3-N2.1; BD PharMingen, San Jose, CA, USA) according to the manufacturer's instructions.

2.4 Cell sorting and T/B cell coculture

For sorting circulating CXCR5⁺CD4⁺ T cells and B cells by flow cytometry, freshly isolated PBMC from asthma patients and healthy controls were stained with CD4-APC-CY7(RPA-T4), CXCR5-PerCP Cy5.5 (TG2), and CD19-FITC (HIB19). The purity of CXCR5⁺CD4⁺ T cells and B cells was greater than 96% and used immediately after isolation. Circulating CXCR5⁺CD4⁺ T cells (5×10^4 cells/well) were co-cultured for 12 days with autologous B cells (5×10^4 cells/well) in U-bottom 96-well plates containing complete RPMI 1640 medium (Lonza, Valais, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (GE Healthcare Life Sciences, Utah, USA) in the presence of *Staphylococcal* enterotoxin B (1 μ g/mL, Sigma-Aldrich, St Louis, MO, USA) and IL-2 (10 U/mL, Thermo Fisher Scientific, MA, USA) [11]. In blocking experiments, the effects of IL-4 and IL-21 were examined by adding anti-IL-4 monoclonal antibody (1 μ g/mL, R&D Systems, Minneapolis, USA), or IL-21R-Fc (20 μ g/mL, R&D Systems, Minneapolis, USA). Supernatants of cell culture were harvested and stored at -80°C for later analysis.

2.5 Ig measurement

The concentrations of IgG, IgA and IgM were measured by ELISA (Abcam, Cambridge, UK), and IgE

by IMAGE® Immunochemistry Systems (Beckman Coulter, Fullerton, CA, USA) according to the manufacturers' protocol. The minimal detectable concentrations were 0.23 ng/mL(IgG), 0.78 ng/mL(IgA), 1.563 ng/mL(IgM), and 5 IU/mL(IgE). All samples were measured in duplicate.

2.6 Statistical analysis

All statistics were performed using the SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA). The data are expressed as mean $\pm$ standard deviations. If a normal distribution of sample means could be assumed, t-test was used; otherwise the Kruskal-Wallis test followed by the Mann-Whitney U test were applied. Correlations between variables were assessed with Pearson's correlation coefficient. All statistical analyses were based on a two-tailed hypothesis test with a significance level of P -value < 0.05 . Data were analyzed with GraphPad Prism 5 software (GraphPad Software Inc., CA, USA).

3. Results

3.1 Circulating CXCR5⁺CD4⁺ T cells are expanded in asthma patients

The characteristics of the study groups are summarized in **Table 1**. Consistent with our previous data [21,22], a significantly higher frequency of CXCR5⁺CD4⁺ T cells was observed in patients with asthma relative to the HC group [(19.5 $\pm$ 4.2)% vs (8.4 $\pm$ 2.1)%, $P < 0.01$, Fig. 1A, B].

3.2 Expression of surface marks by CXCR5⁺CD4⁺ T cells from asthma patients

In order to further identify the differential expression of general surface markers on the circulating CXCR5⁺CD4⁺ T cells in allergic asthma, we then analyzed the surface molecular signatures of Tfh cells, such as PD-1, ICOS, CD69, CD95, and HLA-DR in circulating CXCR5⁺CD4⁺ T cells. As shown in Fig. 2A and 2B, the percentages of PD-1⁺, and ICOS⁺ cells were raised in allergic asthma group when compared to HC group. In addition, there were no differences in the expression of CD69, CD95, or HLA-DR (See Supplementary Figure).

3.3 Higher frequency of IL-4⁺-, and IL-21⁺- producing CXCR5⁺CD4⁺ T cells in allergic asthma patients

Given the importance of cytokines in inducing Ig producing and class-switching, we next extended our analysis to investigate the profile of cytokine production by CXCR5⁺CD4⁺ T cells from patients with asthma.

As shown in Fig. 3, PMA/ionomycin stimulation generated more IL-4⁺, and IL-21⁺-producing CXCR5⁺CD4⁺ T cells in allergic asthma patients compared with HC. This is in contrast to the IL-17⁺, or IFN- γ ⁺-producing CXCR5⁺CD4⁺ T cells, which showed no statistical difference between allergic asthma patients and HC.

3.4 Circulating IL-4⁺, and IL-21⁺-producing CXCR5⁺CD4⁺ T cells participate in IgE production

As several studies have suggested important roles for Tfh cells in inducing Ig production from B cells, we sought to explore the function of activated CXCR5⁺CD4⁺ T cells in helping B cell secret pathogenic IgE in allergic asthma. To this end, sorted CXCR5⁺CD4⁺ T and autologous CD19⁺B cells from allergic asthma patients were cultured together and the secretion of Igs was detected. Consistent with a previous report[11], circulating CXCR5⁺CD4⁺ T cells showed capacity to promote IgM, IgG, IgA, and IgE production. This is in contrast to CXCR5⁻CD4⁺ T cells, which induced these Igs at very low levels (Fig. 4A). Blocking IL-21 in the culture of B cells with CXCR5⁺CD4⁺ T cells resulted in decreased IgA, IgM, and IgE production (Fig. 4B), whereas blocking IL-4 resulted in a substantial inhibition of only IgE production but not of other Igs (Fig. 4B). IgG could not be detected because of cross-reactivity with blocking reagents. Collectively, these results showed that CXCR5⁺CD4⁺ T cells from allergic asthma patients presented the capacity to promote IgE production, which could be suppressed by IL-21 and IL-4 blockage.

3.5 Circulating IL-4⁺, IL-21⁺- producing CXCR5⁺CD4⁺ T cells correlated with IgE level in allergic asthma patients

We next examine whether the different cytokine producing CXCR5⁺CD4⁺ T cells is associated with the atopic status in asthma. To address this issue, serum TIgE levels were obtained from 25 allergic asthma patients. Interestingly, IL-4⁺, IL-21⁺, but not IFN- γ ⁺, or IL-17⁺ positively correlated with total IgE in the blood (Fig. 5).

4. Discussion

Abnormalities in IgE pathway are an identified pathogenesis of allergic asthma which is associated not only with the inception of asthma but also with its acute deterioration[1,2]. The association of allergic asthma with IgE indicates the implication of B helper Tfh cells in the disease pathogenesis and therefore we

thought it would be interesting to study circulating CXCR5⁺CD4⁺ T cells and its biology in asthma.

Alteration of circulating CXCR5⁺CD4⁺ T cells has been mostly reported in patients with autoimmune diseases and correlated with serum concentration of IgG[12-17,19,20] or IgA[18] auto-antibodies. Recently, however, several prospective studies addressed this issue in IgE-mediated diseases[24-28]. In mouse models of airway allergen exposure, two independent research groups demonstrated that Tfh cells supported the sustained production of IgE antibody *in vivo*[24,25]. As to human systems, it was found that increased percentages of Tfh2 cells (CD4⁺CXCR5⁺CXCR3⁻CCR6⁻), which are subsets of Tfh cells, in patients with allergic rhinitis with and without asthma[26] and patients that develop food allergy after liver transplantation[27]. Moreover, CXCR5⁺CD4⁺ T cells in eosinophilic nasal polyp tissues induced IgE production and correlated highly with local IgE levels[28]. Our previous study has shown that peripheral CXCR5⁺CD4⁺ T cells expanded in acute asthma patients and positively correlated with total IgE in the blood [22]. Here in this study we further explore the function of these cells and their relevance as B-cell helpers in allergic asthma.

Circulating CXCR5⁺CD4⁺ T cells are now recognized as a population that has experienced “Tfh cell differentiation programme” *in vivo* and appeared to be a quiescent memory-type in the peripheral[29,30]. The notion that circulating CXCR5⁺CD4⁺ T cell numbers are a reflection of the pool of bona-fide Tfh cells was reinforced by the phenomena that patients with loss-of-function mutations in CD40L[31], STAT3[32,33] or BTK[34] and patients with gain-of-function mutations in STAT1[35], revealed a deficiency of GCs with deficit of circulating CXCR5⁺CD4⁺ T cells. Indeed, in concordance with Tfh cells from GCs, blood circulating CXCR5⁺CD4⁺ T cells induce naive and memory B cells to become Ig-producing cells following *in vitro* stimulation. However, at variance with Tfh cells from GCs, circulating CXCR5⁺CD4⁺ T cells had reduced expression of ICOS, and PD-1, suggesting that they are in a resting state[36,37]. Here in our study, we observed an increased percentage of ICOS⁺ and PD-1⁺ cells in CXCR5⁺CD4⁺ T cells in asthma patients, which indicates that asthma patients not only have increased numbers of circulating CXCR5⁺CD4⁺ T cells, but also are over-activated, which is consistent with the abnormal humoral immune response.

While IL-4 and IFN- γ have been identified as ones that either induce or antagonize IgE production in human and murine B cells respectively[38-40], the effects of IL-21 on IgE secretion by human B cells remain undefined[41-49]. Patients with loss-of-function mutations in IL-21 and IL-21R genes has supported a restraining role of IL-21 in IgE production in humans, as both mutations are associated with increased IgE

levels[41,42]. Considering that there are so many potential phenotype alterations associated with these mutations, we can not exclude the possibility that other components of the immune systems is functionally involved. Nevertheless, *in vitro* results concerning the role of IL-21 in the regulation of IgE synthesis at the B-cell level are conflicting[43-49]. On one hand, it is reported that IL-21 alone had no effects on IgE secretion from stimulated B cells[43,44]. What's more, IL-21 was reported to inhibit IgE secretion of B cells that were induced by IL-4 in allergic rhinitis patients[44]. On the other hand, researchers have found that IL-21 can dramatically enhance IgE production by human mononuclear cells, total, naive, or memory B cells in the presence of IL-4[45-49]. Importantly, the synergy between IL-4 and IL-21 was evident at the concentrations that failed to induce IgE secretion when used alone[49]. The high efficacy of the synergy led us to suppose that these two cytokines may all participate in IgE production. Here we showed evidence that the frequency of IL-4⁺, and IL-21⁺-producing CXCR5⁺CD4⁺ T cells were increased comparably in allergic asthma patients. Moreover, blocking IL-21 in the culture of B cells with CXCR5⁺CD4⁺ T cells resulted in decreased IgA, IgM, and IgE production. In contrast, blocking IL-4 resulted in a uniquely inhibition of IgE production, depicting its distinct profiles in allergic asthma. These results underscore a critical role of IL-4⁺, and IL-21⁺-producing -CXCR5⁺CD4⁺ T cells in IgE production, which was further confirmed *in vivo* by the finding that IL-4⁺, and IL-21⁺-producing -CXCR5⁺CD4⁺ T cells correlated with IgE level in allergic asthma patients.

Taken together, our data indicated that circulating CXCR5⁺CD4⁺ T cells may have a significant role in facilitating IgE production in allergic asthma patients. Therapy that targets circulating CXCR5⁺CD4⁺ T cells may be beneficial for the treatment of asthma.

Compliance with ethical standards

The protocols were approved by the Committee on Ethics of Biomedicine Research, The Third Hospital Affiliated to Nantong University. Informed consent was obtained from all individual participants included in the study.

Conflict of Interest. The authors declare no competing financial interests.

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Fig 1. Flow cytometry analysis of circulating CXCR5⁺CD4⁺ T cells. (A) Representative analysis of the frequency of circulating CXCR5⁺CD4⁺ T cells in asthma patients and healthy controls (HC). The cells were gated initially on CD3⁺CD4⁺ cells. (B) Percentage of circulating CXCR5⁺CD4⁺ T cells in CD3⁺CD4⁺ cells in asthma patients and HC. * $p < 0.05$; ** $p < 0.01$; ns indicating not significant.

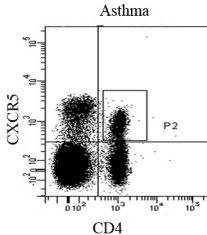
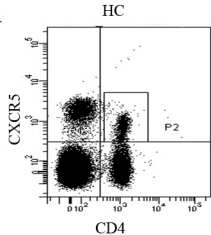
Fig 2. Analysis of receptors expressed by circulating CXCR5⁺CD4⁺ T cells (A) Representative flow cytometry profiles indicating the percentages of PD-1⁺ and ICOS⁺ cells in circulating CXCR5⁺CD4⁺ T cells. The cells were gated initially on CXCR5⁺CD4⁺ T cells. (B) Frequency of PD-1⁺ and ICOS⁺ cells in circulating CXCR5⁺CD4⁺ T cells. * $p < 0.05$; ** $p < 0.01$; ns indicating not significant.

Fig 3. Cytokine production of CXCR5⁺CD4⁺ T cells. (A) Representative flow cytometry data showing cytokines production by CXCR5⁺CD4⁺ T cells stimulated with PMA/ionomycin. Plots were pregated on CD3⁺ CXCR5⁺CD4⁺ cells. (B) Percentage of cytokine-producing cells by CXCR5⁺CD4⁺ T cells. ** $p < 0.01$; ns indicating not significant.

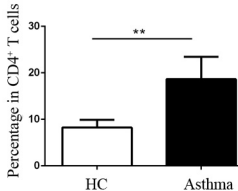
Fig 4. CXCR5⁺CD4⁺ T cells are able to induce IgE production. CXCR5⁺CD4⁺ T cells or CXCR⁻CD4⁺ T cells isolated from allergic asthma patients were co-cultured with autologous B cells for 12 days. Ig concentration in the supernatant was measured by ELISA. Data are presented as mean $\pm$ SDs. (A) CXCR5⁺CD4⁺ T cells and CXCR⁻CD4⁺ T cells purified from 5 allergic asthma patients were compared. (B) Addition of IL-21R-Fc or IL-4 blocking antibody to the cocultures of CXCR5⁺CD4⁺ T cells and autologous B cells from 3 allergic asthma patients. Shown are the concentrations of IgM, IgG, and IgE in the supernatants. IgG levels were not shown because of cross-reactivity with blocking reagents. ** $p < 0.01$; ns indicating not significant.

Fig 5. The frequency of IL-4⁺ and IL-21⁺ CXCR5⁺CD4⁺ cells positively correlated with serum total IgE in the blood from asthma patients

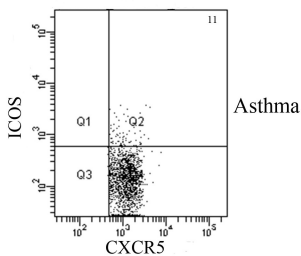
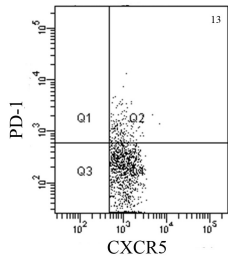
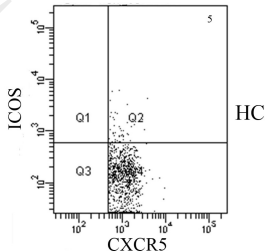
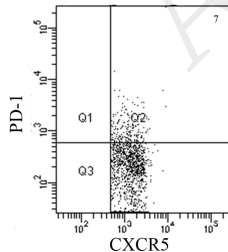
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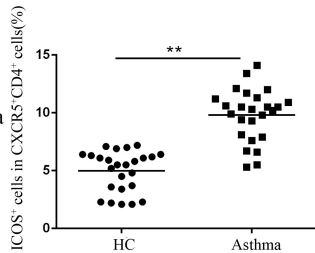
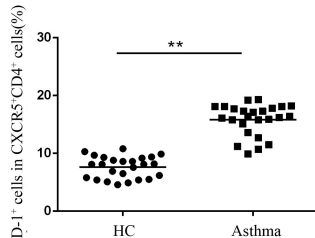
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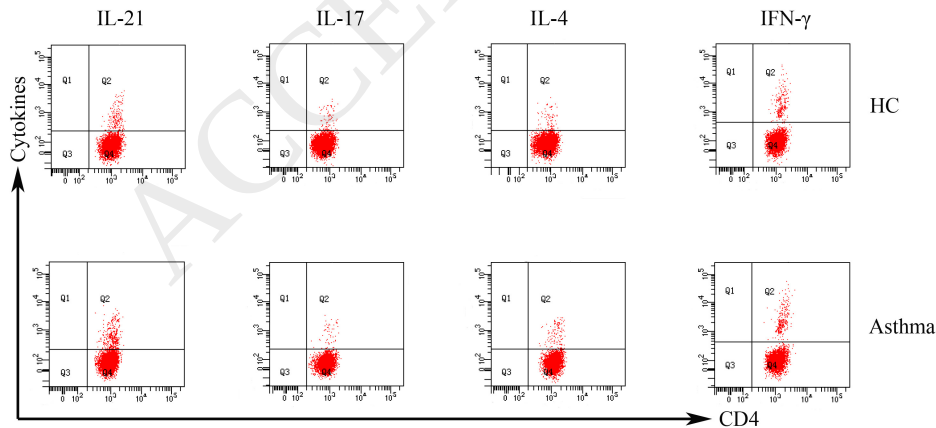
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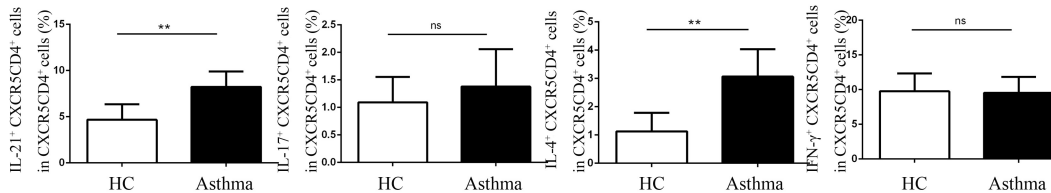
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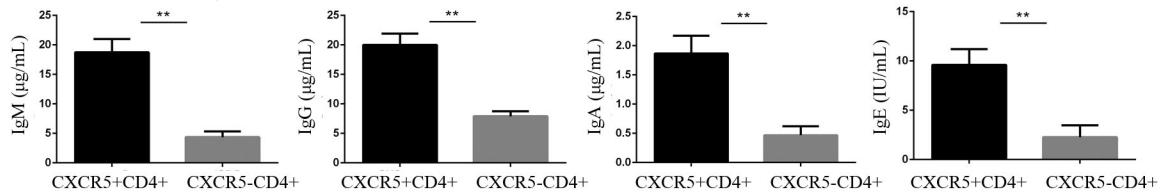
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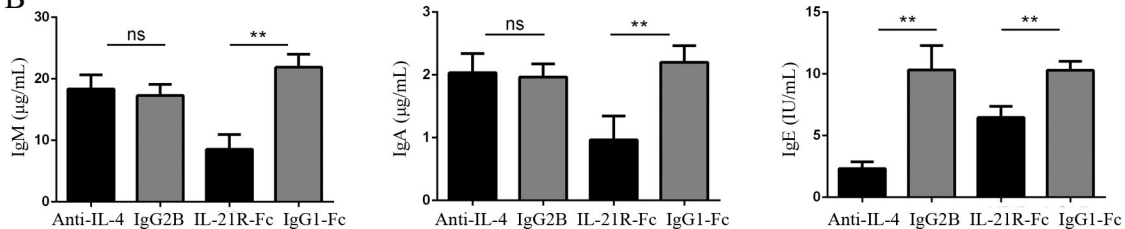
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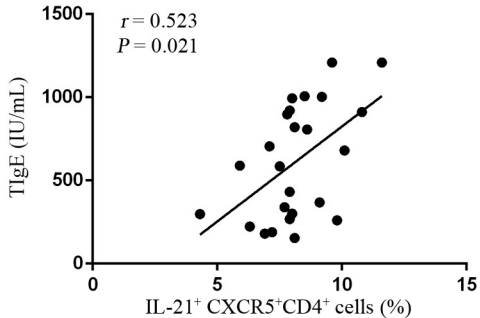
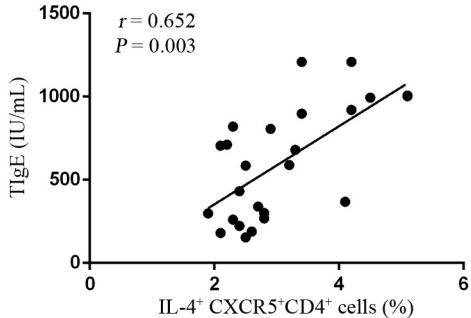


Table 1 Demographic and clinical characteristics of study participants

	Allergic asthma	Healthy control
	(<i>n</i> = 25)	(<i>n</i> = 25)
Age (years)	36.6 ± 10.2	37.5 ± 7.1
Gender (M/F)	15/10	13/12
Inhaled glucocorticoid (no.)	5	0
FEV1%	80.5 ± 7.1*	102.6 ± 10.5
TIgE (U/L)	579.9 ± 257.8*	52.2 ± 38.4

TIgE, total serum immunoglobulin E; FEV1, Forced expiratory volume in 1 second. * $P < 0.05$ compared with healthy control