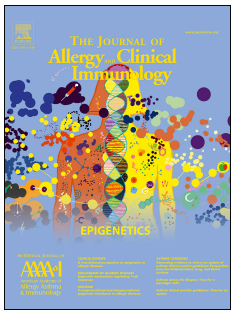
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Cross-talk between Th2 and Th17 pathways in chronic rhinosinusitis with nasal polyps

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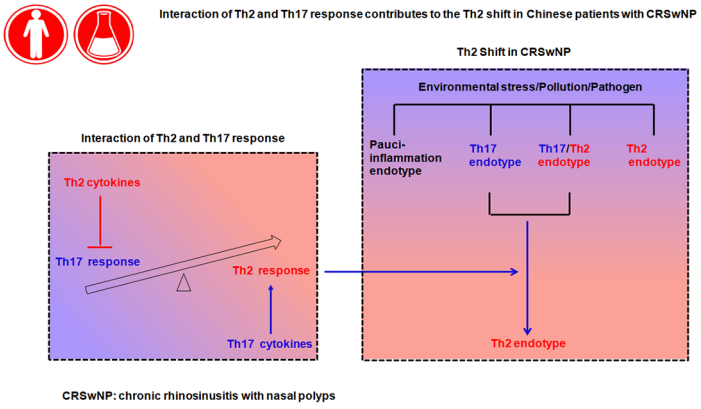
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Title: Cross-talk between Th2 and Th17 pathways in chronic rhinosinusitis with nasal polyps

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ABSTRACT

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a heterogeneous disease with a spectrum of endotypes. Th2 and Th17 related cytokines are two central regulators involved in the inflammation associated with CRSwNP.

Objective: To investigate the inter-regulation of Th2 and Th17 pathways in Chinese patients with CRSwNP.

Methods: Levels of key Th2 and Th17 related factors were measured in homogenates of polyp tissue obtained from CRSwNP patients. The relationship of these factors and their expression in groups classified according to tissue IL-5 and IL-17 concentrations were analyzed. Cross regulation of Th2 and Th17 cytokines and the effects of dexamethasone treatment were studied in dispersed nasal polyp cells (DNPCs). Associations between the Th2- and Th17 related factors and comorbid atopic status and asthma, disease recurrence and edema scores were also explored.

Results: Four CRSwNP groups were classified based on the expression or non-expression of mutually exclusive Th2 and Th17 related factors. The Th2 cytokines IL-4 and IL-13 inhibited the expression of Th17 related factors, while the Th17 cytokines IL-17 and TGF-β1 enhanced the expression of Th2 related factors. Dexamethasone treatment inhibited both the Th2 and Th17 pathways. A patient's atopic status was related to their Th2 immune response. Edema scores were positively correlated with the Th2 pathway and negatively correlated with the Th17 pathway.

Conclusion: Th2 and Th17 pathways are mutually exclusive and regulate each other, favoring the development of a Th2 immune response in Chinese CRSwNP patients.

Clinical implications: This study added new information on the molecular mechanisms driving distinct endotypes of CRSwNP by showing the complex regulation of Th2 and Th17 immunity in CRSwNP, possibly explaining the type 2 shift observed in Asia over time.

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Capsule summary: This study provides useful insights into a possible type 2 endotypes shift in CRSwNP in Asia, as well as endotype classification and treatment criteria for CRSwNP by showing the complex regulation of Th2 and Th17 immunity.

Key words: Chronic rhinosinusitis with nasal polyps (CRSwNP), Th2, Th17, endotypes, shift

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Abbreviations used

CRS: Chronic rhinosinusitis

NP: Nasal polyp

CRSwNP: Chronic rhinosinusitis with nasal polyps

CRSsNP: Chronic rhinosinusitis without nasal polyps

DNPCs: Dispersed nasal polyp cells

DEX: Dexamethasone

HE: Hematoxylin and eosin

HPF: High power field

ECP: Eosinophil cationic protein

MCA: Multiple correspondence analysis

Eos: eosinophil

Neu: neutrophil

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INTRODUCTION

Chronic rhinosinusitis (CRS) is an inflammatory disorder of the nasal and paranasal sinus mucosa. Based on the absence or presence of nasal polyp (NP), CRS can be clinically sub-divided into two sub-groups; CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP). In Europe, CRSwNP was originally characterized by high Th2 cytokine levels and eosinophilic inflammation, whereas CRSsNP presented with low Th2 cytokines levels and thus as a non-eosinophilic disease.[1]

More recently, it is recognized that CRSwNP is a heterogeneous disease comprising a spectrum of endotypes and overlapping T helper cytokine expressions.[2-4] However, a profound understanding of the cytokine pathways and their interplay is required for an appropriate endotype classification and the development of therapeutic strategies.

Th2 and Th17 pathways are two major regulators involved in CRSwNP.[2, 5] In asthma, a reciprocal regulation of Th2 and Th17 pathways has recently been suggested by the mutual exclusiveness of Th2 and Th17 related gene expression signatures in the airways of asthma patients. The amplification of opposing pathways was achieved by targeting Th2 and Th17 cytokines in mouse models of asthma.[6] However, this study was confined to the investigation of IL-13 and IL-17 induced genes, and it remains unclear whether other cytokines that play key roles in Th2 and Th17 inflammation would show the same responses. Thus, we hypothesized that Th2 and Th17 pathways may play interactive regulatory roles in the aetiology of CRSwNP and tested the hypothesis by examining the relationship between the Th2 and Th17 pathways in CRSwNP, focusing on the aspect of key Th2 and Th17 related factors.

Our findings from the current study further support the cross-regulation of Th2 and Th17 pathways in CRSwNP, by showing that key Th2 and Th17 related factors are mutually exclusive in NP tissues of CRSwNP patients. In particular, two endotypes (Th2 and Th17)

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were identified based on the expression of the Th2 marker IL-5 and the Th17 marker IL-17. Th2 cytokine stimulation inhibited the Th17 response while Th17 cytokines enhanced the Th2 response in dispersed nasal polyp cells (DNPCs). A patient's atopic status was related to their Th2 immune response. Dexamethasone (DEX) treatment inhibited both the Th2 and Th17 pathways, and distinct clinical characteristics of patients were associated with those two pathways.

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METHODS

Subjects

Ninety five patients with CRSwNP and twenty four control subjects were recruited from the Rhinology Department of Beijing Tongren Hospital. These CRSwNP and control subjects were same as in previously published study.[2] Full details of the subjects are shown in the Methods section in this article's Online Repository.

Tissue samples were obtained from the inferior turbinates of control subjects and from nasal polyps of CRSwNP subjects, and immediately frozen in liquid nitrogen, prior to being stored at -70ºC until use in immunoassays. Tissue samples used for immunostaining were fixed in 10% neutral buffered formalin for 24h and then embedded in paraffin prior to sectioning for staining.

The study was approved by the Ethics Committee of Beijing Tongren Hospital and written informed consent was obtained from each subject prior to their enrollment in the study.

Peripheral blood eosinophil and neutrophil analysis

Peripheral blood eosinophil and neutrophil numbers were measured with a standard automated cell counter.

Histologic analysis

Paraffin embedded tissue sections were stained with hematoxylin and eosin (HE) and assessed for eosinophil and neutrophil counts and edema. The numbers of eosinophils and neutrophils were counted in 10 randomly selected high power magnification fields (×400), and expressed as the mean number of cells per high power field (HPF).

Edema was evaluated semi quantitatively according to the study of Zhang and colleagues;[5] by scoring on a scale of 0-2, where a rating of 0 indicated no edema, 1 indicated mild/moderate edema, and 2 indicated intense edema. Edema was scored in five randomly

selected high power magnification fields (×200) by an observer blinded to the study protocol, and expressed as the mean score. Considering the subjective nature of this scoring system, Image J analysis was further performed on 50% of randomly selected samples (48 from the 95 CRSwNP patients), for objective evaluation of edema using the Image J software. Since edema is defined as an abnormal accumulation of fluid (seen as white areas) in the extravascular and extraglandular compartments of the NP tissue, Image J analysis was performed on the edematous areas in ten high power magnification fields (×200) in randomly selected pictures of HE stained samples. The edematous areas were graded using the Image J software and expressed as mean percentage of the total area measured in each high power field. The consistency of the subjective and objective measures of edema was further evaluated by analyzing the correlation between the two measures using Spearman's correlation analysis.

Picrosirius red staining for collagen

The collagen content in NP tissue was assessed by using picrosirius red staining, as previously described.[7] For full details, see the Methods section in this article's Online Repository.

Preparation and stimulation of dispersed nasal polyp cells (DNPCs)

NP tissues were harvested from CRSwNP patients who had not taken either systemic or local glucocorticosteroids for at least 4 weeks or antibiotics for at least 2 weeks prior to the surgery. Adequate amount of freshly obtained NP tissue was set aside for measuring cytokines and the remaining NP tissue was used for preparation of DNPCs.

DNPCs were prepared by enzymatic digestion, as previously described.[8] For full details, see the Methods section in this article's Online Repository. DNPCs were suspended in RPMI containing 10% FBS at a concentration of 2×10^6 cells/ml, and 0.5 ml aliquots were cultured in 24-well plates with IL-4 (50ng/mL), IL-5 (50ng/mL), IL-13 (50ng/mL), IL-17

(100ng/mL), IL-23 (100ng/mL), TGF-β1 (100ng/mL) (R&D Systems, Minneapolis, Minn) or DEX (5×10^{-5}mol/L) (Sigma,St. Louis, MO, USA), for 24 h at 37°C in a humidified incubator with 5% CO2. At the end of incubation the supernatant from each culture was collected and assayed for protein.

Immunoassay

Tissue homogenates, prepared as previously described,[9] and supernatants from cultured DNPCs were assessed for the levels of IL-4, IL-5, IL-13, eotaxin, IL-17, TGF-β1, IL-1β, IL-8, TNF-α, G-CSF, IP10, MPO, eosinophil cationic protein (ECP), and total IgE, using commercially available kits. For full details, see the Methods section in this article's Online Repository.

Statistical analysis

The relationships between the Th2 and Th17 related factors were evaluated by performing a multiple correspondence analysis (MCA) and a Spearman's correlation analysis. Before implementing MCA analysis, all the Th2 and Th17 related numeric variables were transformed into categorical variables. Specifically, the original numeric data were defined as "high" or "low" based on a cut-off value set according to their detection limits (for variables which had a high proportion of values under detection limit, i.e. IL-4, IL-5 and IL-17) or their medians (for other variables). For quantitative data, Mann-Whitney U test was used for comparing two groups, and Kruskal-Wallis test with Dunn's correction was used for multiple comparisons in more than two groups. Chi-squared test or Fisher exact test was used for qualitative data. The Wilcoxon matched pairs test was used to compare the expression levels of patient-matched unstimulated and stimulated NP tissues. Benjamini-Hochberg method was applied to control the false discovery rate (FDR) in the multiplicity problems caused by multiple groups (i.e., qualitative data) or multiple testing. The *P* values of 0.05 or less were considered statistically significant. All statistical analyses were performed using IBM SPSS

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20.0 software (SPSS, Chicago, III).

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RESULTS

Patient characteristics

Characteristics of all participants enrolled in this study are shown in Table E1. The control group and CRSwNP group were similar with regard to age, the prevalence of co-morbid asthma and aspirin intolerance. The CRSwNP group had a significantly higher male/female ratio, co-morbid atopic status, CT score and polyp score than the control group.

Negative interrelationships between the Th2 and Th17 inflammatory pathways in CRSwNP patients

IL-5 is a typical Th2 cytokine and IL-17 a typical Th17 cytokine. In the present study, we defined Th2 cytokines IL-4, IL-5 and IL-13, eosinophilic markers (eosinophil counts, ECP and eotaxin) and total IgE as Th2 related factors; and Th17 cytokine IL-17, Th17 differentiation cytokines IL-23, IL-1β and TGF-β1, IL-17 induced cytokines IL-8, TNF-α and G-CSF, and neutrophil related proteins IP10 and MPO as Th17 related factors.

Both IL-5 and IL-17 concentrations were significantly increased in tissue homogenates from CRSwNP patients compared to tissue homogenates from control subjects (Fig. E1). As we have previously shown increased expression of IL-17 in IL-5 low CRSwNP patients; [9] which suggests a negative association of IL-17 with IL-5; we further explored the relationships between Th2 and Th17 related factors in CRSwNP patients in greater detail in this study.

MCA analysis of all patients (Fig. 1) demonstrated that the low Th2 and high Th17 related factors were situated near each other; and the high Th2 variables were situated near low Th17 variables. We next analyzed the correlations among the Th2 and Th17 related factors in all patients. Although most of these factors were positively correlated within the two sets, the two sets were negatively correlated (Fig. E2). These findings were consistent with MCA analysis; with both types of analyses showing negative interrelationships between

the Th2 and Th17 pathways in CRSwNP patients.

The negative interrelationships between the Th2 and Th17 pathways demonstrated above prompted us to classify our CRSwNP patients based on the distribution of typical Th2 cytokines IL-5 and typical Th17 cytokine IL-17. Four CRSwNP groups were obtained based on the detection limits of IL-5 and IL-17 used as cutoff values; including IL-5-IL-17-, IL-5-IL-17+, IL-5+IL-17-, and IL-5+IL-17+ (Figure E3). Characteristics of these four CRSwNP groups are shown in Table E2, and demonstrate that they were similar with regard to age, female/male ratio, CT score and polyp score. Correlation analyses in each of the four CRSwNP groups showed a high number of positive correlations within the Th2 related factors in IL-5+IL-17+ group and within the Th17 related factors in IL-5-IL-17+ and IL-5-IL-17- groups, but negative correlations between those two sets of factors in the IL5+IL-17- group (Figure E4).

Expression of Th2 and Th17 related factors in the four CRSwNP groups defined by tissue IL-5 and IL-17 concentrations

Fig 2 shows the expression levels of Th2 related factors, and indicates that most of these (i.e., IL-13, eotaxin, ECP, ECP/MPO ratio, tissue total IgE concentrations and blood eosinophil counts) were significantly higher in IL-5+IL-17- group than in the two IL-5 negative groups (IL-5-IL-17+ and IL-5-IL-17-). Furthermore, tissue eosinophil counts were higher in the IL-5+IL-17- group than in the IL-5-IL-17+ group; and ECP/MPO ratio was higher in the IL-5+IL-17- group than in the IL-5+IL-17+ group. Different from most Th2 related factors, IL-4 concentrations were lowest in the IL-5-IL-17- group and highest in the IL-5+IL-17+ group; with no significant difference between the IL-5+IL-17- and IL-5-IL-17+ groups (Fig. 2).

In contrast to Th2 related factors, most of the Th17 related factors (i.e. IL-8, IL-1β, G-CSF and TNF-α) were expressed at significantly higher levels in IL-5-IL-17+ group than in

two IL-17 negative groups (IL-5+IL-17- and IL-5-IL-17-). Furthermore, IL-8, IL-1β, G-CSF and TNF-α were higher in the IL-5+IL-17+ group than in the IL-5+IL-17- group. The concentrations of MPO and IL-23 in IL-5-IL-17+ group were higher than those in the IL-5+IL-17- group, but not higher than in the IL-5-IL-17- group (Fig. 3). The four groups showed no significant difference with regard to blood neutrophil counts (Fig. E5A). The tissue neutrophil counts in the IL-5-IL-17- group were lower than neutrophil counts in the other three groups, however no significant differences were reached (Fig. E5B).

Associations of clinical parameters with Th2 and Th17 responses in CRSwNP patients

Edema scores, used to evaluate edema formation in NPs, were positively correlated with most of the Th2 related factors (i.e., IL-5, IL-13, ECP/MPO ratio, ECP and Blood eos counts) and negatively correlated with most of the Th17 related factors (IL-17, IL-1β, TNF-α and G-CSF) (Fig. 4A). Similarly, edema scores in IL-5+IL-17- group were significantly higher than in two IL-5 negative groups (Fig. 4B). High correlation between the edema score assessed by visual evaluation and the percentage of edema area assessed by image J analysis supported the consistency of these two methods for evaluating edema (Figure E6).

Collagen content was used to evaluate fibrosis in NPs, and showed a strong negative correlation with edema scores (r = - 0.752, P < 0.001). Although this finding would suggest that collagen content may be positively correlated with Th17 related factors and negatively correlated with Th2 related factors, we did not find any correlation between collagen content and Th2 or Th17 related factors, including TGF-ß1. The expression of collagen in the four CRSwNP groups was also not significantly different among the groups (Fig. E7A).

The frequencies of atopic status were higher in the two IL-5 positive groups than in the IL-5-IL-17- group, though not reaching statistically significance (P = 0.101 for IL-5+IL-17-; P = 0.078 for IL-5+IL-17+) (Fig. 4C). Accordingly, the levels of most Th2 related factors (i.e., tissue IL-5, ECP and total IgE concentrations and tissue eosinophil counts) were also found to

be significantly higher in allergic patients than in nonallergic patients (Fig. 4D).

The four groups of CRSwNP showed no statistical differences with regard to the frequencies of NP recurrence (Fig. 4E), asthma occurrence or aspirin intolerance (Fig. E7B,C); however, a trend of increased frequency was noted in the IL-5+IL-17+ group (P = 0.1122) for NP recurrence compared with IL-5-IL-17- group.

Interactions of the Th2 and Th17 pathways in DNPCs

Considering the negative interrelationships between Th2 and Th17 related factors in tissues from CRSwNP patients demonstrated above, we hypothesized that Th2 and Th17 responses might interact negatively *in vivo*. Thus, to test this hypothesis, we investigated the interactions between the Th2 and Th17 pathways further using DNPCs, particularly as these presented the advantage of having all of the different cell types in the polyps, compared to preparations of individual cell types. In this regard, all DNPCs used in the present study were from patients comprising both Th2 and Th17 endotypes (i.e., IL-5+IL-17+ group).

We first explored the effect of Th2 cytokines on Th17 response in DNPCs, and found that IL-4 significantly inhibited three Th17 related factors (IL-17, IL-1β and IL-8) (Fig. 5) and IL-13 significantly inhibited six Th17 related factors (IL-1β, IP10, TNF-α, MPO, IL-8 and G-CSF) (Fig. 6). In contrast, no Th17 related factor in DNPCs was significantly inhibited by IL-5 stimulation (Fig. E8).

We next explored the effects of Th17 cytokine stimulation on the Th2 response in DNPCs, and found that IL-17 and TGF-β1 increased the levels of IL-4, IL-13 and eotaxin, but not the levels of IL-5 (Fig. 7). In contrast, IL-23 did not significantly affect the concentrations of Th2 related cytokines (Fig. E9).

Inhibition of both the Th2 and Th17 responses in DNPCs by DEX treatment

Given the widely use of corticosteroids for CRSwNP patients, we investigated the effect of DEX on Th2 and Th17 responses in DNPCs, and found that treatment with DEX

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significantly decreased the concentrations of three Th2 related cytokines (IL-4, IL-5 and eotaxin) and the concentrations of six Th17 related cytokines (IL-17, IL-1β, TNF-α, MPO, IL-8 and G-CSF) in DNPCs (Fig. E10).

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DISCUSSION

Increasing numbers of studies have revealed that CRSwNP is a heterogeneous disease consisting of many endotypes that are driven by distinct molecular mechanisms.[3, 4, 10] Employing a multicenter study design and using the same Chinese subjects as in the present study, we have previously shown that the Th17 pathway, in addition to the Th2 pathway, contributed to the inflammatory process involved in CRSwNP patients, especially in mainland China. This study further demonstrated that polyps in patients from Asia were clearly different from those in Europe; with significantly more Th2 expression being present in polyps from European patients.[2] However, it remains unclear how the Th2 and Th17 pathways interact in CRSwNP patients and how the observed difference between polyps in Asia and Europe might develop.

In the present study, the negative inter-relationship between Th2 and Th17 related factors demonstrated using MCA and correlation analysis, and the exclusive expression of these two kinds of factors in two single positive groups suggest a mutual exclusion of the Th2 and Th17 pathways. These observations may be extended to asthma. The inter-correlations between Th2 and Th17 cytokines were previously explored in nasal polyps with and without cystic fibrosis in Belgium, not demonstrating similar findings to ours; however, cystic fibrosis is a genetically determined disease different from adult nasal polyps.[9] The less pronounced dominance of the Th2 pathway in China compared with Europe[2, 5] makes it preferable to study the Th2 and Th17 pathway inter-relationship in China rather than in Europe. The factors possibly contributing to the Th17 expression in China have been discussed in detail in a previous study.[2] The lack of relationships of TGF-ß1 with Th17 and Th2 related factors observed in our study may be explained by the fact that TGF-ß1, though a Th17 related factor, was also produced prominently by eosinophils.[11]

As expected, stimulation of DNPCs *in vitro* with the Th2 cytokines IL-4 and IL-13

inhibited Th17 cytokine production. However, IL-5 had no obvious effect in this regard, despite the negative correlation between IL-5 and IL-17 in CRSwNP. Conversely, stimulation with the Th17 cytokines IL-17 and TGF-β1, but not IL-23, enhanced Th2 cytokines production. This suppressive effect of Th2 cytokines on Th17 cytokines in DNPCs is consistent with previous studies conducted in murine asthma models, AD models, epithelial cells, and *in vitro* studies of Th17 cell differentiation.[6,12-27] The effects of Th17 cytokines on the Th2 response varied and appeared to be complicated;[6, 14, 18-24] such as the conflicting regulation of IL-17 on Th2 responses in murine models of lung diseases and in vitro culture of epithelial cells and fibroblast,[6, 14, 18, 19] the inhibition of Th2 differentiation by TGF-β1[20-22] and increased eotaxin production in airway fibroblasts by TGF-β1 plus IL-13 stimulation,[23] and the up-regulation of Th2-cell–mediated eosinophilic airway inflammation by IL-23 in mice.[24] Despite this, our findings support a positive regulation of the Th2 response by Th17 cytokines in Chinese CRSwNP patients. As type 17 immune markers enhance the expression of type 2 immune markers, the immune reactions may change the expression pattern from IL-5-IL-17+ group to IL-5+IL-17+ group, stabilizing the new constellation with a high number of positive correlations. Both sides of the interaction that occurs between the Th2 and Th17 pathways favor the development of the Th2 immune response. As environmental factors such as allergen exposure and *Staphylococcus aureus* colonization have been reported to induce Th2 responses,[25,26] the increased Th2 response in allergic CRSwNP compared with non-allergic CRSwNP in our study supports a role of allergy and allergen exposure for the Th2 response in a situation with rather modest Th2 immune responses. In contrast, it appears that *Staphylococcus aureus* does not play a role in the stimulation of a Th2 response, as only 3 of the 95 Chinese CRSwNP patients in our study were *Staphylococcus aureus* enterotoxin specific IgE positive (data not shown). SE-IgE positivity in European CRSwNP patients is associated with a much stronger type 2 immune reaction.[10] Therefore, complex regulation of

the Th2 and Th17 pathways and allergen exposure may both contribute to the overriding Th2 inflammation in CRSwNP patients in China, with 23% and 38% of CRSwNP patients belonging to the IL-5-IL-17+ and IL-5+IL-17- groups, respectively. Moreover, this might provide an explanation for the possible difference towards type 2 CRSwNP endotypes from Asia to Europe,[2] and the recently described shift in Asia, as indicated by the increased eosinophilic signature of CRSwNP in Thailand and Korea over time.[27-30]

Unlike the strong correlation of tissue IL-5 with tissue and blood eosinophil numbers, tissue IL-17 was strongly correlated with tissue MPO, but not tissue or blood neutrophil numbers (data not shown). This provided direct evidence that neutrophil activity may be more important than neutrophil numbers in Th17 inflammation, as has been assumed in a study employing a mouse model of allergen-induced asthma.[6]

The complex regulation of the Th2 and Th17 pathways in CRSwNP also has important implications for CRSwNP treatments. Several Th2 targeted therapies, such as anti-IL-5, anti-IL-4/13, and anti-IgE antibodies, have been developed for CRSwNP.[31, 32] The inhibitory effects of IL-4 and IL-13 on the Th17 response in DNPCs and the increased Th17 response produced by anti-IL-4/IL-13 treatments in an asthma mouse model[6] demonstrate the need to monitor whether long-term anti-IL-4/13 therapies will produce adverse side effects by increasing the Th17 response in CRSwNP patients. Our results would rather not point into that direction. Furthermore, the involvement of a Th17 response and the complex regulation of the Th2 and Th17 pathways in CRSwNP may necessitate the need to treat the disease by targeting Th2 in combination with Th17 related targets. Topical and systemic corticosteroids are generally used as first-line therapy for NPs. The decreased IL-4, IL-5 and eotaxin concentrations in DNPCs treated with DEX in our study confirmed the inhibitory effect of corticosteroids on the Th2 response.[8, 33, 34] The results of previous studies in patients and mice[6, 35] suggest that this type of Th2 inhibition caused by corticosteroids may subsequently

cause an increased Th17 response due to the loss of Th2 cytokine-induced inhibition. However, the decreased concentrations of Th17 related cytokines in DNPCs treated with DEX in our study suggests that DEX suppressed, rather than enhanced, the Th17 response in DNPCs, which is in accordance with results of studies conducted using NP explants[34] and in psoriatic skin.[36] The direct inhibitory effect of a corticosteroid on the Th17 response may override the loss of inhibition by Th2 cytokines following corticosteroid treatment.

Edema formation in NPs has previously been reported to be associated with eosinophil infiltration.[37, 38] In this regard, our study has shown a positive correlation between edema scores and Th2 related factors and a negative correlation between edema scores and Th17 related factors, suggesting that the Th2 pathway promotes edema formation while the Th17 pathway inhibits edema formation. Our finding that edema scores in the IL-5+IL-17- group were higher than those in the IL-5-IL-17+ group further supports this hypothesis. Despite the negative correlation between collagen (a marker of fibrosis) and CRSwNP edema scores in our study, no expected negative correlation between collagen and the Th2 pathway or positive correlation between collagen and the Th17 pathway was seen. This could be explained by evidences showing that both IL-17[39, 40] and IL-13[41] might be associated with fibrosis.

There are several limitations to this study. First, we did not assess the longitudinal intra-individual changes in Th2 and Th17 responses; and thus no direct evidence in patients was provided to support the assumption that the interactions between the Th2 and Th17 pathways ultimately lead to a Th2 shift. Second, IL-5 and IL-17 concentration measurement in NPs is dependent on performing invasive procedures, and this limits the routine use of IL-15 and IL-17 as biomarkers for Th2 and Th17 pathway activities, respectively. Third, the DNPC system used in this study is comprised of a wide variety of cell types rather than a specific cell type, and this made it impossible to explain the specific mechanisms at cellular level. Furthermore, the balance of cell populations in the DNPC system might be altered due to the cells dying

during culture, which might affect the results.

In conclusion, our data show that the Th2 and Th17 pathways in Chinese CRSwNP patients are mutually exclusive and the interaction of these two pathways favor the development of a Th2 response. Atopy may further play a role in the overriding Th2 response in CRSwNP in China. The Th2 and Th17 pathways can both be inhibited by dexamethasone and may inversely affect the formation of edema, with the Th2 pathway being pro-edematous. Overall, this study provides useful insights into a currently observed shift towards type 2 endotypes in CRSwNP in Asia over time and from Asia to Europe, as well as endotype classification and treatment criteria for CRSwNP. [A]

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610 **FIGURE LEGENDS**

611 **Figure 1**. Multiple correspondence analysis plots showing the interrelationships between Th2
612 and Th17 variables in all CRSwNP patients. The distance between variables indicates the
613 approximate relationship between those variables. Variables situated closer together have a
614 stronger relationship.

615

616 **Figure 2**. Expression of Th2 related factors in groups of CRSwNP patients defined by tissue
617 IL-5 and IL-17 concentrations. EOS, eosinophils. IL-5-IL-17-, n=14; IL-5-IL-17+, n=22; IL-
618 5+IL-17-, n=36; IL-5+IL-17+, n=23.

619

620 **Figure 3**. Expression of Th17 related factors in groups of CRSwNP patients defined by tissue
621 IL-5 and IL-17 concentrations. IL-5-IL-17-, n=14; IL-5-IL-17+, n=22; IL-5+IL-17-, n=36;
622 IL-5+IL-17+, n=23.

623

624 **Figure 4.** Associations of edema scores, allergic status and NP recurrence with Th2 and Th17
625 responses in CRSwNP. (A) Correlations of edema scores with Th2 (red) and Th17 (blue)
626 related factors in all CRSwNP patients. The correlations (Spearman's rho) are presented as a
627 heat map; with *P* values greater than 0.05 defined as 0.00. (B) Edema scores and (C) allergic
628 status in different groups of CRSwNP patients. (D) Expression of Th2 related factors in
629 allergic and non-allergic CRSwNP. (E) Frequency of NP recurrence in different groups of
630 CRSwNP patients.

631

632 **Figure 5.** Inhibitory effect of IL-4 stimulation on Th17 cytokines in DNPCs. IL-4, 50ng/mL;
633 n=10. The statistical significance of differences in expression was assessed by using the
634 Wilcoxon matched pairs test.

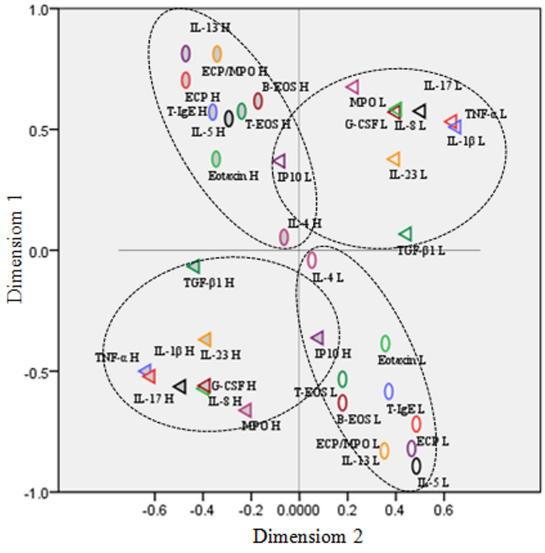
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636 **Figure 6.** Inhibitory effect of IL-13 stimulation on Th17 cytokines in DNPCs. IL-13,
637 50ng/mL; n=10. The statistical significance of differences in expression was assessed by
638 using the Wilcoxon matched pairs test.

639

640 **Figure 7.** Enhancing effect of IL-17 (A) and TGF-β1 (B) stimulations on Th2 cytokines in
641 DNPCs. IL-17, 100ng/mL; n=9; TGF-β1, 100ng/mL; n=10. The statistical significance of
642 differences in expression was assessed by using the Wilcoxon matched pairs test.

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Th2 variables

Th17 variables

L: low (open circles or triangles)

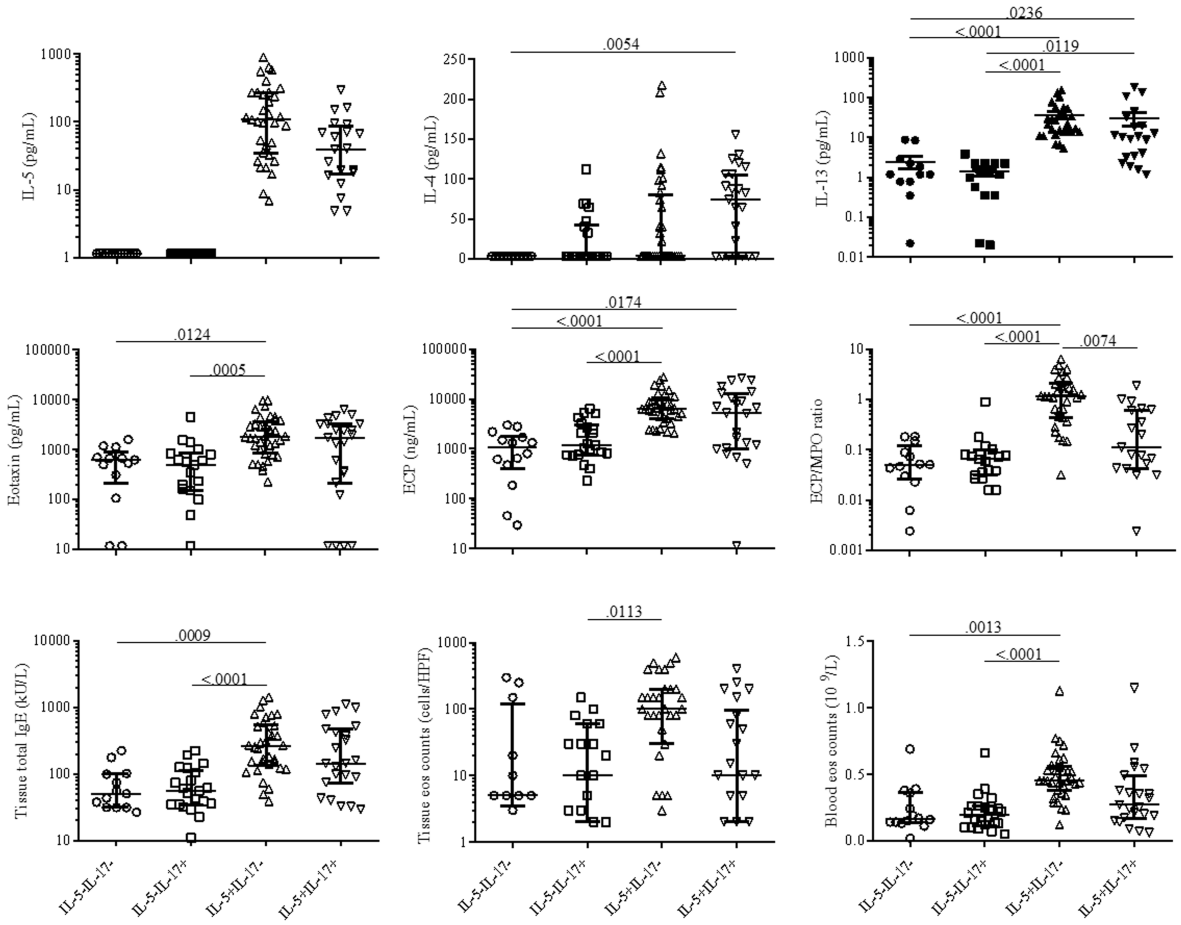
H: high (solid circles or triangles)

B-EOS: blood eos counts

T-EOS: tissue eos counts

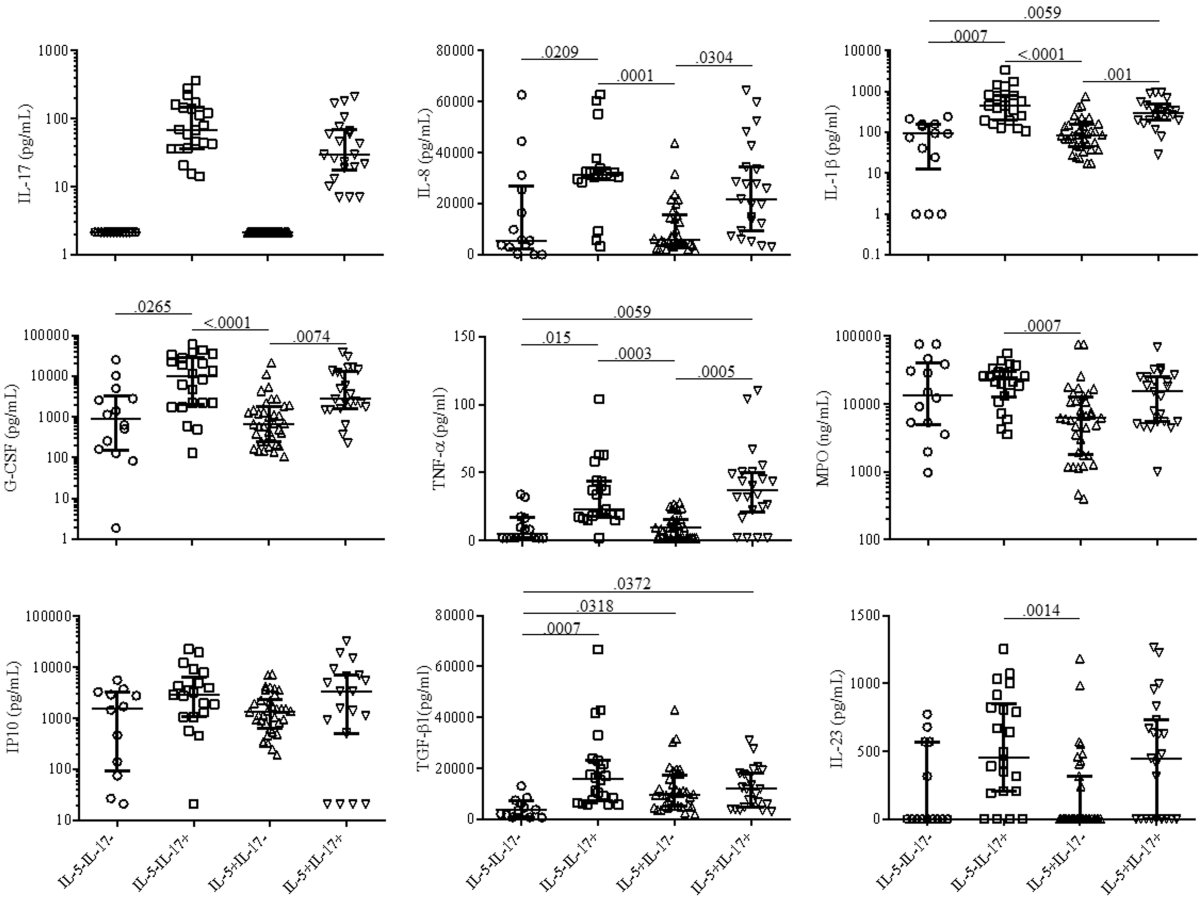
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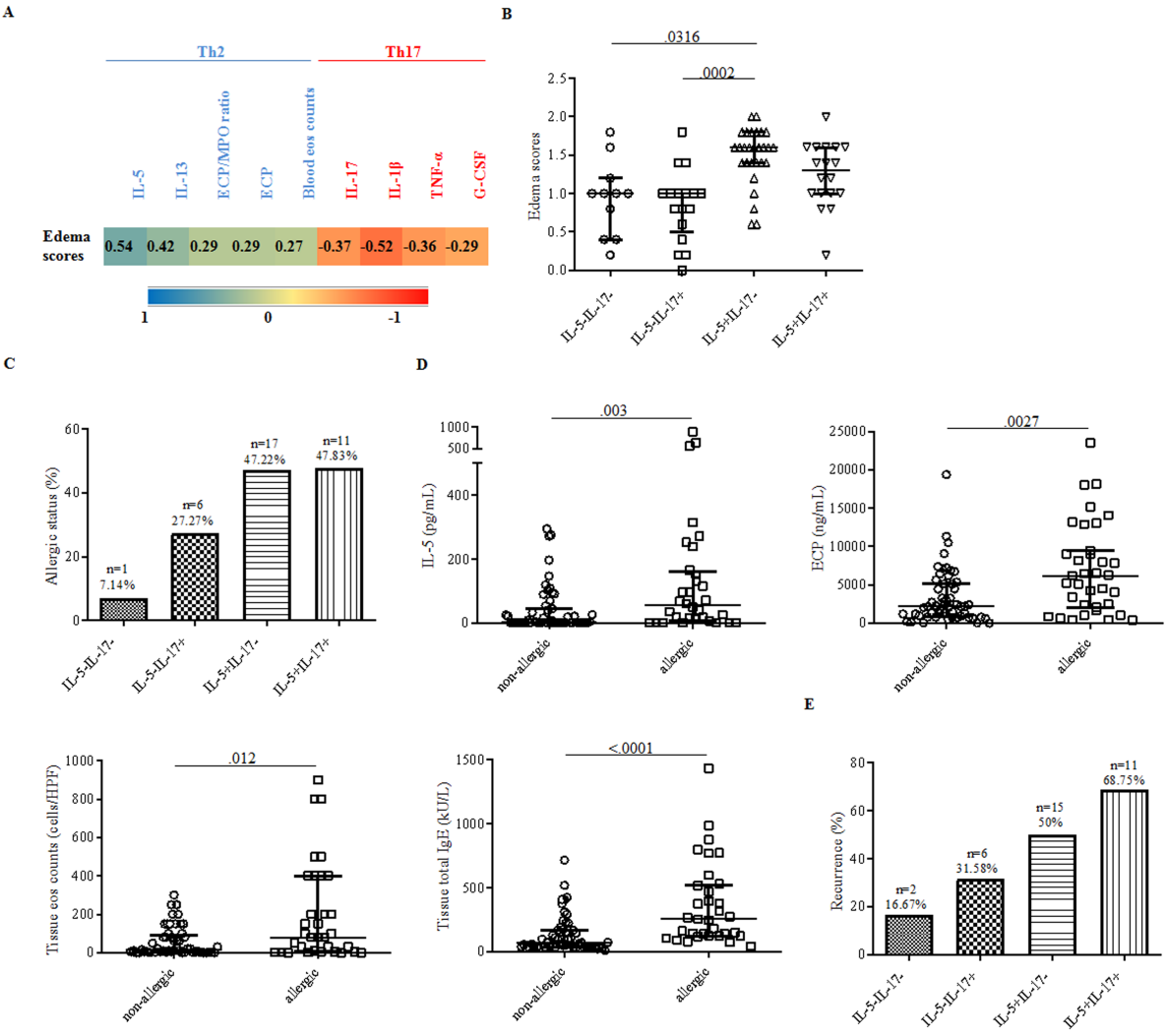
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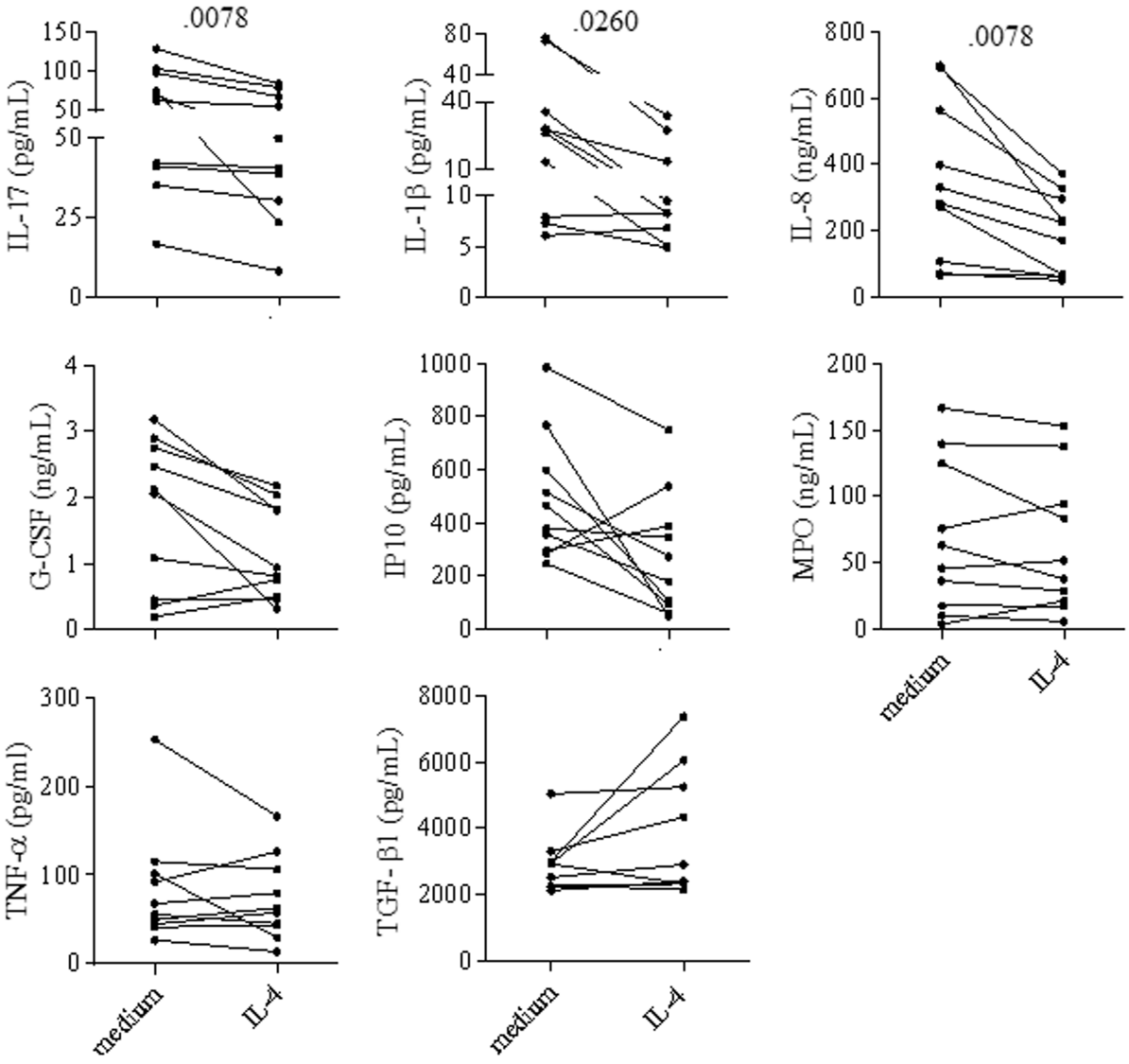
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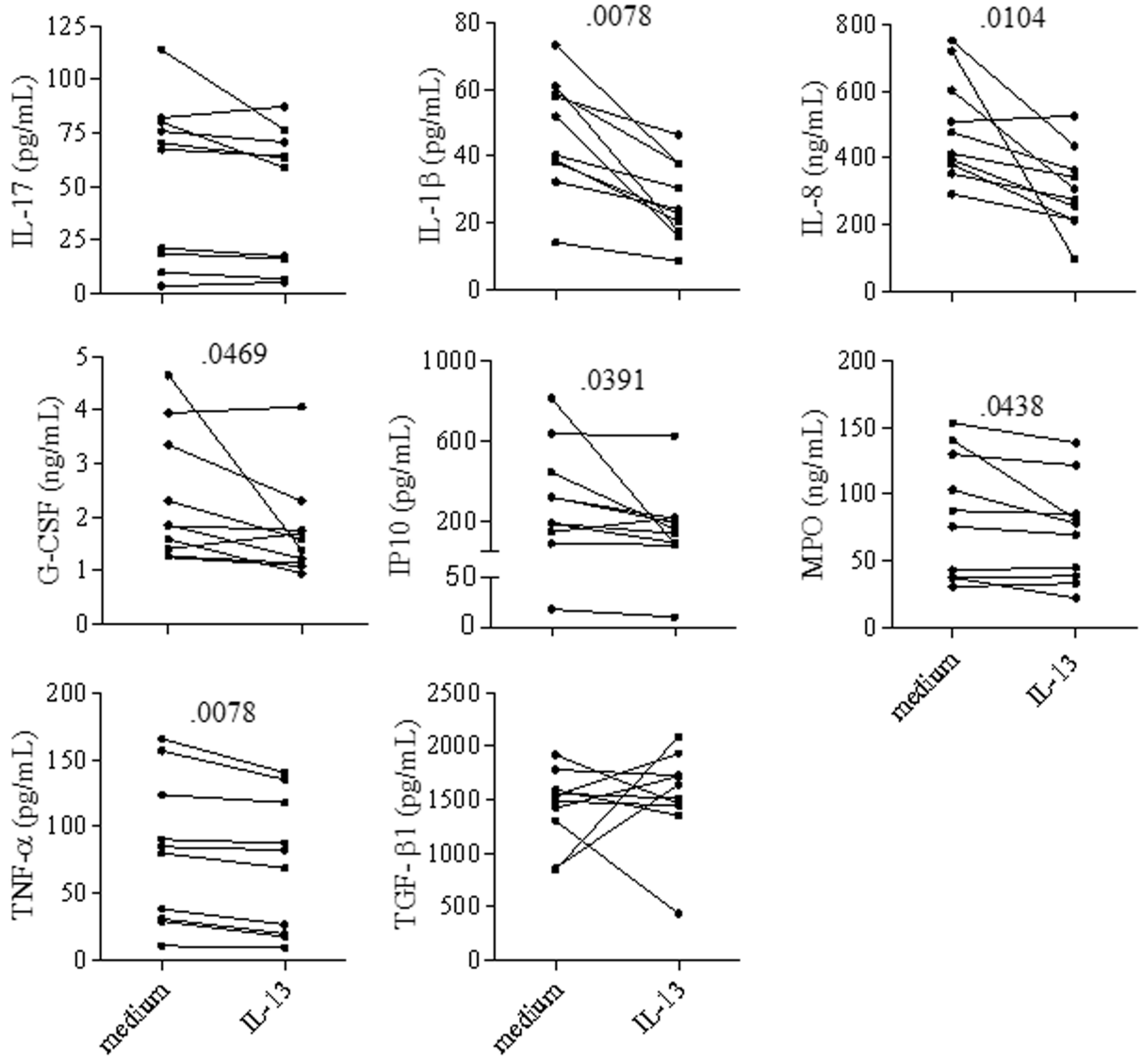


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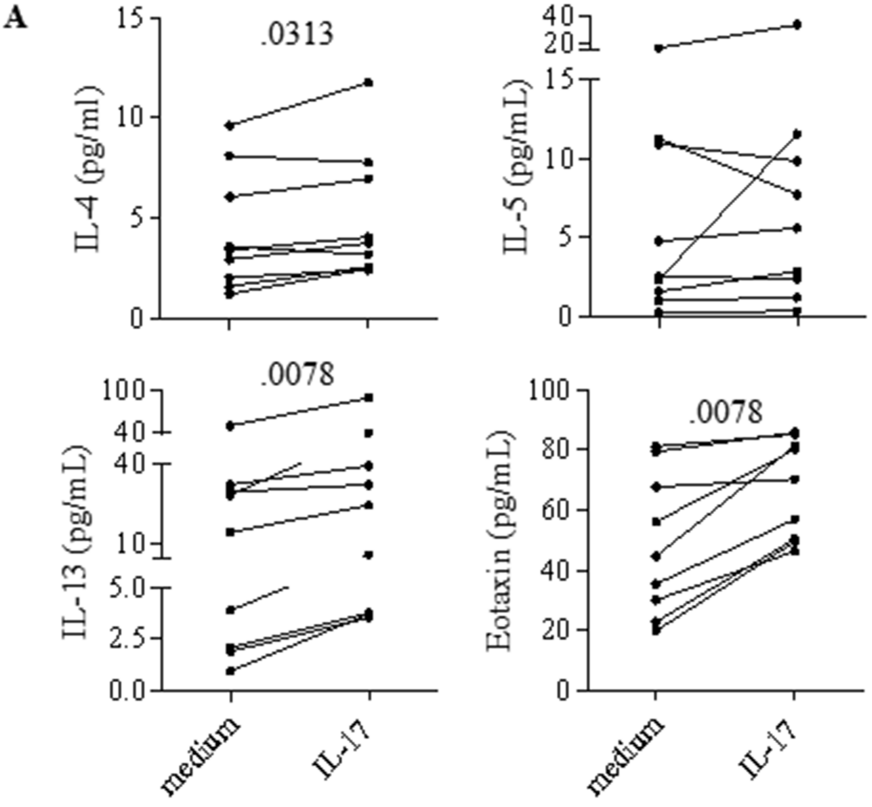
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.0078
IL-17 (pg/mL)
150
100
50
50
25
0
.0260
IL-1β (pg/mL)
80
40
40
10
10
5
0
.0078
IL-8 (ng/mL)
800
600
400
200
0
G-CSF (ng/mL)
4
3
2
1
0
IP10 (pg/mL)
1000
800
600
400
200
0
MPO (ng/mL)
200
150
100
50
0
TNF-α (pg/ml)
300
200
100
0
TGF- β1 (pg/mL)
8000
6000
4000
2000
0
medium
IL-4
medium
IL-4
medium
IL-4

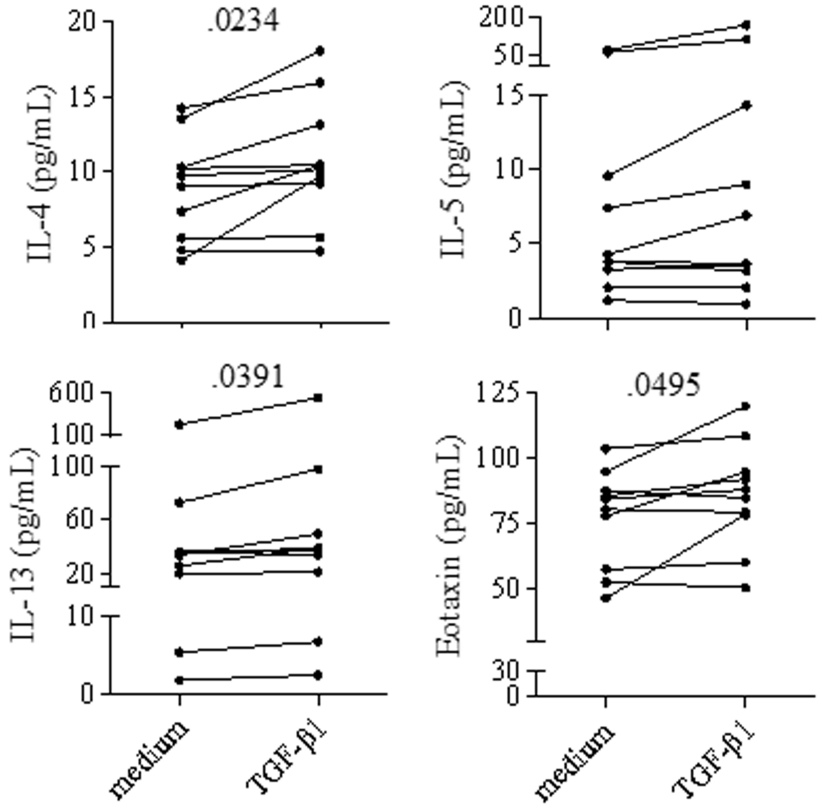

IL-17 (pg/mL)
125
100
75
50
25
0
IL-1β (pg/mL)
80
60
40
20
0
.0078
IL-8 (ng/mL)
800
600
400
200
0
.0104
G-CSF (ng/mL)
5
4
3
2
1
0
.0469
IP10 (pg/mL)
1000
600
200
50
0
.0391
MPO (ng/mL)
200
150
100
50
0
.0438
TNF-α (pg/mL)
200
150
100
50
0
.0078
TGF-β1 (pg/mL)
2500
2000
1500
1000
500
0
medium
IL-13
medium
IL-13
medium
IL-13

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METHODS

Subjects

The diagnosis of CRSwNP was based on history, clinical examination, nasal endoscopy, and CT scanning according to the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS 2012) (E2). Subjects undergoing septoplasty because of anatomic variations and without any sinonasal disease were recruited as controls. Individuals who had used systemic or local glucocorticosteroids within the 4 weeks or antibiotics within the 2 weeks before their surgeries were excluded from the study. The atopic status of each participant was evaluated by skin prick tests and the Phadiotop test (Phadia, Uppsala, Sweden) to detect IgE antibodies against various common inhalant allergens. Patients were diagnosed for asthma and aspirin intolerance by a chest physician. All patients received similar medical therapy post-nasal polyp surgery, and recurrence of polyps was defined as the presence of nasal polyps detected by nasal endoscopy with the postoperative follow-up period of at least 2 years.

Picrosirius red staining for collagen

The collagen content in NP tissue was assessed by using picrosirius red staining, as previously described (E2). Briefly, paraffin embedded sections were stained with Direct Red 80 (Alfa Aesar, Tianjin, China) for 60 minutes; after which, they were washed in acidified water and dehydrated in 100% ethanol. Total collagen was quantified under a bright-field microscope. Image analysis was performed using Image J software and collagen content was expressed as the percentage of positively stained area in the total area as seen in 10 randomly selected fields per sample.

Preparation of DNPCs

DNPCs were prepared by enzymatic digestion, as previously described (E3). Briefly, fresh nasal polyps were cut into small pieces and mixed in a GentleMACs Tube (Miltenyi Biotec, Germany) with Gentle MACs (Miltenyi Biotec, Germany). After centrifugation, the minced pieces of nasal polyps were incubated in RPMI medium containing 2 mg/mL collagenase (Gibco Life Technologies, Paisley, UK) and 0.04 mg/mL DNAse1 (Roche Diagnostics, German) for 45 min at 37°C.The tissue samples were then mixed a second time with GentleMACs and the cell suspension was filtered through a 70 μm cell strainer (BD Falcon, Bedford, MA, USA). Red blood cells in the cell pellet were lysed in erythrocyte lysis buffer.

Immunoassay

The levels of IL-4, IL-5, eotaxin, IL-17, TGF-β1, IL-1β, IL-8, TNF-α, G-CSF, IP10 (Magnetic Luminex Performance Assay, R&D Systems; Minneapolis, USA), IL-13 (Bio-Rad, Hercules, CA, USA), MPO (Oxis International, Portland, OR, USA), eosinophil cationic protein (ECP), and total IgE (UniCAP system, Pharmacia, Uppsala, Sweden) were analyzed in tissue homogenates from 95 CRSwNP and 24 control subjects using commercially available kits. Similarly, the levels of IL-5 and IL-17 (Magnetic Luminex Performance Assay, R&D Systems; Minneapolis, USA) were analyzed in tissue homogenates of NP used for DNPCs preparation, and the levels of IL-4, IL-5, IL-13, eotaxin, IL-17, TGF-β1, IL-1β, IL-8, TNF-α, G-CSF, IP10 (Bio-Rad, Hercules, CA, USA) and MPO (eBioscience, San Diego, CA, USA) in DNPCs supernatants using commercially available kits.

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E3. Derycke L, Zhang N, Holtappels G, Dutré T, Bachert C. IL-17A as a regulator of neutrophil survival in nasal polyp disease of patients with and without cystic fibrosis. *J Cyst Fibros* 2012;11:193-200.

Legends to figures in Online Repository

Figure E1. Increased concentrations of IL-5 (A) and IL-17 (B) in NP tissue homogenates from CRSwNP patients. Bold lines represent medians with interquartile range. Control, n=24; CRSwNP, n=95.

Figure E2. Correlations among the nine Th2 (red), and the nine Th17 (blue) related factors in all CRSwNP patients. The correlations (Spearman's rho) are presented as a heat map; with *P* values greater than 0.05 defined as 0.00. Eos, eosinophil.

Figure E3. Distribution of tissue IL-5 and IL-17 concentrations in all CRSwNP patients. Several values of IL-5 and IL-17 were below their detection limits (36 out of 95 for IL-5, 50 out of 95 for IL-17) and these were assigned as half of their limits. Using the detection limits of IL-5 and IL-17 as cutoff values, four groups were observed; including IL-5-IL-17-, IL-5-IL-17+, IL-5+IL-17- and IL-5+IL-17+.-, below the detection limit; +, above the detection limit.

Figure E4. Correlations among the nine Th2 (red), and the nine Th17 (blue) related factors in the individual IL-5-IL-17- (A), IL-5-IL-17+ (B), IL-5+IL-17- (C) and IL-5+IL-17+ (D) CRSwNP groups. The correlations (Spearman's rho) are presented as a heat map; with *P* values greater than 0.05 defined as 0.00. Eos, eosinophil. -, no value.

Figure E5. Blood (A) and tissue (B) neutrophil count in groups of CRSwNP

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patients defined by tissue IL-5 and IL-17 concentrations. Neu, neutrophil. IL-5-IL-17-, n=14; IL-5-IL-17+, n=22; IL-5+IL-17-, n=36; IL-5+IL-17+, n=23.

Figure E6. Correlation between edema score assessed by visual evaluation and the percentage of edema area assessed by Image J analysis in nasal polyp tissues of CRSwNP patients. N=48.

Figure E7. Collagen expression (A) and frequency of asthma (B) and aspirin intolerance (C) in groups of CRSwNP patients defined by tissue IL-5 and IL-17 concentrations. IL-5-IL-17-, n=14; IL-5-IL-17+, n=22; IL-5+IL-17-, n=36; IL-5+IL-17+, n=23.

Figure E8. Effect of IL-5 stimulation on Th17 cytokines in DNPCs. IL-5, 50ng/mL; n=9. The statistical significance of differences in expression was assessed by using the Wilcoxon matched pairs test.

Figure E9. Effect of IL-23 stimulation on Th2 cytokines in DNPCs. IL-23, 100ng/mL; n=10. The statistical significance of differences in expression was assessed by using the Wilcoxon matched pairs test.

Figure E10. Inhibition of Th2 (A) and Th17 (B) cytokines in DNPCs by Dexamethasone (DEX) treatment. DEX, 5×10^{-5}mol/L; n=10-11. The statistical significance of differences in expression was assessed using the Wilcoxon matched pairs test.

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Repository tables

Table E1. Demographic and clinical characteristics of participants.

	control	CRSwNP
No.	24	95
Age(y), median (range)	39.5 (28.8-47.8)	47 (36-55)
Gender (male/female)	13/11	75/20*
Asthma, no. (%)	0 (0)	13 (13.7)
Atopic status, no. (%)	1 (4.17)	35 (36.8)**
Aspirin intolerance, no. (%)	0 (0)	7 (7.4)
CT score (Lund-Mackay), median (range)	0 (0-1)	17 (11-22)
Polyp score (Davos), median (range)	0	4 (2-5)

The level of significance (*P*) was obtained by means of Mann-Whitney U test (for quantitative data) or the Chi-square/ Fisher exact test (for qualitative data). Range, interquartile range. *, $P<0.05$; **, $P<0.01$.

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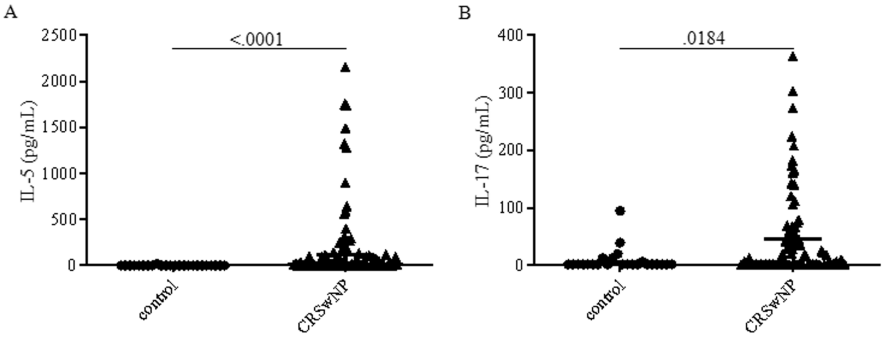
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Table E2. Characteristics of the four groups of CRSwNP patients.

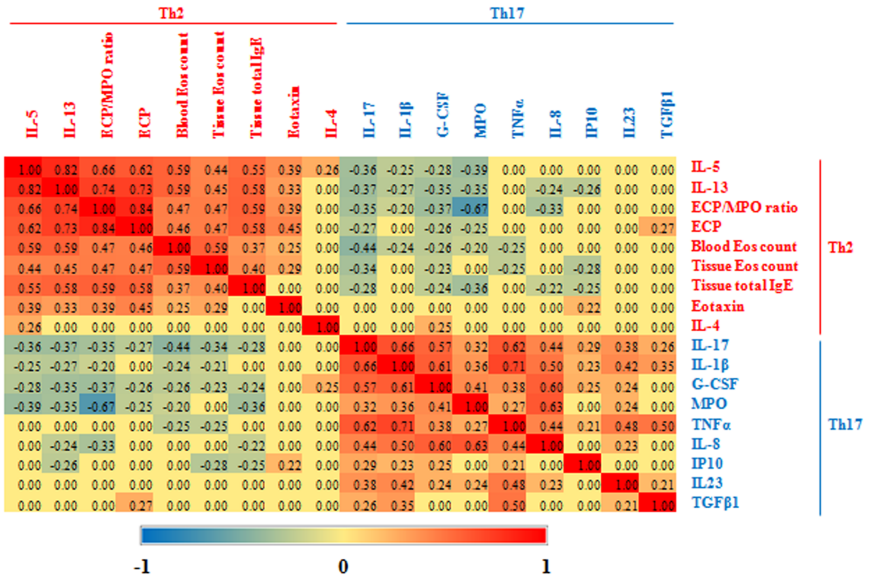
	CRSwNP groups			
	IL-5-IL-17-	IL-5-IL-17+	IL-5+IL-17-	IL-5+IL-17+
No.	14	22	36	23
Age(y), median (range)	51 (42-57.5)	46.5 (35.3-58)	46 (38.3-54)	42 (33-52)
Gender (male/female)	12/2	17/5	29/7	17/6
CT score (Lund-Mackay), median (range)	20 (9.3-23.3)	20 (11.8-23.3)	17 (12.3-22)	16 (8-22)
Polyp score (Davos), median (range)	4.6 (2-6)	4 (2.8-4.2)	4 (2-5)	4 (2-5)

The level of significance (*P*) was obtained by means of Kruskal-Wallis (for quantitative data) or the Chi-square test (for qualitative data). Range, interquartile range.

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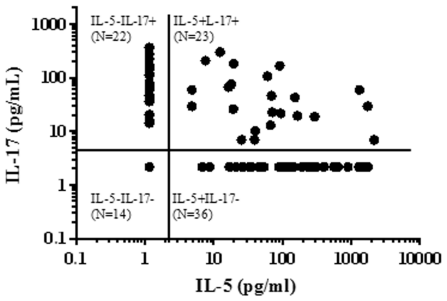


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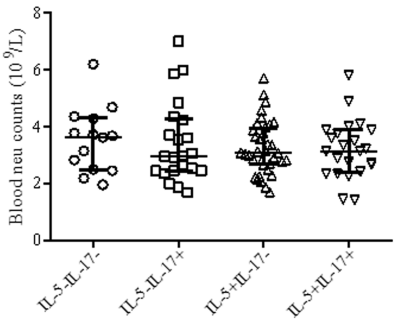
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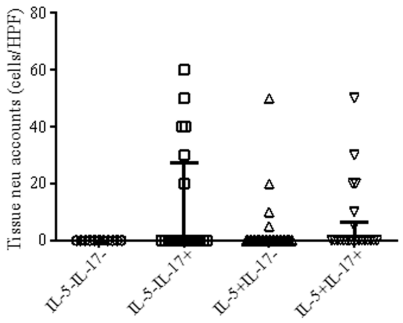


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A

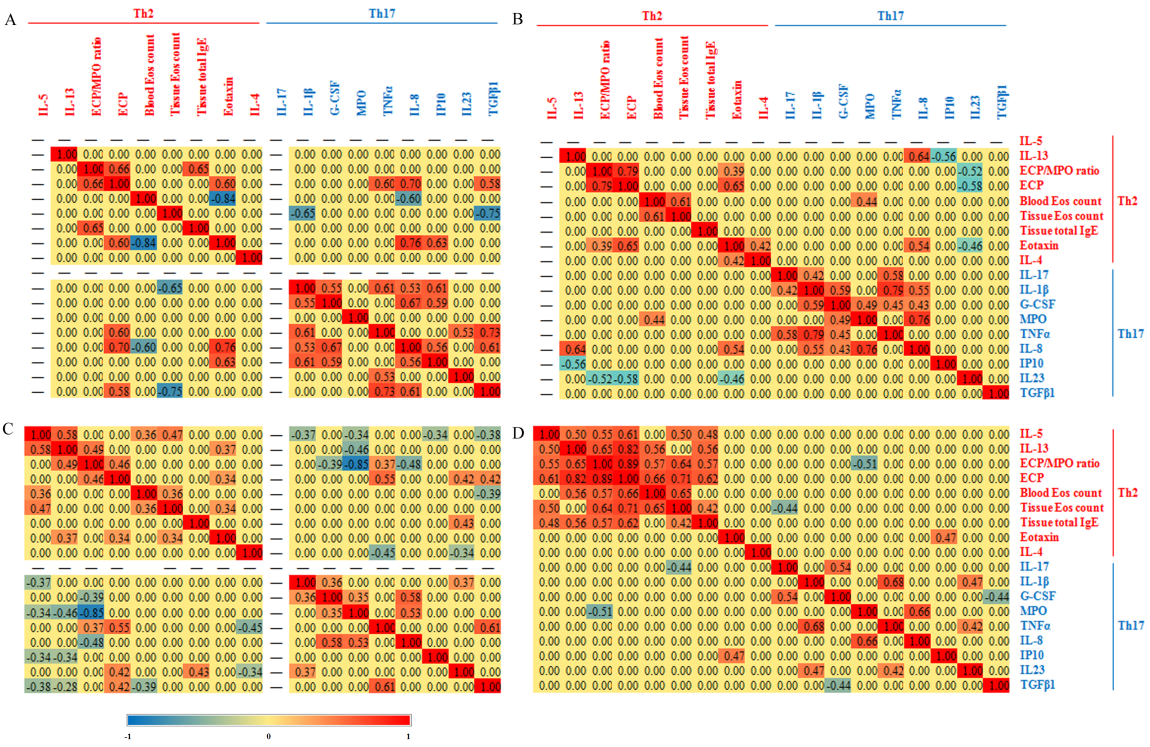


B

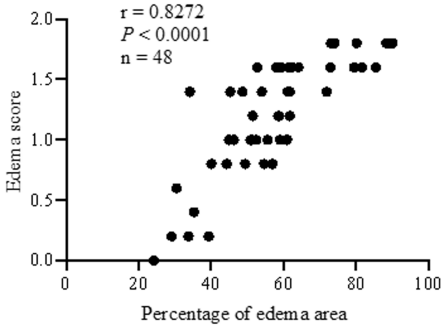


ACCEPTED MANUSCRIPT

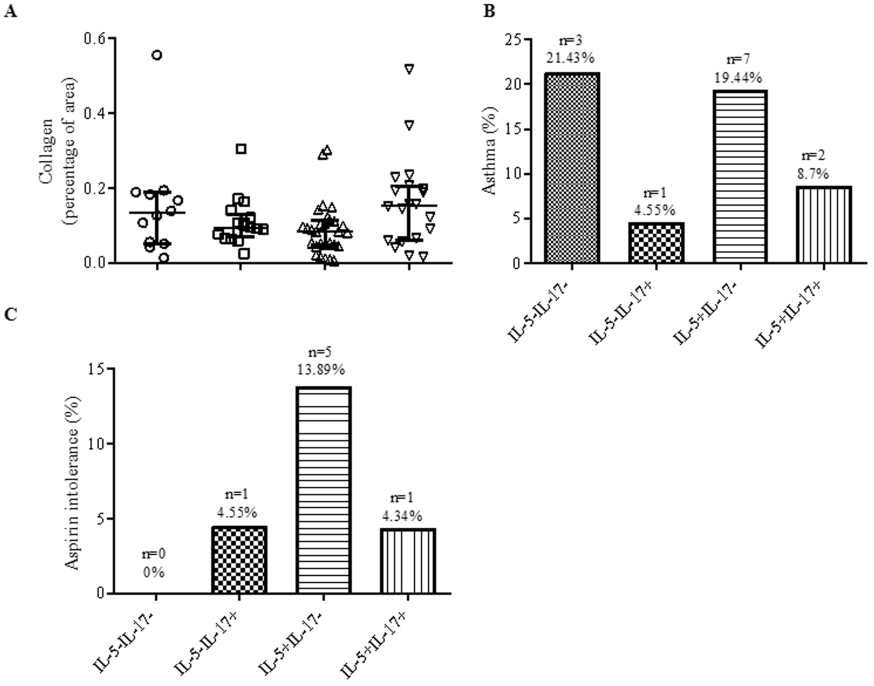
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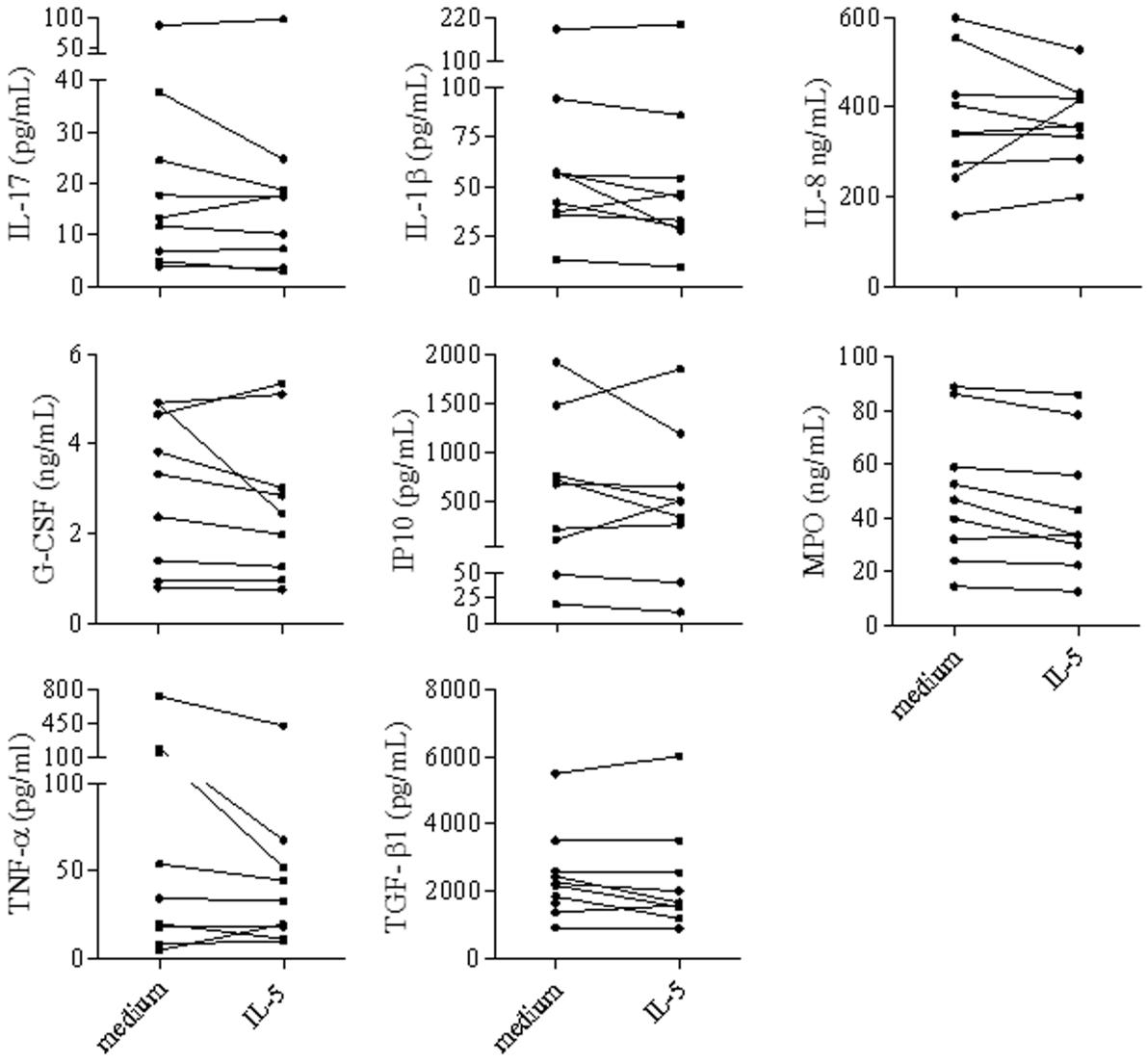
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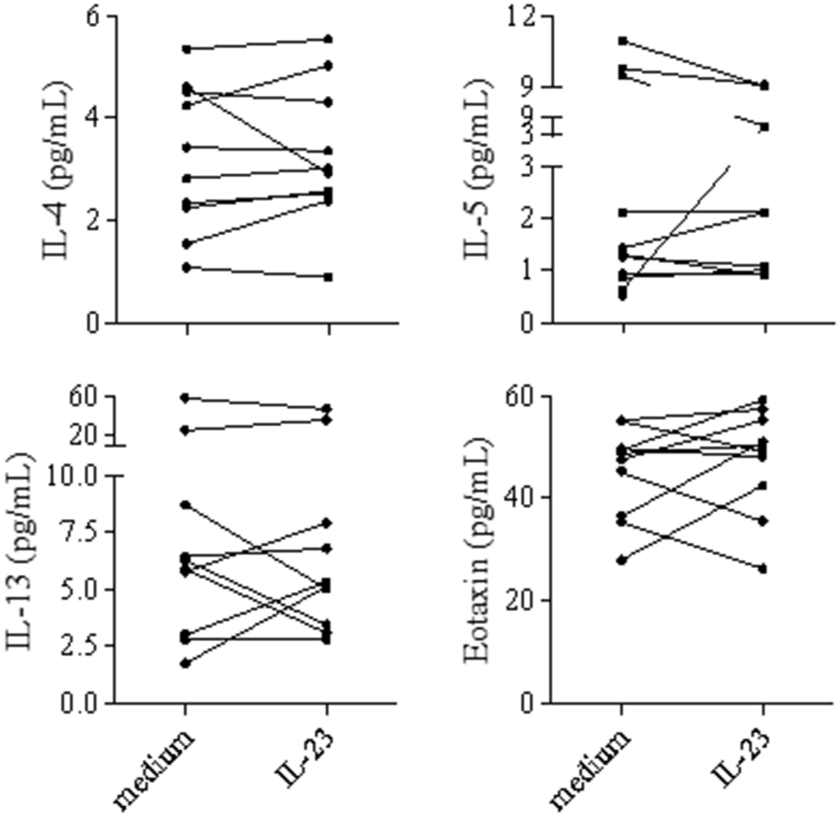
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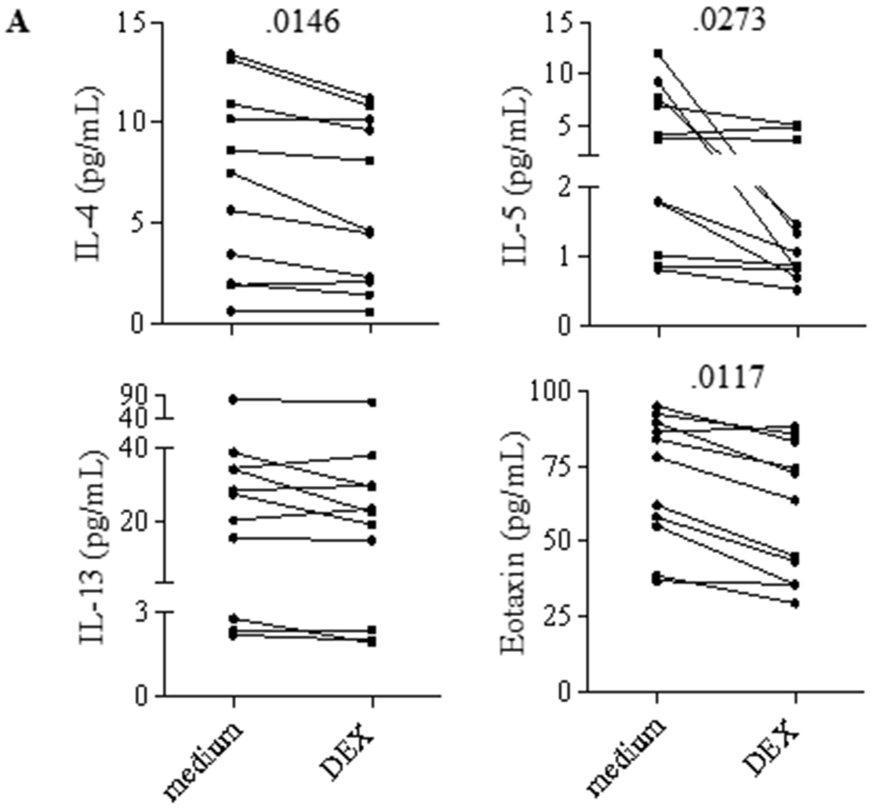
ACCEPTED MANUSCRIPT


IL-17 (pg/mL)
IL-1β (pg/mL)
IL-8 ng/mL)
G-CSF (ng/mL)
IP10 (pg/mL)
MPO (ng/mL)
TNF-α (pg/ml)
TGF- β1 (pg/mL)
medium
IL-5

ACCEPTED

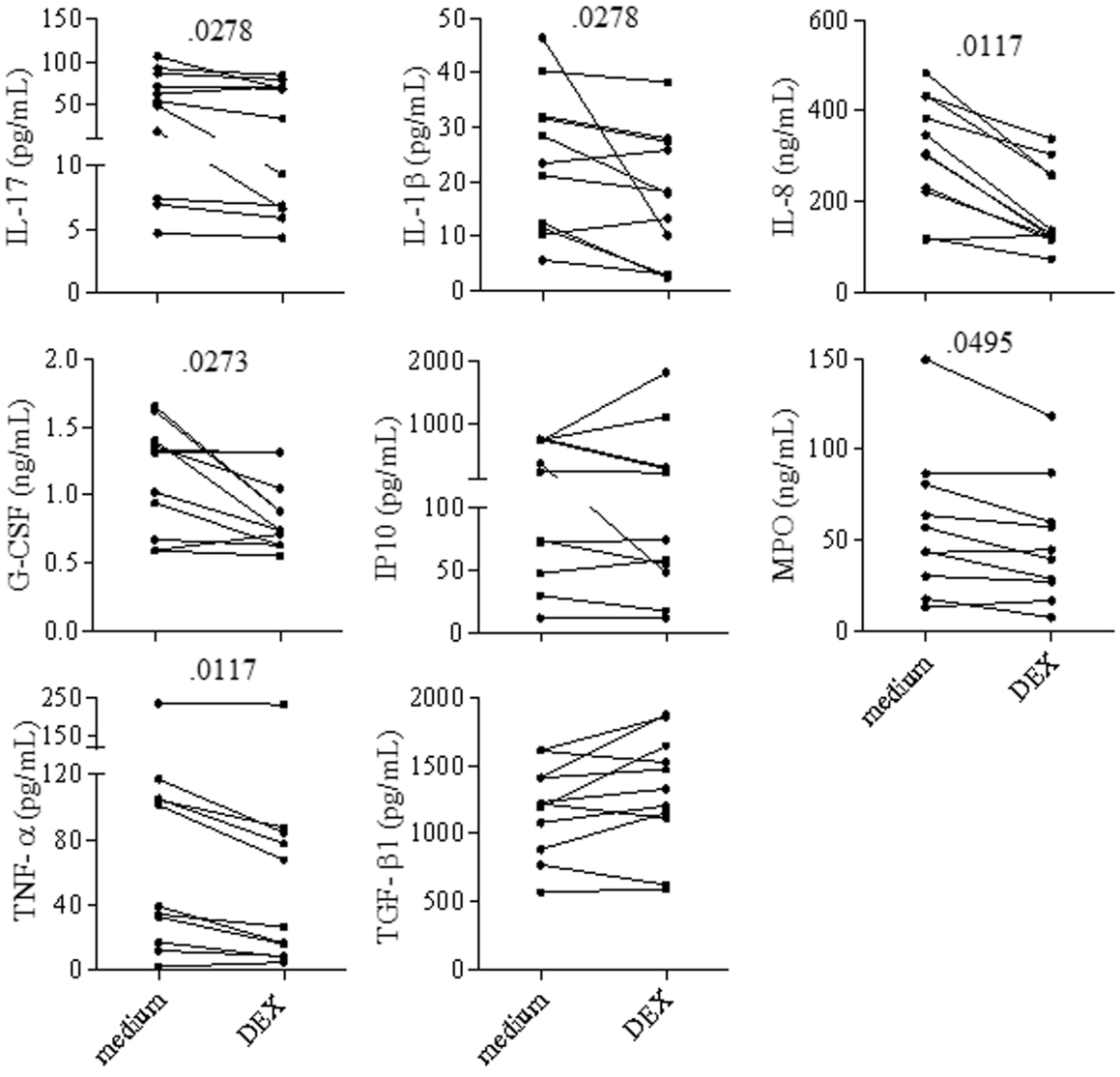


ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT

B


.0278
IL-17 (pg/mL)
150
100
50
10
5
0
.0278
IL-1β (pg/mL)
50
40
30
20
10
0
.0117
IL-8 (ng/mL)
600
400
200
0
.0273
G-CSF (ng/mL)
2.0
1.5
1.0
0.5
0.0
IP10 (pg/mL)
2000
1000
100
50
0
.0495
MPO (ng/mL)
150
100
50
0
.0117
TNF- α (pg/mL)
250
150
120
80
40
0
TGF- β1 (pg/mL)
2000
1500
1000
500
0
medium
DEX
medium
DEX
medium
DEX

ACCEPTED