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# Zinc sulfate improved the unbalanced T cell profiles in Der p-allergic asthma: An ex vivo study

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## Abstract

**Introduction:** In the pathogenesis of asthma, an imbalance between helper T (Th) 1/Th2 and Th17/Treg cells is believed to play a key role in asthmatic inflammatory responses. Some studies indicated that zinc deficiency increases inflammatory factor production and worsens asthma. However, the effects of zinc on T cell profiles to reduce inflammatory response remain unclear.

**Objectives:** We investigated the beneficial effects of zinc on isolated cell populations and cytokine levels from patients with asthma.

**Methods:** Thirty-six individuals *Dermatophagoides pteronyssinus* (Der p)-allergic and 31 healthy subjects were enrolled in the study, and peripheral blood mononuclear cells (PBMCs) were collected. Harvested PBMCs were stimulated with recombinant Der p antigen in the presence or absence of zinc sulfate (25 μM or 50 μM) for 48 h. Cell surface markers and intracellular cytokine levels were examined by flow cytometry. The pro-inflammatory factors in plasma and culture supernatants were measured by commercial enzyme-linked immunosorbent assay.

**Results:** Zinc sulfate dramatically reduced the proportions of Th2 and Th17 cells, but increased that of Th1 and Treg cells. Zinc sulfate also markedly reduced the levels of interleukin (IL)-4 and IL-17, but increased the levels of IFN-γ.

**Conclusions:** Zinc ameliorates the imbalance in T cell profiles and could be a potential adjuvant therapy for Der p-induced allergic hypersensitivity.

KEYWORDS
asthma, house dust mite, inflammation, T cells, zinc sulfate

## 1 | INTRODUCTION

Asthma is a chronic disease characterized by airway hyperresponsiveness, inflammation, and remodeling as well as reversible airway obstruction. Pulmonary exposure to aeroallergens is a key risk factor for the development of allergic asthma.[1] In Taiwan, house dust mites (HDMs), particularly *Dermatophagoides pteronyssinus* (Der p), are a major allergen for asthmatic patients.[2] The mechanisms involved in the induction of asthma by Der p are not well understood. Der p is known to express major IgE-binding proteins, with unknown biological functions, and exposure to Der p can increase IgE production and stimulate T cell proliferation. In allergic asthma, Der p induce helper T (Th) 2 cell response, whereas healthy subjects show a predominantly Th1 response. The possible mechanism is that dendritic cells

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(DCs) synthesize IL-12 efficiently and are potent inducers of Th1 immune responses in healthy subjects.[3] The Der p2 have structural homology with MD-2 and might activate macrophages through the toll-like receptor 4 pathway to induce inflammation response.[4] Another study suggests that the Der p-specific proliferating T cells are mainly Th 2 cells.[5] The possible mechanisms are that Der p interact with T cell receptors (TCRs) to activate phospholipase D (PLD), which activates Th 2 cell responses.[6] Der p induce Th 2 cells to release interleukin (IL)-4, IL-5, and IL-13[7,8] to increase the production of inflammatory cells, including neutrophils, macrophages, mast cells, and eosinophils, as well as infiltration, cytokines (IL-6 and IL-8), smooth muscle cell proliferation, and mucus production in allergic asthma. These responses cause airway inflammation and remodeling, resulting in the development of asthma.[9] In addition, some studies have suggested that Th17 cells may also be induced during the development of asthma,[10,11] while other studies have proposed that regulatory T cells (Tregs) may be involved in regulating airway inflammation.[12,13]

Asthmatic patients suffer from varying degrees of airway inflammation and narrowing, which may be related to the concentration of zinc in the body. A clinical study indicated that subjects with low intake of dietary zinc have allergic symptoms that are 4–5 times worse than those of subjects with a high zinc diet.[14,15] Asthma is a multifactorial disease; zinc deficiency is one factor known to affect the balance between T helper subsets and to worsen airway inflammation.[16] Moreover, zinc supplementation is thought to reduce airway hyperresponsiveness (AHR) and airway inflammation related to allergic inflammation in mice.[17] Some clinical studies showed that zinc homeostasis may play a role in allergic inflammation in asthma.[18,19] Our previous study showed that zinc treatment reduces inflammatory cytokine production in Der p-stimulated smooth muscle cells by reducing activation of the NF-κB pathway.[20] In this study, peripheral blood mononuclear cells (PBMCs) from Der p-allergic asthmatic and non-asthmatic subjects were cultured with Der p to activate T cells. Then, we investigated the beneficial effect of zinc on asthma and T cell profiles and cytokine levels.

## 2 | MATERIALS AND METHODS

### 2.1 | Study subjects

The study subjects were selected from outpatients of Tungs' Taichung MetroHarbor Hospital. The inclusion criteria were[1] allergic asthma in response to Der p (as evidenced by the presence of Der p-specific IgE antibody using the ImmunoCAP-system),[2] no complicated underlying disease[3] no history of upper or lower airway disease during the month before the study, and[4] not having received immunotherapy or oral or intravenous steroids during the 4 weeks before the study (inhaled steroids were allowed). The exclusion criteria were malignancy, infection, pregnancy, or other systematic immune disease during the study, and specific health food intake, Thirty-six patients with allergic asthma (19 women and 17 men) and 31 healthy subjects without allergic asthma (23 women and 8 men) were included. Pulmonary function was assessed by spirometry according to the standards of the American Thoracic Society. All subjects were fully informed about the purpose and nature of the study, which was approved by the medical ethics committee of Tungs' Taichung MetroHarbor Hospital.

### 2.2 | Lung function tests

Diagnosis of asthma was confirmed by assessing lung function using spirometry, including measurement of forced vital capacity (FVC) and forced expiratory volume in 1 s ($FEV_1$). Bronchoprovocation (19 asthmatic patients were assessed by the methacholine challenge test or histamine challenge test) is a part of asthma diagnosis. If the spirometry measurements remained close to the baseline values after inhalation of increasing doses of methacholine, the test was considered negative, and asthma was effectively ruled out. If the spirometry values declined significantly (with $FEV_1$ declining more than 20%) after methacholine inhalation, or if wheezing or other symptoms developed, the test was considered positive.

### 2.3 | Asthma control test

The Asthma Control Test is a tool that helps patients to assess asthma control. Each item is scored from one (poor control) to five (good control), and the scores are added to yield a final score with a maximum value of 25. A cut-off score of 19 is optimal for differentiating between well-controlled and uncontrolled asthma.[21]

### 2.4 | Analysis of blood samples

Anthropometric data, including body weight, height, and blood pressure, were measured using standardized techniques. Blood was collected to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine levels as well as T lymphocyte phenotype. Serum levels of total IgE and Der p-specific IgE were determined using the ImmunoCAP-system (ImmunoCAP CLA-1 Chemiluminescent Allergy System, Phadia, Uppsala, Sweden).

### 2.5 | PBMC stimulation assay

For the stimulation assay, PBMCs were isolated from heparinized blood by Ficoll-Hypaque density centrifugation at 400*g* for 20 min within 1 h of collection, and serum was

stored as aliquots in liquid nitrogen until analysis. PBMCs were washed twice with phosphate-buffered saline (PBS) and resuspended at $2 \times 10^6$/mL in RPMI-1640 medium supplemented with 10% calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Cells were stimulated with Der p2 (20 μg/mL; Allergopharma, Hamburg, Germany) with or without zinc sulfate (25 μM or 50 μM) for 48 h. After incubation, the cells were centrifuged at 1200 rpm for 10 min. The cell pellet was harvested for flow cytometry analysis.

### 2.6 | Flow cytometric analysis for T cell subtypes and intracellular cytokines

After 48 h stimulation, PBMCs were reactivated with phorbol myristate acetate (PMA, 50 ng/mL; Alexis Biochemicals, San Diego, California) and ionomycin (1 μM; Sigma-Aldrich, St. Louis, Missouri) in the presence of monensin (1 μL/mL; BD, Franklin Lakes, New Jercy) for 4 h at 37°C in 5% $CO_2$ (modified form[22]). The cells were incubated with phycoerythrin (PE)-PE conjugated anti-human CD3 antibodies for 20 min at 4°C in the dark. After surface staining, half of the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, CD8, or CD25. The remaining cells were stained with FITC-conjugated anti-human CD4 and resuspended in fixation and permeabilization solution (BD, Franklin Lakes, New Jercy) according to the manufacturer's instructions, and then stained with PE-conjugated anti-human IFN-γ, IL-4, IL-17A, or Foxp3 antibodies. PE-conjugated mouse $IgG_1$ antibodies were used as isotype controls.

### 2.7 | ELISA analysis of cytokines

The supernatants from the PBMC stimulation assay were harvested and frozen at −20°C until use. Samples and serum were assayed for IFN-γ, IL-4, and IL-17A using ELISA kits (eBioscience, San Diego, California) according to the manufacturer's instructions. All assays were performed in duplicate.

### 2.8 | Statistical analysis

The results were expressed as the mean ± SD. SPSS for Windows (SPSS, Inc., Chicago, Illinois) was used for the statistical analyses. Statistical significance was determined by unpaired *t*-test for comparisons of asthmatic and non-asthmatic groups and by one-way ANOVA for comparisons of different treatment groups. LSD post hoc test was used in ANOVA. Two-tailed statistical tests were used, and *P* values less than 0.05 were considered statistically significant.

## 3 | RESULTS

### 3.1 | Patient characteristics

Forty asthmatic patients were recruited; however, complete data were not available for four patients. Thus, 36 asthmatic patients (asthmatic subjects) and 31 healthy volunteers (non-asthmatic subjects) were included in the study. Basic demographic characteristics for the two groups are shown in Table 1. There was no significant difference between the two groups, except for gender, total IgE levels and lung function ($P < .05$). Asthmatic subjects presented lower lung function ($FEV_1$/FVC pr, forced expiratory flow $[FEF]_{(25–75\%)}$ pr, $FEV_1$ po, and $FEF_{(25–75\%)}$ po) than non-asthmatic subjects ($P < .05$). The serum zinc concentration of asthma subjects and non-asthmatic subjects was 813.2 ± 89.2 μg/L and 847.7± 112.3 μg/L, respectively. The serum zinc concentration of asthma subjects was lower than that of non-asthmatic subjects, but the difference was not statistically significant. Using ACT scores to distinguish subjects, we found that serum zinc concentration of subjects with an ACT score greater than 25 was 872.7± 84.9 μg/L, that of subjects with an ACT score of 20–24 was 901.2 ± 32.3 μg/L, and that of subjects with an ACT score lower than 20 was 808.0 ± 38.1 μg/L. However, these differences did not achieve statistical significance.

### 3.2 | Concentration of Th cell cytokines in serum

Heparinized peripheral blood (20 mL) was collected from the two groups of subjects, and separated into serum and PBMCs. Serum levels of IFN-γ, a Th1 cell cytokine, IL-4, a Th2 cell cytokine, and IL-17, a Th17 cell cytokine, were assayed using ELISA kits (Figure 1). The IFN-γ level was lower in asthmatic subjects than in non-asthmatic subjects (12.4 ± 14.1 pg/mL *vs.* 35.9 ± 36.8 pg/mL, respectively; $P < .05$), and the IL-17 serum level was higher in asthmatic subjects than in non-asthmatic subjects (3.95 ± 3.7 pg/mL *vs.* 1.45 ± 1.6 pg/mL, respectively; $P < .05$).

### 3.3 | Zinc sulfate increased the Th1/Th2 ratio

The concentration of zinc in human plasma is 11.6–23 μM. In the in vitro system, the concentration of zinc (16 μM) was similar to normal human plasma zinc level; and 32 μM of zinc was the higher end of plasma zinc level, produced by oral supplementation.[23] We investigated the effects of 25 μM zinc sulfate as the lower limit of plasma zinc level by oral supplementation and 50 μM zinc sulfate as upper limit of zinc by treatment of PBMCs. PBMCs were stimulated with Der p and different concentrations of zinc sulfate (ZN1: 25μM and ZN2: 50μM) for 48 h. The baseline number of $CD3^+$ $CD4^+$ T cells was higher in asthmatic subjects than in

**TABLE 1** Patients' characteristics

| | Asthma subjects ($n = 36$) | Non-asthma subjects ($n = 31$) |
|---|---|---|
| Age | 39.2 ± 15.9 | 39.0 ± 10.9 |
| Sex (M/F) | 17/19 | 8/23* |
| Height (cm) | 164.7 ± 8.7 | 161.1 ± 7.0 |
| Weight (kg) | 65.4 ± 16.9 | 61.7 ± 14.0 |
| SBP(mmHg) | 120.6 ± 13.7 | 119.8 ± 10.5 |
| DBP(mmHg) | 75.3 ± 9.2 | 74.3 ± 9.8 |
| ALT(U/L) | 23.4 ± 8.2 | 22.1 ± 12.2 |
| AST (U/L) | 24.2 ± 14.5 | 28.9 ± 33.2 |
| BUN (mg/dl) | 14.5 ± 5.2 | 13.3 ± 4.4 |
| Creatinine (mg/dl) | 1.1 ± 0.9 | 1.3 ± 0.5 |
| TG (mg/dL) | 137.6 ± 66.1 | 137.4 ± 78.4 |
| Cholesterol(mg/dL) | 192.8 ± 32.5 | 192.4 ± 28.7 |
| WBC($10^3$/uL) | 7.4 ± 2.1 | 6.3 ± 1.8 |
| Total IgE (kUA /l) | 387.2 ± 225.4 | 43.4 ± 26.7* |
| ACT score | 21.3 ± 3.9 | N.D. |
| Zinc (μM) | 12.4 ± 1.36 | 13.0 ± 1.72 |
| FVC pr | 3.4 ± 0.9 | 3.2 ± 0.6 |
| $FEV_1$ pr | 2.6 ± 0.8 | 2.6 ± 0.6 |
| $FEV_1$/FVC pr | 75.1 ± 12.3 | 82.8 ± 8.6* |
| FEF(25-75%) $P^r$ | 2.3 ± 1.2 | 3.0 ± 1.0* |
| FVC po | 3·1 ± 1·2 | 2.9 ± 1.2 |
| $FEV_1$ po | 2.2 ± 0.8 | 2.4 ± 1.3* |
| $FEV_1$/FVC po | 70.4 ± 10.2 | 80.0 ± 13.1 |
| FEF (25∼75%) po | 1.6 ± 0.7 | 2.6 ± 2.0* |

Abbreviations: ACT, asthma control test; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBP, diastolic blood pressure; N.D., no determine; po, post test; pr, predicted; SBP, systolic blood pressure; TG, triglyceride; WBC, white blood cell.
*Significant difference to asthma subjects ($P < .05$).

non-asthmatic subjects, but the difference was not significant (Table 2). In contrast, the baseline number of Th1 cells ($CD4^+$ IFN-$\gamma^+$ cells) was significantly lower in asthmatic subjects (6.7 ± 8.2%) than in non-asthmatic subjects (20.7 ± 21.2%; $P < .05$), whereas the baseline number of Th2 cells ($CD4^+$ IL-$4^+$ cells) was markedly higher in asthmatic subjects (24.8 ± 15.2%) than in non-asthmatic subjects (12.8 ± 18.7%; $P < .05$). In asthmatic subjects, the percentage of Th1 cells was significantly higher in zinc sulfate-treated PBMCs (ZN1: 25.9 ± 30.3% and ZN2: 32.4 ± 31.6%) than in the baseline (untreated) PBMCs (6.7 ± 8.2%; $P < .05$). Conversely, the proportion of Th2 cells was significantly lower in the zinc sulfate-treated PBMCs (ZN1:16.9 ± 13.1% and ZN2:16.7 ± 16.9%) than in the baseline PBMCs (24.8 ± 15.2%; $P < .05$; Table 2, Figure 2). The Th1/Th2 ($IFN^+$/IL-$4^+$) ratio showed a trend similar to that observed for the proportion of Th1 cells ($CD4^+$ IFN-$\gamma^+$ cells).

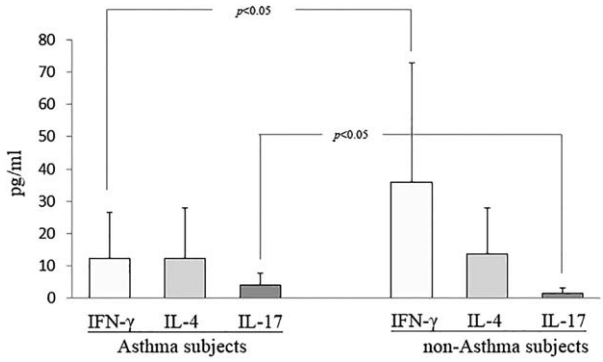


**FIGURE 1** Serum cytokine levels in asthmatic and non-asthmatic subjects. The serum levels of IFN-γ, a helper T (Th) 1 cell cytokine, IL-4, a Th2 cell cytokine, and IL-17, a Th17 cell cytokine, were assayed using ELISA kits

IFN-γ and IL-4 cytokines were also assayed in culture medium. The results were similar to those obtained when determining the proportions of Th1 cells ($CD4^+$ $IFN^+$ cells) and Th2 cells ($CD4^+$ IL-$4^+$ cells; Table 2). There were no significant differences in the non-asthmatic subjects after zinc sulfate treatment.

### 3.4 | Zinc sulfate reduced the proportion of Th17 cells

The baseline proportion of Th17 cells was higher in the asthmatic subjects than in the non-asthmatic subjects (Figure 3). After zinc sulfate treatment, the proportion of Th17 cells ($CD4^+$ IL-$17^+$/$CD4^+$ cells) in PBMCs from asthmatic subjects was significantly lower (ZN1:2.1 ± 1.0% and ZN2:2.2 ± 1.2%) than in the baseline untreated-zinc PBMCs (3.0 ± 2.9%; $P < .05$). IL-17 cytokine levels in culture medium were assayed, the levels in ZN1 group (16.4 ± 15.2 pg/mL) and ZN2 group (19.0 ± 17.7 pg/mL) were lower than the baseline group (30.7 ± 29 pg/mL) in asthmatic subjects ($P < .05$). The results were similar to those obtained when measuring the proportion of Th17 cells.

### 3.5 | Zinc sulfate increased the proportion of Treg cells

The proportion of Treg cells in asthmatic subjects was lower than that in non-asthmatic subjects. In asthmatic subjects, the

**TABLE 2** The changes of T cell subtypes and cytokines after different zinc treated-PBMCs from two groups of subjects

| | Asthma subjects ($n = 36$) | | | Non-asthma subjects ($n = 31$) | | |
|---|---|---|---|---|---|---|
| | **Baseline** | **ZN1** | **ZN2** | **Baseline** | **ZN1** | **ZN2** |
| $CD3^+$ (%) | 45.0 ± 17.5 | 50.8 ± 16.8 | 48.6 ± 18.8 | 36.0 + 14.9 | 41.9 ± 14.8 | 37.4 ± 15.2 |
| $CD3^+$ $CD4^+$ (%) | 26.5 ± 10.3 | 25.7 ± 8.2 | 27.7 ± 11.9 | 22.6 ± 13.0 | 27.9 ± 13.3 | 27.3 ± 12.6 |
| $CD4^+$ $IFN^+$ (%) | 6.7 ± 8.2 | 25.9 ± 30.3* | 32.4 ± 31.6* | 20.7 ± 21.2** | 25.6 ± 28.7 | 27.7 ± 29.8 |
| $CD4^+$ $IL\text{-}4^+$ (%) | 24.8 ± 15.2 | 16.9 ± 13.1* | 16.7 ± 16.9* | 12.8 ± 18.7** | 10.3 ± 15.4 | 10.8 ± 13.8 |
| $IFN^+/IL\text{-}4^+$ | 0.4 ± 0.3 | 1.1 ± 1.5* | 1.3 ± 1.5* | 1.9 ± 1.8** | 2.5 ± 3.5 | 2.6 ± 3.6 |
| EFN (pg/mL)[a] | 6.9 ± 3.5 | 80.4 ± 7.2* | 165.4 ± 18.7* | 318.7 ± 18.1** | 302.9 ± 29.4 | 341.8 ± 31.4 |
| IL-4 (pg/mL)[a] | 57.5 ± 15.5 | 49.2 ± 12.3 | 41.2 ± 8.5* | 37.1 ± 12.9** | 35.6 ± 15.6 | 29.3 ± 10.3 |

*Note*. Baseline, no-treated zinc; ZN1, treated zinc (25μM) for 48 h; ZN2, treated zinc (50μM) for 48 h.
*Significant difference to baseline group of asthma subjects ($P < .05$); **significant difference between asthma subjects and non-asthma subjects ($P < .05$).
[a]Cytokines measured were from culture supernatants by ELISA.

proportions of Treg cells ($CD4^+$ $CD25^+$ cells and $CD4^+$ $Foxp3^+$ cells) were significantly higher in the zinc sulfate-treated groups than in the baseline group ($CD4^+$ $CD25^+$ cells, baseline: 9.4 ± 5.7%, ZN1: 14.4 ± 10.6%, and ZN2: 14.4 ± 10.8%; $CD4^+$ $Foxp3^+$ cells, baseline: 28.6.4 ± 14.6%, ZN1: 35.9± 15.2%, and ZN2: 37.7 ± 16.3%, $P < .05$; Figure 4).

## 4 | DISCUSSION

Zinc may play an important role in the reduction of inflammation by protecting the function and regulation of T cells in the airways and lungs.[24,25] Previous research showed that the concentration of zinc in the sera of asthmatic patients is lower than that in non-asthmatic subjects.[18] In this study, the

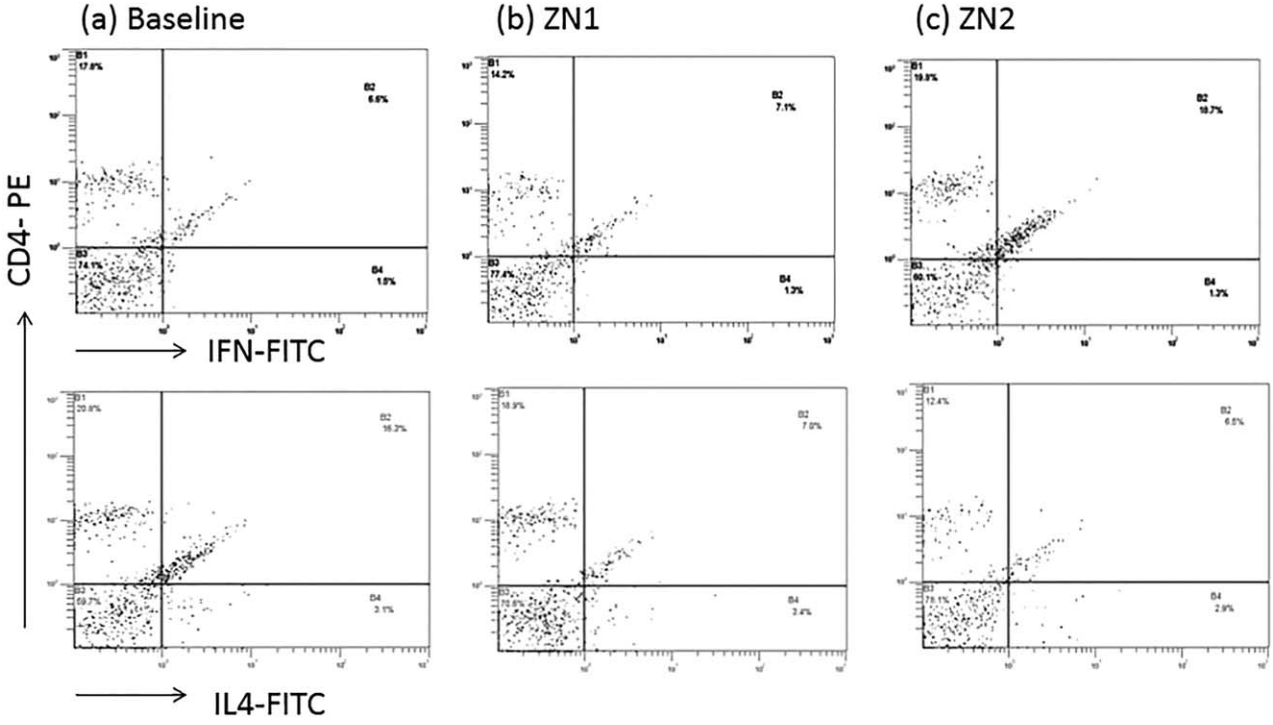


**FIGURE 2** The changes in $CD4^+$ $IFN^+$ and $CD4^+$ $IL\text{-}4^+$ cell populations after zinc treatment of PBMCs from asthmatic subjects. PBMCs from asthmatic and non-asthmatic subjects were treated with Der p2 (20 μg/mL) with or without zinc sulfate (25 or 50 μM) for 48 h. The populations of $CD4^+$ $IFN^+$ and $CD4^+$ $IL\text{-}4^+$ cells were assayed by flow cytometry. (a) baseline: PBMCs not treated with zinc sulfate; (b) ZN1: PBMCs were treated with 25 μM zinc sulfate for 48 h; (c) ZN2: PBMCs were treated with 50 μM zinc sulfate for 48 h. These data were obtained from asthmatic subject no. 17

concentrations of zinc in the sera of asthmatic patients and non-asthmatic subjects were not different. There are studies that also show similar results.[26,27] In this study, all asthmatic subjects had good control over asthma and were on corticosteroid therapy. Corticosteroids decrease the levels of zinc in hair and restore serum zinc levels in asthmatic subjects.[28] Serum zinc levels could indicate zinc deficiency, and zinc could be a negative acute phase reactant that decreases with inflammation.[19] In addition to this, we recruited asthmatic patients with no zinc supplementation; however, we did not evaluate their dietary intake; hence, we do not know whether there were differences between these two groups in their dietary zinc intake.

Zinc deficiency increases the expression of inflammatory factors, which increases asthma progression.[16] In animal models of allergic airway inflammation, mice that consumed a low-Zn diet exhibited greater AHR than mice with normal Zn intake.[17] Related studies also demonstrated that Zn supplementation can reduce the infiltration of inflammatory cells into the airway, thereby reducing airway inflammation. In a clinical study, childhood asthma subjects receiving zinc supplementation (50 mg/day) showed significant improvements in clinical symptoms, such as cough, wheezing, dyspnea, and spirometry parameters (FVC, $FEV_1$, and $FEV_1$/FVC).[29] In this study, though the concentrations of zinc in the sera of asthmatic patients and non-asthmatic subjects showed no difference, asthmatic patients showed lower lung function than non-asthmatic subjects did. In future studies, we will determine the plasma/serum and RBC zinc levels to understand this aspect.

Der p-stimulated T cells tend to be shifted toward Th2 immune responses rather than Th1, and Th1 cells may inhibit the Th2-induced asthmatic response.[30] In other study, Th1 cells may enhance the asthmatic inflammatory response.[31] Therefore, the role of Th1 in allergic asthma needs to be clarified. In this study, zinc sulfate significantly increased the proportion of Th1 cells and IFN-γ levels in asthmatic subjects (Table 2), whereas these parameters did not differ significantly in non-asthmatic subjects. In other words, zinc sulfate increased the Th1 immune response but did not increase inflammation.

Allergic asthma is considered to be a Th2-dominant inflammatory response, although Th1 and Th17 cells can also cause inflammation of the airways. Th17 cells produce IL-17A/F and IL-22, which play important roles in the development of asthma.[32] Th17 cells and IL-17 increase neutrophilic and eosinophilic inflammation in asthmatic patients,[33] and Th17 cells may be involved in the asthmatic response. Additionally, in this study, zinc sulfate significantly decreased the proportion of Th17 cells and IL-17 levels in asthmatic subjects (Figure 3). In this study, because of the limited amount of blood samples, we could not identify the possible mechanisms of zinc for helper T cells. However, in related studies, it was reported that different dosages of zinc could not cause cell antiproliferation or toxicity.[23,34]

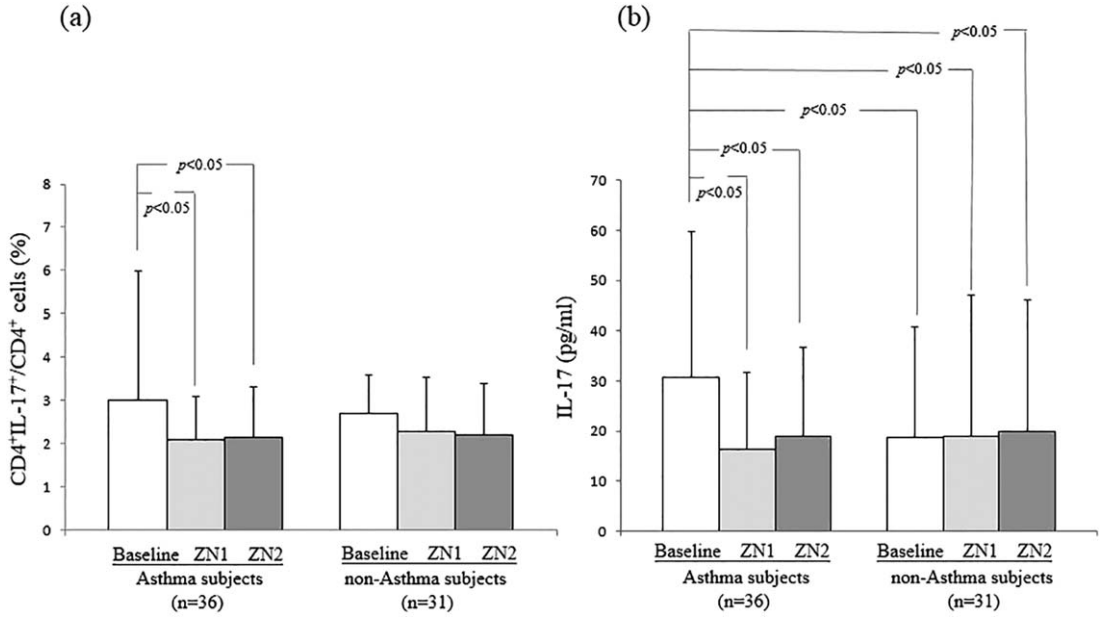


**FIGURE 3** The changes in Th17 cells and IL-17 levels after treatment of PBMCs with different concentrations of zinc sulfate. PBMCs from asthmatic and non-asthmatic subjects were treated with Der p2 (20 μg/mL) with or without zinc sulfate (25 or 50 μM) for 48 h. The populations of Th17 cells ($CD4^+IL\text{-}17^+$ cells) were assayed by flow cytometry, and IL-17 levels were assayed using an ELISA kit. ZN1: 25 μM zinc sulfate; ZN2: 50 μM zinc sulfate

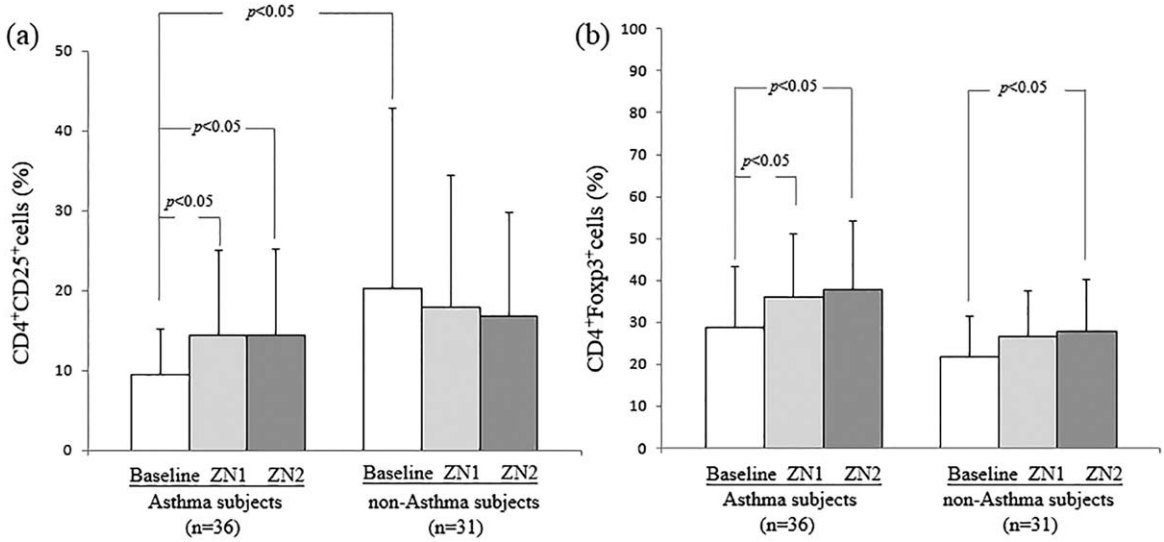


**FIGURE 4** Changes in Treg cells after treatment of PBMCs with different concentrations of zinc sulfate. The PBMCs from asthmatic and non-asthmatic subjects were treated with Der p2 (20 μg/mL) with or without zinc sulfate (25 or 50 μM) for 48 h. The population of Treg cells ($CD4^+CD25^+$ (a) and $CD4^+Foxp3^+$cells (b)) was assayed by flow cytometry. ZN1: 25 μM zinc sulfate; ZN2: 50 μM zinc sulfate

Treg cells express CD25 on the cell surface ($CD4^+$ $CD25^+$ T cells), and $CD4^+$ $CD25^+$ T cells express the forkhead box P3 (Foxp3) transcription factor, which could play a role in the establishment and maintenance of their immune suppressive activity.[35] Treg cells are the major regulators of the suppression of autoimmunity and antigen-specific inflammation.[36] Asthmatic patients on corticosteroids have lower proportions of Treg cells than healthy subjects.[12] In this study, we also found a lower proportion of Treg cells in asthmatic subjects than in non-asthmatic subjects (Figure 4), and zinc sulfate significantly increased these proportions. Treg cells reduce T cell-induced inflammation through the production of IL-10.[37] However, the mechanisms of inflammation suppression in allergic asthma mediated by Treg cells are not yet well understood.

Our results indicate that zinc sulfate has beneficial effects on allergic airway inflammation. We compared the $CD4^+$ T cell profiles of asthmatic and non-asthmatic subjects and assessed the effect of zinc sulfate treatment on these $CD4^+$ T cell profiles. Zinc sulfate ameliorated the imbalance in the proportions of Th1, Th2, Th17, and Treg cells. The limitation of the study is[1]: Because of limited funds, the number of subjects recruited was low[2] Because of the limited amount of blood, we could only use other research to show the relationship between zinc and T cell survival; however, subsequent research will be improved.

## ACKNOWLEDGMENTS

The study was provided necessary equipment by Hungkuang University, Kuang-Tien General Hospital, and Tungs' Taichung MetroHarbor Hospital. The authors had full access to all of the data from the study and take full responsibility for the integrity of the data and the accuracy of the data analysis.

## CONFLICT OF INTEREST

We declare that we have no conflict of interest related to the publication of this manuscript.

## AUTHOR CONTRIBUTIONS

All authors contributed extensively to the work presented in this paper. Wang-Sheng Ko and Ya-Ling Chiou designed the experiment. Yu Lin Tsai, Jung-Lung Hsiao, Hsin-Hung Pan and Ya-Ling Chiou performed the experiments. Wang-Sheng Ko and Ya-Ling Chiou analyzed the data and prepared the manuscript.

## ETHICS

This study involved human subjects. Our study was approved by the ethics committee of Tungs' Taichung MetroHarbor Hospital, and informed consent was obtained from all the patients prior to the commencement of the study.

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