Circulating levels of both Th1 and Th2 chemokines are elevated in patients with sarcoidosis

Shin-ichi Nureki, Eishi Miyazaki, Masaru Ando, Takuya Ueno, Tetsujiro Fukami, Toshihide Kumamoto, Katsunori Sugisaki, Tomiyasu Tsuda

Division of Pulmonary Disease, Third Department of Internal Medicine, Oita University Faculty of Medicine, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan
Division of Neurology and Neuromuscular Disorders, Third Department of Internal Medicine, Oita University Faculty of Medicine, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan
Department of Respiratory Medicine, National Nishi-Beppu Hospital, 4548 Tsurumi, Beppu, Oita 874-0833, Japan
Department of Respiratory Medicine, Shin-Beppu Hospital, 3898 Tsurumi, Beppu, Oita 874-0833, Japan

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Summary
Background: In sarcoidosis, the T helper type 1 (Th1) response tends to predominate at affected disease sites; however, whether Th1/Th2 polarization occurs in the peripheral circulation is unknown.
Methods: Fifty-two patients with sarcoidosis and 21 healthy volunteers were investigated. The concentrations of interferon-inducible protein 10 (IP-10)/CXCL10 and thymus- and activation-regulated chemokine (TARC)/CCL17 in the serum, bronchoalveolar lavage fluid (BALF) and culture supernatant were measured by an enzyme-linked immunosorbent assay. The circulating CXCR3+ CD4+ T cells and CCR4+ CD4+ T cells were assessed by flow cytometry.
Results: The CXCR3+ or CCR4-positive ratios among CD4+ T cells were both higher in sarcoidosis than in healthy volunteers. The serum levels of both IP-10 and TARC of the patients with sarcoidosis were significantly higher than those of the healthy volunteers. In patients with sarcoidosis, a larger amount of IP-10 was generated by the BALF cells, whereas IP-10 production by peripheral blood mononuclear cells did not increase in comparison to the control subjects. The TARC levels produced by peripheral blood mononuclear cells of sarcoidosis patients were significantly higher than those of the controls, while no difference existed between the 2 groups regarding TARC production by BALF cells.
Introduction

Sarcoidosis is a multisystem inflammatory disease of unknown etiology. It is characterized by non-caseating epithelioid cell granulomas and the accumulation of CD4+ T cells and macrophages at sites of inflammation. Previous studies have shown that a polarized T helper type 1 (Th1) response is involved in the granuloma formation, and Th1-associated cytokines, such as interferon (IFN)-γ and interleukin (IL)-2, play important roles in the pathogenesis of sarcoidosis. In addition, IL-12 and IL-18 synergistically induce increased production of IFN-γ primarily in the granulomatous tissue of sarcoidosis. Moreover, studies of intracellular cytokine expression in T cell clones from bronchoalveolar lavage fluid (BALF) strongly support the notion that sarcoidosis is a Th1-mediated disease.

However, the Th1/Th2 profiles associated with sarcoidosis remain controversial. For example, Imai et al. reported that the percentage of IFN-γ and IL-4 producing CD4+ T cells obtained from the peripheral blood do not significantly differ between patients with sarcoidosis and healthy volunteers. Whereas, Wahlström et al. reported that these percentages are higher in the peripheral blood from patients with sarcoidosis compared to healthy volunteers. The elevated circulating IFN-γ, IL-12p40 subunit and IL-18 levels have been observed by several groups. In addition, an increased expression of IL-13mRNA in the peripheral mononuclear cells (PBMC) of patients with sarcoidosis has also been reported.

Chemokines are critical elements for the selective recruitment and activation of various leukocyte subsets in the inflammatory process. IFN-inducible protein 10 (IP-10/CXCL10) is a specific ligand for the CXC chemokine receptor (CXCR3), which is preferentially expressed on Th1 cells. IP-10 expression is strongly induced by IFN-γ. At the sites of sarcoaid granuloma, epithelioid cells and CD68+ macrophages express IP-10, which contributes to the granuloma formation by means of regulating the migration and activation of Th1 cells toward the site of these lesions. In contrast, thymus- and activation-regulated chemokine (TARC) functions as a selective chemoattractant for CC chemokine receptor (CCR) 4 bearing Th2 cells. TARC is therefore involved in Th2-dominant pulmonary disorders, such as bronchial asthma and eosinophilic pneumonia. The measurement of both Th1 chemoattractant IP-10 and Th2 chemoattractant TARC is valuable for further understanding of the peripheral Th1/Th2 balance in sarcoidosis.

This study investigates the IP-10 and TARC concentrations in the BALF and serum from patients with sarcoidosis and healthy volunteers. These data were correlated with clinical or laboratory findings. In addition, the source of the circulating IP-10 and TARC was determined.

Materials and methods

Study population

The study group included 52 patients with sarcoidosis. The sarcoidosis was diagnosed according to the criteria defined in the International Statement on Sarcoidosis based on the typical clinical features, as well as the histologic observation of non-caseating granulomas of in lung, muscle or lymph node biopsies, and the exclusion of other diseases with similar histologic or clinical characteristics. All of the samples from the patients with sarcoidosis were obtained at the time of diagnosis and none of the patients were being treated with corticosteroids or other immunomodulatory drugs at the time of sample retrieval. The control subjects consisted of 21 healthy sex- and age-matched volunteers. None of the healthy volunteers had a history of respiratory disease. In addition, none of study subjects had a history of allergic diseases.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously described after obtaining informed consent. The BAL procedure in healthy volunteers was approved by the ethics committee of Oita University Faculty of Medicine. Bronchoalveolar lavage was performed by injecting 150 ml of saline into the middle lobe or left lingula. The cells were counted in a hemocytometer. A 100-μl aliquot of cells (1×10^6/ml) was cytocentrifuged (Cytospin, Shandon Southern, Runcorn, UK) and air dried to obtain differential cell counts after staining with May-Grünwald-Giemsa staining.

Flow cytometry

After lysis of the red blood cells, peripheral blood leukocytes were incubated for 20 min at 4°C in the dark with the following directly conjugated anti-cell-surface marker and anti-chemokine receptor antibodies: fluorescein isothiocyanate isothiocyanate-conjugated anti-CD4 monoclonal antibody (mAb) (eBiosciences Inc, San Diego, CA) and either phycoerythrin-conjugated anti-CXCR3mAb (R & D Systems Inc., Minneapolis, MN) or phycoerythrin-conjugated anti-CCR4mAb (Becton Dickinson, Mountain View, CA). The cells were washed twice, fixed in 0.2% paraformaldehyde and analyzed in a flowcytometer (FACScalibur, Becton Dickinson) within 24 h. The lymphocytes were easily distinguished based on the forward and side scatters, and were isolated by gating. The frequency of CXCR3 or CCR4+ cells was assessed by gating. The frequency of CXCR3 or CCR4+ cells was assessed by gating. The frequency of CXCR3 or CCR4+ cells was assessed by gating.
the chemokine receptors and cell-surface markers were used for setting positive thresholds. To determine the phenotype of the lymphocytes in BALF, flow cytometry was performed using the direct immunofluorescence method with fluorescein isothiocyanate-conjugated anti-CD2, CD4, and CD8 mAbs. The cell-free supernatants were stored at −80 °C until further analysis.

**Cell culture of peripheral blood mononuclear cells and adherence-purified BALF cells**

PBMC were isolated from the blood using Ficoll-Hypaque (Pharmacia-Biotech, Uppsala, Sweden) gradient centrifugation. The cells were washed 3 times with sterile saline and suspended in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin G, 100 g/ml streptomycin, and 10% fetal calf serum, and incubated in cell culture dishes for 30 min at 37 °C in a 5% CO₂ incubator. The final PBMC concentration was adjusted to 1 × 10⁶ cells/ml and the cell cultures were returned to the incubator. After 48 h, the culture supernatants were collected and stored at −80 °C until further analysis. Adherence-purified BALF cells were cultured as previously described. BALF cells collected by centrifugation were suspended in the above medium and incubated in cell culture dishes for 30 min at 37 °C in a 5% CO₂ incubator. The dishes were washed 3 times with sterile saline to remove any non-adherent cells and the remaining adherent cells were collected. The final concentration of adherence-purified viable BALF cells was adjusted to 0.5 × 10⁵ cells/ml. They were cultured in the above medium for 48 h at 37 °C in a 5% CO₂ incubator.

**Measurement of IP-10 and TARC concentrations**

The chemokine concentration was measured using a commercially available enzyme-linked immunosorbent assay kit according to the manufacturer’s protocol (R & D Systems, Abington, UK). The coefficients of variation for the chemokines assays were within 10%. The minimum detectable levels of IP-10 and TARC were 1.67 and 7 pg/ml, respectively.

**Statistical analysis**

The results were expressed as the median and interquartile range. A statistical analysis was performed using either the Mann–Whitney U-test or the paired Student’s t-test. Any correlations between the 2 groups were examined using Spearman’s rank correlation test.

**Results**

**Patients characteristics**

For the chemokine assays, 52 patients with sarcoidosis were examined (34 women and 18 men, 21–80 years, median 53 years). The control subjects were 21 healthy sex- and age-matched volunteers (12 women and 9 men, 26–87 years, median age 44 years). The characteristics of the patients and healthy volunteers are summarized in Table 1. There was no difference in smoking status between the sarcoidosis and control groups. The patients were divided into groups based on the chest radiographic stages (stage 0: n = 4, stage I: n = 28, stage II: n = 9, stage III: n = 8, stage IV: n = 3).

<table>
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<th>Characteristics</th>
<th>Sarcoidosis</th>
<th>Healthy volunteers</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>Number</td>
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<td>21</td>
<td></td>
</tr>
<tr>
<td>Sex, Female/male</td>
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<td>12/9</td>
<td>NS</td>
</tr>
<tr>
<td>Age (yr), median (min, max)</td>
<td>53 (21, 80)</td>
<td>44 (26, 87)</td>
<td>NS</td>
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<td>7/1/13</td>
<td>NS</td>
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<tr>
<td>ACE (U/ml), median (min, max)</td>
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<tr>
<td>Lysozyme (ng/ml), median (min, max)</td>
<td>12.2 (5.8, 46.5)</td>
<td>ND</td>
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<td></td>
<td></td>
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<tr>
<td>BALF findings</td>
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<td>7/1/6</td>
<td>NS</td>
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<tr>
<td>Number</td>
<td>13/4/29</td>
<td>1.4 (0.8, 3.9)</td>
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<td>TCC (10⁵/ml), median (min, max)</td>
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<td>96.0 (82.9, 98.0)</td>
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<td>AM (%), median (min, max)</td>
<td>76.2 (31.0, 96.8)</td>
<td>2.3 (1.3, 9.5)</td>
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<td>Lym (%), median (min, max)</td>
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<td>1.0 (0.0, 2.8)</td>
<td>NS</td>
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<td>Neut (%), median (min, max)</td>
<td>0.5 (0.0, 60.0)</td>
<td>0.3 (0.0, 4.7)</td>
<td>NS</td>
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<td>Eo (%), median (min, max)</td>
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<td>1.7 (0.2, 4.7)</td>
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<td>CD4/CD8, median (min, max)</td>
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<td>1.2 (0.2, 4.7)</td>
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</table>

Definition of abbreviations: NS = not significant, yr = years, min = minimum, max = maximum, cur = current smoker, ex = ex-smoker, non = non-smoker, ACE = angiotensin converting enzyme, ND = not done, BALF = bronchoalveolar lavage, TCC = total cell concentration, AM = alveolar macrophage, Lym = lymphocyte, Neut = neutrophil, Eo = eosinophil. Comparisons between the two groups were assessed using the Student’s t-test.
BALF analysis showed that the patients with sarcoidosis had significant higher cell concentrations, lymphocyte percentage and CD4/CD8 ratios than did the healthy volunteers. The median value of angiotensin-converting enzyme (ACE) and lysozyme in patients with sarcoidosis was 16.6 U/ml (range, 3.6–54.2 U/ml) and 12.2 mg/ml (range, 5.8–46.5 mg/ml), respectively.

**IP-10 and TARC concentrations in the serum and BALF in patients with sarcoidosis**

IP-10 and TARC concentrations were measured for 52 patients with sarcoidosis and 21 healthy volunteers (Figure 1). The median concentration of IP-10 in the serum from patients with sarcoidosis was 389 pg/ml (range, 74–1362 pg/ml), which was significantly higher than that from healthy volunteers (median, 82 pg/ml, range, 36–190; \( p < 0.0001 \); Figure 1A). The TARC concentrations in patients with sarcoidosis (median, 972 pg/ml, range, 59–18228 pg/ml) were also significantly higher than those in healthy volunteers (median, 171 pg/ml, range, 93–503 pg/ml; \( p < 0.0001 \); Figure 1B). There was a significant correlation between the serum levels of IP-10 and TARC among patients with sarcoidosis (\( r = 0.573, p < 0.0001 \); Figure 1C). However, the serum IP-10/TARC ratio between the 2 groups was not significantly different (sarcoidosis; median, 0.31, range 0.04–3.42, healthy volunteers; median, 0.44, range, 0.11–1.19 pg/ml; Figure 1D).

Figure 1  (A) and (B) Concentrations of IP-10 and TARC in serum obtained from healthy volunteers (HV; \( n = 21 \)) and patients with sarcoidosis (SAR; \( n = 52 \)). The Y-axis shows the log of the serum IP-10 (A) and TARC (B) concentrations. The short horizontal line represents the median value. (C) Correlation between serum IP-10 and TARC concentrations in patients with sarcoidosis. (D) IP-10/TARC ratio in the serum from patients with sarcoidosis and healthy volunteers. The Y-axis shows the log of the serum IP-10/TARC ratio. The short horizontal line represents the median value. (E) and (F) Concentrations of IP-10 and TARC in the BALF obtained from healthy volunteers (HV; \( n = 14 \)) and patients with sarcoidosis (SAR; \( n = 46 \)). The Y-axis shows the log of the BALF IP-10 (E) and TARC (F) concentrations. The short horizontal line represents the median value. Paired comparisons were evaluated using Mann–Whitney \( U \)-test. NS; not significant.
Both the IP-10 and TARC levels were also measured in the BALF of 46 of the 52 patients with sarcoidosis. The BALF from 14 healthy volunteers were used controls. Figure 1E demonstrates that IP-10 was detected in 45 patients with sarcoidosis and 11 controls. The concentrations of IP-10 in the BALF from patients with sarcoidosis (median, 70 pg/ml, range 0–300 pg/ml; \( p < 0.0001 \)) were significantly higher than those from the healthy volunteers (median, 15 pg/ml, range 0–35 pg/ml; \( p < 0.0001 \)). When the sarcoidosis patients were compared, the IP-10 levels in circulation were not significantly correlated with those in the BALF (\( r = 0.261, \ p = 0.0794 \)). Detectable levels of TARC were found in the BALF of only 5 patients with sarcoidosis and 2 healthy volunteers (Figure 1F)

CXCR3 and CCR4 expression on peripheral blood CD4+ T cells

To determine Th1/Th2 polarization in peripheral blood in sarcoidosis, 2-color flow cytometry was performed for 10 patients with sarcoidosis (6 women and 4 men, 33–68 years, median 55 years, current smoker/ex-smoker/non-smoker = 3/2/5) and 10 healthy volunteers (6 women and 4 men, 26–70 years, median age 51 years, current smoker/ex-smoker/non-smoker = 3/1/6) who gave informed consent (Figure 2). There was no difference in age, sex, and smoking status between the sarcoidosis and control groups. Based on the chest X-ray findings, sarcoidosis patients were divided into stage 0 (\( n = 2 \)), stage I (\( n = 5 \)), stage II (\( n = 3 \)). Seven patients had extra pulmonary disease. Data are presented as the positive ratio of each CCR, Th1-associated CXCR3 or Th2-associated CCR4, in CD4+ T cells. Patients with sarcoidosis had significantly higher percentages of CXCR3+ CD4+ lymphocytes (median, 3.3%, range, 2.0–37.8%) than did the healthy volunteers (median, 0.6%, range, 0.2–1.5%; \( p = 0.0002 \)). The percentages of CCR4-positive cells among CD4+ T cells were significantly higher in the peripheral blood lymphocytes (PBL) from patients with sarcoidosis (median, 3.3%, range 1.0%–37.6%) than that in PBL from healthy volunteers (median, 1.2%, range, 0.6–3.2%; \( p = 0.0209 \)). In addition, the CXCR3+/CCR4+ ratios among CD4+ T cells were higher in PBL from the patients with sarcoidosis (median, 1.0%, range, 0.7–2.0%) than those in the PBL from healthy volunteers (median, 0.5%, range, 0.1–1.3%; \( p = 0.0121 \)).

Production of IP-10 and TARC by adherence-purified BALF cells and PBMC from patients with sarcoidosis and healthy volunteers

IP-10 and TARC productions were examined by measuring the concentrations in the culture supernatant of adherence-purified BALF cells and PBMC from 10 patients with sarcoidosis (4 women and 6 men, 24–80 years, median 43 years, current smoker/ex-smoker/non-smoker = 6/1/3) and 10 healthy volunteers (4 women and 6 men, 26–81 years).
years, median 43 years, current smoker/ex-smoker/non-smoker = 4/1/5). There was no difference in age, sex, and smoking status between the sarcoidosis and control groups. Based on the chest X-ray findings, sarcoidosis patients were divided into stage 0 (n = 2), stage I (n = 3), stage II (n = 2), and stage III (n = 3). Eight patients had extra pulmonary disease. The values of chemokine production per cells (pg/10⁶ cells) are shown in Figure 3. IP-10 was detected in the culture supernatant of adherence-purified BALF cells in 9 of 10 patients with sarcoidosis, whereas, it was undetectable in all control subjects. The IP-10 production by adherence-purified BALF cells was significantly higher in the patients with sarcoidosis (median, 100 pg/ml; range, 0.0–520 pg/ml) than in the healthy volunteers (median, 0.0 pg/ml; range, 0.0–0.0 pg/ml; Figure 3A). The sarcoidosis group tended to have lower value of IP-10 in the culture supernatant of PBMC compared with the control group, although there was no statistical difference between the 2 groups (sarcoidosis: median, 43 pg/ml; range, 5–279 pg/ml, healthy volunteer: median, 67 pg/ml; range, 38–1593 pg/ml; Figure 3B).

No difference was observed in TARC production by adherence-purified BALF cells between patients with sarcoidosis (median, 100 pg/ml; range, 0.0–520 pg/ml) and in the healthy volunteers (median, 0.0 pg/ml; range, 0.0–0.0 pg/ml; Figure 3C). In contrast, the TARC production by PBMC from patients with sarcoidosis (median, 34 pg/ml; range, 0.0–150 pg/ml) was significantly higher than that from healthy volunteers (median, 0 pg/ml; range, 0.0–62 pg/ml; Figure 3D).

Discussion

This study demonstrated that circulating levels of the Th2-chemoattractant TARC as well as the Th1-chemoattractant IP-10 were significantly elevated in patients with sarcoidosis. It is important to note that, compared with the healthy controls, IP-10 was predominantly generated by the BALF cells but not by PBMC, whereas an increased TARC production was found in the PBMC but not in BALF cells in the patients with sarcoidosis. Previously, Th2-associated chemokine production had not been reported in the PBMC of the sarcoidosis patients. BALF IP-10 levels in these patients were also considerably higher than those in control subjects. Similarly, increased levels of IP-10 were generated by BALF cells from patients with sarcoidosis than in control subjects. These results were consistent with those of Agostini et al., which documented an enhanced expression of IP-10 in sarcoid tissues and alveolar macrophages and very high levels of IP-10 in the BALF of 24 patients with active pulmonary sarcoidosis, compared with patients with inactive disease or control subjects. In addition, they demonstrated a positive relationship between the BALF IP-10 levels and the degree of infiltrated CD4+ T cells expressing Th1 cytokines (IL-2 and IFN-γ) and high levels of CXCR3. Elevated levels of IP-10 in the BALF of patients with sarcoidosis were also reported by several groups. IP-10 appears to be more important in Th1-type immune responses because its expression is stimulated by IFN-γ and inhibited by IL-4. Increased IP-10 expression in the inflamed lung tissue associated with
Elevated serum TARC and IP-10 in sarcoidosis

Sarcoidosis suggests its pivotal role in attracting T cells to sites of ongoing inflammation. The present study was the first to observe that level of IP-10 produced by PBMC from sarcoidosis patients was comparable to that from healthy volunteers. The correlation between IP-10 levels of serum and BALF was not significant and circulating levels of IP-10 were not associated with the x-ray-based stage of sarcoidosis, indicating that elevated levels of circulating IP-10 are derived from not only the lungs but also extra-pulmonary organs. In contrast to BALF IP-10, which is a marker of T cell alveolitis, serum IP-10 probably reflects systemic disease activity.

In this study, serum levels of the Th2-associated chemokine TARC was significantly elevated in patients with sarcoidosis compared with healthy volunteers. TARC is important in initiating a Th2 response characterized by T cell infiltration. Several studies have documented the involvement of TARC in Th2-associated disorders, such as bronchial asthma, eosinophilic pneumonia and atopic dermatitis. The expression of TARC in Th1-mediated diseases, however, has not been well documented. Moreover, the roles of this chemokine in the pathogenesis of sarcoidosis have not been studied. The increased circulating TARC levels found in this study may support the previous reports demonstrating increased numbers of CD4+ T cells expressing the TARC receptor CCR4 and the elevated IL-13 levels in serum from sarcoidosis patients. The Serum TARC has recently been associated with disease activity in bronchial asthma, atopic dermatitis and eosinophilic pneumonia. Elevated levels of serum TARC have also been reported as a prognostic factor in Hodgkin's disease. However, in contrast to these disorders in which elevated expression of TARC is observed in the tissue of the affected organs, TARC levels in BALF as well as culture supernatants of BALF cells of patients with sarcoidosis were not elevated, despite the high level of TARC in the peripheral blood. The PBMC from patients with sarcoidosis secreted more TARC protein, without cytokine stimulation than that seen in healthy volunteers. Therefore, the elevated concentration of circulating TARC appears to be due to increased production by the PBMC, but not by the inflamed organs.

There is no definitive explanation for the presence of elevated TARC levels in the associated with sarcoidosis, which is a Th1-mediated disease. Generally, the Th1-polarized immune response plays an important role in the onset and development of the granulomas. And elevated TARC levels were not observed in the lungs, which is consistent with previous reports. This suggests that TARC is unlikely to locally contribute to T cell alveolitis or granuloma formation. It is possible that circulating TARC may play a role in compensation for the Th1-polarized response, at the peripheral blood level. This is supported by the observation that the serum TARC levels were correlated with the serum IP-10 levels in the same samples from sarcoidosis patients. Elevated TARC production by the PBMC may lead to normalization of the Th1/Th2 imbalance in circulation and thus contribute to the pathogenesis of sarcoidosis.

One other explanation for the elevated TARC in peripheral blood is that sarcoidosis leads to a general activation of T cells, similar to its effect on B cells. This general activation is non-specific and therefore both Th-1 and Th-2 chemokines are increased. It is only in localized areas that the differentiation occurs.

Recently, Katchar et al. investigated the distribution of CXCR3- or CCR4-bearing CD4+ cells in the BALF and peripheral blood of sarcoidosis patients. They showed that CXCR3 was expressed on the majority of the BALF CD4+ T cells whereas one-third population of PBL CD4+ T cells expressed CXCR3. However, there were significantly lower numbers of BALF CD4+ T cells that expressed CCR4 compared with PBL CD4+ T cells. It is possible that Th2-cell predominance exists at the PBL level in sarcoidosis. The present study compared the proportion of CCR4+ cells as well as CXCR3+ cells in the CD4+ T cell population in peripheral blood, demonstrating higher proportion of both cell types in patients with sarcoidosis than in healthy volunteers. As a result, the CXCR3/CCR4 ratio in the CD4+ cells of sarcoidosis patients was slightly higher than that in the controls, indicating the presence of Th1-predominance in circulation. This might result from the contribution of other CXCR3 ligands, such as monokines induced by IFN-γ/CXCL9 and IFN-γ inducible T cell α-chemoattractants/CXCL11.

Although Th1- and Th2-directed chemokines are involved in the pathogenesis of the specific disorders, Th1/Th2 chemokine profiles in serum and tissue samples are more complicated. For example, IP-10 as well as TARC is highly expressed in bronchial biopsy specimens in bronchial asthma and both IP-10 and TARC levels are elevated in the circulation of patients with systemic sclerosis, though, both are Th2-predominant diseases. In sarcoidosis, elevated levels of both IP-10 and TARC in association with elevated numbers of Th1 and Th2 cells in the peripheral blood also seem to reflect the systemic nature of this disease.

In conclusion, patients with sarcoidosis had extremely high concentrations of TARC as well as IP-10 in their serum. This study suggests that IP-10 is mainly produced at the lung and TARC is produced in the peripheral circulation in sarcoidosis. Both IP-10 and TARC generated at these different sites cooperatively play a role in the pathogenesis of sarcoidosis. Further studies to elucidate the regulatory mechanisms of Th1/Th2 balance in association with chemokine expression in the peripheral blood circulation are thus called for.

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Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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