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INFLAMMATORY MARKERS IN ACTIVE PULMONARY TUBERCULOSIS: ASSOCIATION WITH Th1/Th2 AND Tc1/Tc2 BALANCE

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Abstract [Objective] Few inflammatory markers closely reflect the activity of tuberculosis and only a few surrogate markers are available. The purpose of this study was to clarify the usefulness of measuring the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and serum amyloid A (SAA), and the association between these markers and T-cell profiles. [Methods] One hundred one patients with active pulmonary tuberculosis were enrolled in this study. The associations between ESR, CRP, and SAA values on admission and microbiological and radiological findings and T-cell profiles were assessed. Th1/Th2 and Tc1/Tc2 were determined by analyzing intracellular cytokine staining for IFN-γ and IL-4 in blood CD4$^+$ and CD8$^+$ T cells using flow cytometry after stimulation with PMA and ionomycin. [Results] There were significant correlations between ESR, CRP, and SAA, of which the correlation between CRP and SAA was strong (r＝0.88). CRP values significantly correlated with the sputum smear scale and the extent of lesions, and were higher in bilateral lesions. SAA values correlated with the sputum smear scale, whereas all markers were higher in patients with pleural effusion. Both CRP and SAA levels negatively correlated with the ratio of Th1/Th2. In contrast, ESR negatively correlated with the ratio of Tc1/Tc2. [Conclusion] CRP reflected the disease severity before treatment. CRP and SAA values were associated with helper T-cell proportions whereas ESR was associated with cytotoxic T-cell proportions, both being type 2 predominant.

Key words: Erythrocyte sedimentation rate, C-reactive protein, Serum amyloid A, T-cell profile

Introduction

Few serological markers closely reflect the activity of tuberculosis and only a few surrogate markers are available for monitoring anti-tuberculosis therapy. The erythrocyte sedimentation rate (ESR) has been empirically a classic laboratory index for diagnosis and follow-up of patients with tuberculosis. It particularly reflects changes in the concentration of fibrinogen, which increases 24 to 48 hours after infection[1]. C-reactive protein (CRP) levels are a rapid indicator of inflammation and tissue necrosis. It is an acute phase protein produced by the liver, and promotes phagocytosis in response to bacterial infection. The increased synthesis of acute phase proteins is mediated by IL-6, and CRP levels rise within 6 to 8 hours after events. Serum CRP levels are increased in tuberculosis, particularly in patients with advanced disease, and they decline with therapy[2]~[4]. Serum amyloid A (SAA) protein exists in its native state in the plasma as a high molecular weight form mainly associated with high density lipoprotein and behaving as an apolipoprotein. Compared to CRP, SAA has an even wider dynamic range. Very few studies have examined SAA as an inflammatory marker of pulmonary tuberculosis[5]. Thus, ESR, CRP, and SAA have been used as inflammatory markers in tuberculosis; however, their usefulness in the management of the disease has not been established.

The flow cytometric method to examine intracellular cytokine production at the single-cell level is sensitive and enables the easy analysis of T-cell profiles. In general, tuberculosis is classified as a granulomatous Th1 (IFN-γ-producing CD4$^+$ T cells) inflammatory condition, but not a Th2 (IL-4-producing CD4$^+$ T cells) condition, such as asthma[6]. The association between Tc1 (IFN-γ-producing CD8$^+$ T cells)/Tc2 (IL-4-producing CD8$^+$ T cells) as well as the Th1/Th2 balance and inflammatory markers in tuberculosis has not been studied.

The purpose of this study was to clarify the usefulness of measuring ESR, CRP, and SAA, and the association between these markers and T-cell profiles in active pulmonary tuberculosis.

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Methods

Subjects

One hundred one patients with active pulmonary tuberculosis who were admitted to Shizuoka General Hospital between April 2005 and March 2006 were enrolled in this study. Acid-fast smears of sputum were positive, which was the criterion for admission, and subsequently *Mycobacterium tuberculosis* was isolated by culture in all patients. Patients with miliary tuberculosis, extrapulmonary tuberculosis, other pulmonary diseases, collagen vascular diseases, malignant tumors, diabetes mellitus, hepatic cirrhosis, chronic renal failure with and without hemodialysis, or other apparent infections with bacteria, hepatitis viruses, or human immunodeficiency virus were excluded from the study. We also excluded patients who had received anti-tuberculosis drugs prior to admission, corticosteroids, or other immunomodulators. After admission, all patients were treated with a 3 or 4 anti-tuberculosis drug regimen of rifampicin, isoniazid, and ethambutol with or without pyrazinamide. Microbiological examinations were performed every other week and if sputum smears were negative for 3 successive examinations, discharge was considered. The protocols were approved by the local ethics committee and informed consent was obtained from all patients prior to the study.

Study design

Clinical data were obtained from medical records. Sputum smears were obtained before treatment and classified according to the quantitation scale: ±, 1−2 bacilli/30 fields; 1＋, 1−9 bacilli/10 fields; 2＋, 1−9 bacilli/field; 3＋, 10−90 bacilli/field; and 4＋, ＞90 bacilli/field[7]. Radiological findings were assessed by the site and extent of lesions with or without cavitation and pleural effusion. The extent of lesions was classified into 3 categories: 1, extension within one-third of the unilateral lung; 2, within the unilateral lung field; and 3, beyond the unilateral lung field. Inflammatory markers, including ESR, CRP and SAA, were measured at the time of admission and discharge. ESR was measured using the Westergren tube method and the upper normal limit was 19 mm/hr[8]. CRP and SAA were measured quantitatively by latex agglutination turbidimetric immunoassay[9] and the upper normal limits were 0.3 mg/d*l* and 8 μg/m*l*, respectively.

Determination of peripheral blood T-cell profiles

Heparinized blood was collected and diluted 1 : 20 in RPMI-1640. One milliliter of diluted blood was stimulated with 25 ng/m*l* PMA and 1 μg/m*l* ionomycin in the presence of 10 μg/m*l* brefeldin-A (Sigma, St Louis, MO, USA) for 4 hr at 37°C. Negative controls consisted of unstimulated cells. After stimulation with PMA/ionomycin, the cells were incubated with either peridin chlorophyll protein (PerCP)-conjugated anti-CD4 or anti-CD8 antibody (Becton Dickinson, San Jose, CA, USA) for 15 min at room temperature (RT). In the prepa-

ration of peripheral blood cells, erythrocytes were lysed by adding FACS Lysing Solution (Becton Dickinson). After centrifugation, the cells were washed with 0.1% BSA/PBS and subsequently incubated in FACS Permeabilizing Solution (Becton Dickinson) for 10 min at RT. After washing with 0.1% BSA/PBS, the permeabilized cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ and phycoerythrin (PE)-conjugated anti-IL-4 antibodies for 30 min at RT. After staining, they were washed with 0.1% BSA/PBS, fixed in 1% paraformaldehyde, and analyzed. Negative controls consisted of unstimulated cells. The samples were analyzed on a FACScan flow cytometer (Becton Dickinson). Lymphocytes were first gated, and a second gate was selected. For 3-color analysis, we measured the number of positive cells for anti-IFN-γ (FITC), anti-IL-4 (PE), or both antibodies simultaneously in gated cell populations that were stained with either anti-CD4 or anti-CD8 antibody (PerCP). The number of positive cells for each cytokine was expressed as a percentage of $CD4^+$ or $CD8^+$ T cells. In all experiments, parallel incubations were performed with FITC- or PE-conjugated irrelevant antibodies matched for the isotypes of anti-cytokine antibodies. The cutoff level for the definition of positive cells was thus set so that less than 1% of irrelevant antibody-stained cells were positive.

Statistical analysis

Categorical data were summarized as counts, and quantitative data were summarized as medians with ranges. The Kruskal-Wallis test was used as a global test of the statistical significance of differences among group medians and when significance was found, the Mann-Whitney U test was performed to test post-hoc hypotheses about specific group differences. The chi-square or Fisher's exact test was used to test significance group differences with respect to the percentage of patients in various categories. The Spearman rank correlation coefficient was used to assess pair-wise associations between quantitative outcome variables. Concerning the determination of T-cell profiles, no difference was found in quantitative data and categorical data between the 29 patients with complete data and the remaining 72 patients with incomplete data by the t-test and chi-square test, respectively. Stat View Version 5.0 (SAS Institute, Cary, NC, USA) was used for statistical calculations. A p value of ＜0.05 was considered significant, and all tests were 2 sided.

Results

The clinical characteristics of the study population on admission were shown in Table 1. The frequency distribution of ESR, CRP, and SAA in the 101 patients was shown in Fig. 1. We found that the ESR values were distributed extensively from low to high levels, whereas the peaks of CRP and SAA values were at a lower level. Concerning CRP values, more than 10 patients showed high values of greater than 10 mg/m*l*. As shown in Fig. 2A and 2B, there were significant correlations

Table 1   Characteristics of study patients on admission

Characteristics	Findings (n＝101)
Age, median (range), y	73 (19−95)
Male/female, No.	58/43
Microbiological findings	
Sputum smear scale*, ±/1＋/2＋/3＋/4＋, No.	24/16/19/37/5
Radiological findings	
Site of lesions, unilateral/bilateral, No.	34/67
Extent of lesions[†], 1/2/3, No.	20/67/14
Cavitation, − / ＋, No.	55/46
Pleural effusion, − / ＋, No.	83/18
Length of hospital stay, median (range), d	68 (24−200)
Inflammatory markers	
ESR, median (range), mm/hr	58 (4−112)
CRP, median (range), mg/dl	3.3 (0.3−16.8)
SAA, median (range), μg/ml	108 (8−1327)
T-cell profile[‡]	
Th1: IFN-γ-producing CD4$^+$ T cells, median (range),%	21.0 (7.9−69.2)
Th2: IL-4-producing CD4$^+$ T cells, median (range), %	2.6 (1.0−5.9)
Th1/Th2: IFN-γ-producing/IL-4-producing CD4$^+$ T cells, median (range)	7.2 (2.7−69.2)
Tc1: IFN-γ-producing CD8$^+$ T cells, median (range),%	72.5 (39.7−94.7)
Tc2: IL-4-producing CD8$^+$ T cells, median (range), %	1.5 (0.2−8.5)
Tc1/Tc2: IFN-γ-producing/IL-4-producing CD8$^+$ T cells, median (range)	47.9 (7.0−473.5)

Abbreviations:CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SAA, serum amyloid A
*±, 1−2 bacilli/30 fields;1＋, 1−9 bacilli/10 fields;2＋, 1−9 bacilli/field;3＋, 10−90 bacilli/field;
 4＋, >90 bacilli/field.
[†]1, extension within one-third of the unilateral lung;2, within the unilateral lung field;3, beyond the
 unilateral lung field.
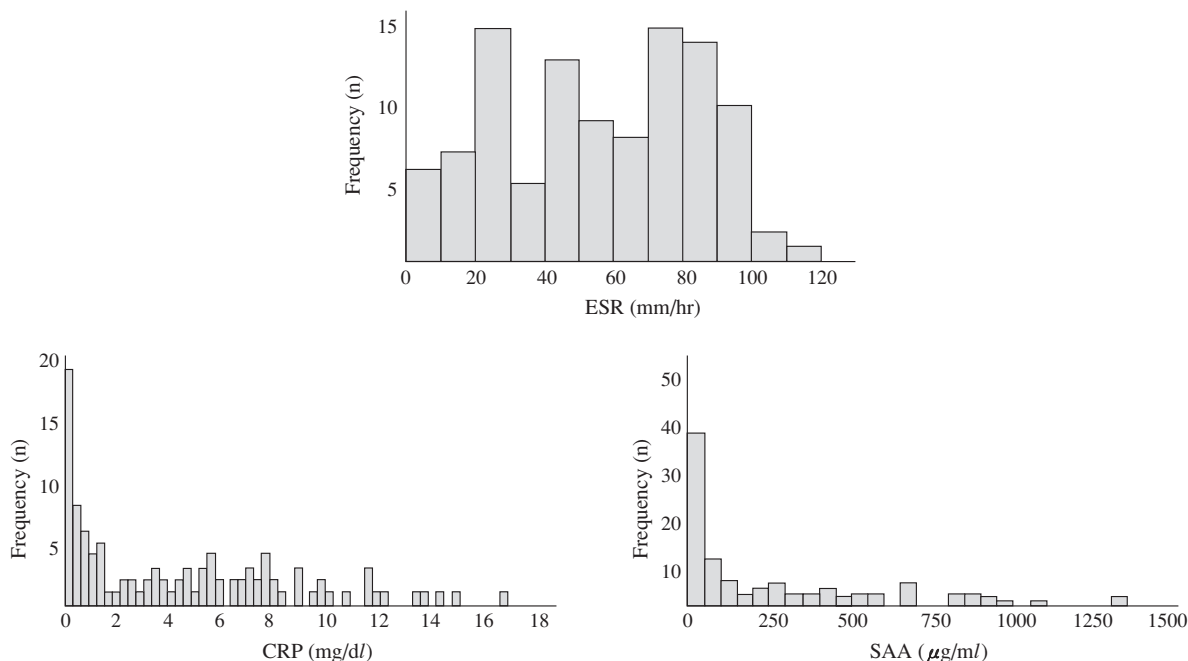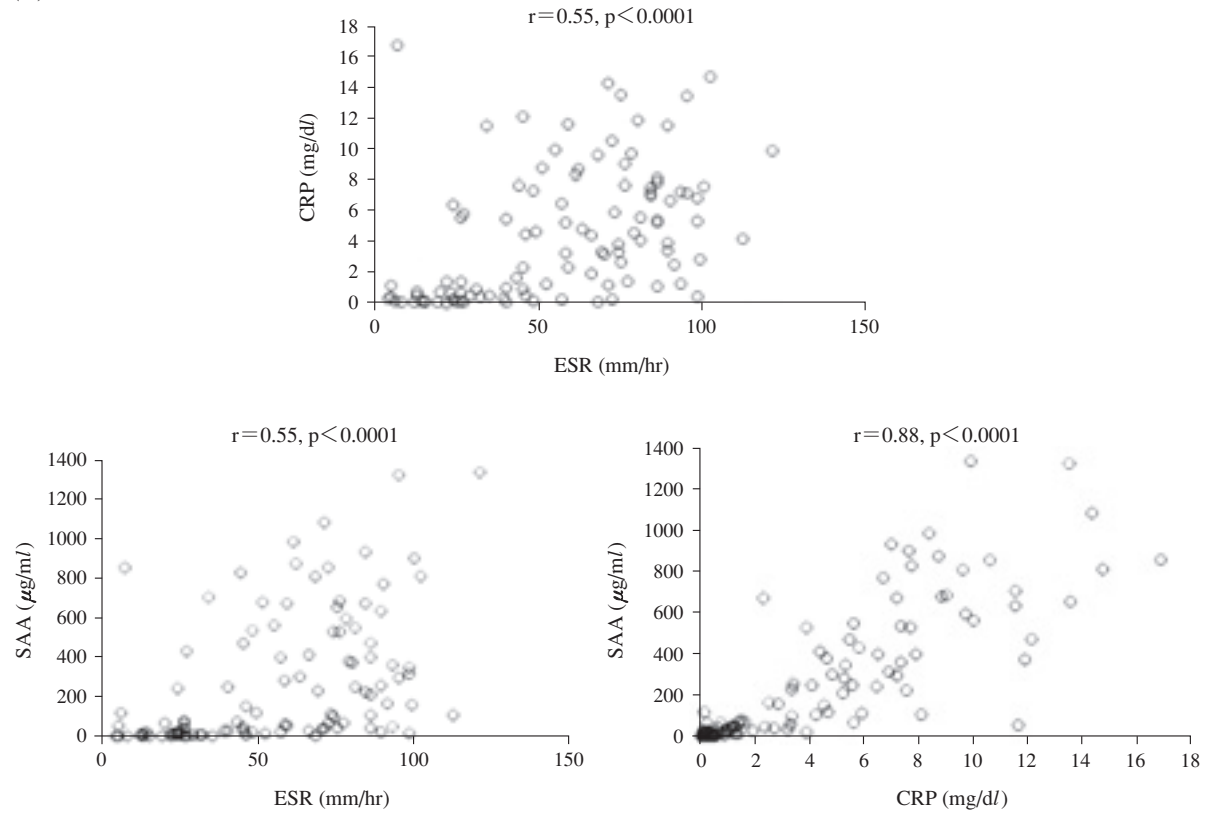[‡]T-cell profiles were analyzed in 29 patients.

Fig. 1   Frequency distribution of ESR, CRP, and SAA. Widths of each bar mean 10 mm/hr for ESR, 0.3 mg/ml for CRP, and 50 μg/ml for SAA, respectively.
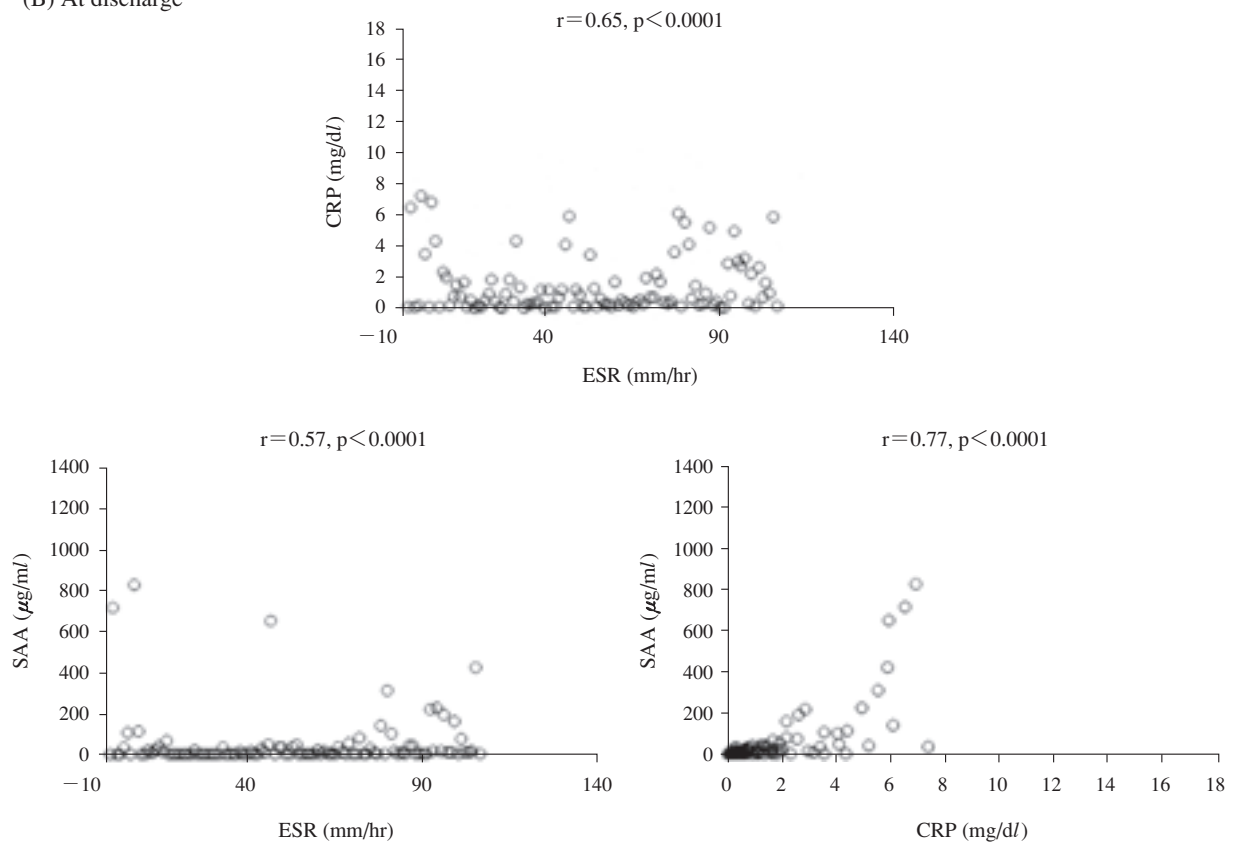
(A) On admission

(B) At discharge

Fig. 2　Correlation between ESR, CRP, and SAA on admission (A) and at discharge (B)

Table 2 Correlation between inflammatory markers and T-cell profiles

	ESR	CRP	SAA
Th1 cells	− 0.12	− 0.21	− 0.42 (P＝0.0339)
Th2 cells	− 0.03	0.46 (P＝0.0161)	0.36
Th1/Th2 ratio	− 0.11	− 0.52 (P＝0.0056)	− 0.60 (P＝0.0024)
Tc1 cells	− 0.56 (P＝0.0029)	− 0.25	− 0.35
Tc2 cells	0.33	0.17	0.11
Tc1/Tc2 ratio	− 0.37 (P＝0.0485)	− 0.17	− 0.12

Abbreviations: Tc1, IFN-γ-producing CD8$^+$ T; Tc2, IL-4-producing CD8$^+$ T;
Th1, IFN-γ-producing CD4$^+$ T; Th2, IL-4-producing CD4$^+$ T
Values are the correlation coefficient and statistical significance. If only the number is shown,
it is not significant.

between ESR, CRP, and SAA both on admission and at discharge. In particular, there was a strong correlation between CRP and SAA. The median ESR, CRP, and SAA values at discharge were significantly lower than those on admission: ESR, 58 mm/hr vs 27 mm/hr; CRP, 3.3 mg/dl vs 0.7 mg/dl; and SAA, 108 μg/ml vs 12 μg/ml, respectively. The percentages of each marker within normal ranges at the time of admission and discharge were as follows: ESR, 8.5％ vs 19.8％; CRP, 23.6％ vs 48.1％; and SAA, 10.4％ vs 46.2％, respectively.

The sputum smear scale positively correlated with CRP (r＝0.43, P＜0.0001) and SAA (r＝0.48, P＜0.0001), but not ESR. Concerning radiological findings, CRP values were significantly higher in bilateral lesions than in unilateral lesions (median, 4.6 versus 1.2 mg/dl, P＝0.0161), but ESR and SAA were not. There was a significant correlation between the extent of lesions and CRP (r＝0.42, P＜0.0001), but not ESR and SAA. ESR, CRP, and SAA values were higher in patients with pleural effusion than in those without pleural effusion (76 vs 51 mm/hr; 7.4 vs 2.3 mg/dl; and 476 vs 70 μg/ml, respectively, P＜0.05), whereas there was no difference in each inflammatory marker concerning the presence of cavitation.

The correlation between inflammatory markers and T-cell profiles was shown in Table 2. The proportion of Th1 cells was negatively correlated with SAA whereas the proportion of Th2 cells was positively correlated with CRP. The ratio of Th1/Th2 was negatively correlated with both CRP and SAA. In contrast, the proportion of Tc1 cells was negatively correlated with ESR and the ratio of Tc1/Tc2 was negatively correlated with ESR.

Discussion

In this study, we found significant correlations among ESR, CRP, and SAA, of which the correlation between CRP and SAA was strong. CRP values significantly correlated with the sputum smear scale and the extent of lesions, and were higher in bilateral lesions. SAA values correlated with the sputum smear scale, whereas all markers were higher in patients with pleural effusion. Both CRP and SAA levels negatively correlated with the ratio of Th1/Th2. In contrast, ESR negatively correlated with the ratio of Tc1/Tc2.

The CRP level (median, 3.3 mg/dl) in this study was comparable with those reported previously[2)10)]. Choi et al. found that CRP levels were lower in 46 patients with tuberculosis than in 67 patients with non-tuberculosis pneumonia (3.2 vs 8.3 mg/dl, respectively)[2)]. Kang et al. reported that the median CRP concentration was 5.27 mg/dl in patients with pulmonary tuberculosis and 14.58 mg/dl in those with bacterial community-acquired pneumonia[10)]. These authors concluded that CRP was useful in the differential diagnosis of pulmonary tuberculosis and pneumonia. Since we had excluded cases of secondary bacterial infections, it was thought that the CRP values in this study represented the inflammatory levels of tuberculosis itself. Meanwhile, high values of CRP greater than 10 mg/ml were found in more than 10 patients in this study. Yanagisawa et al. reported that 42 of 326 patients (13％) with active pulmonary tuberculosis showed high values of CRP greater than 10 mg/ml[11)]. These findings indicated that high values of CRP could occur due to the inflammation of tuberculosis itself. We should keep this in mind in the differential diagnosis of tuberculosis and bacterial infections.

The association between CRP and the sputum smear scale, and the site and extent of lesions suggested that CRP might reflect the severity of the disease before treatment. Previous studies reported similar results. In a study by Pakasi et al., CRP values were significantly higher in severe tuberculosis than in mild tuberculosis[4)]. Djoba Siawaya et al. found that high levels of CRP at diagnosis were associated with the presence of multiple cavities on chest X-rays[3)]. Concerning the presence of cavitation, however, there was no difference in inflammatory markers in this study. Possible explanations for this difference included the small number of patients in their study (n＝20) and the diversity of the studied populations.

In a study reported by Choi et al.[2)], there was no difference in ESR levels between tuberculosis and non-tuberculosis pneumonia (21.5 vs 25.0 mm/hr, respectively), which was lower than in this study (median, 58 mm/hr). Yanagisawa et al. studied the ERS and related factors in 326 patients with pulmonary tuberculosis[11)] and found a significant correlation between ESR and sputum smear scale, the extent of lesions, and CRP. The correlation between ESR and CRP was consistent with this study but otherwise inconsistent. They concluded that ESR together with CRP should be evaluated since CRP was within the normal range in 23％ of the patients. The false

negative results of each marker on admission in this study also suggested the need for combined evaluation of inflammatory markers, including ESR, CRP, and SAA.

De Beer et al. measured CRP and SAA levels in 54 patients with pulmonary tuberculosis and found high levels of SAA as well as CRP and a subsequent decline after treatment[5]; however, they concluded that the rise in each marker was likely to be due to secondary bacterial infection in addition to tuberculosis. In this study, we found a strong correlation between CRP and SAA, similar data distribution, and the superiority of CRP in assessing the severity of disease, thereby suggesting that SAA may be omitted from the inflammatory markers in active pulmonary tuberculosis.

In this study, we found negative correlations between CRP and SAA values and Th1/Th2 ratios and between ESR values and Tc1/Tc2 ratios for the first time. These results suggested several important points. First, inflammatory parameters might reflect peripheral blood T-cell profiles. T cells played a pivotal role in the immune response to *Mycobacterium tuberculosis* infection and the flow cytometric method to examine intracellular cytokine production at the single-cell level was sensitive and enabled the easy analysis of T-cell profiles. In general, ESR, CRP, and SAA indicated inflammatory changes in the whole body induced by tuberculous infection. As shown in this study, inflammatory parameters reflected peripheral blood T-cell profiles, at least partly, before treatment with anti-tuberculosis drugs. Secondly, there were differences in the association with T-cell profiles as well as in the data distribution pattern between ESR, and CRP and SAA. We found that the ESR values were distributed extensively from low to high levels, whereas the peaks of CRP and SAA values were at a lower level. Also, it was unlikely that ESR, not CRP and SAA, reflected the severity of tuberculosis in this study. Considering the correlation coefficients of each paired parameter and the strong correlation between CRP and SAA, ESR seemed different from the other two parameters. In addition to these, ESR negatively correlated with Tc, cytotoxic $CD8^+$ T cells, whereas CRP and SAA correlated with Th, helper $CD4^+$ T cells. Tsao et al. reported that patients with more severe pulmonary tuberculosis had higher percentages of $CD4^+$ lymphocytes and increased $CD4^+/CD8^+$ ratios in bronchoalveolar lavage fluid but lower percentages of $CD4^+$ lymphocytes in peripheral blood[12]. The peripheral blood $CD4^+/CD8^+$ ratio was decreased also because of increased $CD8^+$ lymphocytes. Thus, measuring ESR, CRP and SAA might be useful to evaluate the $CD4^+/CD8^+$ balance in the peripheral blood, but more detailed studies would be needed in this regard. Thirdly, both CRP and SAA, and ESR negatively correlated with the ratios of type 1 to type 2 helper or cytotoxic T cells; that is, the greater each inflammatory parameter was, the more prominent the imbalance toward a type 2 phenotype became. In particular, the high values of CRP and SAA corresponded to more severe disease activity in this study. The polarization of host immune responses in tuberculosis was reported previously. Condos et

al. demonstrated that Th1-predominant host immune responses, as defined by marked alveolar lymphocytosis of IFN-γ-secreting cells detected by bronchoalveolar lavage from radiographically abnormal lung segments in patients with tuberculosis, was associated with milder clinical disease, as defined by lack of cavity formation and a low bacterial burden[13]. In contrast, patients with more clinically advanced disease, as defined by cavitary lesions and sputum smear positivity, did not have evidence of a Th1 response. This study suggested an association between the ability to mount a Th1-type response in the setting of active disease and a more benign clinical course. Other investigators also examined the peripheral blood Th1/Th2 and Tc1/Tc2 balance in active pulmonary tuberculosis[14]~[16]. Van Crevel et al. found an increase in the production of IL-4 in $CD4^+$ as well as $CD8^+$ T cells, especially in patients with cavitary tuberculosis[14]. The type 2 cell predominance demonstrated by van Crevel et al. was consistent with the results in this study. Thus, tuberculosis was generally classified into granulomatous Th1 inflammatory conditions; however, this study suggested that the balance between type 1 and type 2 cell predominance varied according to disease severity.

In conclusion, this study showed that CRP was superior to ESR and SAA in assessing the severity of disease prior to treatment. CRP and SAA were associated with peripheral blood helper T-cell proportions whereas ESR was associated with cytotoxic T-cell proportions, both being type 2 predominant.

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