

SERUM LEVELS OF SOLUBLE CD163 AS A USEFUL MARKER OF MACROPHAGE/MONOCYTE ACTIVITY IN SARCOIDOSIS PATIENTS

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ABSTRACT. *Background:* Monocyte-macrophage lineage cells are the main immunocompetent cells in sarcoidosis. The main cellular elements of sarcoidal granulomas are epithelioid cells and multinucleated giant cells (MGC). MGC are also produced *in vitro* by human blood monocytes following various stimuli. The *in vitro* formation of MGC is a useful tool for understanding granulomas. CD163, a scavenger receptor for the hemoglobin-haptoglobin complex, is expressed on monocytes/macrophages and shed into blood in a soluble form (sCD163) after stimulation from Toll-like receptors and oxidative stress. sCD163 serum levels have been reported to increase in inflammatory or infectious conditions. *Objective:* The aim of the present study was to examine the relationship between serum levels of sCD163 and the conventional disease markers of sarcoidosis, and also to evaluate sCD163 levels in culture supernatants following the formation of MGC by human peripheral monocytes *in vitro*. *Patients and methods:* Twenty sarcoidosis patients and twenty healthy subjects were enrolled in the study. sCD163 serum levels were evaluated using sCD163 ELISA. MGC were formed from peripheral blood monocytes by treatment with supernatant of concanavalin A-stimulated peripheral blood mononuclear cells, and sCD163 levels in the culture supernatants were measured by ELISA. *Results:* sCD163 serum levels were significantly higher in sarcoidosis patients than in healthy controls and correlated with ACE and soluble interleukin-2 receptor serum levels. sCD163 levels in culture supernatants increased with the production of MGC. *Conclusions:* sCD163 may be used as a favorable specific marker of macrophage/monocyte activity in order to more clearly understand the disease activity of sarcoidosis. (*Sarcoidosis Vasc Diffuse Lung Dis* 2015; 32: 99-105)

KEY WORDS: CD163, sarcoidosis, angiotensin converting enzyme, soluble IL-2 receptor, monocyte

INTRODUCTION

The hemoglobin-haptoglobin scavenger receptor, CD163, is a trans-membrane protein of the cysteine-rich super family (1). CD163 is expressed selectively on most macrophages in human tissues and also on at least 10-30% of monocytes (2). The ex-

pression of CD163 was previously shown to be increased by IL-6, IL-10, and glucocorticoids, and decreased by tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- β), and transforming growth factor beta (TGF- γ) (3-8). CD163 is shed by the Toll-like receptor and oxidative stress stimulation (9, 10). The soluble form of CD163 (sCD163) is known to inhibit the activation and proliferation of T-lymphocytes (11), which suggests that sCD163 may play a role in inflammatory disorders mediated by T-lymphocytes and monocyte-macrophage lineage cells. sCD163 has been clinically confirmed as a new macrophage-specific biomarker for cardiovascular disorders (12, 13), infections (14), and collagen

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diseases (15). Sarcoidosis is a systemic granulomatous disease characterized by non-caseating granulomas consisting of activated T-lymphocytes and epithelioid cells. In the present study, we measured serum levels of sCD163 in sarcoidosis patients and evaluated the relationship between these serum levels and the well-known disease markers of sarcoidosis, such as angiotensin-converting enzyme (ACE) and soluble interleukin-2 receptor (sIL-2R).

The main cellular elements of sarcoidal granulomas are epithelioid cells, Langhans type- and foreign body type-multinucleated giant cells (MGC). MGC are also produced *in vitro* by human blood monocytes following various stimuli (16). The *in vitro* MGC formation model is a useful tool to more clearly understand granulomas. We here also investigated sCD163 concentrations in culture supernatants during MGC formation from human peripheral blood monocytes.

MATERIALS AND METHODS

Patients

Twenty patients with sarcoidosis (17 women and 3 men; mean age 62.12 years) were enrolled in

this study (Table 1). All patients were examined in the Departments of Dermatology, Internal Medicine, and Ophthalmology in our university hospital. Skin lesions were examined histologically, and the presence of non-caseating granulomas composed principally of epithelioid cells with occasional MGC was confirmed. Each diagnosis of sarcoidosis was based on more than one clinical finding of sarcoidosis and more than one characteristic laboratory abnormality associated with sarcoidosis, including PPD reaction energy, high serum levels of ACE and lysozyme, and the abnormal accumulation of gallium on scintigraphy, in addition to histological evidence of sarcoidal granulomas. Cardiac lesions of sarcoidosis were diagnosed by electrocardiography, echocardiography, and ⁶⁷Ga-scintigraphy or PET-CT findings. Other systemic disorders such as malignant lymphoma, tuberculosis, and berylliosis were excluded prior to the diagnosis of sarcoidosis. Infectious conditions were eliminated by specific stains for certain micro-organisms: the Ziehl-Neelsen stain as the acid-fast stain and Grocott methenamine silver stain for fungus.

Serum samples were also collected from 20 healthy volunteers. Informed consent was obtained from all patients and control subjects.

Table 1. Summary of 20 patients with sarcoidosis

Case	Patient	Skin lesion	Eye	Heart	Lung	Lymph node	Muscle	ACE	sIL-2R	Lysozyme	sCD163
1	56 M	Plaque	-	-	-	+	-	20.2	546	4.4	1231.5
2	73 M	Plaque	-	+	-	+	-	11.4	578	4.7	1038.7
3	57 M	Infiltration of Scar	-	-	+	+	-	8.8	730	7.8	1045.6
4	79 F	Plaque	-	-	-	+	-	12.5	ND	5.2	3213.0
5	56 F	Erythema	-	+	-	-	-	15.8	350	ND	1056.57
6	32 F	Nodular	+	-	-	-	-	6.2	378	6.2	592.3
7	47 F	Nodular	+	-	-	+	-	13.9	ND	ND	979.0
8	80 F	Plaque	+	-	-	+	-	11.4	879	ND	2194.3
9	32 F	Erythema	-	-	+	+	-	12.3	ND	4.4	1089.5
10	80 F	Plaque	+	-	-	+	+	14.5	260	7.5	1347.9
11	73 F	subcutaneous	-	-	+	+	-	0.5	1310	7.3	2401.3
12	63 F	Nodular	+	-	-	+	-	2.4	844	5.8	1292.0
13	70 F	Plaque	+	-	+	+	-	27.7	933	8.1	3178.2
14	49 F	subcutaneous	+	+	+	+	-	9.3	557	3.6	1568.2
15	66 F	subcutaneous	-	-	-	+	-	17.0	416	3.5	2526.8
16	78 F	Nodular	+	+	-	+	-	25.7	1130	10.9	2353.0
17	74 F	Plaque	+	-	-	-	-	19.8	ND	6.9	2179.2
18	65 F	Nodular	-	-	+	+	-	18.1	627	9.2	2489.2
19	62 F	Infiltration of Scar	-	-	+	+	-	33.9	951	ND	1656.2
20	64 F	Erythema	+	-	-	+	+	42.9	ND	21.4	2603.76

The measurement of sCD163

Serum samples were stored in aliquots at -80°C until analysis. sCD163 levels were measured in the serum of sarcoidosis patients and healthy controls using an enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis, MN, USA).

Generation of MGC

According to our previous study (17), MGC were formed from human peripheral blood monocytes stimulated with the following condition medium. To obtain the condition medium needed to induce MGC, PBMC were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY, USA) and $16\ \mu\text{g/ml}$ concanavalin A (ConA) (Sigma, St Louis, MO, USA) at a density of 2×10^6 cells/ml for 72 h. The cell-free supernatant was used as conditioned medium and stored at -40°C before use. PBMC were isolated from heparinized blood by density gradient centrifugation with LymphoprepTM (Axis-Shield, Oslo, Norway). Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium. Cells were then added to a 6-well tissue culture at a density of 2×10^6 cells per well. After incubation for 2h at 37°C , non-adherent cells were removed by repeated vigorous washing with RPMI 1640. Adherent monocytes were used in the present study. MGC were induced by culturing monocytes in RPMI 1640 supplemented with a final concentration of 25% conditioned medium. The formation of MGC was observed *in situ* using the Giemsa stain (Merck, Tokyo, Japan). The cell-free supernatant was harvested 72 hours after culturing and sCD163 levels were measured.

Statistical analysis

Statistical calculations and analyses were performed using the Prism 5 (GraphPad Software, Inc, La Jolla, CA, USA) statistical software package. The Student's t-test was used to test the significance of differences in the variables. All statistical tests performed were two-sided. The threshold of significance was set at 5% ($\alpha = 0.05$).

RESULTS

Serum levels of sCD163 in sarcoidosis patients

sCD163 serum levels were significantly higher in patients with sarcoidosis than in healthy controls (Mean $1638 \pm 134.8\text{ng/ml}$ vs $550.6 \pm 59.7\text{ng/ml}$, $p < 0.0005$) (Fig. 1). Skin lesions in patients with sCD163 serum levels more than 3000ng/ml were plaques. The sCD163 serum levels of two patients with small skin lesions, infiltration of the scar alone, were lower at $1045.6\ \text{ng/ml}$ and $1656.2\ \text{ng/ml}$. On the other hand, three of four patients with cardiac lesions had lower serum levels of sCD163 than the mean levels in sarcoidosis patients. Thus, no significant differences were observed in sCD163 serum levels regardless of the presence or absence of eye, heart, lung, or lymph node lesions.

Correlation between serum sCD163 levels and the well-known disease markers of sarcoidosis

Serum ACE levels are elevated in 60% of sarcoidosis patients at the time of diagnosis and may correlate with the total body granuloma load. Soluble IL-2R is released by activated T lymphocytes and is also considered to be a useful parameter of the disease activity of sarcoidosis. The relationships between sCD163 serum levels and both well-known disease markers of sarcoidosis were examined. Serum levels of sCD163 positively correlated with other

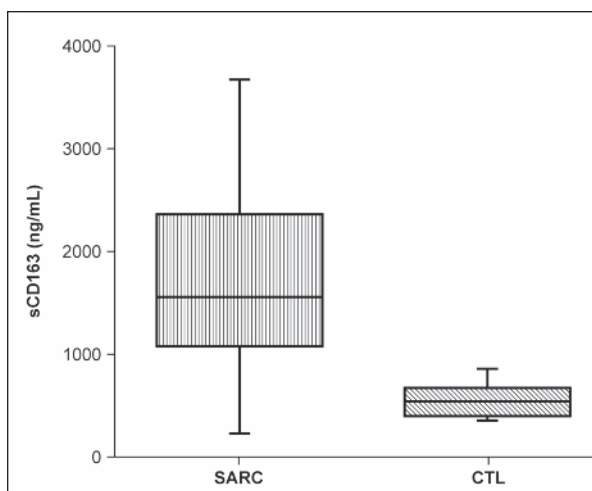


Fig. 1. sCD163 levels in the serum of sarcoidosis patients and controls ($1638 \pm 134.8\text{ng/ml}$ vs $550.6 \pm 59.65\text{ng/ml}$, $P < 0.0005$)

serum parameters of disease activity; ACE ($R^2=0.3818$ $P < .005$) (Fig. 2-a) and sIL-2R values ($R^2=0.2683$ $P < .005$) (Fig. 2-b).

Serial changes in sCD163 serum levels during the clinical course

sCD163 serum levels were serially measured in two cases, Case 5 and Case 20. Both cases were treated with 10 mg/d of prednisolone and sCD163 serum levels were decreased following the treatment (Fig. 3).

sCD163 levels with the formation of MGC

We examined sCD163 levels in culture supernatants of monocytes for the formation of MGC to

investigate the source of sCD163 in sarcoidosis. sCD163 levels significantly increased with the formation of MGC (16.75 ± 3.33 ng/ml vs 32.25 ± 4.19 ng/ml, $P < 0.01$, $N=4$) (Fig. 4).

DISCUSSION

Sarcoidosis is recognized as a disorder of the Th1-type T cell-mediated immune response. IL-2 release from lesional lymphocytes is considered to be important for the accumulation of T cells in sarcoidal lesions. Th17 cells were also recently shown to participate in the development of sarcoidal granulomas (18). On the other hand, monocyte-macrophage lineage cells are also considered to be key cells in the initiation, development, and maintenance of sarcoidal granulomas. Monocytes and macrophages play key roles in the production of chemoattractant factors for the accumulation of T cells in sarcoidal lesions (19). Previous studies have demonstrated the importance of dendritic cells in the pathogenesis of sarcoidosis, and DCs in the lymph nodes have been shown to mature and polarize pathogenic Th1 cells (20). Epithelioid cells and MGC, the main cellular element of mature sarcoidal lesions, are derived from monocyte-macrophage lineage cells.

Monocytes have been classified into subpopulations of $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ cells. We previously reported that the percentage of $CD14^{+}CD16^{+}$ monocytes was significantly higher in sarcoidosis patients than in healthy control subjects (16). We here demonstrated that serum sCD163 levels were significantly higher in sarcoidosis patients than in healthy subjects. Furthermore, sCD163 was positively correlated with serum levels of ACE and sIL-2 receptors, which are parameters used to monitor the disease activity of sarcoidosis. CD163 is known to be expressed selectively on monocyte-macrophage lineage cells and shed into the plasma as a soluble form by external stimuli (21-23). The $CD14^{+}CD16^{+}$ population has the highest shedding of CD163 (24), which suggests that the percentage of $CD14^{+}CD16^{+}$ monocytes in sarcoidosis patients may contribute to the high serum sCD163 levels. Since sCD163 has a direct anti-inflammatory effect on T lymphocytes (11), it may contribute to the suppression of peripheral immune

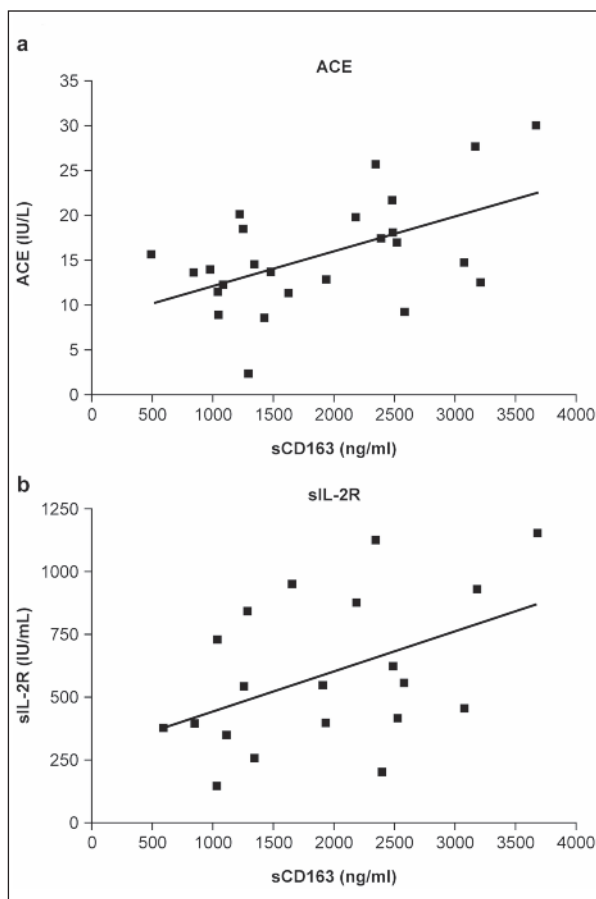


Fig. 2. Relationship between serum levels of sCD163 and ACE (a), or sIL-2R (b) in sarcoidosis patients. A significant positive correlation was observed between sCD163 serum levels and ACE ($R^2=0.3818$ $P < .005$) and sIL-2R ($R^2=0.2683$ $P < .005$)

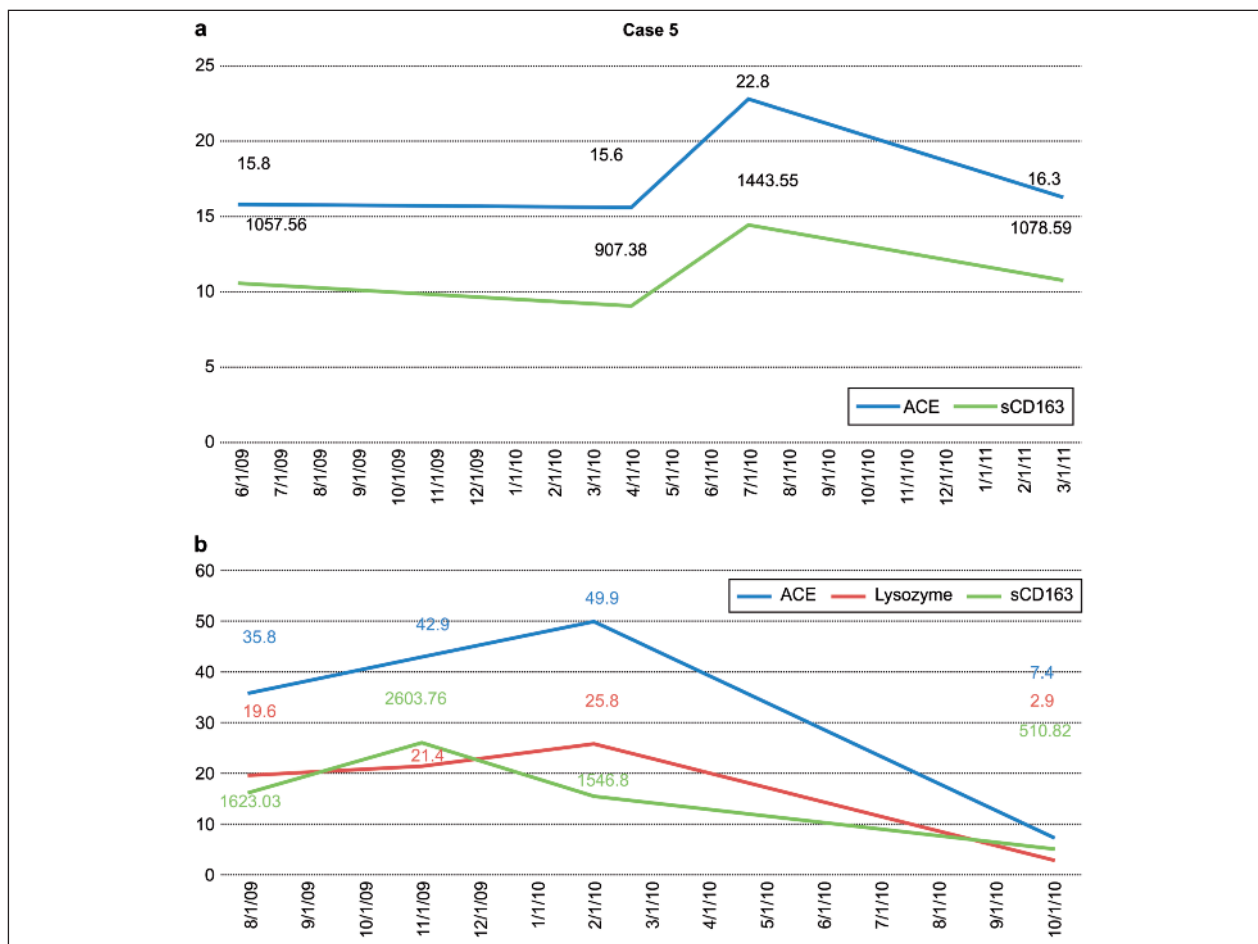


Fig. 3. Serum levels of sCD163 in Case 5 and Case 20. sCD163 serum levels decreased with ACE after the administration of 10 mg/d of prednisolone in both cases

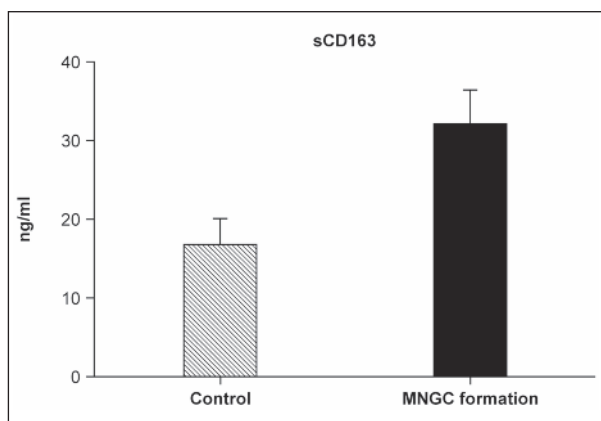


Fig. 4. ELISA of sCD163 concentrations in culture supernatants of peripheral blood monocytes treated with conditioned medium for 72 hours. sCD163 levels were significantly increased ($16.75 \pm 3.326 \text{ ng/ml}$ vs $32.25 \pm 4.191 \text{ ng/ml}$, $P < 0.01$, $N = 4$).

responses in sarcoidosis.

Several explanations have been suggested for the high sCD163 levels observed in sarcoidosis patients. CD163 on macrophages functions as an innate immunosensor for bacteria (25), and its shedding may decrease monocyte activation and inflammation (24). CD163 levels on monocytes have been inversely correlated with *in vitro* levels in tissue cultures medium and *in vivo* levels in plasma (26, 27). CD163 is shed into the blood as a soluble form by TLR2, TLR4 (21, 22), and oxidative stress (23). Although the etiology of sarcoidosis remains undetermined, mycobacterial and *Propionibacterium acnes* (*P. acnes*) have been the most commonly implicated potential etiological agents in sarcoidosis (28, 29). TLR2 and TLR4 have been shown to play an important role in the immune response to mycobacter-

ial infections (30), and *P. acnes* induced the activation of TLR2 and TLR4 *in vitro*. Both expressions of monocytes were shown to be up-regulated in sarcoidosis patients (31, 32), which indicated that high sCD163 serum levels in sarcoidosis patients may be associated with the activation of TLR2 and TLR4 by such microorganisms.

Another CD163-shedding stimulus is oxidative stress. The granulomas of sarcoidosis express ACE and membrane ACE converts angiotensin I into angiotensin II (33). Angiotensin II binds to angiotensin receptor-I, a Gq coupled receptor, and then stimulates NADPH oxidase to produce reactive oxygen species (ROS). Therefore, high serum levels of sCD163 in sarcoidosis patients may also be associated with oxidative stress. Psathakis et al (34) reported that oxidative stress markers such as 8-isoprostane were elevated in sarcoidosis patients. Timmermann and Hogger (23) demonstrated that 8-isoprostane as well as oxidative stress generated by H₂O₂ induced the significant shedding of CD163.

The presence of MGC is one of the signatures of sarcoidosis. MGC have been classified morphologically into Langhans-type cells (LGC) and foreign body-type cells (FGC). When histologically examined, sarcoidal lesions had both types of MGC with a predominance of LGC (35). MGC can also be formed *in vitro* from human blood monocytes by various stimuli. We previously reported that the enhanced ability of monocytes from patients with sarcoidosis to produce MGC (17). An elevation in sCD163 levels was observed in the culture medium used for the formation of MGC in the present study, which suggested that tissue monocytes trend for MGC are one of the sources of sCD163 in sarcoidosis patients.

sCD163 has been documented as a promising marker molecule for macrophage activation in diseases and pathological conditions such as sepsis (14), liver diseases (36), rheumatoid arthritis (15), and multiple sclerosis (37). Sarcoidosis is a disorder characterized by overwhelming macrophage activity. Clinical disease activity has been correlated with serum ACE, lysozyme, and sIL-2R in patients with sarcoidosis. Our study showed that sCD163 serum levels positively correlated with those of ACE and sIL-2 receptors and significantly decreased after treatments were administered. Thus, measuring serum levels of sCD163 may be helpful for monitor-

ing the disease activity of sarcoidosis.

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