Increased release of soluble CD163 by the peripheral blood mononuclear cells is associated with worse prognosis in patients with systemic sclerosis

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ABSTRACT

Purpose: CD163 is a scavenger receptor which is exclusively expressed on monocytes/macrophages and participates in modulation of inflammatory response. We aimed to evaluate ex vivo production of soluble CD163 (sCD163) by peripheral blood mononuclear cells (PBMC) from patients with systemic sclerosis (scleroderma, SSc).

Material/Methods: Concentration of sCD163 was measured by commercially available ELISA kit in the PBMC supernates from 23 SSc patients and 16 age- and sex-matched healthy controls (HC). Eighteen SSc patients were subsequently followed for at least three years or until death whichever happened earlier. Disease progression was defined as death due to SSc-related organ complication, development of a new or progression of pre-existing SSc-related organ involvement.

Results: PBMC from SSc patients released significantly greater amounts of sCD163 as compared with HC ($p<0.05$). No significant associations between release of sCD163 by PBMC and baseline clinical or laboratory parameters of the disease could be found. However, concentration of sCD163 in cell culture supernates was significantly higher in 6 SSc patients who experienced subsequent progression of the disease as compared with 12 SSc patients with stable disease course over a 3-year follow-up period ($p<0.05$).

Conclusions: We show, for the first time, that PBMC from SSc release significantly greater amounts of sCD163 than do PBMC from healthy subjects. Evaluation of sCD163 production by PBMC ex vivo may serve as a new biomarker of disease progression. Further studies are required to evaluate the role of sCD163 in the development of SSc.

Key words: systemic sclerosis, scleroderma, peripheral blood mononuclear cells, biomarker

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is a chronic autoimmune disease characterized by excessive fibrosis of the skin and internal organs. Activation of the immune system, widespread vascular injury and excessive synthesis of extracellular matrix components by activated fibroblasts are believed to play a key role in the pathogenesis of SSc however, detailed interactions between those processes are not yet fully understood [1]. Increasing evidence indicates that monocytes/macrophages play a key role in the development of both autoimmune and fibrotic diseases [2, 3]. Monocytes/macrophages represent functionally and morphologically heterogeneous population. Depending on their functional status, they may exert both

pro-inflammatory and anti-inflammatory effects. Moreover, they play an important role in resolution of the inflammatory response and tissue remodeling [2].

In SSc, monocytes/macrophages are major constituents of inflammatory infiltrates found in the skin and affected organs at early stages of the disease [4, 5]. Many important observations suggest that monocytes/macrophages are activated in SSc [6]. However, more data is still needed to better understand the role of those cells in SSc.

CD163 is a scavenger receptor cysteine-rich domain family member, which is exclusively expressed by monocytes/macrophages [7]. Shedding of membrane-bound CD163 through proteolytic cleavage produces soluble CD163 molecule (sCD163) which can be detected in body fluids [8]. Both membrane-bound and soluble CD163 exert strong anti-inflammatory effects [9]. Uptake of haptoglobin-hemoglobin (Hp-Hb) complexes by CD163-expressing cells leads to a strong increase in IL-10 production as well as increased expression of heme oxygenase -1 (HO-1), with subsequent production of CO that possesses anti-inflammatory functions [10]. Soluble, but not membrane bound, CD163 directly inhibits T cells proliferation [11]. Accordingly, expression of CD163 increases during resolution phase of inflammatory response [12].

Recently, Higashi-Kuwata et al. have found increased expression of CD163 on CD14-positive peripheral blood mononuclear cells (PBMC) and skin macrophages from patients with SSc as compared with healthy subjects [13]. However, the clinical relevance of increased expression of CD163 in SSc has not been addressed. Moreover, almost 20% of SSc patients included in their study were receiving corticosteroid therapy. Since corticosteroids strongly induce expression of CD163 on monocytes/macrophages it can not be excluded that increased expression of CD163 observed by Higashi-Kuwata et al, at least in part, could be due to influence of corticosteroid treatment rather than effect of the disease [14].

We undertook this study to evaluate ex vivo release of sCD163 in cultures of peripheral blood mononuclear cells (PBMC) from SSc patients not treated with immunosuppressive drugs or corticosteroids. Moreover, production of sCD163 by PBMC was correlated with clinical features and outcome of the disease.

PATIENTS AND METHODS

Patients

Twenty three consecutive patients fulfilling the ACR classification criteria for SSc.[15] and/or the definition of early SSc as proposed by LeRoy et al. [16] were included. To avoid modification of the activity of leucocytes by immunosuppressive drugs only patients who had not taken any immunosuppressive therapies or steroids for at least 6 months before blood collection were considered eligible. Control group consisted of 16 age- and sex matched healthy controls.

SSc patients were evaluated as described previously [17, 18]. Patients were classified as having diffuse cutaneous SSc (dSSc) or limited cutaneous SSc (lSSc) based on criteria by LeRoy et al [19]. Duration of Raynaud's phenomenon as well as duration of the disease, calculated from the time of the first non-Raynaud's symptom attributable to SSc, were recorded for every patient. In agreement with generally accepted criteria, early disease was defined as ≤ 3 years in diffuses SSc patients or ≤ 5 years duration in patients with limited SSc measured from the first non-Raynaud- symptom [20-22].

The severity of skin changes was assessed using modified Rodnan skin score (mRSS), as described elsewhere [23]. The presence of scleroderma interstitial lung disease (SLD) was defined based on the presence of features of interstitial fibrosis and/or "ground glass" opacifications in high resolution computed tomography (HRCT) of the lungs. Pulmonary function was assessed based on measurements of forced vital capacity (FVC) and diffusing capacity of the lungs (DLCO), and expressed as percentage of values predicted for sex, high and age. Pulmonary hypertension (PH) was defined as pulmonary artery systolic pressure (PASP) higher than 45 mmHg, as measured by echo-Doppler. Patients without tricuspid regurgitation were considered as having normal pulmonary artery pressure. Heart involvement was defined as the presence of congestive heart failure or arrhythmias/conduction disturbances requiring medications. Scleroderma renal crisis was defined as development of kidney insufficiency with or without arterial hypertension. Gastrointestinal involvement was defined as the presence of dysphagia due to esophageal hypomotylity, chronic diarrhea or malnutrition after excluding other reasons.

Assessment of peripheral vascular involvement included evaluation of the presence of Raynaud's phenomenon, the presence of digital ulcers and/or pitting scars and assessment of microvascular injury in capillaroscopy. Capillaroscopic assessment consisted of evaluation of the four fingers (II-V) of both hands with regard to the presence of megacapillaries, bushy capillaries and/or avascular areas (defined as lack of at least three consecutive capillaries). The severity of capillary injury was classified according to the classification by Maricq as "slow" pattern characterized by regular distribution of the capillaries, preserved architecture of vascular layout and the presence of megacapilaries, or as 'active' pattern characterized by irregular drop-out of capillaries resulting in disrupted architecture of vascular layout, the presence of megacapillaries and/or bushy capillaries [24, 25].

The presence of antinuclear antibodies (ANA) and anticentromere (ACA) antibodies was evaluated by indirect immunofluorescence on Hep-2 cells and the presence of

anti-topoizomerase I (anti-topo, anti-Scl70) antibodies – by ELISA technique. ESR and CRP concentrations were used as laboratory parameters of activity of inflammation.

Disease progression was defined as death due to SSc-related organ complication, development of a new one or worsening of pre-existing SSc-related organ involvement. SSc-related organ complications were defined as described above. As in previous studies, worsening of internal organ involvement was defined as an increase in mRSS of ≥25%, a decreased in FVC of ≥10% in patients with SLD, an increase in dyspnea by at least one WHO/NYHA class in patients with PH or heart failure, the need for chronic dialysis in patient with scleroderma renal crisis or parenteral nutrition in patients with gastrointestinal involvement [21, 26-29].

The study protocol was approved by the local ethics committee and all patients gave appropriate informed consent.

PBMC cultures and measurements of sCD163 in supernates

Peripheral blood mononuclear cells (PBMC) were isolated from the whole blood using density gradient centrifugation on Histopaque, and cultured in RPMI medium supplemented with fetal calf serum for 24 hours as described previously [18]. Subsequently the cells were centrifuged and supernates were collected and frozen at -80°C until measurements.

Evaluation of sCD163 concentration in the PBMC supernates was performed using commercially available ELSA kits (R&D Systems, Inc. MN, USA).

Statistical analysis

For the assessment of between-group comparisons the ANOVA Kruskall Wallis test, the Mann-Whitney U test and Fisher exact test were used, when appropriate. Correlations were assessed using the Spearman correlation test. For multiple comparisons the Bonferroni correction was applied to correct for number of comparisons.

The differences were considered significant at p value lower than 0.05.

All results are expressed as mean ± standard deviation (SD) unless stated otherwise.

RESULTS

Baseline characteristics of patients with systemic sclerosis and control group

The patients' mean age was 46.1 years, and the mean disease duration at enrollment was 2.3 years *(Tab. 1)*. Twenty out of 23 patients (87%) had early SSc while in the remaining three SSc patients disease lasted longer than 5 years. Eleven patients were classified as having diffuse cutaneous scleroderma and the remaining 12 –limited SSc. HRCT of the lungs revealed features of SLD in 12 (52%) patients. However, restrictive

ventilatory defects (defined as FVC<75% of predicted and FEV1/FVC>80%) were found in only two of them at baseline. PH by echo and clinical features of congestive heart failure were found in only one patient while none of the SSc patients had scleroderma renal crisis at the beginning of the study.

There was no significant difference in age and sex distribution between SSc patients and healthy controls.

Detailed clinical characteristics of the SSc patients and the control group is given in *Table 1.*

CD163 in PBMC cultures from SSc patients and healthy controls

The sCD163 was detectable in all samples studied from both SSc patients and healthy subjects. The mean concentration of sCD163 in the supernates of PBMC from SSc patients (2689.4 ± 657.5 pg/mL/10^5 cells) was significantly greater than in healthy controls (2306.38 ± 278.02 pg/mL/10^5 cells; p=0.03) *(Fig. 1)*. There were no significant differences in sCD163 levels between patients with diffuse SSc and those with limited SSc *(Fig. 1)*.

In SSc patients concentration of sCD163 in PBMC culture supernates significantly correlated with serum CRP concentration (R=0.50, p<0.05) *(Tab. 2)*. However, the correlation between sCD163 levels in PBMC cultures and serum CRP concentration lost its significance when corrected for number of comparisons (p=0.09). No other significant correlations between cell culture supernate sCD163 and any other baseline clinical or laboratory parameter could be found, including duration of the disease or Raynaud phenomenon, mRSS, lung function tests, PASP or ESR *(Tab. 2)*. No significant associations could be found between release of sCD163 by PBMC and the presence of specific autoantibodies or features of peripheral vasculopathy such as digital ulcers/pitting scars or capillaroscopic patterns, either (data not shown). Since there were no SSc patients with scleroderma renal crisis and only one patient had pulmonary hypertension, no associations between those severe vascular complications and the concentration of sCD163 in cell culture supernates could be assessed.

Association between release of sCD163 by PBMC and disease progression in patients with SSc

Eighteen SSc patients included in this study were subsequently followed for at least three years or until death whichever happened earlier. Within three years follow-up 6 out of those 18 SSc patients (33%) experienced progression of the disease or died. In the remaining 12 (67%) SSc patients the disease remained stable over three years' time period. Detailed clinical characteristics of SSc patients with available follow-up data is presented in *Table 1.*

Six SSc patients with subsequent progression of the disease had significantly shorter duration of Raynaud phenomenon at baseline and anti-topo I antibodies were more frequently detected in those patients as compared with SSc patients with

Table 1. **Clinical characteristics of the patients with systemic sclerosis and the control group. All data are presented as mean +/- SD unless stated otherwise.**

Parameter	SSc patients (n=23)	SSc patients with disease progression (n=6)	SSc patients with stable disease (n=12)	Control group (n=16)
Female/Male ratio	19/4	4/2	12/0	13/3
Age (mean, range)	46.1 +/- 13.7	49.2 +/-13.9	47.3 +/- 12.7	40.4 +/- 13.6
Disease duration in years	2.3 +/- 2.4	1.0 +/- 0.8	3.0 +/- 3.0	
Duration of Raynaud's phenomenon in years	6.1 +/- 5.4	3.4 +/- 3.5^	8.5 +/- 6.2	
Early disease* (%)	20 (87.0)	6 (100)	9 (75)	
dSSc/lSSc	11/12	4/2	3/9	
mRSS	13.3 +/- 11.6	21.7 +/- 13.7	14.88 +/- 17.9	
ANA positive (%)	23 (100)	6 (100)	12 (100)	
Anty-Scl70 positive (%)	14 (60.9)	6 (100)^	4 (33.3)	
ACA positive (%)	6 (26.1)	0	5 (41.7)	
Raynaud's phenomenon (%)	23 (100)	6 (100)	12 (100)	
SLD by HRCT/Xray (%)	12 (52.2)	4 (66.7)	3 (25)	
Pulmonary hypertension (%)	1 (4.4)	1 (16.7)	0	
FVC (% predicted)	103.9 +/- 17.9	95.2 +/- 28.4	110.9 +/- 16.4	
DLCO (% predicted)	84.6 +/- 21.2	79.8 +/- 20.5	90.9 +/- 23.0	
PASP (mmHg)	30.8 +/- 8.3	21.7 +/- 13.7	14.8 +/- 27.9	
Digital ulcers and/or pitting scars (%)	13 (56.5)	4 (66.7)	7 (58.3)	
Capillaroscopic pattern ("active"/"slow")	11/12	4/2	5/7	
ESR (mm/h)	21.3 +/- 17.2	29.7 +/- 30.6	18.8 +/- 9.5	
CRP (mg/L)	7.3 +/- 10.1	13.9 +/- 18.4	4.4 +/- 1.5	

^$p<0.05$ versus SSc patients with stable disease, Comparisons lost their significance ($p>0.05$) after applying Bonferroni correction for multiple testing.
* Early disease duration was defined as ≤ than 3 years in dSSc patients or ≤ than 5 years duration in patients with lSSc.
ACA=anticentromere antibodies, ACR=American College of Rheumatology, ANA=antinuclear antibodies, dSSc=diffuse systemic sclerosis, HRCT=high resolution computed tomography, lSSc=limited systemic sclerosis, SLD=scleroderma lung disease

Table 2. **Correlations between concentrations of sCD163 in the PBMC cultures and clinical and laboratory parameters of patients with systemic sclerosis.**

Parameter	CD163	
	R Spearman	(p value)
Age	0.13	p>0.05
Disease duration	-0.30	p>0.05
Duration of Raynaud's phenomenon	-0.32	p>0.05
mRSS	0.38	p>0.05
FVC (% predicted)	-0.24	p>0.05
DLCO (% predicted)	-0.32	p>0.05
PASP (mmHg)	0.35	p>0.05
ESR (mm/h)	0.09	p>0.05
CRP (mg/L)	0.52	p<0.05*

*Correlation lost its significance ($p>0.05$) after applying Bonferroni correction for multiple testing.
CRP=C-reactive protein, DLCO=diffusing capacity of the lungs for carbon monoxide, ESR=erythrocyte sedimentation rate, FVC=forced vital capacity, mRSS=modified Rodnan skin score, PASP=pulmonary artery systolic pressure

Figure 1. Concentration of sCD163 in supernates of PBMC from overall group of patients with systemic sclerosis (SSc), patients with diffuse SSc (dSSc), those with limited SSc (lSSc) and healthy controls. PBMC were incubated for 24 hours in RPMI medium supplemented with fetal calf serum.
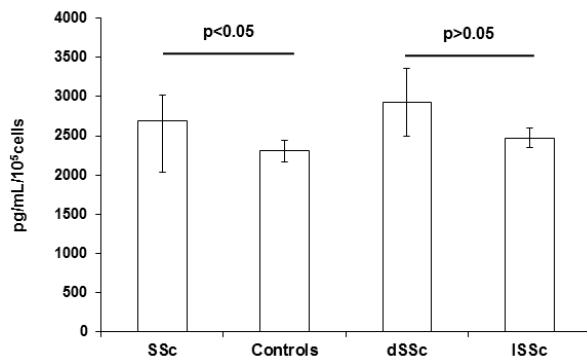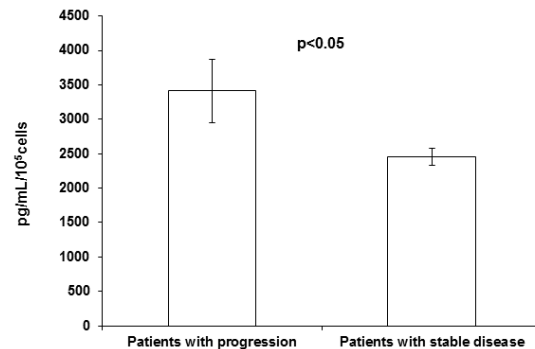

Figure 2. Concentration of sCD163 in supernates of PBMC from patients with SSc with subsequent progression of the disease and those SSc patients who had stable disease. PBMC were incubated for 24 hours in RPMI medium supplemented with fetal calf serum.

stable disease *(Tab. 1)*. However, none of those differences persisted after correction for multiple comparisons.

No more significant differences could be found in other clinical or laboratory parameters between SSc patients with progression of the disease and those with stable disease.

The mean concentration of sCD163 in PBMC cultures of those 6 patients with subsequent progression of the disease was significantly higher (3411.34 +/- 914.42 pg/mL/10^5 cells) as compared with the remaining 12 SSc patients with stable disease (2456.16 +/- 252.08 pg/mL/10^5 cells, p=0.003) *(Fig. 2)*. The difference remained significant after Bonferroni correction for multiple comparisons. The sCD163 levels in 6 patients with disease worsening were also significantly greater as compared with healthy controls while release of sCD163 by PBMC from SSc patients with stable disease did not differ from that in the control group.

DISCUSSION

Our major findings indicate that PBMC from SSc patients spontaneously release ex vivo increased amounts of soluble CD163 and that among SSc patients greater release of sCD163 by PBMC ex vivo is associated with worse clinical outcome.

Our results are in agreement with those presented in two recently published studies from Japanese groups showing that serum concentration of sCD163 is increased in patients with SSc as compared with healthy subjects [30, 31]. Indeed, demonstration that PBMC from SSc patients spontaneously release increased amounts of sCD163 ex vivo may, at least partially, explain elevated levels of sCD163 seen in sera of SSc patients in vivo.

Unlike Nakayama et al. and Shimizu et al. who found significant associations between high serum concentrations of sCD163 and the presence of scleroderma lung disease or pulmonary hypertension, we did not reveal association between greater release of sCD163 in PBMC and lung

involvement in SSc patients [30, 31]. These discrepancies might be caused by the differences in study methodology and/or by the differences in patients populations involved in particular studies. In contrast to studies by Nakayama et al. and Shimizu et al. the majority of patients included in our study had early SSc with only few advanced organ manifestations at baseline. This made analysis of associations between ex vivo production of sCD163 by PBMC and severity of the disease less accurate.

Our study is the first one evaluating associations between expression of sCD163 and clinical outcome in patients with SSc. We found that PBMC from SSc patients with worse prognosis released significantly greater amounts of sCD163 that did PBMC from SSc patients with stable disease or healthy controls. Moreover, in our study concentration of sCD163 in PBMC cultures was the only one parameter associated with clinical outcome in SSc patients. Indeed, no other significant differences could be found between patients who experienced subsequent progression of the disease and those with stable SSc, including skin score, the presence of SLD, PH, pulmonary function tests, autoantibody status or laboratory markers of inflammation. This finding is of great interest and suggests that sCD163 might be a new valuable biomarker of clinical prognosis in SSc. Indeed, several independent studies indicate that increased serum levels of soluble sCD163 is a good predictor of survival in patients with severe infections [32, 33]. Association between high release of sCD163 by PBMC and worse clinical outcome in SSc patients does not, however, explain the role of CD163 in the development of SSc. CD163 is considered a marker of differentiation of monocytes into alternatively activated macrophages (M2 macrophages) which release anti-inflammatory mediators such as interleukin 10 (IL-10) and contribute to resolution of inflammatory response [9]. However, alternatively activated macrophages are also a source of pro-fibrotic cytokines including tumor growth factor beta (TGFbeta) which is considered to play a key role

in the pathogenesis of fibrotic disease, including SSc [2, 34]. It can therefore by hypothesized that increased expression/ release of CD163 observed by us and the others in SSc may be associated with greater activity of fibrosis.

On the other hand, CD163 exerts several actions which can be of potential benefit in SSc. As a scavenger receptor CD163 binds haptoglobin-hemoglobin complexes and in this way contributes to protection of blood vessels against toxic effect of free heme and oxidative stress [35]. This function of CD163 might be of particular importance in SSc since widespread microangiopathy is present in SSc patients from the very beginning of the disease and might lead to increased injury of erythrocytes with subsequent intravascular hemolysis and release of free hemoglobin [36]. CD163-positive cells have also been shown to exert pro-angiogenic activities including release of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [37]. The latter might be of importance in the regeneration of injured blood vessels in SSc since, as discussed above, vascular injury is common and important feature in SSc. Of note, we have previously shown that PBMC from SSc patients release significantly greater amounts of VEGF as compared with healthy controls [38]. Accordingly, injection of PBMC has been shown to improve ischemic diseases through delivery of proangiogenic mediators [39].

Taking into account complexity of CD163 function further studies are required to address the exact role of CD163 in the pathogenesis of SSc. This might be of great interest since CD163-targeted therapies are being developed [40].

CONCLUSIONS

In conclusion, our study demonstrates that increased production of sCD163 by PBMC may be considered as a marker of unfavorable outcome already in the early stages of SSc. Further studies are warranted to elucidate if elevated production of sCD163 ex vivo is reflected by elevated levels of sCD163 in serum of patients with early SSc who have poor clinical prognosis.

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