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ORIGINAL ARTICLE

Downregulation of CD163 in monocytes and its soluble form in the plasma is associated with a pro-inflammatory profile in pregnant women with preeclampsia

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Abstract

Preeclampsia (PE) is a pregnancy-specific syndrome characterized by a systemic inflammatory response that polarizes peripheral blood monocytes to the M1 phenotype. The classically activated M1 monocytes comprise immune effector cells with an acute inflammatory phenotype. CD163 is a scavenger receptor expressed by monocytes/macrophages that may be shed from their cell membrane after proteolytic cleavage, producing the soluble CD163 molecule (sCD163). This study evaluated CD163 expression by monocytes and sCD163 as well as pro- and anti-inflammatory cytokine concentration in the plasma of pregnant women with PE. Fifty-six women with PE and 28 normotensive pregnant women were included. Plasma levels of sCD163, interleukin-1 beta (IL-1β), IL-6, IL-10, transforming growth factor beta (TGF-β1), and tumor necrosis factor-alpha (TNF-α) were determined by ELISA, and CD163 expression by monocytes was assessed by flow cytometry. The expression of CD163 by monocytes was significantly lower in severe and mild PE than in normotensive pregnant. Plasma concentrations of IL-1β, TGF-β1, and TNF-α were higher in severe PE than in mild PE and normotensive pregnant women. Both groups of preeclamptic women showed decreased plasma levels of sCD163 and IL-10. Negative correlations between sCD163 and IL-1β ($r = -0.45$; $P = 0.014$) and between sCD163 and TNF-α concentrations ($r = -0.54$; $P = 0.001$) were observed in the severe PE group. The association between the pro-inflammatory cytokine profile and lower concentrations of sCD163 and IL-10 in plasma from women with severe PE suggests an impairment in the modulation of the systemic inflammatory response in this group of pregnant women with preeclampsia.

Keywords CD163 · Cytokines · Inflammation · Monocytes · Preeclampsia

Introduction

Preeclampsia (PE) is a pregnancy-specific syndrome characterized by an intense systemic inflammatory reaction [1, 2] with great involvement of activated cells from the immune system [3, 4]. The excessive production of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), IL-6 and IL-8, CXCL10/IP-10 and MCP-1 chemokines, has been reported in PE [5–10], as well as decreased levels of the regulatory cytokine IL-10 [9, 10].

The endogenous activation of monocytes from pregnant women with PE is demonstrated by the high production of superoxide anion, hydrogen peroxide, IL-1β, IL-18, and TNF-α by these cells, suggesting that circulating monocytes may represent an important source of inflammatory cytokines during the disease [9, 11, 12].

Monocytes are cells belonging to the monocyte-macrophage lineage, which are considered able to adapt and respond to a variety of signals present in their environment [13]. Macrophages can be classified into at least two subpopulations with distinct phenotypes considered the ends of an activation spectrum, known as classically

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activated/inflammatory (M1) and alternatively activated (M2), according to their functions [13, 14]. These polarized cells differ by the expression of surface receptors, cytokine production, and effector functions [15]. M1 macrophages are activated by IFN-ɣ, TNF-α, or LPS and express opsoninics receptors FcɣRI, RII and RIII (CD16, CD32, CD64), TLR2 and TLR4 receptors, and inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, and IL-23, as well as reactive oxygen and nitrogen species [14–16]. On the other hand, the activation of these cells by IL-4 and IL-13 polarizes them to the M2 profile, which is characterized by the increased expression of CD163 "the scavenger" receptor for the haptoglobin-hemoglobin complex, which also has anti-inflammatory and immunoregulatory properties [17]. Also, M2 macrophages show higher expression of the mannose receptor (CD206), as well as the increased production of IL-10 and TGF-β1 [13]. Thus, macrophages are cells with plasticity because they can change from an M1-activated state to a regulator M2 state and vice versa, depending on the effects of specific signals found in their environment [18, 19]. This M1 and M2 profile classification can be extended to human peripheral blood monocytes, and polarization to a M1 profile has been demonstrated in some diseases such as sepsis [20], type 2 diabetes [21], and atherosclerosis [22]. Also, in vitro studies using modulating agents to polarize human monocytes towards a M1 or M2 phenotype provide better understanding of the role of inflammasomes in the pathophysiology of human inflammatory disorders [23].

In a previous study, we demonstrated that monocytes from pregnant women with PE are polarized to the M1 profile, with the significantly increased expression of endogenous CD64 and TLR4, as well as the increased production of the inflammatory cytokines TNF-α and IL-12. Furthermore, the expression of CD163 and CD206 and the production of IL-10, considered markers of the M2 profile, were significantly lower than in normotensive pregnant women [24]. The CD163 receptor may shed from the cell surface upon inflammatory stimuli and is detectable as a soluble form (sCD163) in human plasma [25]. According to Buechler et al. [26], increased concentrations of sCD163 were detected in the serum of critically ill patients and in chronic inflammatory and infectious diseases, are related to disease severity, and are suitable biomarkers for diagnosis, prognosis, and therapeutic drug monitoring.

Considering that PE may manifest in mild or severe forms, with severity associated with an intense activation of circulating monocytes, the present study evaluated whether there is an association among CD163 expression by monocytes, plasma levels of CD163 soluble receptor (sCD163), and pro- and anti-inflammatory cytokines in pregnant women distributed according to mild or severe forms of the disease.

Methods

Study population

The study comprised 56 patients with PE, with 28 with the mild form and 28 diagnosed with severe PE. Preeclampsia was identified by hypertension and proteinuria from 20 weeks of gestation or by hypertension associated with maternal neurologic or hematologic complications, kidney dysfunction, liver involvement, or fetal growth restriction [27, 28]. Proteinuria was measured using the Technicon RAXT automation system in the Clinical Laboratory, Botucatu Medical School, UNESP. Severe PE was diagnosed as the presence of the following criteria: systolic blood pressure ≥ 160 × 110 mmHg, thrombocytopenia, HELLP syndrome, new-onset cerebral or visual disturbances, renal insufficiency, or acute pulmonary edema [29].

Twenty-eight normotensive (NT) pregnant women with an uncomplicated pregnancy who were non-proteinuric were recruited and matched for gestational age at the time of sampling with the preeclamptic group. These women underwent pregnancy care at the Obstetric Unit of Botucatu Medical School and remained normotensive and non-proteinuric until the end of gestation. The gestational age of the normotensive and preeclamptic pregnant women was calculated from the last menstrual period and confirmed by early (< 12 weeks gestation) ultrasound examination. Exclusion criteria included multiple gestations, prior PE, pregnant women in labor, illicit drug use, and preexisting medical conditions such as diabetes, obesity, chronic hypertension, and infectious and renal diseases.

The study was approved by the Ethics Committee of the Botucatu Medical School (Protocol number 417.350), and written informed consent was obtained from all women involved in the study. For pregnant women younger than 18 years old, the written informed consent was obtained from their parents or guardians.

Blood collection

The blood of preeclamptic women was obtained at the time of diagnosis, while that of normotensive pregnant women was obtained at the time of their attendance. Blood samples (10 ml) were collected by venepuncture from the antecubital vein and were put into a sterile plastic tube containing 10 U/ml EDTA (Becton Dickinson-BD Vacutainer; BD Biosciences, Franklin Lakes, NJ). After centrifugation for 10 min at 3000×*g*, the obtained plasma was removed and aliquots were stored at − 80° until the time of cytokine determination.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque Premium (density =

1.077) (GE Healthcare Biosciences, Uppsala, Sweden) as described previously [30]. The obtained mononuclear cell-rich ring was washed twice with RPMI-1640/HEPES tissue-culture medium (LGC Biotechnology, Sao Paulo, SP, Brazil) with centrifugation between washes at 300×*g* for 10 min. After this procedure, the cells were resuspended in RPMI-1640/HEPES culture medium (LGC Biotechnology) supplemented with 10% inactivated fetal bovine serum (complete RPMI). For identification of the mononuclear cells, 50 μl of the mononuclear cell suspension was incubated for 10 min at 37° with 450 μL of 0.02% neutral red solution. The cell concentration was adjusted to 1×10^6 viable cells/ml, and the cells were distributed (1 ml/well) in 24-well flat-bottomed plates (Falcon, Corning Incorporated-Life Sciences, Durham, NC) and incubated at 37°, in a 5% CO_2 atmosphere for 90 min. Non-adherent cells were discarded by washing the plate wells with RPMI-1640/HEPES culture medium (LGC Biotechnology). Cell viability as determined by 0.2% Trypan blue dye exclusion was > 95% in all experiments. The cell concentration was adjusted to 5×10^5 monocytes/ml.

Flow cytometric analysis of CD163 on monocyte surface

For the determination of CD163 receptors on monocytes from preeclamptic and normotensive pregnant, these cells were distributed into Falcon cytometer tubes (BD Biosciences) at a concentration of 5×10^5 monocytes/mL and incubated for 18 h at 37 °C in a humidified 5% CO_2 atmosphere with complete medium. Cells were washed with wash buffer containing 10% endotoxin-free fetal bovine serum (Sigma-Aldrich) and incubated with the following monoclonal antibodies according to the manufacturer's instructions: phycoerythrin-Cy7 (PECy7)-labeled anti-CD14 (BioLegend, Inc, San Diego, CA, USA) and PE-labeled anti-CD163 (GHI/61-Biolegend). The cells were incubated for 30 min in the dark at room temperature. Background staining was determined by staining cells for 30 min with PE or PECy7-labeled control isotype antibodies at room temperature in the dark. All samples were washed with wash buffer (Sigma-Aldrich) and fixed with saline buffer plus 2% paraformaldehyde in PBS (Sigma-Aldrich) at room temperature. The gating strategy to separate monocytes was defined with respective side scatter (SSC) and CD14 staining characteristics. CD163 expression was obtained from CD14 gate. In the case of the measurements, 30,000 events for each sample were acquired on a FACSCanto™ II flow cytometer (BD Biosciences) with facsdiva software (BD Biosciences). The results were analyzed in the software FlowJo, version vX.10.6 (FlowJo, LLC, Ashland, OR), and expressed as median fluorescence intensity (MFI).

Determination of cytokines and soluble CD163 by ELISA

IL-1β, IL-6, IL-10, TNF-α, TGF-β1, and sCD163 concentrations in plasma were determined using specific commercial kits obtained from R&D Systems (Minneapolis, MN, USA). The reactions were developed according to the manufacturer's instructions. The concentrations of the cytokines and sCD163 were calculated from standard curves made with the respective human recombinant standards. In the tests, the concentrations of monoclonal and polyclonal antibodies, as well as specific recombinant cytokines used in standard curves, were those recommended by the manufacturer (R&D Systems). The sensitivity of the kits for the different cytokines and sCD163 was IL-1β = 1 pg/mL, IL-6 = 0.7 pg/mL, IL-10 = 1.5 pg/mL, TGF-β1 = 15.4 pg/mL, TNF-α = 2.5 pg/mL, and sCD163 = 0.613 ng/ml.

Statistical analysis

The results were submitted to normality test of Kolmogorov-Smirnov. Characteristics of women with PE and normotensive pregnant women were analyzed by nonparametric tests (Kruskal-Wallis), and the data on cytokines, CD163 receptor, and the soluble CD163 receptor were evaluated by parametric analysis of variance (ANOVA). The correlation coefficient (r) between sCD163 and cytokines in plasma and CD163 expressed by monocytes for the different groups studied was determined using Pearson's correlation. Data were analyzed using the Prism Statistical software (Graph Prism for Windows, version 6.01, GraphPad, San Diego, CA, USA). Statistical significance was accepted at $P < 0.05$ for all comparisons and in all correlations.

Results

Characteristics of the study population

Table 1 lists characteristics of the three groups studied. No statistical differences were evident between groups with respect to age and body mass index (BMI). Gestational age was similar between the pregnant groups. However, systolic and diastolic blood pressures were significantly higher in women with PE compared with normotensive pregnant women ($p < 0.05$). The severe PE group had higher systolic blood pressure and proteinuria than the mild PE and normotensive groups.

Table 1 Characteristics of the study population

Characteristics	Mild PE ($n = 28$)	Severe PE ($n = 28$)	Normotensive ($n = 28$)
Age (years)	27 (17–40)	24 (13–43)	23 (15–32)
Gestational age (weeks) BMI (kg/m^2)	37 (30–42)	36(28–40)	35 (25–39)
	24.15 (21.17–25.85)	23.02 (19.15–25.38)	23.56 (20.80–25.57)
Systolic blood pressure (mmHg)	140* (135–160)	160*$^+$ (150–210)	105 (95–110)
Diastolic blood pressure (mmHg)	100* (90–100)	110* (90–130)	60 (60–70)
Proteinuria (mg/24 h)	500 (300–1990)	2680*$^+$ (2500–39,600)	< 300

Results are represented as median (range)

Statistical significance: *$p < 0.05$ vs. normotensive; $^+p < 0.05$ vs. mild preeclampsia (Kruskal-Wallis)

Expression of CD163 on monocyte surface and determination of soluble CD163 in plasma

Monocytes from preeclamptic women with both mild and severe PE showed significantly lower mean fluorescent intensity (MFI) expression of CD163 receptor than the normotensive pregnant group ($p < 0.01$) (Fig. 1a). Concentrations of sCD163 were also significantly lower ($p < 0.05$) in plasma of the mild and severe PE groups compared with those detected in the normotensive pregnant group (Fig. 1b). No significant differences were detected between the two PE groups in relation to this parameter.

Determination of cytokines

The concentrations of IL-1 β, IL-6, TNF-α, IL-10, and TGF-β1 in plasma from women with severe and mild PE and normotensive pregnant women are shown in Fig. 2a–e. The inflammatory cytokines IL-1β and TNF-α were significantly higher in women with severe PE compared with mild PE and normotensive pregnant women ($p < 0.05$). These cytokines were also significantly higher in mild PE than normotensive pregnant women ($p < 0.01$). The levels of IL-6 were similar in both groups of preeclamptic women and were significantly higher than in the normotensive women ($p < 0.01$). TGF-β1 concentrations were significantly higher in severe PE compared to the other groups ($p < 0.01$). On the other hand, the plasma concentration of IL-10 was significantly lower in pregnant women with mild PE and severe PE than in normotensive pregnant women ($p < 0.01$).

The correlations between concentrations of sCD163 and cytokines in plasma for the different groups studied are shown in Table 2. An inverse correlation was observed between concentrations of sCD163 and IL-1β ($r = -0.45$; $p = 0.014$) and between sCD163 and TNF-α ($r = -0.54$; $p = 0.001$) in the group with severe PE. In mild PE, there was no correlation between sCD163 and the different cytokines. A positive correlation between sCD163 and IL-10 was detected in the normotensive pregnant group ($r = 0.61$; $p = 0.001$), while no significant correlation was detected between sCD163 and IL-1β, TNF-α, IL-6, and TGF-β1 in this group of pregnant women. Comparison between sCD163 plasmatic concentration and CD163 expression on the monocyte surface showed a high positive correlation in the normotensive group ($r = 0.78$; $p = 0.0001$) and a moderate positive correlation in the other groups: mild PE ($r = 0.42$; $p = 0.003$) and severe PE ($r = 0.56$; $p = 0.001$).

Discussion

In the present study, we compared the plasma concentration of cytokines and soluble CD163 receptor as well as CD163 expression on the monocyte surface of women with mild and severe PE and normotensive pregnant. The results showed that plasma levels of IL-1β and TNF-α were significantly higher in pregnant women with severe PE compared to the mild PE and normotensive group. Although the levels of these cytokines were lower in the mild than in the severe PE group, they were higher than in normotensive women. These results demonstrate that the systemic inflammatory response is exacerbated in cases of severe PE. In preeclamptic women, peripheral blood mononuclear cells produce higher endogenous levels of IL-1β and TNF-α, which are associated with greater activation of the nuclear transcription factor NF-kB. Cytokine synthesis is regulated by this factor, which is more active in mononuclear cells of pregnant women with PE [7]. NF-kB regulates the transcription of genes related to inflammation [31], and TNF-α, in turn, acts by stimulating NF-kB activation by maintaining a cycle of cell activation [32]. Therefore, activation of mononuclear cells, including monocytes, seem to be responsible, in part, for the elevated plasma levels of these inflammatory cytokines in women with PE.

The higher levels of IL-1 β, IL-6, and TNF-α detected in pregnant women with PE are in line with the results of other authors [1, 7, 10, 11, 33] and suggest that the deleterious effects of high circulating concentrations of TNF-α may be associated with more severe manifestations of PE and with the oxidative stress detected in this disease [11]. An association between higher levels of TNF-α, IL-1β, IL-18, and NLRP3

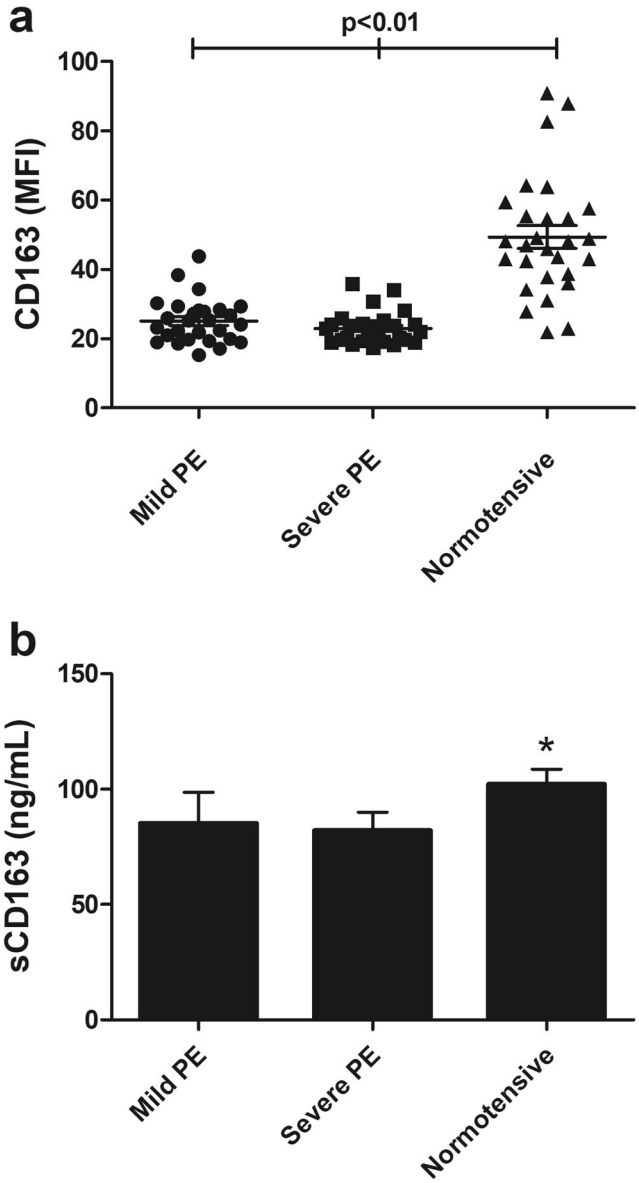

Fig. 1 Expression of CD163 receptor by monocytes (**a**) and plasma concentration of soluble CD163 receptor (sCD163) (**b**) in pregnant women with mild PE, severe PE, and normotensive pregnant women. Data for CD163 receptor are presented as mean fluorescence intensity (MFI ± SD). Statistical significance: *$p < 0.01$ mild PE vs normotensive; severe PE vs normotensive. Plasma concentrations of sCD163 are presented as mean ± SD. Statistical significance: * $p < 0.05$ vs. mild PE and severe PE (ANOVA)

inflammasome activation was reported in peripheral blood monocytes from preeclamptic women. The higher production of TNF-α by monocytes from these patients suggests the involvement of this inflammatory cytokine in inflammasome induction [12]. These results confirmed that endogenous monocyte activation plays an important role in the maintenance of systemic inflammation in pregnant women with PE.

In the present study, the higher plasma levels of TGF-β1 in pregnant women with severe PE compared to mild PE and normotensive pregnant women could be associated with disease severity, as previously suggested [34, 35], and is correlated with decreased platelet numbers and function based on their aggregation [35]. Platelets are structures that store TGF-β1 in their granules and are the main source of circulating TGF-β1 following platelet degranulation [36]. On the other hand, TGF-β1 plays a pleiotropic role on immunity by controlling both anti-inflammatory and pro-inflammatory T cell responses with the presence of other cytokines [37]. Induction of Th17 cells by TGF-β1 requires association with other pro-inflammatory cytokines such as IL-1β, IL-6, and IL-21 [38]. In recent study, we demonstrated higher percentage of Th17 cells expressing RORc transcription factor associated with higher levels of IL-6, IL-17, and TGF-β1 and lower percentage of T cells expressing FoxP3 in severe cases of PE [39]. Therefore, the association between higher levels of TGF-β1 and spontaneous platelet aggregation [35] as well as its role on adaptive immunity generating inflammatory Th17 CD4+ subset, suggest that TGF-β1 may play an important role in the pathophysiology of PE.

On the other hand, analysis of the anti-inflammatory cytokine IL-10 showed significantly lower levels in the plasma of women with mild and severe PE compared to the normotensive pregnant group. The higher production of IL-10 by normotensive pregnant women corroborates with previous findings [33], showing that the cytokine balance is altered in PE with higher levels of TNF-α and IFN-γ and lower levels of IL-10 [8, 9]. The highest levels of IL-10 in normotensive pregnant women suggest the predominance of an anti-inflammatory Th2 response, typical of normal pregnancy, with a predominance of IL-10 over TNF-α to minimize the deleterious effects of the excessive inflammatory response. IL-10 has a potent anti-inflammatory effect that is considered important in the maintenance of pregnancy [40] and is responsible for suppressing the production of inflammatory cytokines by activated monocytes [41].

In this study, a higher concentration of sCD163 was detected in the plasma of normotensive pregnant women compared with women with mild and severe PE and strongly correlates with high CD163 expression by monocytes ($r = 0.78$; $p = 0.001$) as well as with elevated levels of IL-10 ($r = 0.61$; $p = 0.001$) in this control pregnant group. The CD163 receptor is present on the surface of monocytes/macrophages, and the endocytosis of haptoglobin-hemoglobin complexes is the main function of this receptor, leading to the increased production of IL-10, which plays an immunoregulatory role on the immune system [42]. CD163 may shed from the surface of monocytes/macrophages to the plasma, giving rise to its soluble form, which has anti-inflammatory activity [17]. Therefore, our results of the higher expression of CD163 receptor on monocyte surface, as well as higher plasma levels of sCD163 and IL-10 in normotensive pregnant women, may have a down regulatory effect on the inflammatory response to protect the pregnancy development.

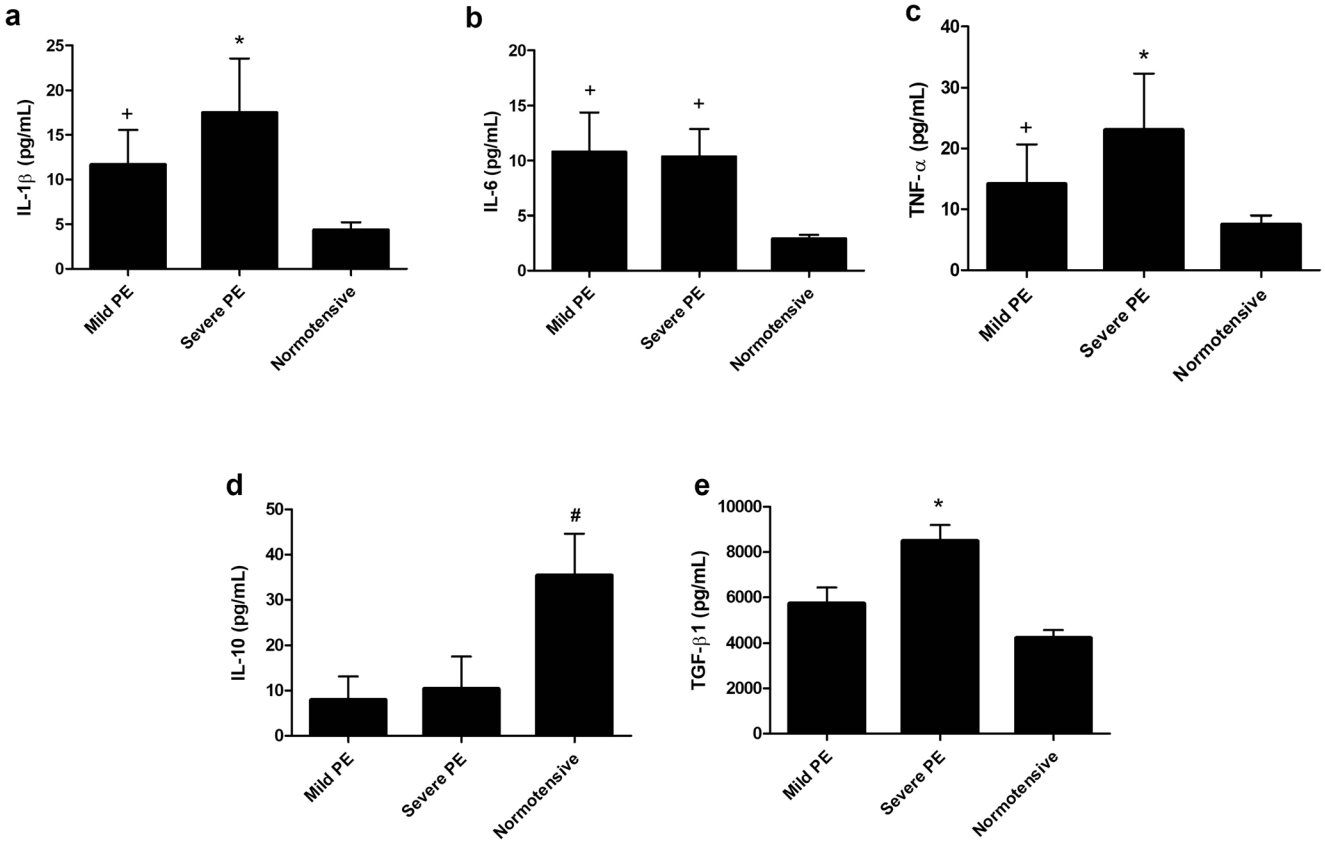

Fig. 2 Concentrations of interleukin-1 beta (IL-1β-a), interleukin-6 (IL-6-b), tumor necrosis factor-alpha (TNF-α-c), interleukin-10 (IL-10-d), and transforming growth factor beta (TGF-β1-e) in plasma of pregnant women with mild PE, severe PE, and normotensive pregnant women. Data are presented as means ± SD. Statistical significance: $*p < 0.05$ vs. mild PE and normotensive; $+p < 0.05$ vs. normotensive; $\#p < 0.01$ vs. mild PE and severe PE (ANOVA)

In our present study, a lower concentration of sCD163 and its negative association with the plasma levels of the inflammatory cytokines IL-1β and TNF-α was observed in women with severe PE. A previous study evaluating sCD163 and neopterin in pregnant women with PE showed that these two serum markers of macrophage activation were not altered in preeclamptic pregnancies [43]. The discrepancy between our results and those from Kronborg et al. [43] might be due to the variations in the techniques used for determining sCD163, differences in population characteristics, and differences in PE severity. Reduced levels of sCD163 in plasma of patients with severe PE could be related to the decreased expression of the CD163 receptor on the monocyte surface, which may be downregulated by the higher levels of IL-1β and TNF-α in the severe PE group. It is well known that endogenous pro-inflammatory cytokines such as IL-1β and TNF-α or chemokines such as IL-8 lead to a decrease in the expression of CD163, whereas IL-10 is responsible for the upregulation of monocyte-macrophage CD163 expression [26, 44]. Thus, the intense inflammatory environment present in PE might be involved in the maintenance of the lower expression of CD163, consequently leading to the decreased shedding of

Table 2 Correlations between concentrations of sCD163 and IL-1β, TNF-α, IL-6, IL-10, and TGF-β1 present in the plasma and CD163 expressed by monocytes of pregnant women with mild PE, severe PE, and normotensive pregnant women

sCD163	Mild PE	Severe PE	Normotensive
IL-1 β	$r = -0.31$ ($p = 0.210$)	*$r = -0.45$ ($p = 0.014$)*	$r = 0.21$ ($p = 0.281$)
TNF-α	$r = -0.32$ ($p = 0.134$)	*$r = -0.54$ ($p = 0.001$)*	$r = 0.32$ ($p = 0.116$)
IL-6	$r = 0.33$ ($p = 0.125$)	$r = -0.25$ ($p = 0.283$)	$r = 0.31$ ($p = 0.183$)
IL-10	$r = 0.23$ ($p = 0.244$)	$r = 0.16$ ($p = 0.445$)	*$r = 0.61$ ($p = 0.001$)*
TGF-β1	$r = -0.28$ ($p = 0.175$)	$r = -0.19$ ($p = 0.512$)	$r = 0.27$ ($p = 0.337$)
CD163	*$r = 0.42$ ($p = 0.003$)*	*$r = 0.56$ ($p = 0.001$)*	*$r = 0.78$ ($p = 0.001$)*

Correlation coefficient (r) was determined by Pearson's correlation. Significant correlation is highlighted in italics, with $P < 0.05$

sCD163 from the monocyte surface and may explain the decrease of power correlation between these two parameters in both groups mild ($r = 0.42$; $p = 0.003$) and severe PE ($r = 0.56$; $p = 0.001$) compared with NT group ($r = 0.78$; $p = 0.001$).

In conclusion, the results of this study show an association between the inflammatory cytokine profile and reduced CD163 expression on the monocyte surface as well as a low plasma concentration of sCD163 in pregnant women with PE. These results were more evident in pregnant women with severe PE and suggest impairment in the modulation of inflammatory response in this group of preeclamptic women. Additional investigations are needed to determine whether sCD163 and mediators of the systemic inflammatory response play a role in the impaired M2 monocyte polarization detected in preeclampsia.

Author contributions M.T.P. and P.R.N. conceived and designed the experiments. P.R.N. and M.R.V. performed the experiments. P.R.N., M.T.P., and M.R.V. analyzed the data and wrote the manuscript. J.C.P., R.A.A.C., L.G.O., and V.T.M.B. recruited the subjects for clinical evaluation and blood collection. All the authors have seen and approved the final version of the manuscript.

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Compliance with ethical standards

This project was approved by the Ethics Committee of the Botucatu Medical School (Protocol number 417.350), and written informed consent was obtained from all women involved in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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