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Brief Conclusive Report

Tumor necrosis factor α-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163

Anders Etzerodt, Maciej Bogdan Maniecki,† Kirsten Møller,‡ Holger Jon Møller,† and Søren Kragh Moestrup*,†,1*

Departments of *Medical Biochemistry, University of Aarhus, and †Clinical Biochemistry, Aarhus University Hospital, Aarhus C, Denmark; and ‡Department of Infectious Diseases, University Hospital Rigshospitalet, Copenhagen, Denmark

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ABSTRACT

CD163 is expressed specifically in the monocyte/macrophage lineage, where it mediates uptake of haptoglobin-hemoglobin complexes, leading to metabolism of the oxidative heme molecule. Shedding of the CD163 ectodomain from the cell surface produces a sCD163 plasma protein, and a positive correlation is seen between the sCD163 plasma level and the severity of various infectious and inflammatory diseases. In the present analysis of the phorbol ester-induced shedding of sCD163 in CD163 cDNA-transfected HEK293 cells, we used metalloproteinase inhibitors and siRNA-mediated inhibition of metalloproteinases to identify TACE/ADAM17 as an enzyme responsible for PMA-induced cleavage of the membrane-proximal region of CD163. As TACE/ADAM17-mediated shedding of TNF-α is upregulated in macrophages subjected to inflammatory stimuli, the present results now provide a likely explanation for the strong empirical relationship between the sCD163 plasma level and infectious/inflammatory diseases relating to macrophage activity. *J. Leukoc. Biol.* 88: 1201–1205; 2010.

Introduction

The scavenger receptor CD163 is a 130-kDa type I transmembrane protein expressed exclusively in cells of the monocytic lineage [1, 2]. The receptor mediates endocytic uptake of haptoglobin-hemoglobin complexes that form upon intravascular hemolysis [3]. This pathway generating anti-inflammatory heme metabolites [4] has linked CD163 to the inflammatory response [5]. Furthermore, CD163 has been reported to mediate intracellular signaling [6] and to function as a receptor for other potential ligands [7], including human pathogenic bacteria [8].

A relatively high concentration of the CD163 ectodomain, consisting of nine scavenger receptor domains, is present in normal plasma [9, 10], and an increased plasma concentration of sCD163 is seen in diseases relating to M2 macrophage activity, such as sepsis and chronic inflammation [11–15]. An association between the sCD163 level and disease severity/mortality has been observed in several conditions, including sepsis [12, 15]. The physiological role of sCD163 is unknown, but it is has been speculated [16] that its binding of haptoglobin-hemoglobin suppresses heme-iron supply to hemolytic bacteria, in line with the inflammation-induced, hepcidin-controlled down-regulation of plasma-iron that may also prevent bacterial growth [17]. Similarly, sCD163 may affect the uptake of haptoglobin-hemoglobin complexes in trypanosomes, which have recently been shown to assimilate the complexes for a supply of heme [18]. On the other hand, sCD163 is a poor competitor for CD163-mediated uptake of haptoglobin-hemoglobin complexes [19], and it is possible that the soluble receptor has functions not related to hemoglobin. Previous studies have reported binding of sCD163 to T-lymphocytes [20], but the physiological implication is unknown.

In accordance with the presence of sCD163 in normal plasma, a constitutive proteolytic shedding of CD163 has been observed in cultured human monocytes [21]. The shedding is strongly induced by inflammatory stimuli such as LPS [16, 22], PMA [21], and FcR cross-linking via activation of TLRs [23]. The PMA-stimulated shedding of CD163 is inhibited by the PKC inhibitor bisindolylmaleimide I [21], as well as the metalloprotease inhibitor TNF protease inhibitor-0 [22] and TIMP-3 [24]. Consequently, the enzyme family of ADAMs [25] and in particular, the TIMP-3-inhibitable ADAM10 and -17 [16, 22, 24, 25] have been considered as inducers of the shedding. In the present study, we have identified the enzyme responsible for CD163 shedding by analyzing how metalloproteinase inhibitors and knockdown of ADAM10 and ADAM17 affect the surface expression and release of recombinantly expressed CD163.

Abbreviations: ADAM=a disintegrin and metalloproteinase domain, HEK=human embryonic kidney, qRT-PCR=quantitative RT-PCR, sCD163=soluble CD163, siRNA=small interfering RNA

1. Correspondence: Department of Medical Biochemistry, University of Aarhus, Ole Worms Allé 3 DK-8000 Aarhus C, Denmark. E-mail: skm@biokemi.au.dk

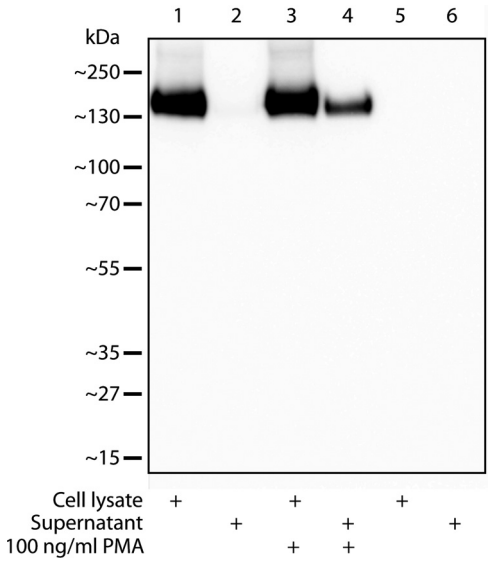

Figure 1. Western blot analysis of sCD163 release in CD163-expressing HEK293 cells. Immunoblot of cell lysate (Lanes 1 and 3) and supernatant (Lanes 2 and 4) from transfected HEK293 cells expressing full-length human CD163, without (Lanes 1 and 2) or with (Lanes 3 and 4) PMA stimulation. Cell lysate (Lane 5) and supernatant (Lane 6) from nontransfected cells were used as negative controls. Immunoreactive bands were visualized using mouse monoclonal anti-CD163 clone Mac2-158 and HRP-conjugated goat anti-mouse IgG.

MATERIALS AND METHODS

Expression of human rCD163 in HEK293 cells

Stably transfected HEK293 cells expressing the human CD163 short tail variant (GenBank Accession No. DQ058615) were established using the FlpIn System (Invitrogen, Taastrup, Denmark).

ELISA, immunoblotting, and flow cytometry

sCD163 was measured by an ELISA assay established already [26]. TNF-α was measured in serum by a commercial ELISA kit (R&D Systems Europe, UK; HSTA00D). Western blotting of cell lysates and cell media was carried out using the anti-human CD163 antibody Mac2-158 (IQ Products, Groningen,The Netherlands) and a secondary HRP conjugate goat anti-mouse IgG antibody (Sigma-Aldrich, Broendby, Denmark). Immunoreactive bands were visualized on a FUJI FLA3000 gel doc (Fujifilm Europe GmbH, Düsseldorf, Germany) using an ECL substrate (Pierce, Rockford, IL, USA).

Flow cytometric analysis was performed as described on monocytic cells [27] using a BD FACSCalibur™ (BD Biosciences, Broendby, Denmark) and PE-conjugated anti-human CD163 antibody Mac2-158 (IQ Products) for detection of CD163.

Preparation of supernatants, single suspension cultures, and cell lysate

To induce shedding of CD163 in culture media, cells expressing CD163 were stimulated in PBS, pH 7.4, with or without 100 ng/ml PMA (Sigma-Aldrich) for 1 h at 37°C. Prior to stimulation, adherent cells were harvested and collected by treating with accutase (Sigma-Aldrich) for 10 min at 37°C. Control FACS analysis of accutase-treated cells and nontreated cells did not reveal any effect on CD163 surface expression. Where indicated, cells were preincubated with 250 μM TIMP inhibitors 1–3 (Sigma-Aldrich) for 30 min at 37°C or siRNA against ADAMs. Cells were pelleted by centrifugation, and supernatants were collected. Cell pellets were used for downstream flow cytometric analysis or lysed in PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) containing EDTA-free protease inhibitor Complete Mini (Roche Applied Science, Hvidovre, Denmark) with 1% Triton X-100

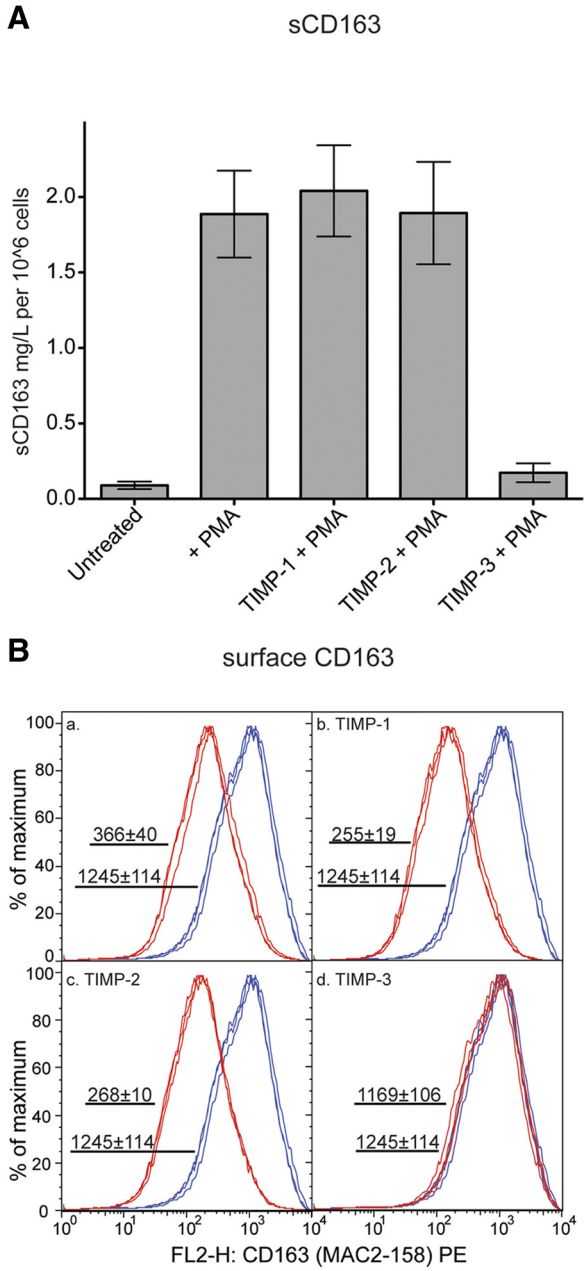

Figure 2. Effect of PMA and TIMP inhibitors on shedding of sCD163 in transfected HEK293 cells. (A) ELISA measurements of sCD163 in the supernatant of cells incubated with and without PMA stimulation and TIMP inhibitors. Bars represent 95% confidence intervals. (B) Flow cytometric analysis of TIMP inhibition on PMA-stimulated shedding of surface CD163 in transfected HEK293 cells. After gating, using FlowJo for Mac, Version 8.8.6 (TreeStar, San Carlos, CA, USA), in forward-scatter- versus side-scatter-plot, cells were replotted in a histogram showing CD163 mAb (PE-Mac2-158) fluorescence. Histograms have an overlay of untreated (blue) versus cells stimulated with PMA (red). Cells were preincubated with no inhibitor (a), TIMP-1 (b), TIMP-2 (c), or TIMP-3 (d). Calculated mean fluorescent intensities are indicated. FL2-H, Fluorescent 2-height.

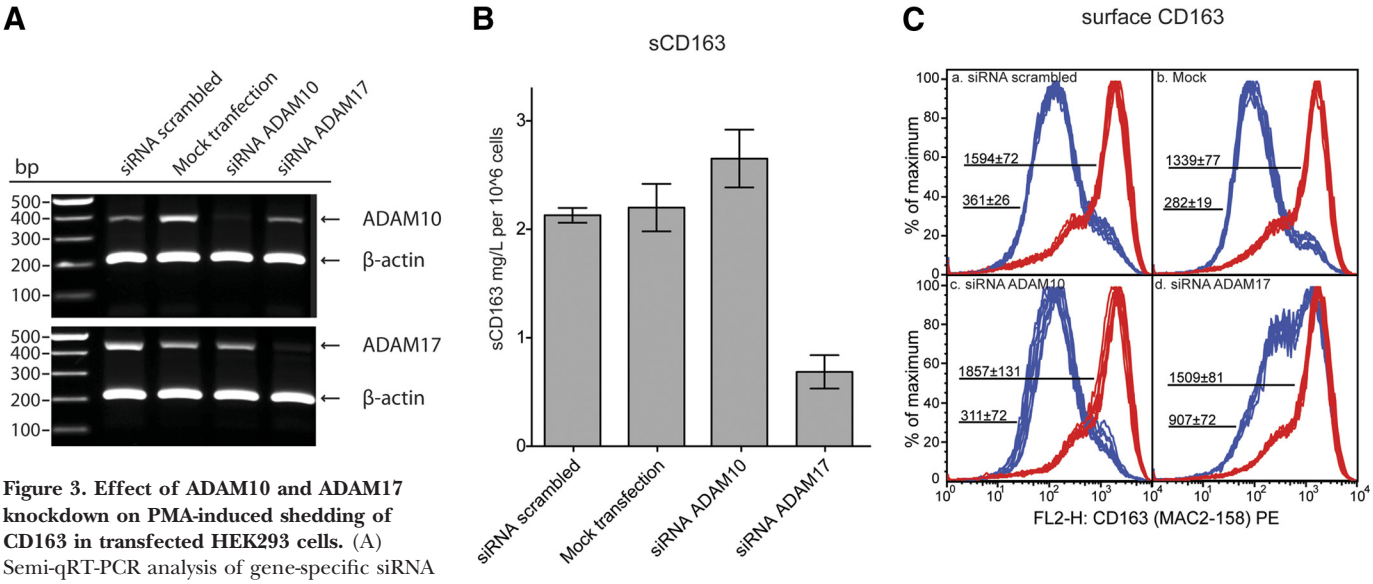

Figure 3. Effect of ADAM10 and ADAM17 knockdown on PMA-induced shedding of CD163 in transfected HEK293 cells. (A) Semi-qRT-PCR analysis of gene-specific siRNA silencing of ADAM10 and ADAM17 in CD163-expressing HEK293 cells, which were mock-transfected or transfected with siRNA against ADAM10, ADAM17, and scrambled. Cells were harvested 48 h post-transfection and subjected to RT-PCR analysis using β-actin as control. (B) ELISA measurements of sCD163 in the supernantant of PMA-stimulated cells transfected with siRNA against ADAM10, ADAM17, or controls. Bars represent 95% confidence intervals. (C) Flow cytometric analysis of surface CD163 on siRNA-transfected cells. After gating, using FlowJo for Mac, Version 8.8.6 (TreeStar), in forward-scatter-versus side-scatter-plot, cells were replotted in a histogram showing CD163 mAb (PE-Mac2-158) fluorescence. Cells were transfected with scrambled siRNA (a), mock (b), siRNA against ADAM10 (c), or siRNA against ADAM17 (d), with (red) or without (blue) PMA stimulation. Calculated mean fluorescent intensities are indicated.

(Merck, Hellerup, Denmark). Lysate was clarified by centrifugation at 14,000 rpm for 10 min at 4°C.

Knockdown of ADAM10 and -17 by siRNA

For siRNA knockdown of ADAM10 and ADAM17, the following ON-TARGET-plus SMARTpool (Dharmacon, Lafayette, CO, USA) siRNA oligonucleotides were used: ADAM17, GAAGAACACGUGUAAAUUA, GCACAAAGAAUUAUGGUAA, UAUGGGAACUCUUGGAUUA, GGAAAUAUGUCAUGUAUCC; ADAM10, CAUCUGACCCUUUAAACCAAA, CAAGGGAAGGAAUAUGUAA, GAACUAUGGGUCUCAUGUA, CGAGAGAGUUAUCAAAUGG. The siRNA transfections were performed according to the Dharmacon protocol. Transfections were incubated for 72 h before inducing shedding.

Semi-qRT-PCR analysis

RT-PCR analysis was done using the RNeasy RNA purification kit (Qiagen, Copenhagen, Denmark), One-Step RT-PCR kit (Qiagen), and gene-specific primers targeted to ADAM10 (400 bp), ADAM17 (421 bp), or β-actin (202 bp) as internal control (ADAM10: sense ACATGCTGCTAATGGTCCAGA, antisense CAACCCAAGCCAGACCAAG; ADAM17: sense GCACACCTTT-

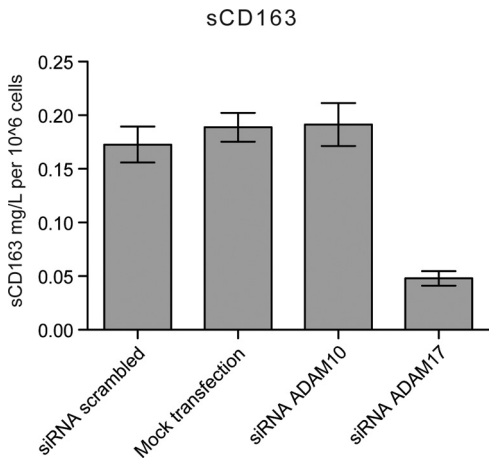

Figure 4. Effect of ADAM10 and ADAM17 knockdown on constitutive shedding of CD163 in transfected HEK293 cells. sCD163 was measured from supernatants of siRNA-transfected cells or mock-transfected using a sCD163-specific ELISA assay. Error bars represent 95% confidence intervals.

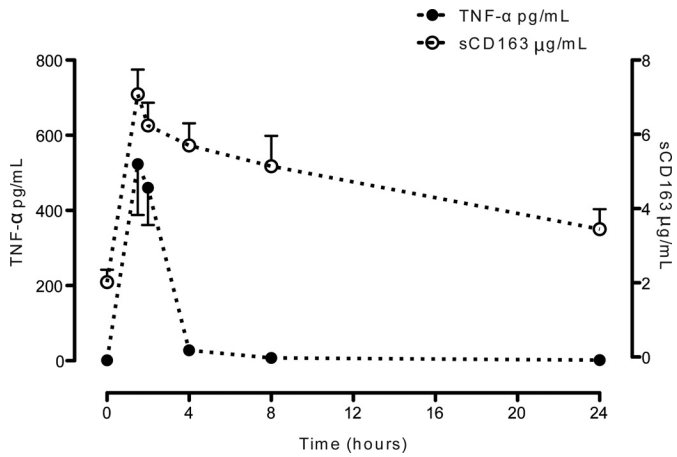

Figure 5. Endotoxemia induces a fast increase in serum sCD163 and TNF-α. Serum levels of sCD163 and TNF-α in human volunteers (n=8) after an i.v. bolus injection of endotoxin/LPS. sCD163 and TNF-α were measured in serum by ELISA.

TCACATACCA, antisense CTCTTCTCCTTCATCCACCCTC; β-actin: sense GGCGGCACCACCATGTACCCT, antisense AGGGGCCGGACTCGTCATACT). Amplified PCR products were separated on 1.0% agarose gels with 1× SYBR® Safe Stain (Invitrogen) and visualized on a FUJI FLA3000 gel doc (Fujifilm Europe GmbH). Intensity of ADAM10 or ADAM17 RT bands was normalized to intensity of β-actin using the Multi Gauge software, Version 3.0 (Fujifilm Europe GmbH). Knockdown was estimated by comparing relative intensities of ADAM10 or ADAM17 in siRNA-transfected cells with mock-transfected controls.

Response on endotoxin in human volunteers

Injection of human volunteers with endotoxin/LPS was carried out as described previously [28]. The study was approved by the Scientific-Ethical Committee of Copenhagen and Frederiksberg Municipalities in Denmark [jr. number (KF) 01-144/98 with amendment (KF) 11-095/00].

RESULTS AND DISCUSSION

To study the CD163 shedding in a model available for a comprehensive analysis, including the use of RNA interference for protein knockdown, we monitored the CD163 shedding in HEK293 cells expressing full-length human rCD163. These cells exhibited a low degree of constitutive release into the medium, whereas stimulation by PMA increased the concentration of sCD163 several-fold (**Figs. 1** and **2A**). This increase in sCD163 corresponds to a strong decrease in surface expression of CD163 (Fig. 2B, a). As demonstrated previously in monocytes [24], the PMA-induced shedding of CD163 in the transfected HEK293 cells is strongly inhibited by the metalloprotease inhibitor TIMP-3, whereas no effect is seen using TIMP-1 and TIMP-2 (Fig. 2). In conclusion, the shedding of CD163 in monocytes and in the CD163 transfectants shows similar characteristics in the sense that shedding is PMA-inducible and inhibited by TIMP-3 but not by TIMP-1 and -2.

ADAM10 and ADAM17 are candidate enzymes for mediating shedding, as both are inhibited by TIMP-3. Moreover, these enzymes are ubiquitously expressed and are also present in the HEK293 CD163 transfectants (**Fig. 3A**). By using siRNA directed against ADAM10 and ADAM17 mRNA, we reduced the mRNA expression of both enzymes to ~80% of controls (Fig. 3A). Knockdown of ADAM17, but not of ADAM10, highly prevented the PMA-inducible shedding of CD163, as shown by flow cytometric analysis of surface CD163 and ELISA measurements of sCD163 in culture medium of unstimulated and PMA-stimulated cells (Fig. 3B and C). In nonstimulated cells, the siRNA-mediated knockdown of ADAM10 or -17 had no significant effect on the level of surface-exposed CD163 (Fig. 3C). However, a reduction in the amount of sCD163 in medium was seen in the ADAM17 knockdowns (**Fig. 4**), thus suggesting that this enzyme also contributes to the constitutive CD163 shedding.

ADAM17 is known to have more than 30 different substrates in a broad spectrum of tissues [29]. One of the most prominent substrates is membrane-bound TNF-α (hence, the alternative name TACE), which is released and activated by ADAM17 cleavage in macrophages. As ADAM17-mediated TNF-α release is stimulated by in vitro proinflammatory stimuli such as LPS, PMA, and FcR cross-linking [29], the present data now provide an explanation for the same stimuli to cause shedding of CD163 in vitro. As released TNF-α is a strong mediator of inflammatory symptoms during conditions such a sepsis and chronic inflammations [30], it is likely that the increased levels of sCD163 measured under these conditions are a result of concomitant ADAM17-induced release of CD163 and TNF-α in macrophages. In the context of inflammation, sCD163 may therefore be regarded as a long-circulating surrogate marker of the rapidly cleared TNF-α released from macrophages. In line with this, LPS injection in humans causes a simultaneous increase of TNF-α and sCD163. Both protein concentrations peak after ~1.5 h [16, 30], but whereas TNF-α is abolished from the circulation after ~3 h, >25% of the LPS-induced increase of sCD163 persists after 24 h (**Fig. 5**).

AUTHORSHIP

A.E. carried out the major part of the experiments and contributed to the study design and paper writing. M.B.M. contributed to the flow cytometry experiments. K.M. contributed to the LPS data. H.J.M. performed the ELISA measurements and reviewed the paper. S.K.M. contributed to study design and paper writing.

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KEY WORDS:
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