Only the soluble form of the scavenger receptor CD163 acts inhibitory on phorbol ester-activated T-lymphocytes, whereas membrane-bound protein has no effect

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Received 18 January 2002; revised 12 July 2002; accepted 23 July 2002

First published online 2 August 2002

Edited by Beat Imhof

Abstract The extracellular moiety of the hemoglobin/haptoglobin scavenger receptor CD163 (RM3/1 antigen) can be shed from monocytes and is a normal plasma component. We found that in a dose-dependent manner soluble CD163 induces a decrease in CD69 expression, a reduced [^{3}H]thymidine uptake and a down-regulated matrix metalloproteinase-9 RNA expression in phorbol myristate acetate-stimulated T-cells. Co-culturing T-cells on transgenic fibroblasts, expressing membrane-bound CD163, yielded no differences compared to culture on non-transfected cells. We conclude that CD163 has at least two distinct functions: the clearance of hemoglobin in its cell-bound form and participation in anti-inflammation as a soluble factor, exhibiting cytokine-like functions.

Key words: Scavenger receptor; Anti-inflammation; CD163; T-Lymphocyte

1. Introduction

CD163, previously described as RM3/1 antigen or M130, belongs to the cysteine-rich scavenger receptor superfamily type B and has recently been identified as a receptor for the hemoglobin/haptoglobin complex [1]. Earlier observations have linked the expression of CD163 on monocytes to anti-inflammatory functions. In vitro, CD163 can be up-regulated by glucocorticoids and interleukin-10 [2,3], whereas proinflammatory stimuli like lipopolysaccharide or interferon-γ suppress its expression [2,4]. In vivo, CD163-positive macrophages are found during the late phase of acute inflammation [5] as well as in chronic inflammation [6] and in wound healing tissue [7]. CD163-positive monocytes were also shown to inhibit proliferation of CD4-positive T-lymphocytes (TL) in co-culture [8], however, nothing is known about a direct participation of CD163 in this process. Another not further characterized anti-inflammatory factor has been described to be secreted by CD163-positive monocytes [9] as well as increased amounts of histamine [10]. Therefore it is still an open question whether expression of CD163 represents just a certain anti-inflammatory phenotype, or the cell-bound molecule directly contributes to anti-inflammatory effects. Recently, it was shown that CD163 can be shed from the cell membrane of glucocorticoid-stimulated monocytes [11] after an inflammatory stimulus and that CD163 is a normal component in the plasma of healthy donors [12,13]. In addition, soluble CD163 has been shown to inhibit phorbol ester-induced proliferation of TL [14].

In this study we show that soluble CD163 acts anti-inflammatory on transcription, surface marker gene expression and growth of phorbol myristate acetate (PMA)-stimulated T-cells, whereas the membrane-bound form has no effect. This is the first evidence that a scavenger receptor can have cytokine-like functions and implicates distinct functions for the membrane-bound and shedded protein.

2. Materials and methods

2.1. Construction of CD163-expressing HEK cells

The cDNA of CD163 was obtained by reverse transcription (RT)-PCR with primers spanning nt 81–103 and 3194–3215 of the published mRNA sequence [15] with attached recognition sites for *Sal*I (forward primer) and *Xho*I (reverse primer). It was then cloned into pBK-CMV (Stratagene) and sequenced (Sequenase 2.0 kit, Amersham). 15 μg of linearized DNA was transfected into 10^7 HEK293 cells by electroporation (pulse 290 V, 950 μF, 40 s), transfected cells were selected with 500 μg/ml G418 for 10 passages. CD163 expression was fluorescence-activated cell sorter (FACS)-analyzed with the monoclonal antibody RM3/1 (our lab) and a FITC-labeled goat anti-mouse antibody (Dianova).

2.2. CD163-containing supernatant and purification of cellular CD163

CD163 was purified from lysates of fluticasone propionate (FP)-stimulated monocytes by affinity columns with the monoclonal antibody RM3/1 as described elsewhere [11]. Purity was controlled by gel electrophoresis and subsequent silver staining or Western blotting using the monoclonal antibodies RM3/1 and Ki-M8 (Bachem, Heidelberg, Germany), a horseradish peroxidase-conjugated secondary antibody (Dako Diagnostics, Hamburg, Germany) and the ECL kit (Amersham Pharmacia Biotech, Freiburg, Germany) (Fig. 1). Alternatively, shedding of CD163 was induced by 10^{-7} M PMA. This supernatant was then washed three times with phosphate-buffered saline (PBS) and 10-fold concentrated in a 50 kDa filter unit (Vivascience).

2.3. Purification of recombinant CD163

The extracellular moiety of CD163 was amplified by PCR and cloned into the expression vector pcDNA6/V5-His (Invitrogen, Karlsruhe, Germany). HEK293 cells were transfected as described above,

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Abbreviations: FP, fluticasone propionate; MMP, matrix metalloproteinase; PMA, phorbol myristate acetate; TL, T-lymphocyte

0014-5793/02/$22.00
PII: S0014-5793(02)03142-3

the supernatant was investigated by Western blot analysis to confirm the integrity of the recombinant protein. Purification of the recombinant protein was performed by binding of the carboxy-terminal His-Tag to Talon™-Matrix, according to the manufacturer's protocol (BD Biosciences Clontech, Palo Alto, CA, USA). Purity was then controlled by gel electrophoresis and subsequent silver staining and Western blotting (Fig. 1). Densitometric analysis of both the silver-stained and the Western blotting bands (Adobe Photo Shop software) showed a decreased (64%) antibody binding to the recombinant protein, as compared with the CD163 purified from cell lysates. This reduced antibody binding was not observed with non-purified recombinant CD163 (not shown) and is therefore probably related to the purification protocol, which includes a drastic pH change for elution of the protein.

2.4. Purification of TL

T-cells from healthy donors (Blutbank, University of Münster, Münster, Germany) were purified from the lymphocyte fraction obtained after Ficoll and subsequent Percoll density gradient centrifugation. Contaminating monocytes and B-lymphocytes were allowed to adhere to a nylon matrix as previously described [16], eluted cells were about 80% CD4-positive and more than 98% CD14-negative as determined by FACS analysis. Cells were always held in RPMI 1640 medium supplemented with 10% fetal calf serum and standard amounts of antibiotics.

2.5. Inhibition of CD69 expression

5×10^5 TL were incubated in 96-well plates in the presence of different concentrations of CD163, either 10% 10-fold concentrated CD163 supernatant, purified CD163 or recombinant CD163, and PMA (10^{-7} M for 3 h or 10^{8} M for 20 h). If indicated, the monoclonal antibody RM3/1 was supplemented at a concentration of 2.5 μg/ml. CD69 expression was determined by FACS with a FITC-conjugated antibody (Dako Diagnostics, Hamburg, Germany). For co-culture experiments 10^6 TL were incubated on $>95\%$ confluent HEK cells (pre-treated with 100 μg/ml mitomycin C for 3 h) in 24-well plates. The increases of fluorescence were calculated by subtracting the values of non-stimulated cells, using the Lysis-II software. Mean values of multiple independent experiments ($n\geq3$) were then statistically analyzed, significance in this study was always confirmed by a Student's t-test.

2.6. Proliferation assay

Proliferation of activated TL was determined by incorporation of tritium-labeled thymidine. 5×10^5 TL were incubated with 10^{-9} M PMA, 10^{-8} M FP and different concentrations of CD163 and the monoclonal antibody RM3/1, as indicated, for 43 h. In a pre-experiment 10^{-9} M PMA had shown to be sufficient to initiate proliferation of TL under these conditions. Then 1 μCi of [^{3}H]thymidine was added. After 5 h the cells were harvested, washed with PBS three times and lysed by three freeze/thaw cycles. Counts per minute were then determined in a scintillation counter. For co-cultivation HEK cells were pre-treated as described above.

2.7. Semi-quantitative RT-PCR

PCR conditions and primers were taken from Giambernadi et al. [17]. Briefly, 2×10^5 TL were incubated with PMA, FP (10^{-8} M each) and different concentrations of CD163, as indicated, for 24 h. Then total RNA was extracted with the RNeasy kit (Qiagen, Hilden) according to the manufacturer's instructions. 500 ng of RNA was reverse-transcribed (Superscript, Gibco, volume 20 μl), 6 μl was then used as template for a 120 μl PCR mix, which was divided into six 20 μl probes. From cycle 15 on one probe was taken out of the cycler every five cycles and analyzed by agarose gel electrophoresis.

3. Results and discussion

Expression of CD69 is accepted as an early marker for qualitative and quantitative analysis of TL activation [18]. Fig. 2 shows the proportional increase in CD69 expression after stimulation of TL with PMA and the inhibition by soluble CD163. CD163-containing supernatant of FP-stimulated, washed, and PMA-treated monocytes was used after diluting and concentrating the solution to eliminate the PMA. The mean inhibition was 29.4%. To exclude the influence of contaminants, antibody-purified CD163 at a concentrations of 0.1 and 0.5 μg/ml as well as recombinant CD163 at a concentration of 0.5 μg/ml were used in the same assay and exhibited a mean inhibition of 15.6, 23.6 and 9.3%, respectively. Addition of the monoclonal antibody RM3/1 (2.5 μg/ml) reduced the effect of the CD163-containing supernatant from 29.4 to 8.3%. Although stimulation with 10^{-7} M PMA for 3 h generally yielded lower CD69 expression than with 10^{-8} M PMA for 20 h, the relative inhibition by CD163 was in the same range. The fact that the recombinant CD163 did not yield as striking effects as the purified CD163 from cell lysates might be related to the observation that there is a reduced antibody binding to the recombinant CD163 after purification (see Materials and methods). The purification procedure includes a pH 5.5 step to elute the protein from the matrix, this might have modified the structure of the protein. Therefore, the recombinant protein was not used in further experiments.

To elucidate the question if membrane-bound CD163 has the same effect, HEK293 cells were stably transfected with the vector pBK-M130. After selection with G418 for 10 passages we received a population of cells expressing similar amounts up to 10-fold more CD163 as FP-stimulated monocytes, as determined by FACS analysis (data not shown). The last two bars of Fig. 2 show that co-culturing of PMA-stimulated TL with these CD163-expressing HEK cells yields no difference in CD69 expression, compared to co-culture with non-transfected cells.

To confirm the inhibitory effect of solely the soluble form of CD163 we investigated the thymidine uptake of TL after PMA stimulation (Fig. 3a). Similar to the previous results, TL activation is suppressed in a concentration-dependent manner: CD163 in a concentration of 0.1 μg/ml leads to a slight but not significant inhibition ($P>0.1$), whereas the increase of thymidine uptake after PMA stimulation in the presence of 0.5 μg/ml CD163 is diminished to 61% ($P<0.05$). Although this effect is not as dramatic as the suppression observed by addition of FP (a reduction to 17.6%), it can be partly reversed by adding 2.5 μg/ml RM3/1 (81.7%) and thus confirms the inhibitory impact of CD163 on activated TL.

Fig. 3b shows that no difference in thymidine uptake is observable when seeding PMA-stimulated TL on CD163-transfected or untransfected HEK cells. Although the HEK cells were mitomycin-treated, they still showed some thymidine incorporation, with no difference between transfected and non-transfected cells. FACS analysis during the experiments confirmed stable CD163 expression under the experimental conditions (not shown). During the co-culture 20–30% of the feeder cells were killed (as determined by propidium iodide staining), however, no CD163 was shed from the cells and could be identified in the medium by Western blotting (not shown).

These data show that the inhibitory effect is limited to the soluble form of CD163. Membrane-anchored protein has no effect in this study, although it can mediate hemoglobin/haptoglobin incorporation as shown before [1]. Therefore, the previous observation that CD163-positive monocytes inhibit the proliferation of activated TL [8] is probably triggered by factors which are associated with the CD163-positive phenotype and not with the membrane-bound CD163 itself. The observed significant but low inhibition of TL activation by

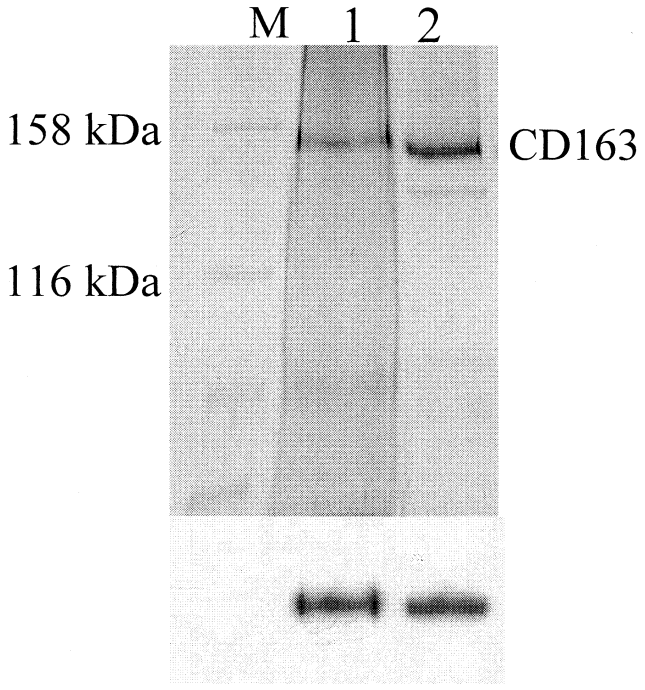

Fig. 1. Analysis of purified CD163 with silver staining (top) and Western blotting (bottom). M: Marker. 1: CD163 purified from cell lysates. 2: Recombinant CD163 after Talon purification. Although the intensity of band 1 is lower after silver staining, compared with 2, it is stronger in the Western blot analysis.

soluble CD163 also suggests a synergistic effect together with other factors. Together with the previous observation that inflammatory stimuli induce the shedding of CD163, we propose the following model: in the late phase of an inflammation, CD163-positive macrophages migrate to the corresponding tissue. Once they have contact to inflammatory factors, the CD163 is shed to act synergistically with other mediators of anti-inflammation. In the intercellular space the concentration of CD163 should be high enough to mediate certain functions in the present, inflammatory primed, T-cells and help to restore the normal, healthy conditions.

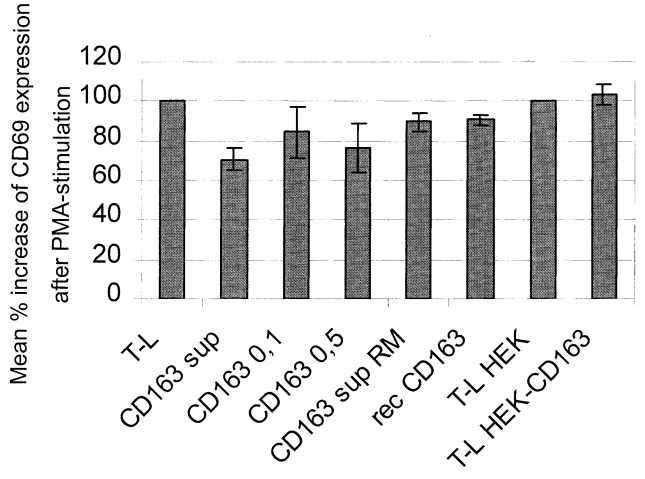

Fig. 2. Inhibition of CD69 expression on PMA-activated TL by soluble CD163. TL were stimulated with 10^{-7} M PMA for 3 h and 10^{-8} M for 20 h in the presence of either CD163-containing supernatant (±the monoclonal antibody RM3/1) from FP-stimulated monocytes after PMA-induced shedding, two different concentrations of purified CD163 from monocyte lysates or recombinant CD163. The mean increase in CD69 expression, determined by FACS analysis, after PMA stimulation alone in comparison to unstimulated cells is defined as 100% (T-L). The last two bars show the CD69 expression on CD163-expressing or native HEK293 cells after PMA stimulation. Sup: Supernatant, RM: RM3/1 2.5 μg/ml, CD163 0,1/0,5: 0.1 or 0.5 μg/ml, rec CD163: recombinant CD163 0.5 μg/ml.

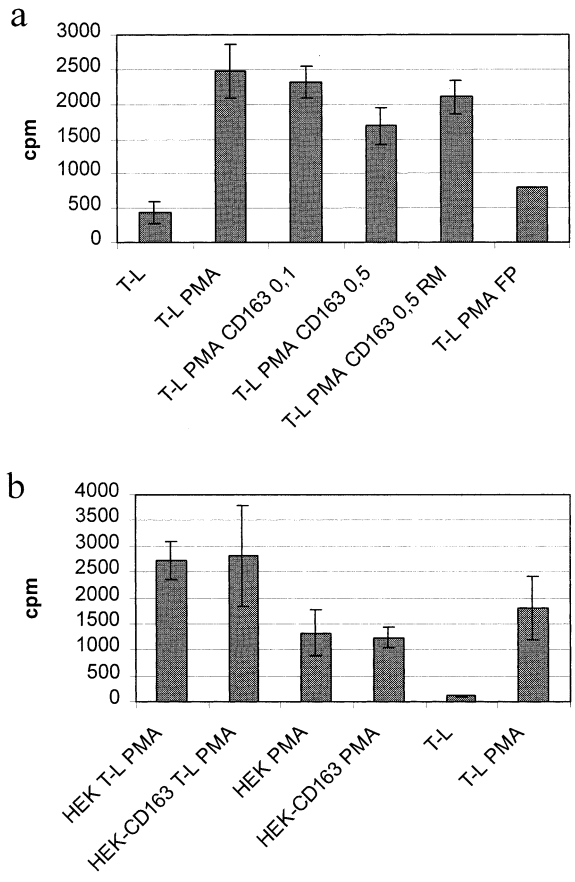

Fig. 3. Reduction of thymidine incorporation into PMA-activated TL is mediated solely by soluble CD163. TL were incubated for 2 days with 10^{-9} M PMA and (a) different concentrations of purified CD163 or (b) co-cultured on native or CD163-expressing, mitomycin-treated HEK293 cells. Cells were chased with [^{3}H]thymidine and analyzed in a scintillation counter. T-L: T-lymphocytes, cpm: counts per minute, 0,1/0,5: 0.1 or 0.5 μg/ml, RM: monoclonal antibody RM3/1, FP: fluticasone propionate.

As soluble CD163 is a normal plasma protein, its function there might also be to suppress TL and take part in the regulation of immune responses under healthy conditions. To investigate this, we measured the RNA expression of gelatinases, which are necessary for transmigration through vessel walls [19]. The gelatinase matrix metalloproteinase (MMP)-9 is constitutively expressed in TL with an enhanced expression during activation of the cells [20]. In contrast, MMP-2 needs adherence of TL to endothelial cells to be expressed, but is upregulated by monocytes/macrophages after inflammatory stimuli and is therefore suitable as a control for the purity of the investigated TL [21,22]. We performed a semi-quantitative RT-PCR for these transcripts after PMA stimulation and found a concentration-dependent inhibition of MMP-9 expression, mediated by CD163 (Table 1). Even the lower concentration (0.1 μg/ml) mediated the same gene expression as the anti-inflammatory glucocorticoid FP. CD163 alone

Table 1
Down-regulation of MMP-9 expression by CD163

	Distinct band after cycle number			
	β-Actin	MMP-2	MMP-9	Rating
TL	20	–	35	++
TL+PMA	20	–	30	+++
TL+PMA+FP	20	–	35	++
TL+PMA+CD163 0.1 μg/ml	20	–	35	++
TL+PMA+CD163 0.5 μg/ml	20	–	40	+
TL+CD163 0.5 μg/ml	20	–	(40) slight band	(+)

TL were incubated in the presence of PMA and FP (10^{-8} M each) or different concentrations of CD163. A semi-quantitative RT-PCR was performed with probes for β-actin and MMP-2 as controls.

(0.5 μg/ml), which was used as a control without PMA, was able to reduce the basic MMP-9 expression to a level which was hardly detectable by this method. MMP-2 expression could not be observed in our assay, indicating the absence of contaminating macrophages in the samples. The normal level of soluble CD163 in plasma has recently been shown to be in the range of 0.7–4.69 μg/ml [13]. Thus, the here observed dose-dependent effects might be even more distinct under natural conditions. However, this remains speculative, as further experiments with higher concentrations could not be performed due to limiting amounts of purified CD163.

In this study we show that the hemoglobin scavenger receptor CD163 also has cytokine-like functions, which have never been described for a scavenger receptor before. However, sequencing and analysis of the human genome [23] pointed out that there are fewer genes existing than estimated before. Therefore, it seems not unlikely that certain gene products can have a tremendously variable function. Probably many more of such diverse functions of a unique gene product will be observed in the future.

As only soluble CD163 has an inhibitory function on the activation of TL, it is likely that TL have a specific receptor which mediates the uptake of CD163, as binding to the receptor alone on transfected, CD163-expressing cells initiates no signal. Identification of this receptor would be of great help to understand the intracellular reactions in TL mediated by CD163, and further studies will have to show how CD163 fits into the existing network of the numerous inflammatory and anti-inflammatory factors.

Acknowledgements: The technical assistance of Heike Berheide and Ulla Nordhues is greatly appreciated. We thank Claus Kerkhoff for helpful discussions and Wolfgang Nacken for critical reading of the manuscript.

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