

Identification of the Integral Membrane Protein RM3/1 on Human Monocytes as a Glucocorticoid-Inducible Member of the Scavenger Receptor Cysteine-Rich Family (CD163)¹

Petra Högger,^{2*†} Jens Dreier,^{*} Anne Droste,^{*} Friedrich Buck,[‡] and Clemens Sorg^{*}

The RM3/1 Ag is a membrane glycoprotein restricted to human monocytes and macrophages that evolve in the late phase of inflammation. Peptide sequence analysis of the RM3/1 protein revealed similarity to CD163, a member of the scavenger receptor cysteine-rich family. Using specific Abs (RM3/1, Ki-M8), we demonstrate an identical cellular regulation for the RM3/1 and the CD163 protein. Most notably, we show for the first time that CD163 is significantly up-regulated by glucocorticoids. In contrast, the protein is down-regulated by the immunosuppressant cyclosporin A and by phorbol esters, while the inflammatory mediator LPS has no significant influence on the expression. We describe the first isolation of a full-length cDNA of CD163 and expression of the corresponding protein. Several splice variants of CD163 exist, and we elucidated the kinetics of induction of three major mRNA splice variants by fluticasone propionate; another splice variant was proved to be unresponsive to this glucocorticoid. Taken together with a previous result showing an involvement of RM3/1 in adhesion of monocytes to the activated endothelium, we discuss that CD163 might play an important role in inflammatory processes. *The Journal of Immunology*, 1998, 161: 1883–1890.

The RM3/1 mAb was developed in search of specific differentiation markers for mononuclear phagocytes (1). The surface Ag detected by this Ab was found to be expressed exclusively on human monocytes and macrophages, predominantly in the late phase of inflammatory processes, e.g., in experimental gingivitis (2) and in allergic contact dermatitis (1). Elucidation of the tissue distribution of the RM3/1 Ag revealed a high abundance in the human term placenta (3). It is present in skin histiocytes, livers' Kupffer cells, spleen macrophages of the red pulp, and some thymus macrophages. In addition, it is found regularly in acute and chronic inflammatory lesions (1).

The Ag is present on the surface of 15 to 30% of freshly isolated monocytes of healthy donors. Both the Ag density and the number of positive cells can be increased significantly in vitro within 48 h of incubation with dexamethasone. In contrast, inflammatory mediators such as LPS or TPA³ were not able to enhance RM3/1 expression (4, 5). Up-regulation of the RM3/1 Ag was also demonstrated in vivo: injection of corticosteroids into primates (6) or human volunteers (4) resulted in an increase of RM3/1-positive blood monocytes up to 80% within 6 h. Induction of the RM3/1 protein was shown to be mediated by glucocorticoid receptors since a glucocorticoid antagonist inhibited up-regulation (5).

Biochemical characterization of the RM3/1 Ag revealed a 130/150-kDa glycoprotein under nonreducing and reducing SDS-PAGE conditions, respectively. The carbohydrates were N-linked and the glycoportion contributed about 25 kDa to the apparent molecular mass (5).

During inflammatory processes, migration of blood mononuclear leukocytes into tissues is an essential step. The recruitment of monocytes requires specific cell adhesion molecules (for review, see Ref. 7). The adhesion of different human monocyte subsets to vascular surfaces has been described for different monocyte subsets (8). RM3/1-positive monocytes exhibited pronounced adhesion to endothelial cells stimulated by IL-6 (8). Blocking experiments with the RM3/1 Ab suggested that the RM3/1 Ag, together with CD14, is involved in the adhesion of monocytes to activated endothelial cells (9).

Along with these findings, monocytes expressing the RM3/1 Ag were reported to produce an antiinflammatory factor (10, 11). This observation is coherent with earlier suggestions that the RM3/1-positive monocytes might be associated with the process of down-regulation of inflammation.

Due to the restricted availability of human monocytes, isolation and partial sequencing of the RM3/1 Ag were not feasible to date. Therefore, we developed an improved isolation procedure based on the addition of divalent cations to the solubilization mixture. The data reported in this work identify the RM3/1 protein as a member of the scavenger receptor cysteine-rich (SRCR) superfamily. Although several members of this ancient and highly conserved family have been described, possible functions of most of these proteins remain speculative (for review, see Ref. 12). We now report that the protein designated as CD163 (13) is identical with the glucocorticoid-inducible RM3/1 Ag. This is the first study showing regulation of a SRCR family member type B by various stimulants. In addition, we demonstrate the kinetics of the mRNA induction by the glucocorticoid fluticasone propionate of four major splice variants of the CD163 mRNA (14). We also describe the expression of the rCD163 protein after transfection of the predominant full-length clone into a mammalian cell line.

Institutes of ^{*}Experimental Dermatology and [†]Pharmaceutical Chemistry, Westfälische Wilhelms-University Münster, Germany; and [‡]Institute of Cell Biochemistry and Clinical Neurobiology, University of Hamburg, Hamburg, Germany

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² Address correspondence and reprint requests to Dr. P. Högger, Institut für Experimentelle Dermatologie, Westfälische Wilhelms-Universität, Von-Esmarch-Str. 56 48149 Münster, Germany. E-mail address: hogger@uni-muenster.de

³ Abbreviations used in this paper: TPA, 12-O-tetradodecanoyl-phorbol-13-acetate; BCIP, 5-bromo-4-chloro-indoyl-phosphate; CHO, Chinese hamster ovary; DIG, digoxigenin; NBT, nitroblue-tetrazolium-chloride; SRCR, scavenger receptor cysteine-rich; TFA, trifluoroacetic acid.

Materials and Methods

Isolation of blood monocytes

Monocytes were isolated from pooled buffy coats (Blutbank, Münster, Germany) by Ficoll-Paque (Pharmacia, Freiburg, Germany) and subsequent Percoll (Pharmacia) density-gradient centrifugation. The monocytes purity was >90%, as quantified by FACScan analysis. Monocytes were cultured at a density of 2×10^6 cells/ml in hydrophobic Teflon bags (Heraeus, Hanau, Germany) in McCoy's 5a medium (Biochrom, Berlin, Germany) supplemented with 15% FCS.

Stimulation of monocytes

Monocytes for FACScan analysis were cultivated for 16 h in presence of 10^{-7} M dexamethasone (Sigma, Deisenhofen, Germany) or 10^{-8} M of the more potent fluticasone-17-propionate (a generous gift from Glaxo Wellcome, Greenford, U.K.) (5).

Stimulation with cyclosporin A (Sigma) was performed for 16 h at a concentration of 0.1 μ g/ml; stimulation with the phorbol ester TPA (Sigma) was performed for 1 h at a concentration of 10 nM.

Isolation of the RM3/1 Ag

Cells were lysed and solubilized in a one-step procedure. Monocytes were incubated with 1 to 10 mM Pefabloc SC (Boehringer Mannheim, Mannheim, Germany), 1 mM CaCl_2 , 1 mM MnCl_2 , and octylthioglucopyranoside (Sigma) at a protein-detergent ratio of 0.2 for 30 min at room temperature under gentle rotation. After centrifugation, the supernatant was collected. The protein extract was separated by preparative SDS-PAGE under reducing conditions, according to Laemmli (15), using an 8% running gel. Gels were stained with 0.1% Coomassie brilliant blue R250 (Sigma), and slices containing the RM3/1 Ag (5) were excised and prepared for sequencing.

Protein sequencing

In-gel digestion was conducted according to the procedure described by Eckerskorn and Lottspeich (16), with the following modifications: the gel pieces were not lyophilized before digestion, but incubated in reaction buffer (25 mM Tris-HCl, pH 8.5, 1 mM EDTA) for 30 min at 37°C. Digestion was achieved overnight at 37°C with 1 μ g of proteinase LysC (sequencing grade; Boehringer Mannheim) in reaction buffer. The reaction was stopped by the addition of 1 μ l trifluoroacetic acid (TFA), the supernatant was collected, and the gel pieces were subsequently incubated for 1 h with 100 μ l of reaction buffer, TFA/acetonitrile (50:50), and acetonitrile. The supernatants were combined, concentrated to a volume of about 100 μ l in a vacuum concentrator, and extracted twice with an equal volume of heptane/isoamyl alcohol (4:1) to remove traces of SDS. The peptides were then separated by narrowbore HPLC (130A; PE Applied Biosystems, Weiterstadt, Germany) on a reversed phase column (Vydac C4; 300 Å pore size; 2.1×250 mm). Peptides were eluted by a linear gradient (2 to 80% B in 45 min; A, water/0.1% TFA; B, 70% acetonitrile/0.85% TFA; flow rate, 200 μ l/min). Peptide-containing fractions detected at 210 nm were collected manually and subjected to rechromatography on a second reversed phase column (Nucleosil C8; 300 Å; 1.6×250 mm; gradient as above; flow rate, 130 μ l/min). Protein sequences were determined by standard Edman degradation on an automatic sequencer (473A; PE Applied Biosystems).

SDS-PAGE, Western blot, and dot blot

Proteins were separated by SDS-PAGE under reducing conditions, according to Laemmli (15), using an 8% running gel. For nonreducing and non-denaturing conditions, mercaptoethanol or DTT (both Sigma) was omitted, and the SDS concentration in the sample buffer was reduced to 0.1%.

For Western blotting, the nonstained gel was transferred to Protran nitrocellulose membrane (0.45 μ m; Schleicher and Schüll, Dassel, Germany) in a semidry blotting unit (Höfer/Pharmacia) applying 0.8 mA/cm² membrane. Subsequently, proteins were detected immunochemically. Nonspecific binding to the membrane was blocked by incubation with 1% skim milk powder in PBS for 1 h under gentle shaking. After washing the membranes with PBS, the primary Ab (12 μ g/ml) in TBS buffer (50 mM Tris-HCl, pH 7.6, 145 mM NaCl) containing 0.1% (BSA) was added for 1 h. Subsequently, membranes were washed with TBS containing 0.05% Tween-20 (TBST) and incubated with the alkaline phosphatase-labeled secondary Ab goat anti-mouse IgG1 (75 ng/ml in TBS) for another hour. After washing of the membrane with TBST and HP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl_2), colorimetric detection was performed by addition of 0.15 mg/ml BCIP and 0.3 mg/ml NBT in HP buffer.

Dot blots were conducted according to the Western blot protocol, but proteins were applied directly onto the nitrocellulose membrane without preceding gel electrophoresis. Densitometric analysis of dot intensities was performed after scanning of the membrane using the National Institutes of Health ImageQuant software.

Antibodies

The mAb RM3/1 was previously generated and characterized by our group (1). The Ki-M8 Ab, mouse anti-human phagocytic macrophage II, was purchased from Bachem Biochemica (Heidelberg, Germany). The mouse isotype control IgG1 and the FITC-, Texas Red-, and alkaline phosphatase-labeled secondary Abs (goat anti-mouse IgG1) were purchased from Dianova (Hamburg, Germany).

FACScan analysis

For indirect immunofluorescence analysis, monocytes were washed with cold PBS, and incubated with 1% BSA for 30 min at 4°C. Then cells were incubated with the primary Ab (3 μ g/ml) for 45 min at 4°C. Mouse IgG1 was included as isotype control at the same concentration. Subsequently, monocytes were washed with PBS and incubated with FITC-labeled secondary Ab goat anti-mouse IgG1 in 1% BSA for 30 min at 4°C. Propidium iodide (1 mM in PBS) was added for the last 2 min of incubation to determine cell viability and exclusion of dead cells. The fluorescence intensity of 10^4 vital cells was measured by FACScan analysis (Becton Dickinson, Heidelberg, Germany). The parameters used were 488 nm excitation wavelength, 250 mW, and logarithmic amplification. The Ag density and the number of RM3/1-positive cells corrected for isotype control were obtained from the main fluorescence channel at 510 to 530 nm using Lysis software (Becton Dickinson).

Cell culture and transfection of CHO cells

CHO DUKX B1 cells (ATCC CRL 9010) were maintained in alpha medium with desoxy- and ribodesoxynucleosides (Life Technologies, Gaithersburg, MD) complemented with 10% FCS (PAA Laboratories, Linz, Austria) and 2 mM glutamine (Biochrom). Cells were transfected using the cationic lipid reagent Dotap (Boehringer Mannheim). Transfection was performed as described in the product protocol. Briefly, 5×10^5 cells were seeded onto 60-mm dishes 1 day before transfection. Immediately before transfection, cells were rinsed with medium without FCS. For each 60-mm dish, 5 μ g of plasmid DNA (clone CD163A-6) and 30 μ l Dotap were diluted to 50 μ l and 100 μ l, respectively. The diluted DNA and Dotap were mixed gently and incubated for 15 min at room temperature. The DNA/Dotap mixture was then diluted with 3 ml medium without serum and added to the cells. After incubation at 37°C in humidified CO₂ incubator for 2 h, 3 ml of Ham's F12 medium with 20% FCS was added. The cells were fixed for immunofluorescence after further incubation at 37°C for 24 h.

Immunofluorescence

For immunofluorescence studies, transfected CHO cells were plated on coverslips and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. For localization of transiently expressed rCD163, cells were incubated with 4 μ g/ml Ki-M8 in blocking solution for 60 min at room temperature. This was followed by a 20-min incubation with 1:150 Texas Red-conjugated anti-mouse IgG (stock concentration 1.5 mg/ml) in blocking solution. Cells were viewed using a fluorescence microscope (Axioskop; Zeiss, Jena, Germany); the enlargement scale was 1:8750.

Northern hybridization

Total RNA was prepared from fluticasone propionate-stimulated and control monocytes, as previously described (17). A quantity amounting to 10 μ g of total RNA per sample was fractionated on 1% agarose formaldehyde gels and transferred to Hybond N nylon membranes (Amersham, Arlington Heights, IL) with $20 \times \text{SSC}$ ($1 \times \text{SSC}$: 0.15 M NaCl and 15 mM sodium citrate) using a LKB 2016 VacuGene blotting apparatus. Antisense RNA probes for Northern hybridization were generated by the DIG RNA labeling kit (Boehringer Mannheim) using linearized DNA templates and T7 RNA polymerase, as described by the manufacturer. Prehybridizations were performed at 68°C for 1 h in a high SDS hybridization buffer (7% SDS, $5 \times \text{SSC}$, 50% formamide, 50 mM sodium phosphate, pH 7, 2% casein, and 0.1% *N*-lauroylsarcosine). The heat-denatured probe (10 min at 95°C) was added to the prehybridization solution (100 ng/ml), followed by gentle agitation at 68°C for 16 h. The nylon membranes were washed twice for 10 min at room temperature in a $2 \times \text{SSC}$, 0.1% SDS solution, and twice for 15 min at 68°C in a solution consisting of $0.1 \times \text{SSC}$ and 0.1% SDS. The hybridization results were visualized by chemiluminescent detection

Table I. Sequences of five RM3/1 peptides after LysC digestion and their positions in the published CD163 sequence

Determined Peptide Sequence	Published Sequence ^a	Position no.
ELRLVDGENK	KELRLVDGENK	45–54
XSYQVYSK	KTSYQVYSK	417–424
XXMXP	KAMSIPT	878–883
A/X M/P D/X I/T P/L M/X Q/X V/X P/(D)	KGPDTLWQCP	893–901
RLAXPXE (E)	KRLASPSSE	908–915

^a Sequence from Reference 14. Amino acids which could not be determined with certainty are marked with "X."

with anti-DIG F(ab')₂ fragments conjugated with alkaline phosphatase and substrate CSPD, as described by the manufacturer (Boehringer Mannheim). Equal loading of samples was controlled by hybridization of RNA with an actin antisense RNA probe.

Construction and screening of a monocyte cDNA library

Human monocytes were cultured in Teflon bags at cell densities of 2×10^6 /ml, as previously reported (1). After 12 h of incubation, cells were stimulated with fluticasone propionate (10^{-8} M) and cultured for 1 day. Pure monocyte populations were isolated by immunomagnetic separation using Dynabeads M-450 CD14, succeeded by direct poly(A⁺) RNA isolation with Dynabeads oligo(dT)₂₅ (DynaL GmbH, Hamburg, Germany), according to the instructions of the manufacturer.

Construction and ligation of cDNA into the vector Lambda Uni-ZAP Express were prepared according to the manufacturer's specifications (Stratagene, Heidelberg, Germany). The DNA was packaged by Gigapack III gold extract and amplified in *Escherichia coli* strain XL1-Blue MRF'. An amplified library with 6.5×10^5 independent clones and a mean insert size of 3.5 kb (0.5 to 6 kb) was obtained.

The library was spread as a monolayer on the XL1-MRF' strain with a titer of $\sim 2.5 \times 10^4$ clones/138-mm plate, and the plaques were subsequently transferred to Hybond N filters (Amersham). The filters were screened with a DIG-labeled PCR fragment, generated by RT-PCR with primers CD163un 5'-CCCGTCGACAATGAGCAAACCTCAGAATG GTG-3' (CD163A, positions 86 to 107) CD163ln 5'-CCCGTCCTTG GAATTTGATCTCTATT-3' (CD163A, positions 599 to 623). Filters were washed at high stringency (0.1 × SSC, 0.1% SDS, 68°C). Colorimetric detection was performed with NBT and BCIP. The resulting 29 positive phages were plaque purified, and the pBK-CMV (Stratagene) phagemid vectors containing CD163 inserts were recovered by in vivo excision, according to the manufacturer's instructions. After restriction endonuclease mapping of the cDNA clones, the nucleotide sequences were established by sequencing both strands of the DNA insert. Two full-length cDNA clones were obtained, representing the coding region of the major variant of CD163 (accession number Z22968 (14)). The clone CD163A-6 (positions 38 to 3703) was used for transfection and expression experiments.

cDNA synthesis and RT-PCR

cDNA was synthesized from 2.5 μg of total RNA with oligo(dT)_{12–18} (Pharmacia) as template primer using M-MuLV reverse-transcriptase Superscript II (Life Technologies), as described by the manufacturer. The reaction was conducted in a final volume of 50 μl containing 1 μl of the transcribed cDNA probe (5%), 200 μM of each dNTP, 1 × PCR buffer including 1.5 mM MgCl₂ (TaKaRa Biomedicals, Shiga, Japan), 0.4 μM forward and reverse primers, and 2.5 U *Taq* polymerase (TaKaRa) covered with two drops of mineral oil (M 3516; Sigma). All CD163 amplifiers were amplified simultaneously with β-actin as internal standard (multiplex PCR). Forward and reverse primers for β-actin amplification were chosen from different exons to detect a possible contamination with nuclear DNA, resulting in PCR products of 222 bp (cDNA) and 430 bp (nuclear DNA), respectively. The respective primer pairs were for human β-actin (GenBank-EMBL accession number M10277): β-ACTu (5'-TTCCAGCCTTC CTCC-3'; positions 2449 to 2464 (exon 3)) and β-ACTi (5'-TTGCCT CAGGAGGAGCAA-3'; positions 2861 to 2879 (exon 5)), for CD163 variant E1 (accession number Z22971 (14)) CD163E1 u (5'-CCCAAGCTT CTCAAGTAAGACCCAGAAAA-3'; positions 1817 to 1836) and CD163E1l (5'-CCCGAATTCCAAGCGAATTTCTGTGTATC-3'; positions 1902 to 1921), for CD163 variant AC2 (accession number Z22970 (14)) CD163AC2u (5'-CCCAAGCTTAATCACACATGCTTTTCTTC-3'; positions 4478 to 4497) and CD163AC2l (5'-CCCGAATTCTTATA AATTCAGCAGCAGTC-3'; positions 4850 to 4869), and for CD163 variant A (accession number Z22968 (14)) and CD163 variant AC1 (accession

number Z22969 (14)) CD163RTu (5'-GCATTATTCTTCTTGACTA-3'; positions 3285 to 3303) and CD163RTl (5'-CCTTGAAAGTCT CATATAC-3'; CD163A, positions 3656 to 3674, and CD163AC1, positions 3739 to 3757). Restriction sites added to the primers are underlined. PCR was assayed at two different cycle numbers by removing one-half of the reaction volume at appropriate time points during amplification. This allowed the control of saturation effects of the PCR, which made it possible to better judge quantitative differences between samples. As control, PCR reactions were performed without addition of template and on RNA samples that had been incubated in the absence of reverse transcriptase. The reactions were conducted in a RoboCycler 40 temperature cycler (Stratagene). The reactions were incubated for 3 min at 94°C, followed by 22 to 32 cycles of denaturation for 1 min at 94°C, annealing for 1.5 min at 50°C, and extension for 1 min at 72°C. An aliquot of the PCR reaction (5 μl) was loaded in parallel with the m.w. marker (pBR322, *AluI* digested; MBI Fermentas, St. Leon-Rot, Germany) on a 1.5% agarose gel containing ethidium bromide, and the gel was photographed under UV light. The relative intensity of bands was measured densitometrically, and the results were expressed as a ratio of the CD163:β-actin band intensities.

Results

Isolation and sequencing of the RM3/1 Ag

The most potent glucocorticoid, fluticasone propionate, was used to increase both the Ag density and the number of positive human monocytes in vitro (5). Monocytes were extracted with detergent-containing buffers of varying concentrations of divalent cations. Subsequently, the amount of intact RM3/1 as recognized by the mAb was determined in the supernatant. Experiments with addition of EDTA as inhibitor of metalloproteinases resulted in reduced reactivity of the RM3/1 Ag in dot blots. Systematic evaluation of the effect of EDTA, calcium, magnesium, and manganese on protein yield showed a clear dose-dependent correlation between amounts of divalent cations and improvement of RM3/1 yield (data not shown). Addition of 1 to 10 mM calcium and manganese was statistically significantly superior vs control (no additives) and vs equimolar concentrations of EDTA (ANOVA and subsequent Fisher PLSD test, $p \leq 0.05$). Thus, for subsequent experiments, 1 mM calcium chloride and 1 mM manganese chloride were added to the solubilization mixture.

After preparative SDS-PAGE (5), gel slices containing the RM3/1 protein were excised and prepared for sequencing. Following digestion with LysC and HPLC separation, five peptide sequences were obtained (Table I). Sequences revealed homology with the CD163 protein sequence. Based on 1116 amino acid residues (1076 amino acids in the processed protein) of CD163, the derived sequences were well distributed over the protein and showed either complete identity or, in cases in which the amino acids could not be determined with certainty, a high degree of similarity with the CD163 protein.

Western blots with RM3/1 and Ki-M8 Abs

Western blots with the RM3/1 Ab were not successful to date, and it appeared that the Ab detected only the nondenatured protein. The protocols for SDS-PAGE and subsequent blotting were optimized for detection of RM3/1 Ag in Western blots. The SDS-PAGE was performed under nondenaturing and nonreducing

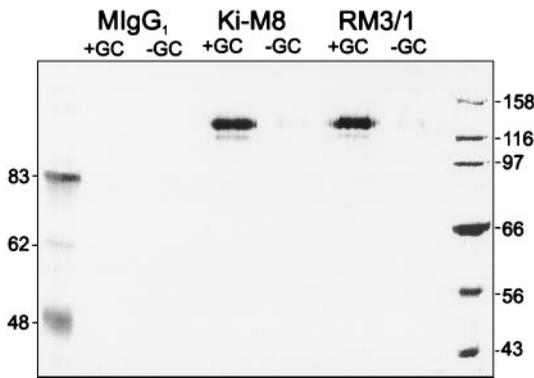


FIGURE 1. Western blot of glucocorticoid-treated and nontreated human monocytes. Glucocorticoid-induced (GC^+) and noninduced (GC^-) monocyte extracts were immunostained with Abs Ki-M8, RM3/1, and MlgG1 for nonspecific control.

conditions. Nonstimulated and glucocorticoid-stimulated monocytes were solubilized in the presence of calcium and manganese and used in Western blots (Fig. 1). The Ab Ki-M8 has been described to recognize CD163 (14). Both Abs, RM3/1 and Ki-M8, clearly detected the same protein, which has an apparent molecular mass of 130 kDa, as described before for the RM3/1 Ag under non-reducing conditions (5). Nonstimulated monocytes gave a weak signal with both the RM3/1 and the Ki-M8 Ab, whereas stimulated monocytes gave an intensive signal after immunostaining.

FACS analysis of the regulation of CD163 expression

FACS analysis of nonstimulated monocytes (control) and stimulated monocytes revealed that all stimulants used influenced the Ag expression in the same manners. Although the extent of Ag density was not identical after immunostaining with RM3/1 and Ki-M8, there was a significant increase in the number of positive cells after addition of fluticasone propionate, whereas a clear decrease of cells carrying this Ag was observed after TPA and cyclosporin A stimulation (Fig. 2).

Screening of a monocyte cDNA library for CD163 full-length clones and expression of CD163 in CHO cells

As the full-length clone of CD163 had not been isolated before (14), we attempted to clone it. The cDNA library was constructed

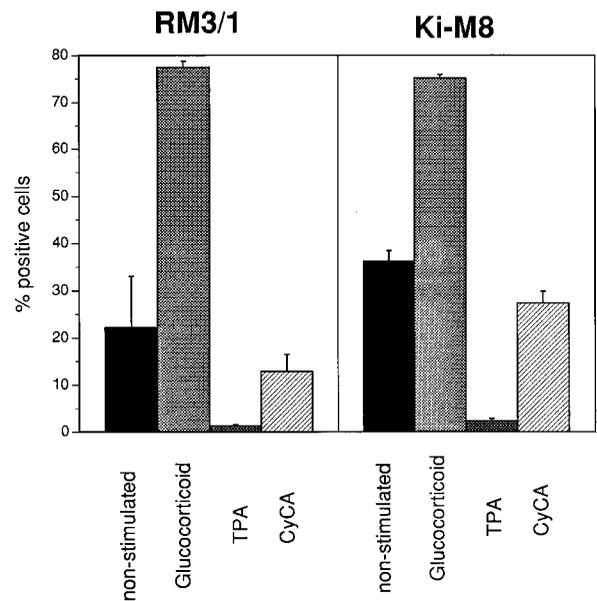


FIGURE 2. Modulation of CD163 expression. Result of a FACSscan measurement of 10^4 vital human monocytes treated with the glucocorticoid fluticasone propionate (16 h, 10^{-8} M), the phorbol ester TPA (1 h, 10 nM), and cyclosporin A (CycA, 16 h, 0.1 μ g/ml), and immunostaining with Abs RM3/1, Ki-M8, and MlgG1 for isotype control. Number of positive cells, corrected for isotype control, was obtained from the main fluorescence channel using Lysis software. Results indicate mean and SD of four independent measurements of a representative experiment.

in vector Lambda ZAP Express (Stratagene) made from oligo(dT)-primed cDNA of fluticasone propionate-stimulated human monocytes. The library was screened with a 0.5-kb PCR-generated DNA fragment (primer pair CD163un/CD163ln) located in the 5' region of the CD163 sequence, as indicated in Figure 3. Positive Lambda clones were purified, and the pBK-CMV phagemid vectors containing CD163 inserts were recovered by *in vivo* excision. After restriction endonuclease mapping of the cDNA clones, the nucleotide sequences were determined. Of 29 isolated cDNA clones, two contained the complete nucleotide sequence, representing the coding region of the major CD163 variant (accession number

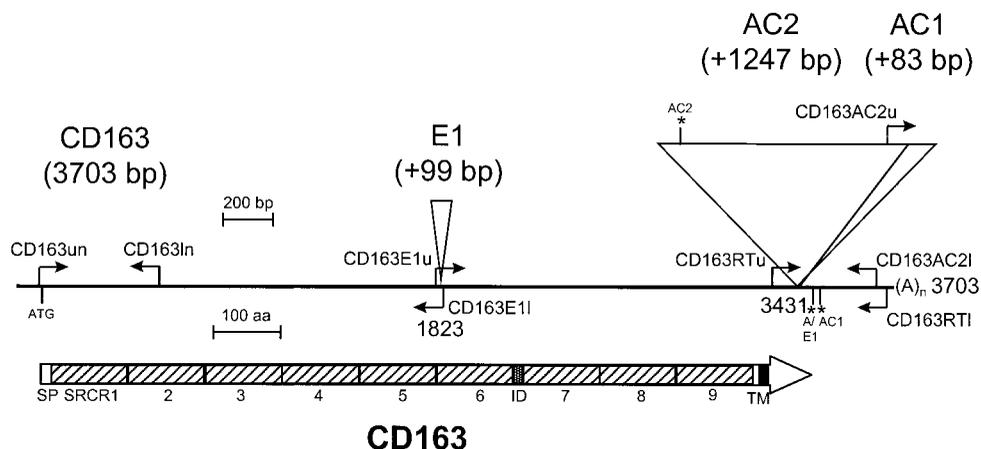
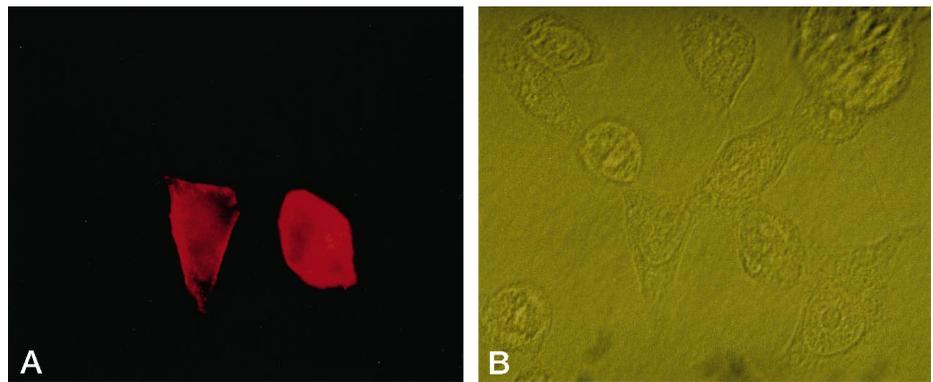


FIGURE 3. Map of CD163 cDNA and its splice variants. Insertions at position 1823 (E1, 99 bp) and position 3431 (AC2, 1247 bp; AC1, 83 bp) are marked. Start (ATG) and stop codons (*) are indicated for each splice variant. The positions of oligonucleotides used for mRNA expression studies (RT-PCR) and probe synthesis for screening the cDNA library are indicated by arrows. The region encoding the structural gene (CD163A, a major splice variant) is marked on the linear map consisting of a putative signal peptide (SP; 40 amino acids), nine scavenger receptor cysteine-rich domains (SRCR; approximately 110 amino acids), and a transmembrane segment (24 amino acids), followed by a cytoplasmic domain (49 amino acids). The interdomain spacer (ID) consisting of 31 amino acids is located between the sixth and seventh SRCR domain.

FIGURE 4. Expression of rCD163 in transiently transfected CHO cells. Transfection with CD163A-6 cDNA resulted in a positive immunostaining with Ki-M8 and RM3/1 Abs after 24 h. Cells were not permeabilized before immunostaining (A, Ki-M8 Ab) to demonstrate Ag expression at the cells' surface. B is a phase contrast image of the same cells. Scale, 1:8750.



Z22968 (14)). The clone CD163A-6 (positions 38 to 3703) was used for transfection and expression experiments. The intactness of the sequences was confirmed by nucleotide sequencing and expression experiments in CHO cells. Transient transfection of CHO cells with CD163A-6 resulted in positive immunostaining of cells with Ki-M8 Ab (Fig. 4A). A control experiment with nonpermeabilized cells confirmed that the Ag was expressed at the cells' surface (Fig. 4B). Cells immunostained with RM3/1 Ab were of identical appearance as cells stained with Ki-M8. There was no signal observed after immunostaining with isotype control mouse IgG1 and with nontransfected cells (data not shown).

Glucocorticoids induce CD163 mRNA in monocytes

Northern blot analysis revealed CD163 transcripts of about 3.7 kb for splice variant CD163-A, and 3.8 kb for splice variant CD163-AC1. Both transcripts appear as a single band (Fig. 5). Transcripts' sizes are in agreement with the predicted size of the cDNA (14). Stimulation of peripheral blood monocytes with the glucocorticoid fluticasone propionate leads to a significant increase of mRNA expression encoding CD163. Hybridization with variant specific probes revealed identical time courses for induction of splice variants A and AC1 (data not shown). Time-course experiments detected maximal induction of CD163 gene transcription after 8 h. Then the level markedly decreased toward 24 h. Analysis of additional time points (10, 12, 16, and 36 h) indicated a decrease of expression 8 h after induction (data not shown). Weak expression was detected at 0 h, with a slight increase during incubation of the cells without glucocorticoid stimulation.

CD163 splice variant expression

RT-PCR was used for analysis of the expression of CD163 splice variants at the mRNA level. Several splice variants were described and detected in an LPS-stimulated human monocyte library (14). The insertion at position 3431 of 1247 bp (AC2) and 83 bp (AC1), respectively, results in protein variants with alternative cytoplasmic domains as compared with the major form (A). In addition, the surface Ag variant E1 exhibits a 99-bp insertion at position 1823 (Fig. 3).

Our aim was to analyze the expression and relative abundance of the major form (A) and its splice variants (AC1, AC2, E1) in glucocorticoid-stimulated and control monocytes. As outlined in the experimental procedures, all of our experiments were standardized by β -actin coamplification in multiplex PCR (222 bp). Splice variant-specific sequences were amplified by RT-PCR using primers based on the cDNA sequences deposited in the GenBank/EMBL database (Fig. 3). Using the primer pair CD163RTu/CD163RTl, PCR products with expected size of 390 bp (A, E1), 454 bp (AC1), and 1718 bp (AC2) should be amplified. Because

PCR conditions were chosen for amplification of DNA fragments below 500 bp, no AC2 product was detected with these primers. As shown in Figure 6A, two CD163-specific products of predicted size were amplified. For AC2 detection, primer pair CD163AC2 u/CD163AC2l was used, amplifying a 392-bp fragment (Fig. 6B). To quantify E1 transcripts, primers CD163E1 u and CD163E1l were used, generating a 124-bp PCR product. After 32 cycles, the E1 PCR product was detected in significant amounts, whereas 22 cycles amplified the E1 product at comparatively low levels (Fig. 6C).

As shown in Figure 6, glucocorticoid stimulation of monocytes resulted in an up-regulation of mRNA expression of variants A, AC1, and AC2. Maximal induction was observed after 8 h, and expression declined by 24 h. As already observed for Northern blot analysis, later time points (10, 12, and 16 h) revealed a decrease of expression after 8 h (data not shown). In contrast to these results, no significant increase in the amount of mRNA was observed for splice variant E1 (Fig. 6C).

The relative abundance of CD163 splice variants is shown in Figure 7, with maximal mRNA expression after 8 h of glucocorticoid stimulation (A, 100%; AC1, 75.7%; AC2, 38.5%; E1, 6.8%) and subsequent decrease (24-h stimulation; A, 86.8%; AC1, 68%; AC2, 25.9%; E1, 3.7%).

Discussion

In the present study, we identified the RM3/1 Ag, previously only described immunologically and biochemically, as a member of the scavenger receptor cysteine-rich superfamily, recently designated as CD163 (13). Most notably, we show for the first time that CD163 is induced significantly by the antiinflammatory glucocorticoids, whereas it is down-regulated by the immunosuppressant

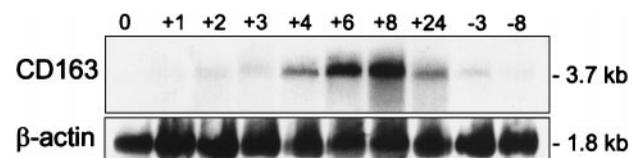


FIGURE 5. Time course of glucocorticoid-induced increase of CD163 mRNA levels. Northern blot analysis was performed with total RNA extracted from peripheral blood monocytes treated with (+) or without (–) fluticasone propionate after 1 to 24 h. Denatured RNAs were fractionated through a formaldehyde agarose gel, blotted to nylon membrane, and probed with DIG-labeled antisense RNAs. The blot was subsequently probed for β -actin transcript. The approximate size of specific transcripts was determined against an RNA molecular size ladder. What appears to be a single band at 3.7 kb are the splice variants A with 3.7 kb and AC1 with 3.8 kb.

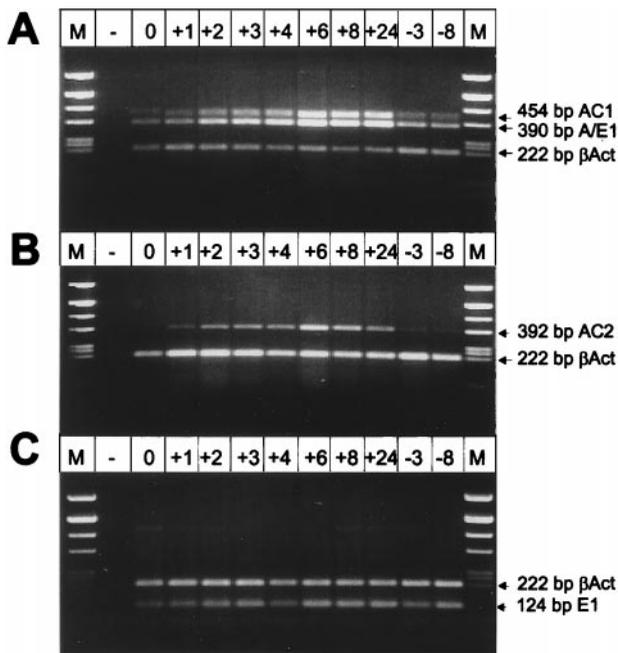


FIGURE 6. mRNA expression of CD163 splice variants after glucocorticoid stimulation analyzed by RT-PCR. Total RNA for cDNA synthesis was isolated from monocytes treated with (+) or without (-) fluticasone propionate after 1 to 24 h. Human monocytes expressing gene transcripts for CD163 variants A and AC1 (A), for AC2 (B), and for E1 (C). Coamplifications with specific primers for β -actin and CD163 variants were performed for 22 (A and B) and 32 cycles (C), respectively. As negative control (-), no template was added to the PCR reaction. Ethidium bromide stain of the amplification products. Results of one representative experiment are given. Each sample was repeated a minimum of three times, and parallel coamplifications of the same cDNA samples were performed to detect CD163 splice variants.

cyclosporin A. The surface expression is clearly decreased by the inflammatory mediator TPA, whereas LPS has almost no influence on the expression (5). In addition, we can now assign an involvement in adhesion processes of monocytes to the activated endothelium (9) to CD163, a member of the SRCR superfamily.

The discovery of a significant increase in recovery of the Ag by addition of divalent cations, preferentially calcium and manganese, to the solubilization mixture initiated the successful isolation. Thus, calcium or manganese is required for structural integrity, enhancing protein stability during isolation and/or preserving the epitope recognized by an Ab. A protection from proteolysis and enhancement of structural stability by divalent cations have been shown for some proteins (18, 19).

Sequencing of peptides derived from the purified RM3/1 Ag revealed sequence identity with the previously described M130 (= CD163) protein (14, 20). This Ag was only detected on cells of the monocyte/macrophage lineage. This is in complete agreement with results previously obtained for the RM3/1 Ag in normal human tissues (1). Interestingly, only few cell lines express CD163. Expression has been described for U937 cells after prolonged stimulation with phorbol esters and for SU-DHL-1 cells (20).

Several experiments were performed to confirm that the previously defined RM3/1 Ag is identical with the CD163 protein. A Western blot of solubilized nonstimulated and glucocorticoid-stimulated human monocytes resulted in superimposable bands originating from staining with RM3/1 and Ki-M8 Abs, respectively. In addition, immunostaining and subsequent FACScan analysis revealed corresponding results for both Abs. Regulation

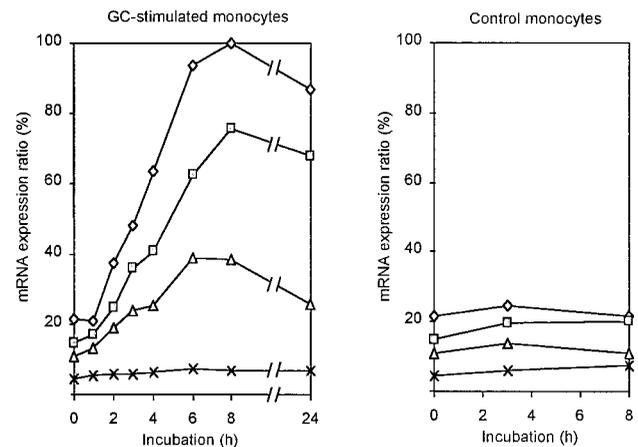


FIGURE 7. mRNA expression ratio of CD163 splice variants. Relative abundance of splice variants A (diamond), AC1 (square), AC2 (triangle), and E1 (cross) was estimated as a ratio of the CD163: β -actin band intensities at 22 PCR cycles, as determined by densitometry. Results are presented as percentage of the normalized intensity of the RT-PCR band with maximal intensity (CD163A, +8 h). Results of one representative experiment are given.

of protein surface expression by the glucocorticoid fluticasone propionate, the phorbol ester TPA, and the immunosuppressant cyclosporin A was equivalent for the protein recognized by RM3/1 and Ki-M8, respectively.

For further molecular characterization of the CD163 gene, we isolated the cDNA from a fluticasone propionate-stimulated monocyte library and transfected CHO cells with the predominant clone variant CD163A. Law et al. (14) isolated only partial cDNA fragments of CD163. Thus, our study is the first description of cloning and expression of a full-length CD163 clone.

The genomic localization and composition of the exon-intron structure of the CD163 gene are not known yet, but the existence of different splice variants for CD163 has been reported previously (14). Alternative splicing is widespread, it is described for several human surface receptors, and it appears to be a common property among proteins with scavenger receptor domains, e.g., for the scavenger receptor class B type 1 (SR-B1), a sponge scavenger receptor, and CD6 (21–23). Alternative splicing of the same transcript resulted in different cytoplasmic domains (21, 23) that could potentially modify signal transduction. Evaluation of molecular recognition motifs for protein kinases (24) in the cytoplasmic domain of CD163 revealed that, for example, the variants A and E1 of CD163 have one potential substrate site for protein kinase C, and the variants AC1 and AC2 each have two protein kinase C phosphorylation sites. Further studies are in progress to determine the functional role of CD163 isoforms.

Using RT-PCR, we analyzed kinetics of induction and relative abundance of four CD163 splice variants. After stimulation with fluticasone propionate, mRNA expression increased rapidly for variant A, AC1, and AC2, peaking after 8 h. Similar expression kinetics have been described for other glucocorticoid-induced gene transcripts (25, 26). The expression of the variant E1, however, appears to be constitutive at low levels and is not inducible by glucocorticoids. No time-specific differences in the relative abundance of CD163 splice variants were apparent. In human monocytes, variant A represented the major mRNA species with 44% of total CD163 mRNA, whereas variants AC1 and AC2 represented 31 and 20%, respectively. The minor mRNA species, denoted E1, was detected at low levels (5% of total CD163 mRNA). In Northern blots, splice variants A and AC1 were detected. Time course of

induction is in complete agreement with data derived from RT-PCR experiments. Since the alternative transcripts A, AC1, and AC2 were detected at significant levels, it may be presumed that the variant proteins derived from these transcripts are also produced. However, this could not be proven yet.

Based on the spacing of the cysteine residues in the CD163 molecule, it has been assigned to the group B of the SRCR family (12). Within this group, it shares structural homology with the WC1 Ag, CD5, CD6, and Sp α (12, 27). There is not much information available about the regulation of these structurally closely related proteins. The effect of dexamethasone has been investigated in bovine lymphocyte populations that express the WC1 Ag (28). The WC1 Ag density on PBMC increased during glucocorticoid injections, while there was a loss in circulating WC1-positive lymphocytes (28). In humans, a similar increase of RM3/1 (CD163)-positive monocytes has been reported 6 h after injection of dexamethasone (4); there is, however, no information about the further time course.

As for the less closely related scavenger receptors, e.g., the SR-B1, glucocorticoids were reported to decrease the expression of these proteins (29, 30), whereas inflammatory mediators such as phorbol esters increased receptor expression in smooth muscle cells (31). In contrast, it has been documented that the expression of CD163 decreases upon stimulation with phorbol esters (5), and also with cyclosporin A (9).

The function of members of the type B SRCR family is not defined conclusively and fully understood yet. WC1 is involved in $\gamma\delta$ T cell regulation (32–34). CD5 and CD6 modulate T cell activation; Sp α is thought to regulate monocyte functions (27). These proteins obviously exert their functions after binding to a specific ligand: CD5 binds to CD72 (35, 36), CD6 to ALCAM (37, 38), a new member of the family, and gp340 binds calcium dependently to lung surfactant protein D (39). No ligand has been defined yet for CD163 and the related WC1 and Sp α .

However, CD163 seems to be involved in adhesion to activated endothelium cells (8, 9). Together with CD14, it has been shown to promote adhesion via a selectin- and integrin-independent pathway, which could be blocked by addition of Abs. Therefore, CD163-positive monocytes have been discussed to promote monocyte infiltration into inflammatory tissues by a nonclassical adhesion mechanism (9). In contrast, glucocorticoids have been reported to inhibit cellular adhesion by down-regulation of several adhesion molecules and to inhibit leukocyte binding to endothelium (40).

It has been proposed early that the RM3/1 (CD163) protein might have some function in the down-regulatory phase of the inflammatory process (1). RM3/1-positive monocytes were later found to produce a novel antiinflammatory factor (11, 41). Recently, it has been reported that RM3/1 (= CD163)-positive monocytes actively inhibit proliferation of CD4⁺ T cells and PBL (42). In conclusion, CD163 appears to play an important regulatory role in immunologic processes.

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