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BRIEF COMMUNICATION

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Molecular cloning and characterization of the mouse CD163 homologue, a highly glucocorticoid-inducible member of the scavenger receptor cysteine-rich family

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CD163 is a glycoprotein belonging to the scavenger receptor cysteine-rich superfamily (SRCR) expressed on cells of the monocyte/macrophage lineage. The protein is induced by the anti-inflammatory mediator, dexamethasone, and is proposed to be associated with the downregulatory phase of inflammatory reactions. However, the biological properties of the protein are poorly characterized. In the present report, the mouse *CD163* cDNA (*mCD163*) was cloned from dexamethasone-treated peritoneal macrophages using a reverse transcription-PCR-based screening method. The predicted polypeptide sequence of the type I transmembrane glycoprotein consists of a 38-amino acid signal peptide, nine SRCR domains, one transmembrane domain, and a short cytoplasmic tail. Sequence variance analysis of all mouse and human CD163-SRCR domains defined a spatial cluster of evolutionarily conserved residues, which could be mapped to a distinct surface area putatively involved in inter- or intramolecular interactions. Determination of *mCD163* mRNA expression by quantitative real-time PCR and RNAse protection assay revealed a more than tenfold induction by dexamethasone and three- to fivefold induction by interleukin-10. In situ hybridization of mouse tissues revealed constitutive expression of *mCD163* mRNA in cells of the monocyte/macrophage series, with the highest expression level observed in Kupffer cells of the liver.

Since the cloning of the mouse type I scavenger receptor in 1990 (Freeman et al. 1990), several cDNAs have been cloned encoding proteins that are homologous to the scavenger receptor cysteine-rich (SRCR) domain found at the C-terminus of this receptor. Members of the SRCR superfamily are cell surface or secreted proteins containing one or more domains highly homologous to the SRCR domain. The SRCR superfamily can be divided in two groups, A and B, based on the number and pattern of cysteine residues in each SRCR domain. Group A proteins include the macrophage scavenger receptor A (Freeman et al. 1990), Mac-2-binding protein (M2BP) (Koths et al. 1993), complement factor I (Goldberger et al. 1987), enterokinase (Kitamoto et al. 1995), lysil oxidase-related protein (Saito et al. 1997), the sea urchin speract receptor (Dangott et al. 1989), and MARCO (Elomaa et al. 1995). Group B includes CD5 (Jones et al. 1986), CD6 (Aruffo et al. 1991), WC1 (Wijngaard et al. 1992), Spα (Gebe et al. 1997), Pema-SPERG (Mayer and Tichy 1995), Ebnerin (Li and Snyder 1995), CRP-ductin (Cheng et al. 1996), Hensin (Takito et al. 1996), DMBT1 (deleted in malignant brain tumors) (Mollenhauer et al. 1997), gp-340 (Holmskov et al. 1999), and CD163 (Hogger et al. 1998; Law et al. 1993).

The functional properties of many members of the group B SRCRs are relatively poorly characterized.

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Several of these proteins are expressed by leukocytes and have been implicated in the development and regulation of the immune system, either by modulating effector cell activity or effector cell abundance by the inhibition of apoptosis (Miyazaki et al. 1999). Others have been proposed as candidate tumor suppressor genes implicated in the pathogenesis of brain tumors and various tumors of the lung and gastrointestinal tract. (Holmskov et al. 1999; Mollenhauer et al. 1997; Mori et al. 1999; Wu et al. 1999).

Human CD163 is a type I cell surface protein belonging to the group B SRCR superfamily and is expressed on cells of the human monocyte/macrophage lineage (Hogger et al. 1998; Law et al. 1993). Expression of the CD163 antigen is regulated by anti-inflammatory mediators in vivo and in vitro (Buechler et al. 2000; Zwadlo-Klarwasser et al. 1990). Glucocorticoids and the anti-inflammatory cytokine interleukin (IL)-10 induce high levels of human CD163 (hCD163) expression on monocytes and macrophages, while the expression is completely abrogated by IL-4, IL-13 and the nuclear receptor PPARγ (peroxisome proliferator-activated receptor γ) agonist 12-deoxy-prostaglandin J2 (Schaer et al., unpublished data). Immunohistological studies have shown an accumulation of hCD163-positive macrophages during the healing phase of acute inflammatory reactions and in chronic inflammatory diseases such as psoriasis (Djemadji-Oudjiel et al. 1996; Zwadlo et al. 1987), suggesting a role for this glycoprotein in the downregulation of the inflammatory process. However, the underlying mechanism resulting in the observed downregulation of the inflammatory process has been poorly characterized due to the lack of a suitable animal model. Glucocorticoids are the most widely used immunosuppressive and anti-inflammatory agents in clinical medicine, yet many of their pharmacological activities involved in the modulation of the immune system are still poorly understood. In this report, we present the cloning and molecular characterization of the mouse *CD163* homologue, and propose this system as a valuable model for the improved characterization of the CD163 glycoprotein in vivo.

We chose an RT-PCR-based cloning strategy using a set of gene-specific primers randomly distributed over the *hCD163* sequence. Total cellular RNA was isolated from cultured mouse peritoneal macrophages with the QIAgen RNA Mini Kit (Qiagen) and reverse transcribed into cDNA with oligo(d)T-primers and M-MuLV Reverse Transcriptase (Stratagene first-strand synthesis kit). Amplification of cDNA from dexamethasone-treated macrophages with 1 out of 15 primer pairs by a standard PCR procedure (forward-primer: 5′-CTCACTGGGACATAGAAGATGC; reverse-primer: 5′-GCCTCTGTAATCTGCTCAGG) yielded a 220-bp fragment with high homology to exon 8 of the *hCD163* gene (mFAT 1.2). The sequence of this fragment served as a template for primer extension PCR toward both ends of the cDNA (SMART RACE kit; Clontech). Two fragments of 2 and 2.5 kb were amplified in the 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE PCR reactions, respectively (5′-RACE primer: 5′-TCCAGTGCAGT GGAACATGTGACTCCAGAC; 3′-RACE primer: 5′-TGGGGTTGCCCAATCTATTCCAGAAGGAGC; anchor primers: provided with the kit) (Fig. 1). Sequence specificity was demonstrated for both fragments by nested PCR using internal primer pairs (5′ nested primer: 5′-CTGACCAGCTCCTTTCCCAAAA TGTGCTCC; 3′ nested primer: 5′-AGGTCTGGA GTCACATGTTCCACTGCACTG). All fragments were cloned into a suitable PCR cloning vector (pCR2.1 Topo-TA; Invitrogen). Two independent clones from two different PCRs were isolated and sequenced from both termini by primer walking to determine the definite nucleotide sequence of

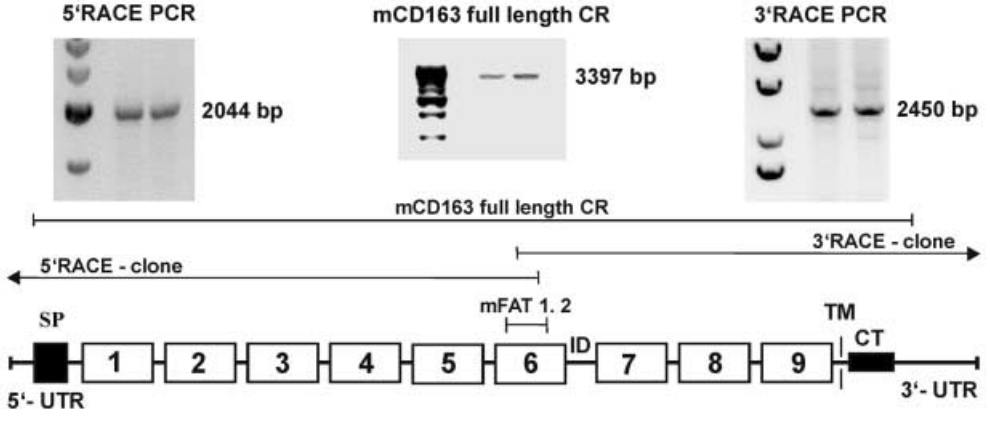

Fig. 1 Structure of the mouse *CD163* cDNA. The 5′- and 3′-RACE-derived cDNA clones used to establish the sequence are shown together with the amplified full-length coding region and a schematic representation of the domain structure of *mCD163*. The positions of the RACE cDNA clones are indicated as *solid dashes*. The respective PCR products (two independent reactions) are shown. The initial cDNA clone (mFAT 1.2) isolated by random PCR screening and the amplified full-length coding sequence are indicated as *solid lines* (*SP* signal peptide, *1–9* scavenger receptor cysteine-rich domains, *ID* scavenger interspersed domain, *TM* transmembrane domain, *CT* cytoplasmic tail, *3′/5′-UTR* untranslated regions)

mCD163 (Microsynth, Balgach, Switzerland). The resulting full-length coding cDNA was subsequently cloned by long-distance PCR using primers near both ends of the sequence of interest (forward primer: 5′-ATGGGTGGACACAGAATGGT; reverse primer: 5′-TTCCATTTAGCTGGCTGTCC). To determine the 5′ extent of the cDNA, six clones from three independent 5′-RACE reactions were isolated and sequenced from the 5′ end. After sequence alignment the longest 5′ extent was used to designate the start of transcription (+1).

The sequence of the *mCD163* cDNA is 4379 nucleotides long (Fig. 2). It contains a single long open reading frame encoding a 1121-amino acid sequence and a 934 nucleotide-long 3′ untranslated region containing four putative polyadenylation signal sequences, 12, 184, 192, and 391 bp upstream of the poly(A) tail. The predicted start of translation at the first ATG codon of the open reading frame is in position +76. In contrast to the human *CD163* where translation preferentially starts at the second ATG (Law et al. 1993) due to a very weak context of the first ATG and consequent leaky scanning of the ribosomal unit, the first ATG in the mouse sequence fulfills the basic requirements for efficient ribosomal binding and subsequent initiation of translation (Kozak 1996).

Computer-assisted analysis of the deduced amino acid sequence revealed a putative N-terminal signal peptide of 38 amino acids (SignalP-Website http://www.cbs.dtu.dk/services/SignalP-2.0/), one transmembrane-spanning region of 23 amino acids at position 1046–1068, followed by a KRRR basic stretch-starting 53 amino acid-long intracellular domain. The extracellular domain contains 16 putative N-linked glycosylation sites, 9 of which are conserved in the human sequence. The extracellular domain organization features nine SRCR domains, which are contiguous, except for a 31 amino acid-long scavenger interspersed domain (SID) containing one disulfide bond separating SRCR domains 6 and 7.

While different fold prediction models failed to recognize the CD163 SRCR domains, probably because they have not yet included the only suitable template structure currently available in the Protein Structure Database (PDB) in their template list, a FastA search of the Brookhaven PDB (at http://www.rcsb.org/) with any of the nine SRCR repeats in the mCD163 extracellular sequence revealed the X-ray structure of the M2BP SRCR domain (PDB entry 1BY2, 2 Å resolution; Hohenester et al. 1999) as a potential template for homology modeling. With sequence similarities between 37% (mCD163 SRCR8 to PDB1BY2) and 62% (mCD163 SRCR7 to PDB1BY2), reasonable models could be generated for all nine domains. The three-dimensional protein structures of the mCD163 SRCR domains were predicted by homology modeling using the Homology, Biopolymer and Discover modules of the program InsightII version 98 (Biosym/MSI, San Diego, Calif.). While the M2BP SRCR domain

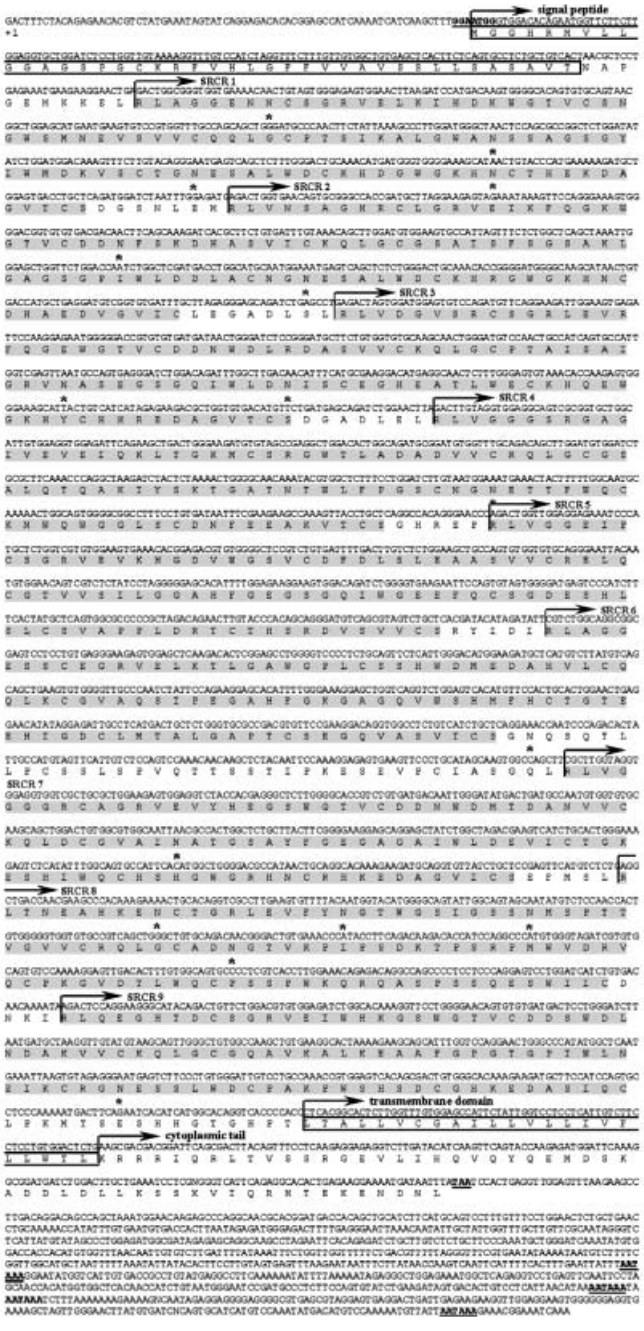

Fig. 2 Complete cDNA sequence, deduced amino acid sequence, and domain structure of mouse *CD163*. The Kozak consensus ribosomal-binding sequence is highlighted in *bold* and indicated as translation initiation site (ATG). The stop codon and four putative polyadenylation signals (AATAAA) are *underlined*. The predicted signal peptide, scavenger receptor cysteine-rich domains 1–9, and the transmembrane domain are marked. *Asterisks* indicate the 16 potential N-linked glycosylation sites

contains only three of the four disulfide bridges commonly found in group B SRCR domains, the coordinates of the residues corresponding to the remaining pair of cysteines were compatible with the remaining disulfide without major structural adjustments

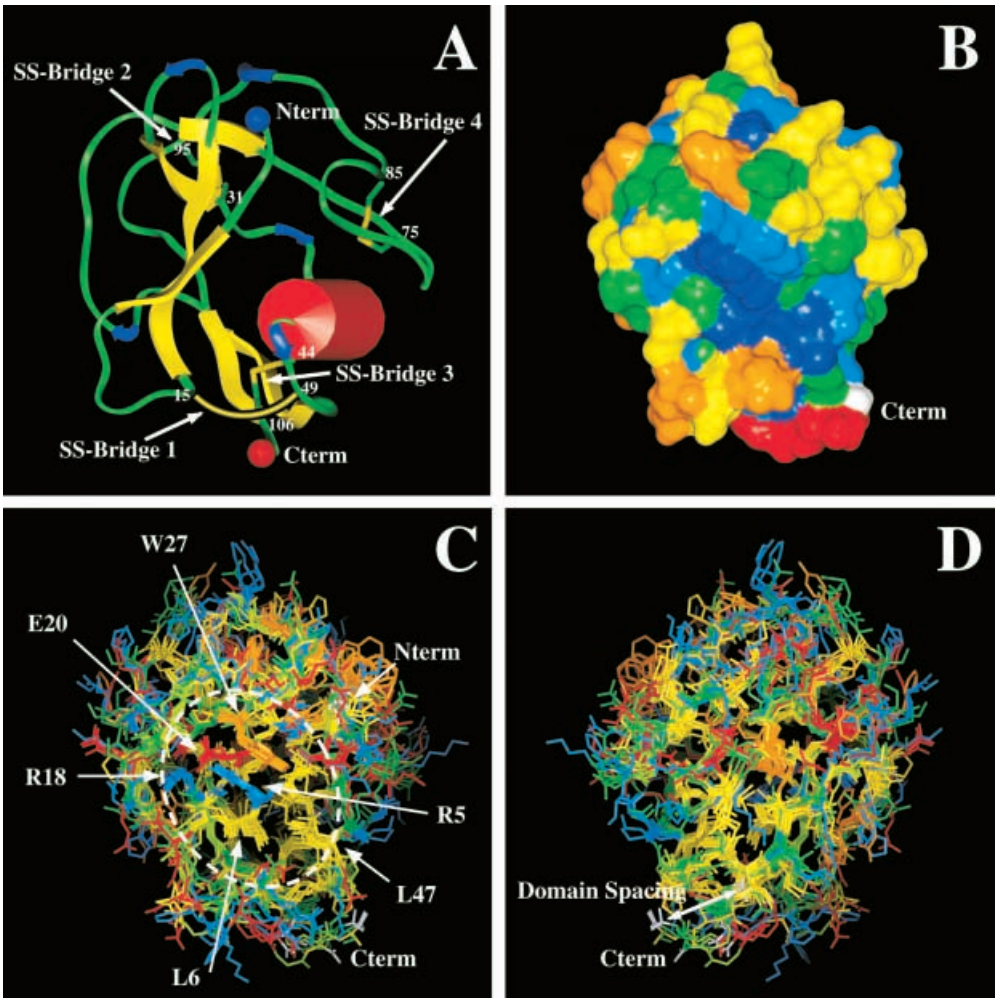

Fig. 3 **A** Cartoon representation of the M2BP SRCR domain structure (*red* α helix, *yellow* β sheets, *blue* turns, *green* random coil, *blue sphere* N-terminal end of the domain, *red sphere* C-terminal end). The residue numbers of the cysteine positions correspond to the generic numbering shown in Fig. 5A. The termini of the M2BP SRCR domain X-ray structure (PDB entry 1BY2, 2 Å resolution; Hohenester et al. 1999) have been truncated back to the domain boundaries suggested by the sequence alignment shown in Fig. 5. SS-bridge 1 is missing in the M2BP SRCR domain, as it is in other group A SRCR domains, although it is conserved in most group B SRCR domains. However, the position and orientation of the corresponding residues in the M2BP SRCR domain are compatible with a disulfide bond easily accommodated without any major structural changes. **B** Surface variability of the CD163 SRCR domains. The variability of individual amino acids (Fig. 5) is mapped onto a space-filling model of a representative mCD163 SRCR domain [*dark blue* identical in all nine domains of human and mouse CD163, *blue* strongly conserved, *green* somewhat conserved, *yellow* variable, *orange* very variable, *red* length variability (insertions/deletions)]. **C,D** Superposition of the nine mouse CD163 SRCR domain models. Two opposite views of all nine superimposed mCD163 SRCR domains are shown [*orange* aromatic residues (W, Y, F), *yellow* other hydrophobic residues (V, L, I, P, A, M, C), *green* uncharged hydrophilic residues (G, S, T, N, Q), *red* acidic residues (D, E), *blue* basic residues (H, K, R), *white* N- and C-terminal of the domains)]. While most of the domain surface shows the variability expected for the degree of sequence divergence between the different domains, the surface area indicated by the *dashed circle* (residues R5, L6, R18, E20, W27, and L47) is disproportionally conserved (**C**). The domains are closely spaced; maximally four residues are conformationally flexible in the area between the last Cys and the N-terminal of the next domain (indicated by the *double arrow* in **D**)

(Fig. 3A). Based on these models, all the disulfide pairings in the CD163 extracellular domain could be assigned. Some minor length variability had to be accommodated by loop remodeling, but all steric clashes introduced by side chain substitutions could be relieved by selecting appropriate side chain rotamers of the substituted or neighboring side chains. SRCR domain 4 of the mouse, but not the human CD163, lacks the first of the four disulfide bridges, since cysteine (Cys)381 has been replaced by a glycine residue, leaving Cys410 unpaired. SRCR domain 8 lacks disulfide bond 2 in both the mouse and the human protein.

To determine structurally and functionally important residues in the CD163 molecule we identified evolutionary conserved residues in the aligned sequences of all mouse and human CD163 SRCR domains (Fig. 4). This approach is based on two

observations: First, multiple homologue domains of a single protein derive from a common ancestor by gene duplication and subsequently undergo sequence divergence by random mutations. Second, as active-site residues are under evolutionary pressure to maintain their functional integrity they undergo distinctly fewer mutations than less functionally important amino acids, implying that evolutionarily related sequences can be compared with one another to extract structural and functional data (Lichtarge et al. 1996). The interdomain variability of each amino acid at a given position was calculated using the algorithm described by Wu and Kabat (1970) (variability=number of different amino acids at a given position/frequency of the most common amino acid at this position). After mapping of the calculated and color-coded sequence variability of each amino acid residue onto a space-filling SRCR domain model, a spatial cluster of highly conserved amino acids could be identified (Fig. 3B). The same conserved surface patch was identified by analysis of the superposition of the nine CD163 SRCR domain models and is indicated by the dashed circle in Fig. 3C. Given the progressive sequence divergence among homologue domains in the absence of an evolutionary pressure to maintain functional integrity, the observed degree of structural conservation implies an involvement of the described surface region in important intra- or intermolecular interactions. One may speculate that the identified surface patch is critically involved in ligand binding or in the three-dimensional arrangement of the nine SRCR domains. The conservation of core and structurally important residues corresponds to what one would expect for structurally homologous domains at the observed level of sequence divergence.

After confirmation of the strong inductive potential of dexamethasone on the expression of *mCD163* mRNA by RNAse protection assay (Fig. 5A), we used quantitative real-time RT-PCR to further determine the regulation of *mCD163* mRNA abundance by the anti-inflammatory mediators IL-4 and IL-10. Ribonuclease protection assays were performed with the Ambion Hybspeed kit according to the manufacturer's instructions, using a 300-bp *mCD163*-specific ^{32}P-labeled RNA probe (forward primer: 5′-TAATACG ACTCACTATAGGGAGGACCTGAGCAGATGAC AGAGG; reverse primer: 5′-ACCTGAGCAGATGA CAGAGG). For quantitative RT-PCR, equal amounts of total RNA from differently treated cells were reverse transcribed and amplified with gene-specific primers by real-time PCR using the LightCycler System (Roche) with SYBR Green I fluorescence (Roche) as described elsewhere (Staege et al. 2000) (*mCD163* forward primer: GGGAAGAGTGGAGCT CAAGA, reverse primer: ACCAGCTCCTTTCCCA AAAT; *hCD163* forward primer: 5′-ACATAGATC ATGCATCTGTCATTTG, reverse primer: 5′-CATTC TCCTTGGAATCTCACTTCTA). These experiments confirmed a more than tenfold induction of *mCD163* mRNA upon incubation with dexamethasone for 16 h compared to untreated cells. While IL-10 similarly induced *CD163* mRNA, the expression was only slightly suppressed below baseline levels by IL-4 after 16 h (Fig. 5B). The suppressive effect of IL-4 compared to control cells increased after prolonged culture of macrophages in the presence of IL-4, as the expression of *CD163* mRNA increases over time in untreated cells (data not shown). Further experiments revealed a parallel regulation of both mouse and human *CD163* genes (Fig. 5B, C).

The tissue distribution of *mCD163*-expressing cells was studied by in situ hybridization using a 1.5-kb *mCD163*-specific ^{35}S-CTP-labeled sense and anitsense RNA probe, as described elsewhere (Mueller et al. 1988). The highest level of constitutive *mCD163* expression was repeatedly observed in Kupffer cells of the liver. Furthermore, we identified a subset of red pulp macrophages and cells of the extrafollicular area of mesenteric lymph nodes that expressed *CD163*. Scattered *mCD163*-positive cells were located in the lamina propria of the colon and in the thymic cortex (data not shown). These results confirm the monocyte/macrophage-restricted expression pattern of *mCD163*.

Glucocorticoids induce a distinct subpopulation of alternatively activated macrophages expressing a specific set of molecules enabling them to actively participate in anti-inflammatory processes, immunosuppression, tolerance induction, and wound healing (Goerdt and Orfanos 1999). Despite the growing knowledge about the molecular repertoire of these suppressor macrophages involving the expression of various anti-inflammatory mediators and the lack of immunostimulatory effector molecules, the immunomodulating armature of these cells is still not fully understood.

The CD163 molecule can be assigned to a subgroup of group B SRCR members consisting almost entirely of a series of contiguous SRCR domains. Members of this subgroup are CD5, CD6, WC1, Spα, and several proteins encoded by the *DMBT1* gene locus. The most closely related proteins are WC1, Spα, and DMBT1. WC1 is involved in γδT-cell regulation (Kirkham et al. 1998; Wijngaard et al. 1992), CD5 and CD6 modulate T-cell activation after specific binding to their ligands CD70 and ALCAM (activated leukocyte cell adhesion molecule), respectively (Osorio et al. 1997; Whitney et al. 1995). Spα, a soluble protein consisting of three SRCR domains, is expressed by macrophages and suggested to inhibit thymocyte apoptosis upon various proapoptotic stimuli in mice (Gebe et al. 1997; Miyazaki et al. 1999). The *DMBT1* gene locus encodes the human DMBT1 and gp-340. While gp-340 is suggested to be a putative opsonin receptor for lung surfactant protein (Holmskov et al. 1999), DMBT1 has been proposed as a candidate tumor suppressor gene implicated in the pathogenesis of brain and various lung and gastrointestinal tumors. This conclusion is based on the finding of homozygous deletions and lack of expression in glioblastoma multiforme and

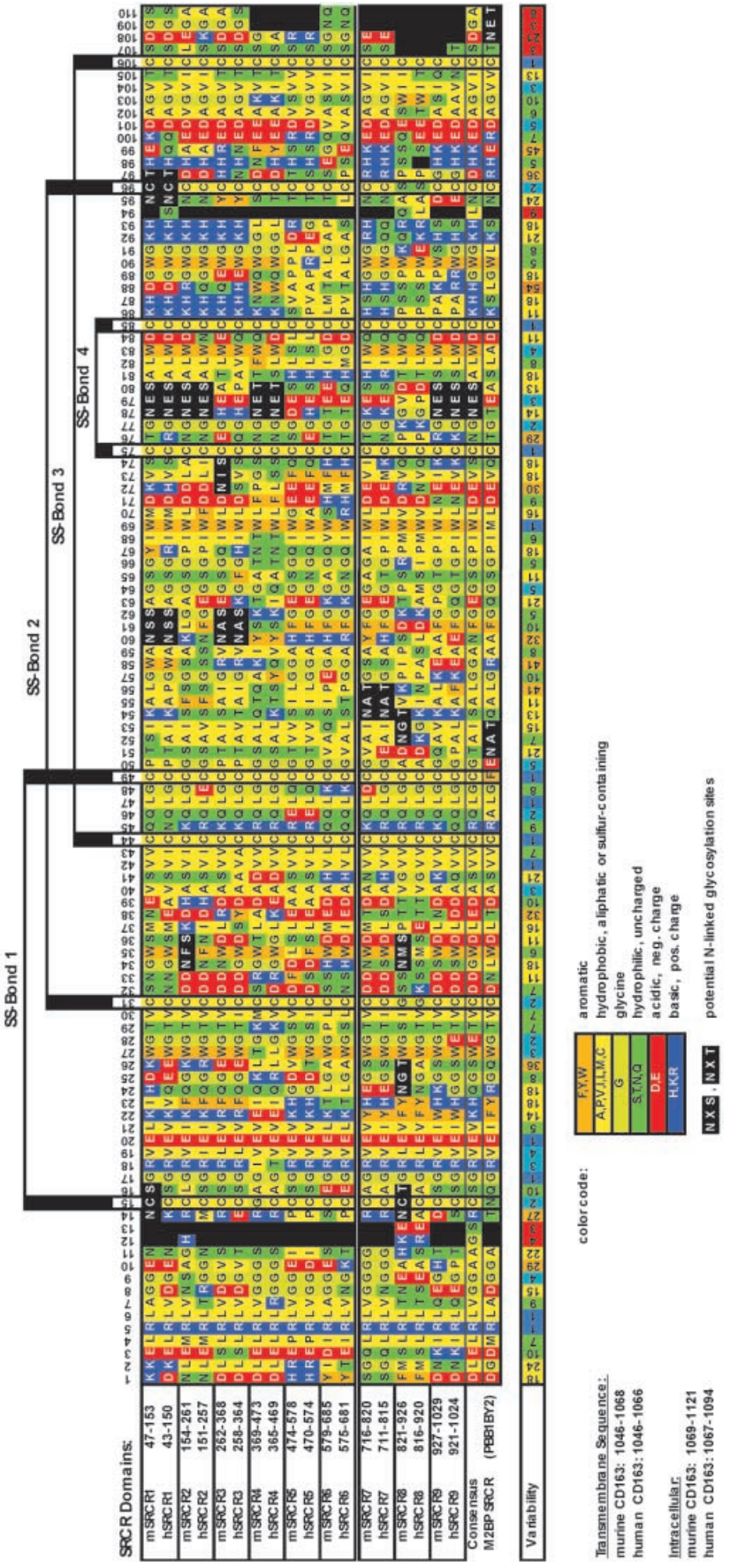

Fig. 4 CD163 SRCR sequence alignment and interdomain amino acid variability. Sequence alignment of the human and mouse CD163 scavenger receptor cysteine-rich domains and predicted disulfide bridges based on the homology to the M2BP SRCR domain structure (PDP entry 1BY2). Potential N-linked glycosylation sites are highlighted. Of the two potential sites of the M2BP SRCR domain, only the first is actually glycosylated. For each residue, the variability among all mouse and human CD163 was calculated using the Kabat algorithm and is indicated as 1 (identical in all nine domains of human and mouse CD163) to 54 (highest observed variability) (*dark blue* identical in all nine domains of human and mouse CD163, *blue* strongly conserved, *green* somewhat conserved, *yellow* variable, *orange* very variable)

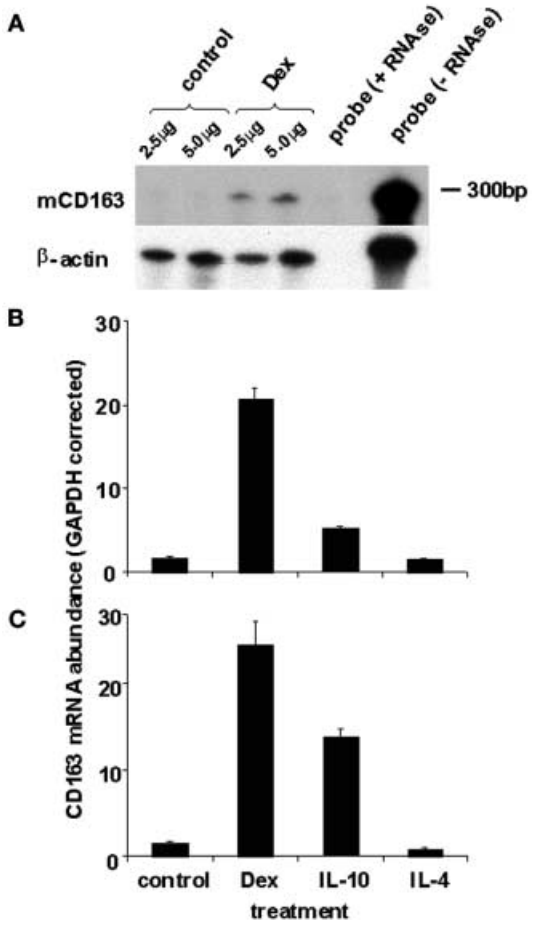

Fig. 5A–C Regulation of mouse and human *CD163* mRNA abundance by anti-inflammatory mediators. **A** RNAse protection assay of dexamethasone-treated and control mouse peritoneal macrophages: the 300-bp RNA probe consisting of the whole cDNA sequence of SRCR6 was incubated with 5.0 µg and 2.5 µg of total cellular RNA. As positive and negative controls the assay was performed without tester RNA, with and without RNAse treatment. **B,C** Real-time quantitative RT-PCR [mouse CD163 (**B**) human CD163 (**C**)]: relative glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *CD163* mRNA abundance were determined for each sample by inclusion of a serial dilution of cDNA in each PCR run as a standard curve. All values of *CD163* mRNA abundance are corrected for variations in *GAPDH* concentration. Indicated are the mean and SD of triplicate values from one representative experiment. Cells were incubated for 16 h with the indicated stimuli (dexamethasone 2.5×10^{-7} M, IL-4 10 ng/ml, IL-10 10 ng/ml)

medulloblastoma (Mollenhauer et al. 1997). Similar results were obtained in a significant fraction of gastrointestinal and lung tumors (Mori et al. 1999). Currently, data indicating putative functions of CD163 are very limited. In addition to the strong induction of *CD163* by glucocorticoids and the anti-inflammatory mediator IL-10, macrophages expressing high levels of CD163 have been identified in chronically inflamed tissues and during the wound-healing process (Zwadlo et al. 1987), indicating a possible role of this protein in the downregulation of the inflammatory process. Furthermore, exceptionally high levels of CD163 are expressed in human placental and alveolar macrophages. In the healthy organism, these alternatively activated macrophages function to protect the respective organ from detrimental inflammatory and immune reactions (Chang et al. 1993; Mues et al. 1989).

Our identification and characterization of the mouse *CD163* sets the basis for the development of transgenic and knockout mouse models. These powerful tools may soon unravel the biological significance of CD163 in the observed downregulation of the inflammatory response associated with the occurrence of high CD163-expressing macrophages.

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