Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar

Spanish Journal of Agricultural Research 2008, 6 (Special issue), 59-72
ISSN: 1695-971-X

Cloning and expression of porcine CD163: its use for characterization of monoclonal antibodies to porcine CD163 and development of an ELISA to measure soluble CD163 in biological fluids

C. Pérez, E. Ortuño, N. Gómez, M. García-Briones, B. Álvarez, P. Martínez de la Riva, F. Alonso, C. Revilla, J. Domínguez and A. Ezquerra*

Departamento de Biotecnología. INIA. Ctra. de A Coruña, km 7,5. 28040 Madrid. Spain

Abstract

CD163 is a member of the family of proteins with *scavenger receptor cysteine-rich* domains, whose expression is restricted to monocytes and macrophages. It functions as a receptor for haemoglobin/haptoglobin complexes and it has also been implicated in the regulation of inflammatory processes. Treatment of monocytes/macrophages with phorbol esters, LPS or cross-linking of Fc gamma receptors, causes shedding of CD163 from cell surface by a protease-dependent mechanism. The level of soluble CD163 (sCD163) in serum and other body fluids has been considered a useful marker of macrophage activation. In addition, porcine CD163 has been shown to play a role in the infection of monocytes/macrophages by the African swine fever virus. The porcine CD163 cDNA has been cloned, and expressed in transfected CHO cells. Clones of CHO cells stably expressing porcine CD163 have been used for the characterization of three new mAbs against porcine CD163. Using two of these mAbs, that bind to different epitopes on CD163 molecule, a sandwich enzyme-linked immunoassay (ELISA) has been developed to measure levels of sCD163 in porcine sera and biological fluids. The assay was calibrated using lysates of CD163 transfectants, showing a sensitivity of 10^5 cells mL^{-1}, that allowed to detect sCD163 in sera and in culture supernatants of activated alveolar macrophages. This assay can be a useful method of monitoring the degree of activation of macrophages in a variety of inflammatory and infectious diseases of swine.

Additional key words: macrophage, SRCR family, swine.

Resumen

Clonación y expresión de CD163 porcino: aplicación en la caracterización de anticuerpos monoclonales frente a CD163 porcino, y desarrollo de un ELISA para cuantificar CD163 soluble en fluidos biológicos

CD163 es un miembro de la familia de proteínas con dominios de receptores «scavenger» ricos en cisteína, con una expresión restringida a células del linaje monocítico. Se ha demostrado su función como receptor de complejos hemoglobina/haptoglobina, y se le ha implicado en la regulación de procesos inflamatorios. El tratamiento de monocitos/macrófagos con ésteres de forbol, LPS, o mediante entrecruzamiento de receptores Fc gamma, provoca la liberación de CD163 de la superficie celular por un mecanismo dependiente de proteasas. La cantidad de CD163 soluble (sCD163) en suero y otros fluidos corporales se considera un indicador de la activación de los macrófagos. Recientemente, nuestro grupo demostró que CD163 está implicado en la infección de monocitos/macrófagos porcinos por el virus de la peste porcina africana. En este trabajo se describe la clonación del cDNA de CD163 porcino, y su expresión en células CHO transfectadas. Se obtuvieron clones de células CHO transfectadas que expresan de forma estable la molécula CD163 porcina, y se utilizaron para caracterizar tres nuevos anticuerpos monoclonales que reconocen específicamente CD163 porcino. El uso de dos de estos anticuerpos, que reconocen epítopos diferentes en CD163 porcino, ha permitido desarrollar un ensayo inmunoenzimático (ELISA) para medir la cantidad de sCD163. El ensayo se calibró con lisados de las células transfectadas que expresan CD163 porcino, mostrando una sensibilidad equivalente a 10^5 células mL^{-1}, suficiente para detectar la presencia de sCD163 en suero y sobrenadantes de cultivo de macrófagos alveolares activados. Este ensayo será de utilidad para determinar el grado de activación de los macrófagos porcinos en procesos inflamatorios y enfermedades infecciosas porcinas.

Palabras clave adicionales: cerdo, familia SRCR, macrófago.

* Corresponding author: ezquerr@inia.es
Received: 07-08-07; Accepted: 18-01-08.

Introduction[1]

CD163 is a cell-surface glycoprotein with expression restricted to cells of monocyte/macrophage lineage, that belongs to the family of molecules with *scavenger receptor cysteine-rich* domains (SRCR) (Law *et al.*, 1993). Human CD163 is a 130 kDa glycoprotein containing a single transmembrane element, a cytoplasmic tail and a large extracellular region containing nine SRCR domains. Four splice variants have been identified that differ in the length of their cytoplasmic tail (Ritter *et al.*, 1999; Fabriek *et al.*, 2005).

Hemoglobin-haptoglobin complexes formed after red blood cell lysis are the only documented ligand of CD163, that upon binding is internalized. CD163 is also believed to contribute to clearance of free hemoglobin (Kristiansen *et al.*, 2001; Schaer *et al.*, 2006), playing a major role in iron metabolism.

CD163 must play an important role in the control of inflammatory processes as suggested by the tightly regulation of its expression by pro- and anti-inflammatory mediators (Kristiansen *et al.*, 2001; Moestrup and Moller, 2004). Thus, surface expression of CD163 is up-regulated by glucocorticoids, IL-6 and IL-10 whereas it is downregulated by IFN-γ, TNF-α, IL-4 and IL-13 (Buechler *et al.*, 2000; Schaer *et al.*, 2002; Sulahian *et al.*, 2004). Moreover, signalling through CD163 leads to production of pro- and anti-inflammatory cytokines (Van den Heuvel *et al.*, 1999; Phillippidis *et al.*, 2004). On other hand, inflammatory stimuli lead to a protease-dependent shedding of CD163 ectodomain (Hintz *et al.*, 2002; Timmermann and Hogger, 2005). This soluble form of CD163 (sCD163) can be detected in plasma and has been shown to inhibit lymphocyte proliferation (Hogger and Sorg, 2001; Timmermann *et al.*, 2004). Plasma levels of sCD163 are considered a good indicator of macrophage activation and may have prognostic value in a variety of disease states (Hiraoka *et al.*, 2005; Schaer *et al.*, 2005; Aristoteli *et al.*, 2006).

In swine, in addition, CD163 has been shown to play a role in the infection of monocytes/macrophages by the African swine fever virus (Sánchez-Torres *et al.*. 2003). To better understand the role of CD163 in these phenomena, and to have a tool to search for ligands cloning of porcine CD163 cDNA was undertaken.

At present, very few mAbs are available against porcine CD163. The first mAb against porcine CD163, named 2A10/11, was characterized by our group. Later, the clone SwNL517.2, binding to the same epitope, was assigned to this cluster (Thacker *et al.*, 2001). More recently, two anti-human CD163 mAbs have been shown to cross-react with porcine cells but data of epitope analysis are lacking (Saalmuller *et al.*, 2005; Komohara *et al.*, 2006).

In the present report the cloning of porcine CD163 cDNA, the expression of this molecule in CHO cell line transfectants, and their use in the characterization of three new mAbs against porcine CD163, are described. Using two antibodies against different epitopes, an ELISA to quantify sCD163 in cell culture supernatants and biological fluids has been developed. The effect of various porcine cytokines on CD163 expression has also been analysed. Like in humans, surface expression of CD163 is upregulated by IL-10 and suppressed by IL-4 and GM-CSF.

Material and Methods

Cells

Large White pigs with an average weight between 30 and 40 kg were used as donors of blood. Peripheral blood mononuclear cells (PBMC) were isolated on Percoll discontinuous gradients after blood sedimentation in dextran as has been previously described (González *et al.*, 1990). Cells were suspended at a density of 10^6 mL^{-1} in RPMI 1640 medium containing 10% foetal calf serum (FCS), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 30 μg mL^{-1} gentamicin (complete medium). Bone marrow cells were obtained by perfusing ribs with PBS. Red cells and non-viable cells were removed by centrifugation at 400 g, 10 min on lymphocyte separation medium (Biowhittaker, USA) at 1.077 g mL^{-1}. Swine granulocytes, alveolar macrophages, platelets

[1] Abbreviations used: AA (amino acid), ASF (African swine fever), CSF (classical swine fever), DMEM (Dulbecco's modified Eagle´s minimal essential medium), FACS (fluorescence activated cell sorter), FB (fluorescence buffer), FCS (foetal calf serum), IF (interfollicular area), mAb (monoclonal antibody), MFI (mean fluorescence intensity), PAGE (poly acrilamide gel electrophoresis), PALS (periarteriolar lymphoid sheath), PBMC (peripheral blood mononuclear cells), PBS (phosphate buffer saline), PE (Phycoerythrin), PMA (phorbol 12-myristate 13-acetate), PPRS (porcine respiratory and reproductive syndrome), PWMS (post-weaning multisystemic wasting syndrome), RP (red pulp), RT (room temperature), SRCR (scavenger receptor cysteine–rich), SS (subcapsular sinus), TM (transfection medium).

and erythrocytes were obtained as has been previously described (Álvarez *et al.*, 2000).

Cloning of porcine CD163 cDNA

RNA was extracted from porcine alveolar macrophages with TriPure Isolation reagent (Roche, Basel, Switzerland) by a method based on that described by Chomczynski and Sacchi (1987). First-strand cDNA was obtained from 5 μg of total RNA in a total volume of 50 μL, containing 50 mM Tris-HCl (pH 8.6), 75 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 50 μM each dNTP, 0.5 mM oligo(dT), 20 U of RNasin ribonuclease inhibitor (Promega, Madison, WI, USA) and 12.5 U of Moloney murine leukaemia virus RT (Epicentre, Madison, WI, USA). The reaction mixtures were incubated for 1 h at 37°C. For PCR, a variable amount of the cDNA (typically 1 μL) was used in a total volume of 25 μL of a PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet P-40, 0.5 to 1 mM $MgCl_2$ (depending on the oligonucleotide pair), 50 μM each dNTP, 10 pmol each of specific oligonucleotides (forward and reverse primers) and 1 U of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). The primer sequences and expected sizes of amplified fragments are shown in Table 1. Each cycle of PCR consisted of denaturation at 94°C for 45 s, annealing for 45 s at temperature dependent of primer pair and extension at 72°C for 1 min. PCR products were analysed on 1% agarose gels. G3PDH expression was used as a control for RNA content and integrity. The PCR products were cloned into pcDNA3.1/TOPO vector using a TA cloning kit (Invitrogen, San Diego, CA, USA). The plasmid DNAs were purified using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany) and sequenced with plasmid specific oligonucleotides and sequence specific primers (Table 1). Sequences of cDNA fragments were used to design new oligonucleotide primers (Table 1).

Expression of CD163 constructs, and generation of stable transfectants

CHO cells were transfected with plasmid pCD163 encoding full-length porcine CD163 by using the LipofectAMINE PLUS reagent (Invitrogen, San Diego, CA, USA) accordingly to the manufacturer's instructions. Briefly, the day prior to transfection CHO cells were seeded into 6-well plates (3×10^5 cells $well^{-1}$) and grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (BioWhittaker, Verviers, Belgium) supplemented with 30 μg mL^{-1} gentamicin, 2 mM L-glutamine and 10% foetal calf serum (BioWhittaker, Verviers, Belgium). Immediately before transfection, cells were rinsed with medium without serum and antibiotic (transfection medium, TM). For each well, 1 μg of plasmid DNA and 6 μL of PLUS reagent were mixed, diluted with 500 μL of TM and incubated for 15 min at room temperature. Then 4 μL of Lipofectamine were mixed with 500 μL of TM and added to the former mixture. After 15 min incubation, the DNA/LipofectAMINE PLUS complexes were added to the cells. Cells were incubated for 3 h at 37°C in 5% CO_2, and then 1 mL of fresh growth medium was added.

Table 1. Oligonucleotide primers used for PCR and sequencing of porcine CD163

Primer name	Sequence	Tm (°C)	PCR product size (bp)
PCR primers			
N1ATG (forward)	ATGGTGCTACTTGAAGACT	54	3,400
2A103520 (reverse)	TAGTCCAGGTCTTCATCAAGG	62	
			Position in sequence
Sequencing primers			
N2F	GATCATGCTGAGGATGC		727-743
N3R	TTACATGCGACAGCGGCATC		877-896
N4F	CCAGCGTGCTGTGCAGGG		1,517-1,534
N5R	GCTACATGTCCCGTCA		1,671-1,686
N6F	AATTGCAGGCATAAGG		2,401-2,416
N7R	TCACAGCAGCATCCTCCTT		3,043-3,061

Transfected cells were harvested at 24 and 48 h post-transfection and analysed on a FACScalibur flow cytometer for expression of CD163. CHO cells transfected with the empty expression vector (pcDNA3.1+) were used as a negative control.

To obtain CHO transfected cells permanently expressing porcine CD163, transfected cultures were selected by adding Geneticin G-418 sulphate (Gibco BRL) at a concentration of 0.8 mg mL^{-1} in DMEM. Selection medium was kept for 2 wk with changes every 4 d. Survivors were analyzed by cytofluorometry and cells expressing CD163 cloned twice by limiting dilution.

Antibodies

Hybridomas secreting mAbs 1C6/BM, 2H12/BM and 3E10/BM were derived from a fusion of Sp2/0 myeloma cells with spleen cells from a Balb/c mouse immunized with bone marrow cells, using established protocols (Köhler and Milstein, 1975). Class and subclass of mAbs were determined by ELISA, using rabbit antisera specific for mouse heavy and light chains and a peroxidase-conjugated goat anti-rabbit Ig (Bio-Rad, USA). Mab 1C6/BM was a IgG2a, and 2H12/BM and 3E10/BM were of IgG_1 isotype. For labeling, mAbs were purified from hybridoma cell culture supernatants by affinity chromatography with Protein G-Sepharose CL 4B (Pharmacia, Sweden). Biotinylation of mAbs was performed as described previously (Domínguez *et al.*, 1990).

Other mAbs used in this study were: 2A10/11 (anti-CD163, IgG1), 3C3/9 (anti-CD45RA, IgG1), BL1H7 (anti-CD172a, IgG1), 2F4/11 (anti-wCD11R3, IgG1) and BL4A2 (IgG1) developed and produced in our laboratory; BB23-8E (anti-CD3, IgG2b), and 76-2-11 (anti-CD8α, IgG2a) kindly provided by M. Pescovitz (Indiana University, USA).

Flow cytometric analysis

Cells (5×10^5/well) were incubated in a V-shaped 96-well microplate with 50 µL of hybridoma supernatants for 30 min on ice. After 2 washes in PBS containing 0.1% BSA and 0.1% sodium azide (fluorescence buffer, FB), cells were incubated for 30 min at 4°C in 50 µL of FITC-conjugated rabbit $F(ab')_2$ anti-mouse Ig (Dako, Denmark) diluted 1/50 in FB. Cells were washed three times in FB and fixed in 0.3% paraformaldehyde prior to be analysed in a Facscalibur (Becton Dickinson, USA).

In cross-bloking experiments, cells were incubated with 25 µL of hybridoma supernatant. After 10 min on ice, and without washing, 25 µL of the optimal dilutions of biotin-labeled mAbs were added for an additional 30 min. Cells were then washed and incubated for 10 min with PE-conjugated streptavidin. An irrelevant isotype-matched mAb was used as negative control.

Immunohistochemical analysis

Samples from spleen, mesenteric lymph nodes and thymus were collected from healthy, conventionally reared, 3-month-old Large-White pigs that had been euthanized with anaesthesia (azaperone and ketamine). Tissue samples were snap frozen in isopentane/liquid nitrogen and stored at –80°C. Frozen sections were cut at 6 mm thick, mounted on poly-L-lysine coated glass slides, and air-dried overnight at room temperature. Slides were then fixed for 10 min in acetone and stained according to previously described protocols (Doménech *et al.*, 2003).

Immunoprecipitation analysis

Cells (10^8) were washed three times in PBS and resuspended in 5 mL of PBS. Sulfo-NHS-biotin (Pierce, USA) (0.4 mg mL^{-1}, final concentration) was added to the cells and incubated for 15 min at 4ºC. After washing three times with PBS, cells were lysed with 1 mL of lysis buffer consisting in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5mM EDTA, 1% NP40, 10 µg mL^{-1} aprotinin and 1 mM phenylmethylsulphonylfluoride. The lysate was precleared twice with 50 µL of a 25% (v/v) suspension of protein G-Sepharose (Pharmacia, Sweden) in lysis buffer and then incubated with the different mAbs. To 0.3 mL of hybridoma supernatant, 0.1 mL of lysate sample was added and incubated for 2 h at room temperature with continuous mixing. Then, 40 µL of a 25% (v/v) suspension of Protein G-Sepharose, previously coupled with rabbit anti-mouse Igs, was added and incubated for 1 h. Beads were washed three times with lysis buffer. Immunoprecipitates were finally boiled in electrophoresis sample buffer with or without 2-mercaptoethanol (reduced or unreduced conditions respectively), run in SDS-7.5% PAGE and transferred to nitrocellulose filters. Filters were incubated

with streptavidin-peroxidase (Pierce, USA) and bands visualized with the ECL detection assay (Amersham, UK).

In vitro CD163 shedding

Cultures of alveolar macrophages (1×10^6 cells/well) in 12-well plates were exposed to 10^{-8} M PMA (Sigma Sigma Chemical Co., St. Louis, Mo). Supernatants were collected at different times (0-8 h), and stored at –70°C until used.

Quantitation of sCD163 by ELISA

Nunc F96 Polysorp plates were coated with 1 µg per well of mAb 2H12/BM in PBS, by overnight incubation at 4°C. After 5 washes with washing solution (PBS containing 0.1% Tween 20), wells were blocked with PBS-0.5%BSA for 2 h at room temperature. Plates were then incubated overnight at 4°C, with serial dilutions of cell lysates, culture supernatants or pig serum, in dilution buffer (PBS, 0.1% Tween 20, 0.1% BSA, 0.01% merthiolate). After five washes, 100 µL of biotinylated mAb 2A10/11 diluted 1/1000 were added to each well and incubated for 1 h. Plates were washed again and 100 µL of 1/2000 dilution of streptavidin-peroxidase (Pierce) added. After 1 h incubation, plates were washed five times and 100 µL of 1,2 o-phenylendiamine (2 mg mL^{-1}) in 0.1 M citrate buffer, pH 5.0 and 0.03% H_2O_2 were added to each well. Colour development was stopped by adding 100 µL of 2 N H_2SO_4 after 10-15 min. Plates were read at 492 nm using a Titertek Multiskan.

Alternatively a chemiluminiscence ELISA was performed, using Nunc white microwell 96F Polysorp plates. Peroxidase activity was determined using the Supersignal ELISA Pico Chemiluminiscent, luminol-based, substrate (Pierce, Rockford, IL, USA). Plates were incubated on a plate shaker at RT for 1 min. The chemiluminescence intensity was measured on a Tecan Genius Microplate Luminometer with a 10 ms read time per well.

Isolation and culture of monocytes with recombinant porcine cytokines

Monocytes were isolated from PBMC using the magnetic cell separation system of Miltenyi Biotec (Bergisch-Gladbach, Germany). First, PBMC (4×10^8) were incubated with an antibody cocktail containing 15 mL anti-CD3 (BB23-8E, IgG2b), 15 mL anti-CD45RA (3C3/9, IgG1) and 15 mL anti-CD8a (76-211, IgG2a) and after 30 min at 4°C washed with PBS containing 5% FCS and 2 mM EDTA. Subsequently, anti-mouse Ig magnetic microbeads were added and incubated for 15 min on ice. Then, suspension of cells was washed and passed through a MACS CS separation column. The effluent fraction was collected and analyzed by flow cytometry resulting in ≥ 90% pure monocyte population.

Freshly isolated monocytes were cultured at 2.5×10^6 cells mL^{-1} in complete medium (RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 30 µg mL^{-1} gentamicin) supplemented with different cytokines or in medium alone. The recombinant porcine INFγ (400 U mL^{-1}) and IFNα (0.8 U mL^{-1}) were generously provided by F. Lefevre (INRA, France); the recombinant porcine IL10 (50 ng mL^{-1}), IL4 (50 ng mL^{-1}) and GM-CSF (50 ng mL^{-1}) were obtained from BioSource (CA, USA). After culture for 24 or 48 h, cells were harvested, stained as indicated and analyzed by flow cytometry.

Results

Cloning of porcine CD163 cDNA

After several attempts for PCR amplification of the porcine CD163 cDNA from alveolar macrophage mRNA, using oligonucleotide primers designed from CD163 sequences of other species, it was achieved by using primer N1ATG, together with primer 2A103520 (Table 1). A 3400 bp cDNA containing the full coding sequence of porcine CD163 was amplified, and cloned, and three independent clones were completely sequenced. The complete sequence of this cDNA is shown in Figure 1, and has been submitted to the EMBL database and assigned accession number AJ311716.

The 3,400 bp cDNA contained an 3,333 bp open reading frame from the initial ATG to the final TGA, coding for a 1,110 amino-acids (AA) protein.

The N-terminal sequence of the porcine CD163 protein isolated by immunoaffinity with mAb 2A10/11 had been previously determined (Sánchez *et al.*, 1999); this sequence (GKDKELRLTG) was found in the translated sequence after the first 40 AAs, which therefore comprise the signal peptide (Fig. 1). Analysis of the protein sequence reveals the presence of nine SRCR domains as previously shown in human protein (Law

et al., 1993), each of which is highlighted in a different color in Figure 1. First six domains are continuous and separated from last three by a sequence of 31 AAs. After the SRCR domains a sequence of 12 AAs leads to the hydrophobic transmembrane region that is followed by a short (48 AAs) intracytoplasmic tail. The abundance of cystein, characteristic of SRCR domains, is not restricted in this protein to those, but six additional cystein residues are found in the rest of the molecule, two in the signal peptide, two more in the region between SRCR domains, and one in each of the transmembrane and cytoplasmic regions.

The porcine CD163 sequence was compared to the orthologous molecules available in data bases (human, primate, bovine, dog, rat, and mouse), and found that the conservation of this molecule is very high, 84-85% identity among non-rodent mammals and 74-75% of identity of porcine sequence to rodent sequences. Consevation is even higher for the SRCR domains, rising up to 95% identity between porcine and human or 83% between pig and rat in fifth SRCR domain. In contrast, identity drops, in the non SRCR sequences, to 60% when comparing the signal peptide of dog and pig or to 53% in the comparison of swine and rat segment between SRCRD6 and D7 (Table 2).

Expression of porcine CD163 in transfected cells. Establishment of stable transfectants

Plasmid containing porcine CD163 was transfected into CHO cells. Expression of the CD163 protein in those cells was analyzed by the reactivity of 2A10 mAb in cytofluorometry. Results of these analyses showed that transfected cells were reactive with the 2A10 mAb, while CHO controls, transfected with empty plasmid, did not show any reactivity (not shown).

To establish cell lines permanently expressing porcine CD163, transfected CHO cells were selected for resistance to Geneticin. Resistant cells were tested by cytofluorimetry for the expression of porcine CD163, and positive cultures were cloned by limiting dilution. After this process two clones (named ML20 and ML11, Fig. 2) of CHO cells expressing different amounts of porcine CD163 in their cell surface were selected.

Monoclonal antibodies 1C6/BM, 2H12/BM and 3E10/BM recognize porcine CD163

Hybridoma producing mAbs 1C6/BM, 2H12/BM and 3E10/BM were derived from a fusion using bone marrow cells as immunogen. These antibodies showed a staining pattern restricted to cells of monocyte-macrophage lineage. They reacted with a subset of blood monocytes and the majority of alveolar macrophages, whereas they did not stain lymphocytes, granulocytes, platelets or erythrocytes (Fig. 3).

On tissue sections, all three antibodies gave almost identical patterns, staining red pulp macrophages in the spleen, cortical and medullary macrophages in thymus, macrophages in the subcapsular sinus and interfollicular areas of lymph nodes and Kupffer cells in the liver (Fig. 4).

Table 2. Comparison of pig CD163 molecule and segments with other species ortologs (percentage of identity)

	Human	Vervet monkey	Cattle	Dog	Rat	Mouse
Whole molecule	84	84	84	85	75	74
Signal peptide	75	72	65	60	—[a]	60
SRCR D1	89	88	84	89	79	80
SRCR D2	87	92	90	90	75	79
SRCR D3	89	89	89	89	79	75
SRCR D4	90	89	87	88	79	75
SRCR D5	93	94	88	92	83	82
SRCR D6	86	87	84	86	76	76
Inter domain segment	70	70	74	76	53	56
SRCR D7	86	86	84	88	87	83
SRCR D8	83	83	81	83	72	72
SRCR D9	84	83	91	89	80	78
Trans-membrane + cytoplasmic segment	66	66	—[b]	73	56	58

[a] Available rat sequence is very different in lenght of all others. [b] Sequence of the same splicing variant not available for comparison.

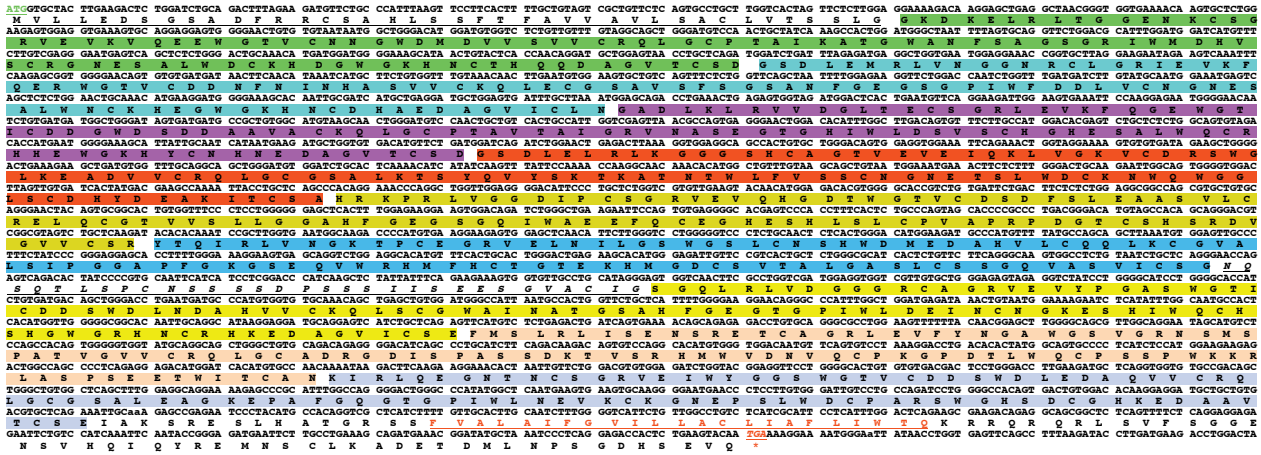

Figure 1. Complete nucleotide sequence (3,400 nt) of cDNA coding for porcine CD163. The translated aminoacid sequence, using the single letter code, is presented under the nucleotide sequence. Initial ATG codon (green and underlined) and end TGA codon (red, italics and underlined) are indicated. The different segments of the protein sequence are highlighted as follows: Signal Peptide, underlined; SRCR domains 1-9 background in different colours; segment between SRCR D6 and D7, underlined and italics; transmembrane segment, red and underlined.

All of them precipitated, from lysates of biotin-labeled alveolar macrophages, a band with relative molecular size of 150,000 under reducing conditions (Fig. 5).

These features resembled that of anti-CD163 mAb 2A10/11 (Bullido *et al.,* 1997; Sánchez *et al.*, 1999). Confirmation of the reactivity of these mAbs with porcine CD163 was obtained by flow cytometric analyses on ML20 cells expressing porcine CD163 (Fig. 6).

Epitope analysis

To determine whether these mAbs were directed against different epitopes on CD163 molecule, cross-blocking experiments were carried out (Fig. 7). MAb 2A10/11 and 3E10/BM blocked the binding of biotinylated 2A10/11, but not that of biotinylated 2H12/BM, to peripheral blood monocytes, as determined by FACS analysis. On the other hand, mAbs 1C6/BM and 2H12/BM blocked the binding of biotinylated 2H12/BM but not of biotinylated 2A10/11. These results indicate that these mAbs react at least with two different epitopes on the CD163 molecule.

Quantitation of sCD163

Next the suitability of a combination of two of the mAbs reacting with different CD163 epitopes, to be used in a sandwich ELISA for detection of sCD163 was evaluated. MAb 2H12 was used for capture of antigen and biotinylated mAb 2A10/11 was used for detection.

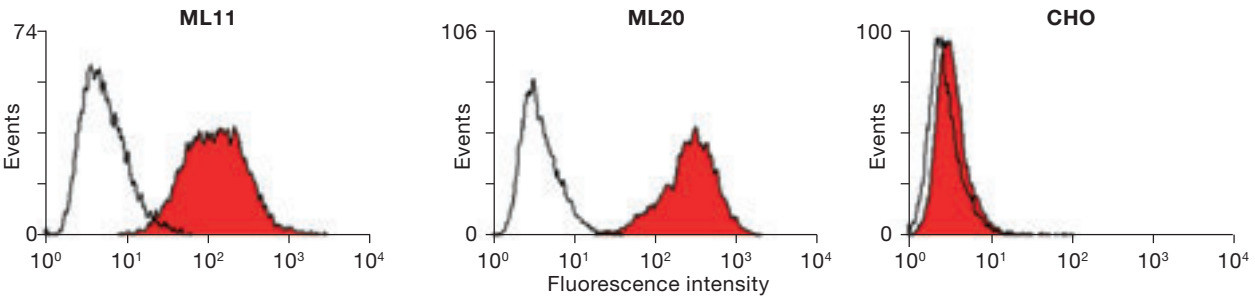

Figure 2. Histograms showing staining with anti-CD163 mAb 2A10/11 (red filled histograms) of CHO transfected (ML11 and ML20) cell lines expressing porcine CD163. Non-transfected CHO cells are shown as control. In each panel, open histogram shows staining with isotype control mAb.

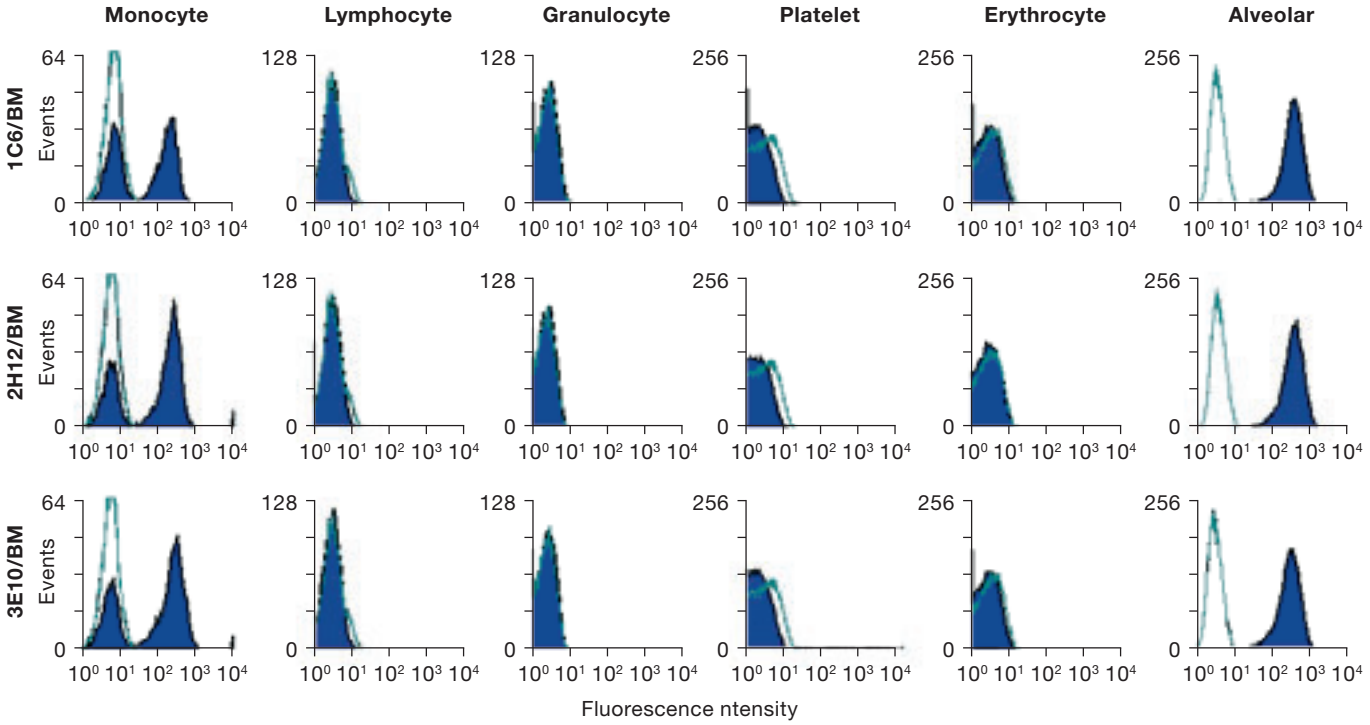

Figure 3. Flow cytometric analysis of porcine peripheral blood monocytes, lymphocytes, granulocytes, platelets, erythrocytes and alveolar macrophages stained by mAbs 1C6/BM, 2H12/BM and 3E10/BM as indicated. Open histogram corresponds to the negative control staining using an isotype-matched irrelevant mAb. Representative results of three independent experiments are shown. All cell types were purified for the analysis, except monocytes and lymphocytes that were discriminated in the PBMC by their light scattering properties (Bullido *et al.,* 1996).

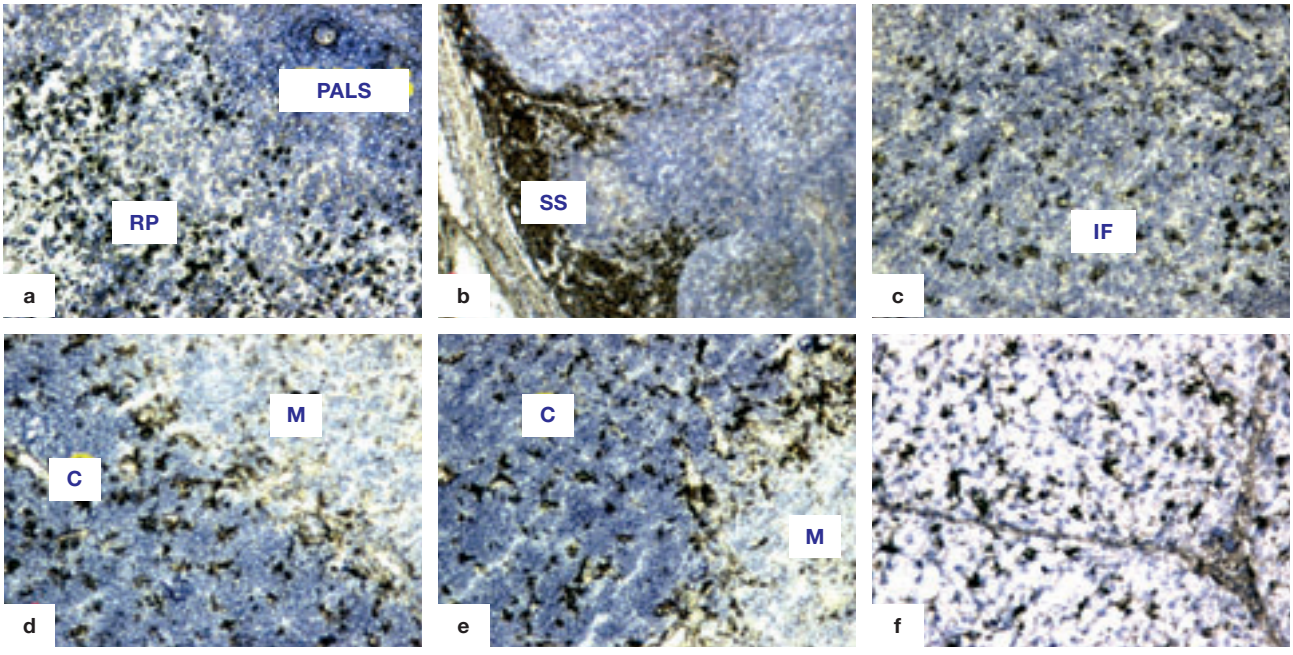

Figure 4. Cryostat sections of porcine spleen (a), mesenteric lymph node (b, c), thymus (d, e) and liver (f) stained with mAb 1C6 (a, b, f), 3E10 (c, e), and 2H12 (d) by the immunoperoxidase method. The periarteriolar lymphoid sheath (PALS) and red pulp of spleen (RP), the interfollicular areas (IF) and subcapsular sinus (SS) in lymph nodes and the cortex (C) and medulla (M) in thymus are indicated. Original magnification: (a, c, d, e, f) × 100; (b) × 40.

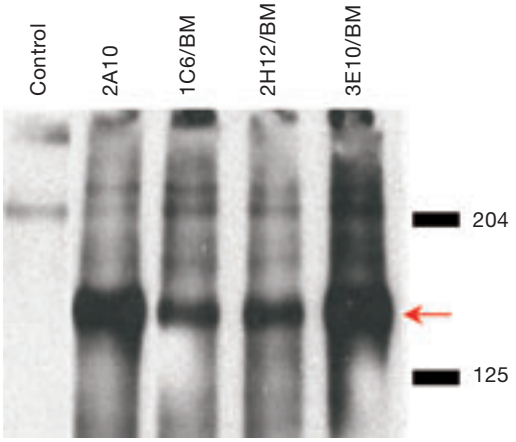

Figure 5. Lysates from biotin labeled alveolar macrophages were immunopreciptated with the indicated antibodies and analyzed by SDS-7.5% PAGE under reducing conditions. The position and size in kDa of the molecular weight markers is given on the right side of the figure. The arrow indicates the position of the specific band.

The dilution of biotinylated mAb 2A10/11 giving the best signal to background ratio was first determined, by using different concentrations of the antibody in assays with lysates of ML-20 cells and CHO cells as negative contol. A dilution of 1:1000 gave the best ratio (not shown) and was used thereafter in following experiments.

Sensitivity of the assay was determined using serial dilutions of lysates from alveolar macrophages and ML-20 cells; lysates from granulocytes, which do not express CD163, and non-transfected CHO cells were used as negative controls. It could be determined that the assay was able to detect the CD163 present in lysates of as few as 10^5 ML-20 cells mL^{-1}, or 3×10^4 macrophages mL^{-1} (Fig. 8A) using a chemiluminiscent substrate; this sensitivity was 3 to 10 times lower when a colorimetric substrate was used.

Then the capacity of this ELISA to detect sCD163 in the supernatant of macrophage cultures was evaluated. Since treatment with PMA causes rapid shedding of CD163 (Droste *et al.*, 1999) the levels of sCD163 in supernatants of cultures of macrophages, treated or not with PMA, were compared. As shown in Fig. 8B sCD163 immunoreactivity is already detected in supernatants from PMA-treated macrophages, after 2 h of treatment, staying at the same level until the end of the experiment after 8 h of treatment.

To determine if this ELISA was able to detect sCD163 present in serum, samples from six different healthy donors were titrated in blocking buffer. As seen in Fig. 8C, sCD163 immunoreactivity was detectable in five of the sera tested at 1:2 dilutions, while only one was negative. Of those positive, three were still positive at 1:4 dilution, and one of these showed high reactivity at the higher dilution tested (1:8).

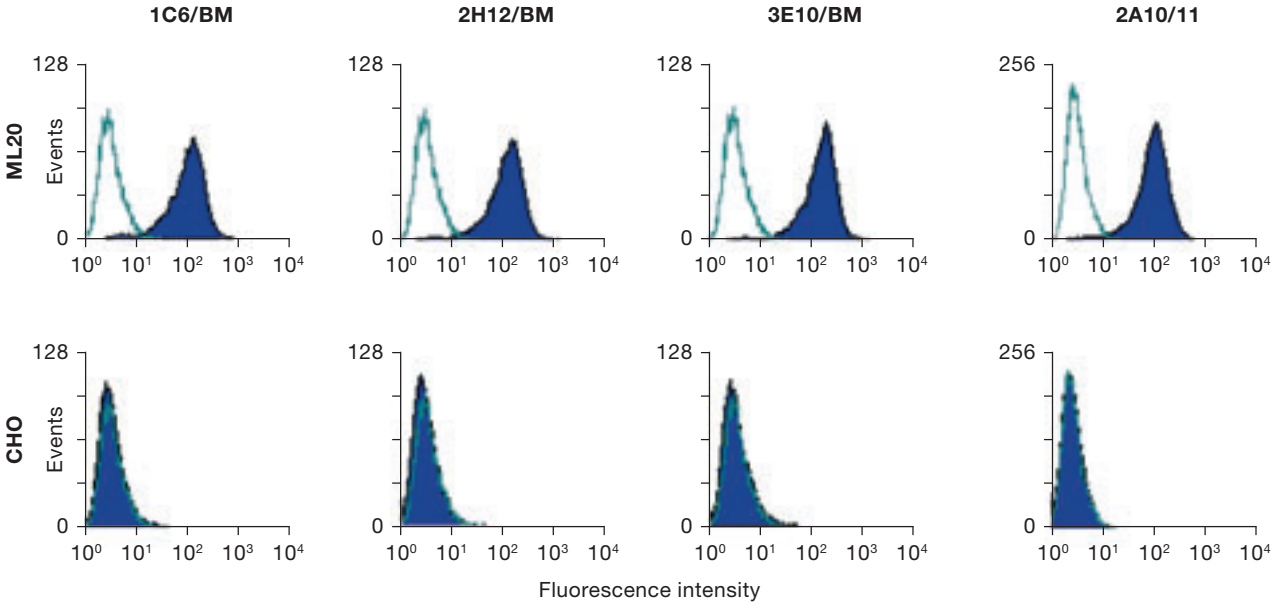

Figure 6. Porcine CD163-expressing CHO cell line, ML20, and control CHO cells were stained with 1C6/BM, 2H12/BM, 3E10/BM and 2A10/11 as indicated. The open histogram corresponds to the negative control staining using an isotype-matched irrelevant mAb.

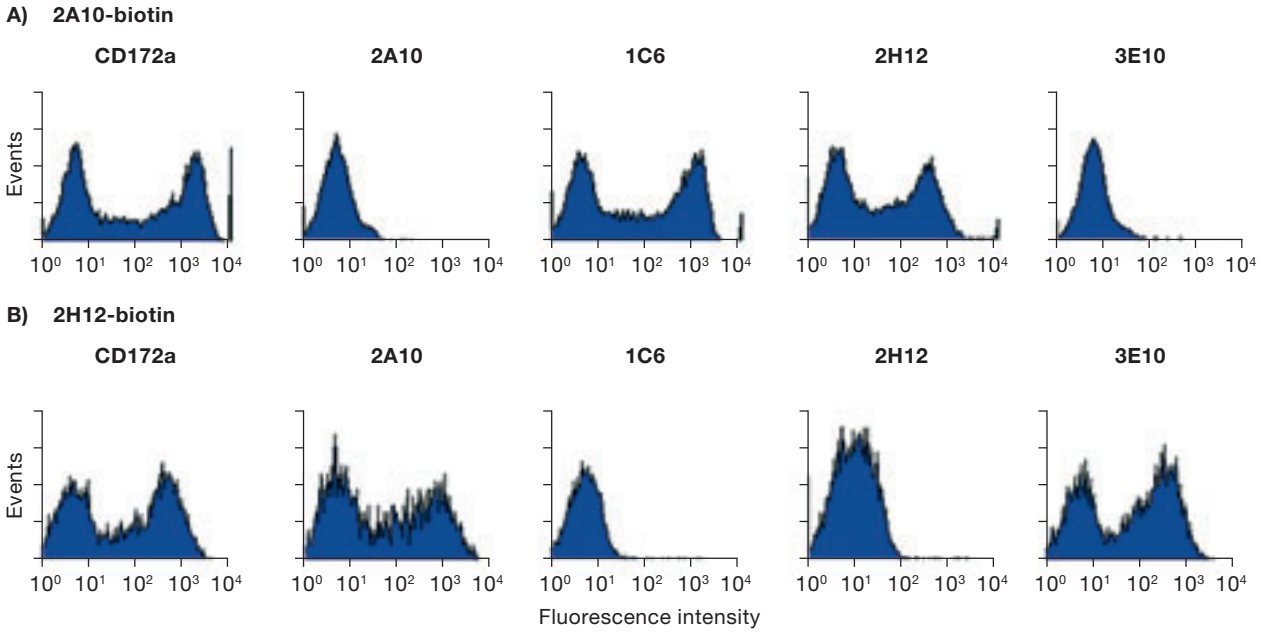

Figure 7. Epitope analysis. Panels represents histograms from peripheral blood monocytes preincubated for 10 min with the indicated unlabeled mAbs, followed by 2A10-biotin (A) or 2H12-biotin (B) and streptavidin –PE. A mAb to CD172 was used as irrelevant control.

Cytokine regulation of CD163 expression

Finally, the effect of different cytokines on the expression of CD163 was analysed. Monocytes were cultured for 24 and 48 h in their presence at the concentration indicated in Material and Methods. Cells were then harvested and analysed by flow cytometry. As shown in Figure 9, treatment of monocytes with IL-10 resulted in a two or three-fold increase of CD163 surface expression that was already detectable at 24 h, whereas treatment with either IL-4 or GM-CSF, alone or in combination (not shown), caused a substantial reduction of CD163 expression, that was more pronounced at 48 h. No significant effect on monocyte CD163 expression was observed with either IFNα or IFNγ at the concentrations used.

Discussion

CD163 is one of the most reliable markers for cells of monocyte/macrophage lineage, being expressed on

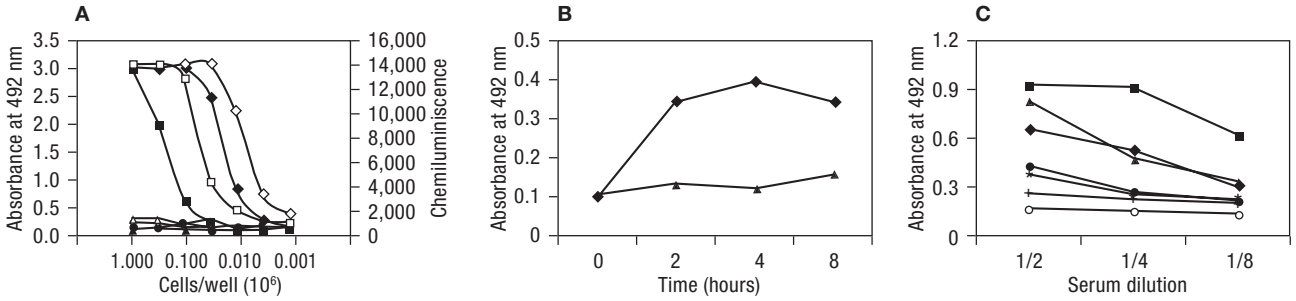

Figure 8. A) Estimation of ELISA sensitivity by titration of porcine CD163-expressing ML20 cells (□) or alveolar macrophages (◇) lysates by using a colorimetric (filled symbols) or a chemiluminiscent substrate (open symbols). Lysates from CHO cells (△) or granulocytes (○) were used as negative controls. Plates were coated with 10 mg mL^{-1} of mAb 2H12 and bound CD163 detected using biotin-labeled mAb 2A10. B) Alveolar macrophages (1×10^6 well) were exposed (◆) or not (▲) to PMA (10^{-8} M) and the presence of sCD163 in the supernatants determined at different times. C) Levels of sCD163 in serum samples from six different healthy donors; FCS (○) was used as negative control.

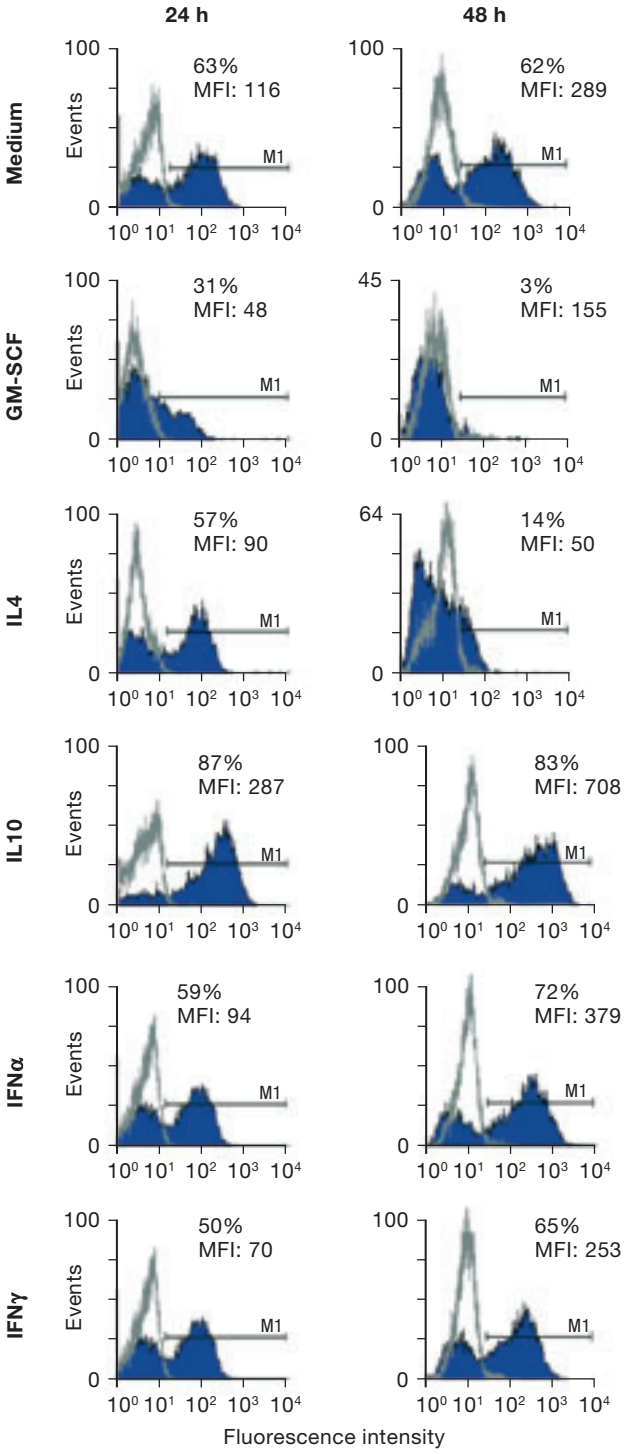

Figure 9. Cytokine regulation of CD163 expression. Monocytes were cultured with the indicated cytokines for 24 h or 48 h and CD163 surface expression (filled histograms) was determined by flow cytometric analysis. Open histogram corresponds to the negative control staining using an isotype-matched irrelevant mAb. The percentage and mean fluorescence intensity (MFI) of CD163-positive cells are indicated. Results are representative of two independent experiments.

most tissue macrophages but not on other myeloid cells. Not much is known about CD163 ligands, being haemoglobin-haptoglobin complexes the only well documented ligand of this molecule. CD163 must play an important role in the control of inflammatory processes as its expression is tightly regulated both by pro- and anti-inflammatory mediators (Buechler *et al.*, 2000; Kristiansen *et al.*, 2001; Schaer *et al.*, 2002; Moestrup and Moller, 2004; Sulahian *et al.*, 2004). Moreover, signalling through CD163 leads to production of cytokines (Van den Heuvel *et al.*, 1999; Phillippidis *et al.*, 2004). In addition, inflammatory stimuli lead to a protease-dependent shedding of CD163 ectodomain (Hintz *et al.*, 2002; Timmermann and Hogger, 2005).

In swine, we reported a role for CD163 in African swine fever (ASF) virus infection of monocyte/macrophage (Sánchez-Torres *et al.*, 2003). Recently, Calvert *et al.* (2007) have shown that expression of CD163 made cells susceptible to porcine respiratory and reproductive syndrome (PRRS) virus infection.

In order to be able to further explore these issues on porcine CD163 a cDNA coding for the porcine CD163 molecule has been cloned and characterized. The cloned cDNA coded for a 1,110 AAs protein that conserved all the features described for the human molecule (Law *et al.*, 1993). The sequence contained the peptides that served to identify the molecule recognized by mAb 2A10/11 with porcine CD163 (Sánchez *et al.*, 1999), these include the n-terminal peptide of the molecule that was located after the first 40 AAs, that therefore comprise a rather long signal peptide. Comparison of this sequence with those of other species showed a high conservation of these molecules, what most likely indicates a conserved function.

The cDNA coding for porcine CD163 has been expressed in CHO cells after transfection, and CHO cell lines stably expressing porcine CD163 have been generated. These cell lines are expected to become a valuable tool to search for porcine CD163 ligands.

In the present study it is also described the characterisation of a set of three new anti-porcine CD163 mAbs. These antibodies precipitate a molecule with a size of 150 kDa and react in flow cytometric analyses with the majority of alveolar macrophages and a subset of blood monocytes. In immunohistochemical analyses on frozen sections they stained various populations of tissue macrophages including Kupffer cells in the liver, cortical and medullar macrophages in the thymus, red pulp macrophages in the spleen, and interfollicular and sinus macrophages in lymph nodes. Altogether, the

reactivity pattern of these antibodies closely resemble that previously reported with the anti-CD163 mAb 2A10/11 (Bullido *et al.*, 1997; Sánchez *et al.*, 1999). This specificity was confirmed by the staining of CHO cell lines expressing the porcine CD163 antigen.

These anti-CD163 mAbs will increase the range of available reagents for the study of macrophage populations in both normal and pathological conditions.

Cytokines are potent modulators of macrophage activation, inducing the expression of a distinctive range of surface receptors and other phenotypic changes. CD163 expression is strongly upregulated by IL-10, a cytokine which induces a deactivation state in macrophage, associated with an anti-inflammatory function. On the other side, IL-4, the archetypal cytokine for «alternative activation of macrophages» (Gordon, 2003) and GM-CSF suppress CD163 expression. These results agree with those reported in humans by Buechler *et al.* (2000), Sulahian *et al.* (2000) and Schaer *et al.* (2002), and with previous results from our group showing a decline in CD163 expression along the *in vitro* differentiation of monocytes into dendritic cells induced by GM-CSF and IL-4 (Chamorro *et al.*, 2004). Like Sulahian *et al.* (2000), no effect of IFNα or IFNγ, which induces classical activation of macrophages, on CD163 expression has been observed. This contrasts with the results of Buechler *et al.* (2000) who found that IFNγ suppressed its expression. Variations in the treatment of cells and culture conditions may explain these different results.

In addition to the membrane-bound variants of CD163, there is a soluble form of CD163 which corresponds to the extracellular domain of CD163 that is shed after treatment of monocytes/macrophages with inflammatory stimuli. In healthy human volunteers, following an injection of LPS, the levels of soluble CD163 in plasma showed a sharp increase that peaked at 2 h and began to decline by 4 h (Hintz *et al.*, 2002), behaving as an early-phase reactant. Elevated plasma levels of sCD163 could be therefore a good indicator of an ongoing inflammatory or activation process of tissue macrophages. Besides, sCD163 appears to have immunosuppresive effects, inhibiting the activation and proliferation of T lymphocytes at concentrations not higher than physiological levels (Hogger and Sorg, 2001; Frings *et al.*, 2002).

The availability of mAbs against at least two non-overlapping epitopes of CD163 molecule has allowed to develop an ELISA for determination of the concentration of sCD163 in body fluids. Although purified CD163 has not been used for calibration and these values can not be compared with those reported in humans, this assay has enough sensitivity to detect sCD163 present in sera of healthy animals as well as in supernatants from macrophage cultures. Consistent with the shedding of CD163 after treatment with inflammatory stimuli, the supernatants from PMA-treated macrophages contained more soluble CD163 than those from non-treated cells.

Some of the most economically important diseases of swine, such as ASF, classical swine fever (CSF), PRRS or post-weaning multisystemic wasting syndrome (PWMS) are caused by viruses that replicate in cells of monocyte/macrophage lineage. Permissiveness of monocytes to ASF virus infection correlates with expression of CD163, and this receptor appears to be involved in the entry of the virus into the cell (Sánchez-Torres *et al.*, 2003). On the other hand, increased levels of IL-10 have been reported in PRRS and PWMS-affected pigs (Darwich *et al.*, 2003; Suradhat *et al.*, 2003; Thanawongnuwech *et al.*, 2004). As seen above, IL-10 induces a strong upregulation of CD163 expression. Signaling through CD163 can, in turn, lead to the secretion of high levels of IL-10 in macrophages, resulting in a positive feedback loop (Philippidis *et al.*, 2004). In this respect, the interaction of PRRSV with CD163 recently reported by Calvert *et al.* (2007) might play a role in the increased levels of IL-10 in PRRS.

The ELISA here described will allow to evaluate the usefulness of sCD163 serum levels for monitoring the degree of activation of macrophages in these infections and the potential contribution of this molecule to the impaired immune responses associated with these infections.

In summary, a cDNA coding for the porcine CD163 molecule has been cloned and characterized. Expression of this cDNA in CHO cells has allowed to generate cell lines stably expressing porcine CD163 molecule. We have also described the generation of three new specific mAb against porcine CD163 that proved to be useful in a wide range of techniques, such as flow cytometry, immunohistochemistry, immunoprecipitation and ELISA. All these tools are already valuable means for studying the role of this molecule in the pathogenesis of a variety of swine diseases.

Acknowledgements

This work was supported by grants AGL2000-1441, and CSD2006-0007 from Spanish Ministry of Education

and Science, and CPE03-021-C4-2 of the National Institute for Agricultural and Food Research and Technology (INIA). C.R. was recipient of a RyC contract of the Spanish Ministry of Education and Science.

References

ÁLVAREZ B., DOMÉNECH N., ALONSO F., SÁNCHEZ C., GÓMEZ DEL MORAL M., EZQUERRA A., DOMÍNGUEZ J., 2000. Molecular and functional characterization of porcine LFA-1 using monoclonal antibodies to CD11a and CD18. Xenotransplantation 7, 258-266.

ARISTOTELI L.P., MOLLER H.J., BAILEY B., MOESTRUP S.K., KRITHARIDES L., 2006. The monocytic lineage specific soluble CD163 is a plasma marker of coronary atherosclerosis. Atherosclerosis 184, 342-347.

BUECHLER C., RITTER M., ORSO E., LANGMANN T., KLUCKEN J., SCHMITZ G., 2000. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. J Leukoc Biol 67, 97-103.

BULLIDO R., ALONSO F., GÓMEZ DEL MORAL M., EZQUERRA A., ÁLVAREZ B., ORTUÑO E., DOMÍNGUEZ J., 1996. Monoclonal antibody 2F4/11 recognizes the alpha chain of a porcine beta 2 integrin involved in adhesion and complement mediated phagocytosis. J Immunol Methods 195(1-2), 125-34.

BULLIDO R., GÓMEZ DEL MORAL M., ALONSO F., EZQUERRA A., ZAPATA A., SÁNCHEZ C., ORTUÑO E., ÁLVAREZ B., DOMÍNGUEZ J., 1997. Monoclonal antibodies specific for porcine monocytes/macrophages: macrophage heterogeneity in the pig evidenced by the expresión of surface antigens. Tissue Antigens 49, 403-413.

CALVERT J.G., SLADE D.E., SHIELDS S.L., JOLIE R., MANNAN R.M., ANKENBAUER R.G., WELCH S.K., 2007. CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. J Virol 81(14), 7371-7379.

CHAMORRO S., REVILLA C., GÓMEZ N., ÁLVAREZ B., ALONSO F., EZQUERRA A., DOMÍNGUEZ J., 2004. In vitro differentiation of porcine blood CD163– and CD163+ monocytes into functional dendritic cells. Immunobiol 209, 57-65.

CHOMCZYNSKI P., SACCHI N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162, 156-159.

DARWICH L., BALASCH M., PLANA-DURÁN J., SEGALES J., DOMINGO M., MATEU E., 2003. Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. J Gen Virol 84, 3453-3457.

DOMÉNECH N., RODRÍGUEZ-CARRENO M.P., FILGUEIRA P., ÁLVAREZ B., CHAMORRO S., DOMÍNGUEZ J., 2003. Identification of porcine macrophages with monoclonal antibodies in formalin-fixed, paraffin-embedded tissues. Vet Immunol Immunopathol 94(1-2), 77-81.

DOMÍNGUEZ J., HEDRICK R.P., SÁNCHEZ-VIZCAÍNO J.M., 1990. Use of monoclonal antibodies for detection of infectious pancreatic necrosis virus by the enzyme-linked immunosorbent assay. Dis Aquat Org 8, 157.

DROSTE A., SORG C., HOGGER P., 1999. Shedding of CD163, a novel regulatory mechanism for a member of the scavenger receptor cysteine-rich family. Biochem Biophys Res Commun 256(1), 110-113.

FABRIEK B.O., DIJKSTRA C.D., VAN DEN BERG T.K., 2005. The macrophage scavenger receptor CD163. Immunobiology 210(2-4), 153-160.

FRINGS W., DREIER J., SORG C., 2002. Only the soluble form of the scavenger receptor CD163 acts inhibitory on phorbol ester-activated T-lymphocytes, whereas membrane-bound protein has no effect. FEBS Lett 526(1-3), 93-96.

GONZÁLEZ S., MENDOZA C., SÁNCHEZ-VIZCAÍNO J.M., ALONSO F., 1990. Inhibitory effect of African swine fever virus on lectin-dependent swine lymphocyte proliferation. Vet Immunol Immunopathol 26, 71-80.

GORDON S., 2003. Alternative activation of macrophages. Nature Rev Immunol 3, 23-35.

HINTZ K., RASSIAS A.J., WARDWELL K., MOSS M.L., MORGANELLI P.M., PIOLI P.A., GIVAN A.L., WALLACE P.K., YEAGER M.P., GUYRE P.M., 2002. Endotoxin induces rapid metalloproteinase-mediated shedding followed by up-regulation of the monocyte hemoglobin sacvenger receptor CD163. J Leukoc Biol 72, 711-717.

HIRAOKA A., HORIIKE N., AKBAR S.M., MICHITAKA K., MATSUYAMA T., ONJI M., 2005. Soluble CD163 in patients with liver diseases: very high levels of soluble CD163 in patients with fulminant hepatic failure. J Gastroenterol 40, 52-56.

HOGGER P., SORG C., 2001. Soluble CD163 inhibits phorbol ester-induced lymphocyte proliferation. Biochem Biophys Res Commun 288, 841-843.

KÖHLER G., MILSTEIN C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-497.

KOMOHARA Y., HIRAHARA J., HORIKAWA T., KAWAMURA K., KIYOTA E., SAKASHITA N., ARAKI N., TAKEYA M., 2006. AM-3K, an anti-macrophage antibody, recognizes CD163, a molecule associated with an anti-inflammatory macrophage phenotype. J Histochem Cytochem 54, 763-771.

KRISTIANSEN M., GRAVERSEN J.H., JACOBSEN C., SONNE O., HOFFMAN H.J., LAW S.K., MOESTRUP S.K., 2001. Identification of the haemoglobin scavenger receptor. Nature 409, 198-201.

LAW S.K.A., MICKLEM K.J., SHAW J.M., ZHANG X.P., DONG Y., WILLIS A.C., MASON D.Y., 1993. A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. Eur J Immunol 23, 2320-2325.

MOESTRUP S.K., MOLLER H.J., 2004. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. Ann Med 36, 347-354.

PHILIPPIDIS P., MASON J.C., EVANS B.J., NADRA I., TAYLOR K.M., HASKARD D.O., LANDLIS R.C., 2004. Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxigenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. Circ Res 94, 119-126.

RITTER M., BUECHLER C., LANGMANN T., ORSO E., KLUCKEN J., SCHMITZ G., 1999. The scavenger receptor CD163: regulation, promoter structure and genomic organization. Pathobiology 67(5-6), 257-261.

SAALMULLER A., LUNNEY J.K., DAUBENBERGER C., DAVIS W., FISCHER U., GOBEL T.W., GRIEBEL P., HOLLEMWEGUER E., LASCO T., MEISTER R., SCHUBERTH H.J., SESTAK K., SOPP P., STEINBACH F., XIAO-WEI W., AASTED B., 2005. Summary of the animal homologue section of HLDA8. Cell Immunol 236, 51-58.

SÁNCHEZ C., DOMÉNECH N., VÁZQUEZ J., ALONSO F., EZQUERRA A., DOMÍNGUEZ J., 1999. The porcine 2A10 antigen is homologous to human CD163 and related to macrophage differentiation. J Immunol 162, 5230-5237.

SÁNCHEZ-TORRES C., GÓMEZ-PUERTAS P., GÓMEZ DEL MORAL M., ALONSO F., ESCRIBANO J.M., EZQUERRA A., DOMÍNGUEZ J., 2003. Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection. Arch Virol 148, 2307-2323.

SCHAER D.J., BORETTI F.S., SCHOEDON G., SCHAFFNER A., 2002. Induction of the CD163-dependent haemoglobin uptake by macrophages as a novel anti-inflammatory action of glucocorticoids. British J Haematol 119, 239-243.

SCHAER D.J., SCHLEIFFENBAUM B., KURRER M., IMHOF A., BACHLI E., FEHR J., MOLLER H.J., MOESTRUP S.K., SCHAFFNER A., 2005. Soluble hemoglobin-haptoglobin scavenger receptor CD163 as a lineage-specific marker in the reactive hemophagocytic syndrome. Eur J Haematol 74, 6-10.

SCHAER D.J., SCHAER C.A., BUEHLER P.W., BOYKINS R.A., SCHOEDON G., ALAYASH A.I., SCHAFFNER A., 2006. CD163 is the macrophage scavenger receptor for native and chemically modified haemoglobins in the absence of haptoglobin. Blood 107, 373-380.

SULAHIAN T.H., HOGGER P., WAHNER A.E., WARDWELL K., GOULDING N.J., SORG C., DROSTE A., STEHLING M., WALLACE P.K., MORGANELLI P.M., GUYRE P.M., 2000. Human monocytes express CD163, which is upregulated by IL-10 and identical to p155. Cytokine 12, 1312-1321.

SULAHIAN T.H., PIOLI P.A., WARDWELL K., GUYRE P.M., 2004. Cross-linking of FcgR triggers shedding of the hemoglobin-haptoglobin scavenger receptor CD163. J Leukoc Biol 76, 271-277.

SURADHAT S., THANAWONGNUWECH R., 2003. Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. J Gen Virol 84, 2755-2760.

THACKER E., SUMMERFIELD A., MCCULLOUGH K., EZQUERRA A., DOMINGUEZ J., ALONSO F., LUNNEY J., SINKORA J., HAVERSON K., 2001. Summary of workshop findings for porcine myelomonocytic markers. Vet Immunol Immunopathol 80, 93-109.

THANAWONGNUWECH R., THACKER B., HALBUR P., THACKER E.L., 2004. Increased production of proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae.* Clin Diagn Lab Immunol 11(5), 901-908.

TIMMERMANN M., HOGGER P., 2005. Oxidative stress and 8-iso-prostaglandin F(2alpha) induce ectodomain shedding of CD163 and release of tumor necrosis factor-alpha from human monocytes. Free Radic Biol Med 39, 98-107.

TIMMERMANN M., BUCK F., SORG C., HOGGER P., 2004. Interaction of soluble CD163 with activated T lymphocytes involves its association with non-muscle myosin heavy chain type A. Immunol Cell Biol 82, 479-487.

VAN DEN HEUVEL M.M., TENSEN C.P., VAN AS J.H., VAN DEN BERG T.K., FLUITSMA D.M., DIJKSTRA C.D., DÖPP E.A., DROSTE A., VAN GAALEN F.A., SORG C., HÖGGER P., BEELEN R.H., 1999. Regulation of CD163 on human macrophages: cross-linking of CD163 induces signalling and activation. J Leukoc Biol 66, 858-866.