The Journal of Immunology

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J Immunol 2012; 188:2399-2409; Prepublished online 25 January 2012;
doi: 10.4049/jimmunol.1103150
http://www.jimmunol.org/content/188/5/2399

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

CD163-L1 Is an Endocytic Macrophage Protein Strongly Regulated by Mediators in the Inflammatory Response

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CD163-L1 belongs to the group B scavenger receptor cysteine-rich family of proteins, where the CD163-L1 gene arose by duplication of the gene encoding the hemoglobin scavenger receptor CD163 in late evolution. The current data demonstrate that CD163-L1 is highly expressed and colocalizes with CD163 on large subsets of macrophages, but in contrast to CD163 the expression is low or absent in monocytes and in alveolar macrophages, glia, and Kupffer cells. The expression of CD163-L1 increases when cultured monocytes are M-CSF stimulated to macrophages, and the expression is further increased by the acute-phase mediator IL-6 and the anti-inflammatory mediator IL-10 but is suppressed by the proinflammatory mediators IL-4, IL-13, TNF-α, and LPS/IFN-γ. Furthermore, we show that CD163-L1 is an endocytic receptor, which internalizes independently of cross-linking through a clathrin-mediated pathway. Two cytoplasmic splice variants of CD163-L1 are differentially expressed and have different subcellular distribution patterns. Despite its many similarities to CD163, CD163-L1 does not possess measurable affinity for CD163 ligands such as the haptoglobin–hemoglobin complex or various bacteria. In conclusion, CD163-L1 exhibits similarity to CD163 in terms of structure and regulated expression in cultured monocytes but shows clear differences compared with the known CD163 ligand preferences and expression pattern in the pool of tissue macrophages. We postulate that CD163-L1 functions as a scavenger receptor for one or several ligands that might have a role in resolution of inflammation. ***The Journal of Immunology*****, 2012, 188: 2399–2409.**

CD163-L1 (M160) is a group B scavenger receptor cysteine-rich (SRCR) molecule that is expressed by cells of myeloid origin (1). CD163-L1 belongs to the SRCR superfamily, which is an ancient group of receptors that have been identified in a wide range of animal phyla and in certain unicellular algae (2–4). The domain organization of CD163-L1 features a large extracellular region containing 12 group B SRCR domains, followed by a transmembrane region and a cytoplasmic tail that occurs in two forms: the predominant full-length variant CD163-L1α of 71 residues and the short-tail variant CD163-L1β of 39 residues.

We have previously shown by conventional RT-PCR that CD163-L1 mRNA is expressed by alveolar macrophages and by the myeloid cell lines HL60, U937, and THP1 but not by Jurkat or Raji cells. Moreover, stimulation of U937 cells with phorbol ester resulted in an increase of expression. RT-PCR analysis of 19 different tissues showed that the full-length CD163-L1α is expressed in all tissues, whereas expression of CD163-L1β appears confined to the spleen (1).

The five membrane-proximal SRCR domains of CD163-L1 constitute a so-called long-range repeat, which shows up to 75% sequence identity to that of CD163 (1, 5). The high structural similarity to CD163, together with the assignment of the gene encoding CD163-L1 to chromosome 12p13.3 adjacent to the CD163 gene, suggests that the primate-specific CD163-L1 has emerged via a recent gene duplication of CD163 (1, 6).

CD163 is a well-characterized transmembrane glycoprotein that comprises nine extracellular SRCR group B domains, followed by a transmembrane region and a cytoplasmic tail that occurs in three forms (7). CD163 has been identified and characterized as an endocytic receptor for the haptoglobin–hemoglobin (Hp–Hb) complex and is believed to be essential for the removal of free hemoglobin by macrophages during hemolysis (8–10). In addition, CD163 has also been identified as a potential erythroblast adhesion receptor and as a novel pattern recognition receptor for bacteria (11, 12). Indeed, the ability to bind microbial microorganisms has been shown for several members of the SRCR superfamily including SR-AI, gp-340, MARCO, CD5, CD6, and Spα, suggesting a possible unifying function as pattern recognition receptors in host defense (13–18).

At present, little is known about the expression and function of CD163-L1. In the current study, we have developed and used novel Abs directed against the protein for characterization of the CD163-L1 tissue distribution and regulation. Moreover, we have used various recombinant protein expression approaches to study the endocytic properties of CD163-L1 and potential differences of two splice variants of the protein.

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Received for publication November 3, 2011. Accepted for publication December 19, 2011.

This work was supported by the Danish Medical Research Council, the Novo Nordisk Foundation, the Lundbeck Foundation, Fonden til Lægevidenskabens Fremme, and Overlægerådets Legat-udvalg.

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Abbreviations used in this article: AU, arbitrary unit; Hp–Hb, haptoglobin–hemoglobin; qRT-PCR, quantitative RT-PCR; SPR, surface plasmon resonance; SRCR, scavenger receptor cysteine-rich.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1103150

Materials and Methods

Buffers

Buffers used were PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), PBS/BSA [PBS, 0.5% (w/v) BSA], TBS [140 mM NaCl, 10 mM Tris-HCl, 0.02% (w/v) NaN_2, pH 7.4], TBST (TBS, 0.05% Tween 20), sample buffer (100 mM Tris, 2% SDS, 10% glycerol, 0.0145% bromophenol blue, pH 8.9), and TEG buffer (10 mM Tris, 0.5 mM EDTA, pH 9).

Establishment of stable transfectant cells

HEK293 cells transfected with CD163 cDNA were obtained from a previous study (19), and vectors containing CD163-L1α or CD163 cDNA (1, 8) were used as templates for the generation of all other constructs. Full-length CD163-L1β cDNA was obtained by PCR mutagenesis of the CD163-L1α cDNA using the 5′-phosphorylated primers 5′P-GATGACACCCCCAA-CCATGGTTGTGAAGATGC-3′ and 5′P-TCTGAGGGGCAGATGTTTTT-GTTTCTGAACTC-3′. Mutagenesis of the di-leucine internalization motif of CD163-L1α was performed using the QuickChange II Site-Directed Mutagenesis Kit according to the manufacturer's recommendations (Stratagene). cDNA fragments of the CD163-L1 splice variants, CD163-L1 SRCR 1–7, and CD163-L1 ectodomain were produced by PCR using the forward primer CD163-L1FL 5′-GACTCAGGAAGAGATAGACC-3′ and the corresponding reverse primers CD163-L1 FL 5′-GGACAAGTTTTCCA-TAGG-3′, CD163-L1 SRCR 1–7 5′-AATCTGCCTGTGGGCTGAGCA-3′, and CD163-L1 ectodomain 5′-CAGCGACTGTCCAGAGCACCT-3′, respectively. The PCR products were cloned into the expression vector pcDNA5/FRT/V5-His-TOPO TA vector (Invitrogen) and sequenced in their entirety. The fusion construct encoding the extracellular and transmembrane regions of CD163 (aa 1–1067) fused to the cytoplasmic tail of CD163-L1 (aa 1383–1453) was generated by PCR using the CD163 primers 5′-CCCGGTACCGAATTCTTAGTTGTTTTC-3′ and 5′-CCC-GGATCCAGTCAAGAAGAATAATGC-3′ and the CD163-L1 primers 5′-GAGTCTGGATCCTGCCGAGTTCAGAAACAAAAAC-3′ and 5′-GAGGAGGCGGCCGCTTGGACAAGTTTTCCATAGGGC-3′. The generated cDNA fragments were inserted into the KpnI and NotI sites of the pcDNA5/FRT vector, respectively, and fused to one another by ligation, taking advantage of a primer-generated BamHI site situated in the 3′ end of the CD163 cDNA fragment and 5′ end of the CD163-L1 cDNA fragment. The resulting fusion construct was sequenced in its entirety. The produced pcDNA5/FRT vectors were transfected into Flp-In HEK293 cells (Invitrogen) using either jetPEI (Polyplus-transfection) or FuGENE 6 (Roche Diagnostics). Stable transfectants were selected with 150 μg/ml Hygromycin B (Invitrogen), and protein expression was verified by Western blotting of cell culture supernatants or cell lysates. Stably transfected clones were cultured and maintained in DMEM, 2 mM L-glutamine, 10% FBS, 150 μg/ml Hygromycin B, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

Expression and purification of recombinant CD163-L1

Stably transfected clones expressing recombinant CD163-L1 SRCR 1–7 or ectodomain were grown to confluence. At confluence, cells were washed three times in PBS and cultured without FBS in DMEM, 2 mM L-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin (Invitrogen). Supernatants were harvested after 96 h, and recombinant CD163-L1 SRCR 1–7 and ectodomain were purified using a HisTrap HP column (GE Healthcare) as described by Schlosser et al. (20).

Development of mAbs

BALB/c mice were immunized s.c. with 25 μg purified recombinant CD163-L1 SRCR 1–7 in 400 μl 1:1 (v/v) PBS/Freund's incomplete adjuvant per injection. The injections were repeated at intervals of 14 d. Mice with high Ab titer against CD163-L1, as determined by ELISA on CD163-L1 SRCR 1–7 coated microtiter plates, were selected for fusion with spleen cells. B cell hybridomas were produced by fusion with myeloma cells (X63Ag8.6.5.3) using polyethylene glycol 4000 as the fusogen. Recombinant CD163-L1 SRCR 1–7 was used to identify positive anti–CD163-L1 clones by ELISA. Hybridomas producing Abs against CD163-L1 were cloned three times and adapted to serum-free hybridoma medium containing 20 units/ml penicillin and 20 μg/ml streptomycin (Invitrogen). Abs were purified using a protein G column (GE Healthcare) as described by Schlosser et al. (20). ELISA and biotinylation of Abs were performed essentially as described by Leth-Larsen et al. (21).

Generation of Fab

Purified mAbs were dialyzed against 100 mM acetate buffer, 1 mM EDTA, and 50 mM cysteine pH 5.5 and incubated for 5 h with 10 μg/mg (Ab) of papain (Sigma-Aldrich). The generated Fab fragments were separated by size exclusion chromatography using PBS pH 7.4 on a Superdex 200 HiLoad 16/60 column (GE Healthcare).

Immunoprecipitation of CD163-L1

mAbs were immobilized using cyanogen bromide-activated Sepharose 4B (GE Healthcare). Coupling was performed as recommended by the manufacturer using 10 mg Ab per milliliter of gel. The monoclonal anti-OVA Ab (Hyb 099-01) was used as a control. Immunoprecipitation was performed using Triton X-100 lysates of placenta precleared against Sepharose 4B (GE Healthcare) for 1 h at 4°C. In brief, 200 μl Sepharose 4B-immobilized Ab slurry was combined with 10 ml placenta lysate and incubated for 2 h at 4°C with gentle agitation. The immobilized Ab matrices were washed twice in 10 ml lysis buffer and twice in 10 ml TBS containing 500 mM NaCl and 5 mM $CaCl_2$. Elution was performed using 100 mM glycine HCl, pH 2.7.

Alexa Fluor 488 labeling of Abs

Alexa Fluor 488 labeling of mAbs and Fab fragments was performed using the Alexa Fluor protein labeling kit, as described by the manufacturer (Invitrogen).

SDS-PAGE and Western blotting

Protein samples were diluted in sample buffer, denatured, and separated on 4–12% polyacrylamide gradient gels using a discontinuous buffer system (Bio-Rad). Separated proteins were blotted onto Amersham Hybond-P polyvinylidene difluoride membranes (GE Healthcare). The membranes were incubated overnight at 4°C with 1 μg/ml monoclonal mouse anti–CD163-L1 (HG-Hyb1-3), polyclonal rabbit anti–CD163-L1 (Protein Atlas ID: HPA015663), polyclonal rabbit anti-CD163 Ab (8) or polyclonal rabbit anti–gp-340 (22) diluted in TBST containing 2.5% (w/v) dry milk. The membrane was washed extensively in TBST and incubated for 1 h in TBST with either alkaline phosphatase-coupled rabbit anti-mouse IgG (Sigma-Aldrich) diluted 1:2000, HRP-coupled goat anti-rabbit IgG (Sigma-Aldrich) diluted 1:10,000, or HRP-coupled rabbit anti-mouse IgG (Sigma-Aldrich) diluted 1:10,000. The membranes were washed extensively and developed as described by Schlosser et al. (20) or using the ECL Plus kit, as described by the manufacturer (GE Healthcare).

Deglycosylation of CD163-L1

Deglycosylation of CD163-L1 expressed by HEK293 cells was performed by enzymatic digestion with PNGase F (unreduced), as recommended by the manufacturer (New England Biolabs).

Quantification of gene expression by real-time PCR

The relative tissue distribution of CD163-L1 mRNA was analyzed using a commercial cDNA library (PrimerDesign). Total RNA from cultured cells was prepared using TRIzol reagent, as recommended by the manufacturer (Invitrogen). cDNA synthesis was performed from 1 μg purified RNA using an oligo$(dT)_{18}$ primer and the Superscript III reverse transcriptase according to the manufacturer's recommendations (Invitrogen). Relative quantification of CD163-L1 and CD163 gene expression was achieved using custom-made TaqMan assays with the primer sequences CD163-L1exon19 (forward) 5′-GCCTCTGAAGCCACAAAATGAC-3′, CD163-L1 exon19 (reverse) 5′-TCCTCTTTATTCATTTAAAAGTTGTTGTCTCCT-3′, CD163 exon5 (forward) 5′-TGAAGATGCTGGCGTGACA-3′, and CD163 exon5 (reverse) 5′-CACACCTTCCCTAACAGTCTCT-3′ (Applied Biosystems). Analysis was performed in triplicate using the cycling conditions 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Quantification of CD163-L1α and CD163-L1β was performed using custom-made PerfectProbe assays with the primer sequences CD163-L1α (forward) 5′-TCAGAGTTTCAACCAGAAGGAG-3′, CD163-L1α (reverse) 5′-TGTCATCTGAGGTTCTTGTCC-3′, CD163-L1β (forward) 5′-TGCCCCTCAGAGATGACAC-3′, and CD163-L1β (reverse) 5′-AGG-TTGATCTGGTGAGCCC-3′ (PrimerDesign). Analysis was performed in triplicate using the cycling conditions 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 15 s. A standard curve was included in each run prepared from dilution series of commercial assay specific copy number controls (PrimerDesign). The absolute and relative abundance of the CD163-L1 splice variants was quantified by normalization to the measured standard curves. All studies were performed on a 7500 Real-Time PCR System (Applied Biosystems) using 18S RNA (tissue distribution) or the reference genes GAPDH, B2M, and TBP (cultured cells) for normalization. The relative expression was analyzed using the qBase software (Biogazelle), and the results were assessed with the two-tailed Student *t* test.

Immunohistochemistry

Normal human tissues were obtained from the tissue bank at the Department of Pathology, Odense University Hospital (Odense, Denmark). Four-micrometer parallel tissue sections were cut from neutral-buffered formaldehyde-fixed paraffin blocks, mounted on ChemMate Capillary Gap slides (Dako), de-paraffinated, and hydrated by conventional methods. Endogenous peroxidase activity was blocked using TBS containing 1.5% hydrogen peroxide for 10 min. Ag retrieval was performed by microwave heating in TEG buffer. Three Tissue-Tek containers (Miles), each with 24 slides in 250 ml buffer, were placed on the edge of a turntable inside the microwave oven. Slides were heated 11 min at full power (900 W), then for 15 min at 400 W. After heating, slides remained in the TEG buffer for 15 min. Immunostaining was automated using a TechMate 500 autostainer (Dako). Detection of CD163-L1 was performed using the PowerVision+ HRP detection system DPVB+500HRP (ImmunoVision Technologies), and immunostaining of CD163, CD11c, and CD68 was performed using the EnVision+ HRP detection system K4001 (Dako). Incubation was performed for 1 h at room temperature using Dako REAL Ab Diluent (Dako) and the following Abs: 1 μg/ml monoclonal mouse anti-human CD163-L1 (HG-Hyb1-3), 0.6 μg/ml affinity-purified polyclonal rabbit anti-human CD163-L1 (Protein Atlas), 0.2 μg/ml anti-CD163 clone 10D6 (Novocastra), 2.7 μg/ml anti-CD11c clone 5D11 (Novocastra) or 0.1 μg/ml anti-CD68 clone PG-M1 (Dako). Tissue sections were counterstained with Mayer's hematoxylin for 2 min and mounted with Aquatex (Merck). The local ethical committee in Odense approved the use of the human tissue sections (Ref. No. VF20050070).

Isolation and culture of human monocytes

Human peripheral blood monocytes were isolated from normal donor buffy coats by density gradient purification followed by $CD14^+$ selection. In brief, buffy coats were diluted 1:1 in sterile PBS containing 2 mM EDTA before lymphocytes were isolated over Ficoll-Paque Plus (GE Healthcare) at 800 × *g* for 20 min at room temperature. Isolated lymphocytes were washed twice to remove platelets and suspended in PBS, 2% heat-inactivated FBS, and 1 mM EDTA. Monocytes were isolated from the total lymphocyte population using a $CD14^+$ selection kit according to the manufacturer's recommendations (StemCell Technologies). Purified $CD14^+$ monocytes (>98% pure, as determined by FACS analysis) were seeded in 6-well culture dishes (TPP) at a density of 1.5×10^5 cells/cm^2. Macrophages were obtained by culturing monocytes for 7 d in RPMI 1640, 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (Invitrogen) supplemented with 100 ng/ml M-CSF (Immunotools). Dendritic cells were obtained by culturing monocytes for 6 d, substituting M-CSF with 100 ng/ml GM-CSF and 20 ng/ml IL-4 (Immunotools). Cells were cultured in 5% CO_2 in air at 37°C. Fresh media supplemented with M-CSF or GM-CSF/IL-4 was added at day 3. Stimulation of the generated macrophages was performed for 24 h using either 20 ng/ml IL-4, IL-6, IL-10, IL-13, IFN-γ, TNF-α (Immunotools), 100 ng/ml *Escherichia coli* serotype 0111:B4 LPS, or 40 ng/ml dexamethasone (Sigma-Aldrich).

Flow cytometry

All measurements of fluorescence intensity were determined on a FACSCalibur flow cytometer (BD Biosciences). Collected data were analyzed using the FlowJo software (Tree Star). The isotype control (IgG_1) anti-OVA Ab (Hyb 099-01) was used in all experiments. Staining of cells was performed as follows.

Peripheral blood leukocytes. Blood from healthy volunteers was collected and diluted 1:1 (v/v) in PBS. Removal of erythrocytes was performed using a standard hyposmolar lysis protocol. Approximately 10^6 cells were resuspended in 200 μl PBS/BSA and incubated with 10 μg mAb (HG-Hyb1-1 or HG-Hyb1-3) for 2 h at 4°C. Cells were washed twice in PBS/BSA and subsequently stained for 1 h at 4°C with FITC-conjugated polyclonal $F(ab')_2$ goat anti-mouse Ig (Dako) diluted 1:20. The cells were washed three times in PBS/BSA before analysis.

Subcellular distribution of CD163-L1. HEK293 cells were detached using Alfazyme (PAA Laboratories), washed, and suspended in PBS/BSA prior to the experiments. Surface expression of CD163-L1α and CD163-L1β was determined as described for peripheral blood leukocytes. The total subcellular distribution of CD163-L1α and CD163-L1β was determined as follows: Cells were washed twice in PBS and fixed for 20 min in PBS containing 2% paraformaldehyde. After fixation, the cells were permeabilized for 10 min using PBS/BSA containing 0.5% saponin. Staining was performed essentially as described for peripheral blood leukocytes except for the addition of 0.2% saponin to all incubation buffers.

Internalization of CD163-L1. HEK293 cells expressing CD163-L1α were detached using Alfazyme (PAA Laboratories), resuspended in media, and stained for 2 h at 4°C using unlabeled or Alexa 488-conjugated HG-Hyb1-3 (IgG and Fab). Cells were washed twice in media before incubation was continued at 37°C. At time intervals (0–20 min), cells were removed and kept on ice before cells stained with unlabeled HG-Hyb1-3 were stained for 1 h at 4°C with FITC-conjugated polyclonal $F(ab')_2$ goat anti-mouse Ig (Dako) diluted 1:20. The cells were finally washed three times in PBS/BSA before analysis.

Confocal microscopy

HEK293 cells were seeded on coverslips (Hounisen) at a density of 1×10^5 cells/coverslip 48 h prior to the experiments.

Surface distribution of CD163-L1. For the surface staining experiments, living cells were incubated in media containing 20 μg/ml CD163-L1 mAb (HG-Hyb1-1) for 2 h at 4°C. Cells were washed three times in PBS and fixed for 20 min in PBS containing 2% paraformaldehyde. Cells were washed three times in PBS/BSA and incubated for 1 h at room temperature with Alexa 488-conjugated $F(ab')_2$ goat anti-mouse IgG (Invitrogen) diluted 1:200. Cells were washed three times in PBS, counterstained with 0.2 mM DAPI (Invitrogen), washed twice in PBS, and mounted using ProLong Gold antifade reagent (Invitrogen).

Surface distribution of CD163 and the CD163–CD163-L1 fusion. Surface staining of cells transfected with CD163 and the CD163–CD163-L1 fusion was performed on coverslips essentially as described by Nielsen et al. (23) using 10 μg/ml rabbit polyclonal anti-CD163 Ab (8) and Alexa 488-conjugated secondary goat anti-rabbit IgG (Invitrogen) as the primary and secondary Abs, respectively. Coverslips were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen).

Total cellular distribution of CD163-L1. The total cellular distribution of CD163-L1 was visualized on cells fixed and permeabilized prior to staining. Living cells were washed twice in PBS, fixed for 20 min in PBS containing 2% paraformaldehyde, and permeabilized using PBS/BSA containing 0.5% saponin for 10 min. Fixed and permeabilized cells were stained for 1 h at room temperature using 20 μg/ml CD163-L1 mAb (HG-Hyb1-1) in PBS/BSA containing 0.2% saponin. Staining with Alexa 488-conjugated $F(ab')_2$, DAPI staining, and mounting of the slides was performed essentially as previously described except for the addition of 0.2% saponin to all buffers.

Internalization of CD163-L1. Visualization of internalization was performed using living cells incubated in media containing 20 μg/ml Alexa 488-conjugated mAb or Fab (HG-Hyb1-3) for 2 h at 4°C. The cells were washed twice before incubation was continued for 20 min at either 37°C or 4°C. Cells were washed twice in PBS and fixed for 20 min in PBS containing 2% paraformaldehyde. Cells were washed twice, then counterstained for 10 min with 5 μg/ml Alexa 633-conjugated wheat germ agglutinin (Invitrogen) before being stained with DAPI and mounted as previously described. Analysis of immunostained cells was performed using an Olympus FV1000 confocal microscope.

Endocytosis of ^{125}I-labeled Hp–Hb

For endocytosis analysis with radiolabeled ligand, complexes of Hp(2-2) and Hb A_0 (both from Sigma) were labeled with [^{125}I] using the chloramine-T method. Untransfected HEK293 cells and HEK293 cells transfected with CD163, CD163-L1, or CD163–CD163-L1 fusion cDNA were seeded in 24-well plates coated with poly-D-lysine according to the manufacturer's instructions (Sigma-Aldrich). Endocytosis experiments were performed essentially as described (8, 23). Briefly, triplicates of cells were incubated in serum-free DMEM (supplemented with 2 mM glutamine) containing 4000 cpm ^{125}I-labeled Hp(2-2)–Hb for various time intervals at 37°C. By the end of the incubation period, the medium was collected and precipitated with 12.5% TCA to separate soluble fragments of degraded Hp(2-2)–Hb from intact Hp(2-2)–Hb. Degradation of Hp(2-2)–Hb was determined as the cell-mediated increment in TCA-soluble radioactivity in the medium. Subsequently, cells were lysed with 0.5 M NaOH, and cell-associated radioactivity was determined by counting the radioactivity of the lysate.

Bacterial binding experiments

The bacterial strains *Escherichia coli*, *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus agalactia* were clinical isolates obtained from patients at Odense University Hospital. The isolated bacteria were cultured on blood agar plates under aerobic conditions at 37°C for 48 h prior to the experiment. Bacteria were harvested, washed twice in TBS, 5 mM $CaCl_2$, 1%

BSA, and finally resuspended to a concentration of 10^8 bacteria/ml. One microgram recombinant CD163-L1 SRCR 1–12 or gp-340 (24) was added to 10^8 bacteria before incubation for 2 h at 4°C with gentle agitation. Bacteria were pelleted by centrifugation at 3000 × *g* for 5 min, and supernatants were collected. The bacteria pellets were washed twice in TBS, 5 mM $CaCl_2$, 1% BSA, and finally resuspended in sample buffer. Supernatants and cell pellets were analyzed by Western blotting using the mAb anti–CD163-L1 (HG-Hyb1-3) or polyclonal rabbit anti–gp-340 (22) essentially as described above.

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) analysis of the binding of the Hp–Hb complex to CD163 or CD163-L1 was carried out using a Biacore 3000 instrument (GE Healthcare). CD163 purified from human spleen was immobilized, and sensorgrams were generated as described by Madsen et al. (9). Purified recombinant CD163-L1 SRCR 1–12 was immobilized at a concentration of 5 μg/ml in 10 mM sodium acetate, pH 4. The SPR signal generated from the immobilized recombinant CD163-L1 protein corresponded to 55 fmol protein/mm^2. Sample and running buffer was 10 mM HEPES, 150 mM NaCl, 3.0 mM $CaCl_2$, 1.0 mM EGTA, and 0.005% Tween 20, pH 7.4.

Results

Recombinant CD163-L1 and mAbs

A schematic representation of full-length CD163-L1α (canonical sequence) is shown in Fig. 1A. We expressed a number of different recombinant CD163-L1 variants in HEK293 cells, including variants comprising the extracellular SRCR domains 1–7, the entire ectodomain of CD163-L1 (SRCR 1–12), both fused to a V5-6xhis epitope, and the two full-length splice variants CD163-L1α and CD163-L1β. Recombinant CD163-L1 SRCR 1–7 was used to develop mAbs resulting in two hybridomas HG-Hyb1-1 and HG-Hyb1-3 with non-overlapping epitopes (Fig. 1B). HG-Hyb1-3 was applied in Western blotting, where it recognized single protein bands in the unreduced supernatants of HEK293 cells stably transfected with either CD163-L1 SRCR 1–7 or ectodomain cDNA (Fig. 1C). The two identified secreted protein variants migrated with the approximate masses of 95 and 160 kDa, respectively, which were equivalent to protein bands identified by an anti-V5 Ab (data not shown). Western blot analysis of crude cell lysates prepared from HEK293 cells stably transfected with CD163-L1α or CD163-L1β cDNA gave rise to protein bands with the expected molecular mass of ~160 kDa (Fig. 1D). No cross-reaction with HEK293 cells transfected with full-length CD163 cDNA (19) was observed. Fig. 1E summarizes the characteristics of the two mAbs and the commercially available polyclonal Ab used in this study.

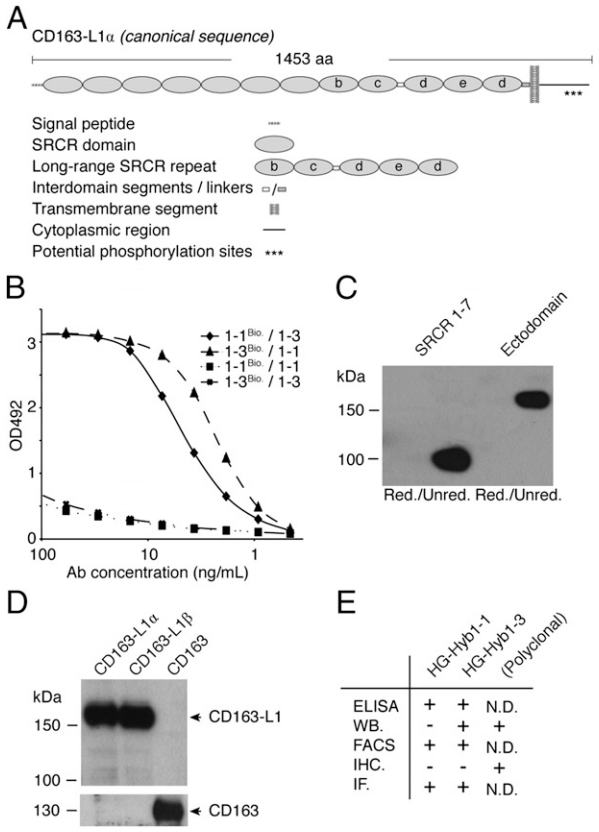

FIGURE 1. Recombinant CD163-L1 and mAbs. (**A**) Schematic presentation of the domain organization of CD163-L1. (**B**) Direct ELISA identifying HG-Hyb1-1 and HG-Hyb1-3 as mAbs with non-overlapping epitopes. Biotinylated Abs were applied in serial dilutions onto CD163-L1 SRCR 1–7 coated microtiter plates preincubated with saturating amounts of unlabeled Ab (100 ng/ml). (**C**) Western blot analysis (reduced/unreduced) of recombinant CD163-L1 SRCR 1–7 and ectodomain from HEK293 supernatants. The blot was developed using the mAb HG-Hyb1-3. (**D**) Western blot analysis (unreduced) of Triton X-100 cell lysates of HEK293 cells transfected with full-length CD163-L1α, CD163-L1β, and CD163 cDNA. The blots were developed using HG-Hyb1-3 (*upper panel*) or a polyclonal rabbit anti-CD163 Ab (*lower panel*). (**E**) Functional properties of the mAbs HG-Hyb1-1, HG-Hyb1-3, and the polyclonal affinity-purified anti–CD163-L1 Ab used in this study.

Tissue distribution of CD163-L1 mRNA

We have previously demonstrated the presence of CD163-L1 mRNA in a number of human tissues by endpoint RT-PCR (1). To extend and quantify these results, we performed a quantitative RT-PCR (qRT-PCR) analysis on various human tissues using TaqMan assays recognizing all annotated membrane-spanning splice variants of CD163-L1 or CD163 (Fig. 2A). As expected, expression of CD163-L1 and CD163 mRNA was detected in all tested tissues. The CD163-L1 mRNA showed high expression in tissues such as the spleen and the small intestine, whereas low expression was observed in the brain, liver, and lung. In comparison, CD163 showed high expression in tissues such as the lung and spleen but low expression in the brain. Absolute quantification by qRT-PCR of CD163-L1α and CD163-L1β showed expression levels ranging from 30 to 1300 arbitrary units (AU) and from undetected to 50 AU, respectively (Fig. 2B, 2C). The expression profile of CD163-L1α was indistinguishable from the relative quantification by the assay detecting both splice variants (Fig. 2A), whereas the expression profile of CD163-L1β showed pronounced variations in several tissues. The relative abundance of CD163-L1β mRNA accounted for 0–13% of the total CD163-L1 mRNA (Fig. 2D).

Immunohistochemical localization of CD163-L1

The tissue expression of CD163-L1 was analyzed by immunohistochemical analysis on human tissues. The immunostaining of CD163-L1 was compared with that of CD163 and with the myeloid markers CD11c and CD68 in parallel tissue sections (Fig. 3A). The analysis was performed using an affinity-purified polyclonal Ab that specifically recognized recombinant full-length CD163-L1 expressed by HEK293 cells (Fig. 3B, 3C). CD163-L1 primarily localized to lymphoid tissues such as the spleen, thymus, and tonsils and to the small and large intestine. In the spleen, immunostaining was observed in the red pulp macrophages and in cells residing within the follicles, whereas no immunostaining was observed in cells residing within the marginal zone. In the thymus, CD163-L1 was present in medullary and cortical macrophages, whereas the tonsils primarily showed immunostaining of perifollicular macrophages. Cells residing in the lamina propria of both the small and the large intestines showed pronounced CD163-L1

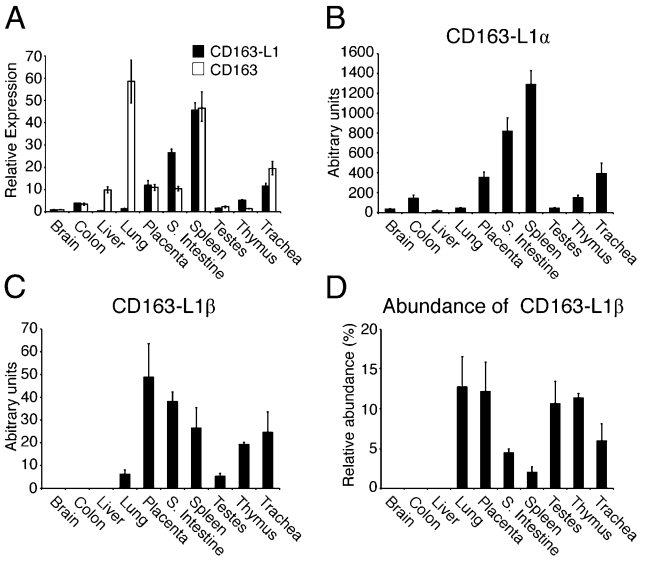

FIGURE 2. CD163-L1 mRNA expression profile in human tissues. (**A**) qRT-PCR analysis of CD163-L1 and CD163 mRNA expression in 10 different human tissues. Primers and probes were designed to recognize all annotated splice variants of CD163-L1 and CD163. (**B** and **C**) Absolute quantification of CD163-L1α and CD163-L1β mRNA expression by qRT-PCR. (**D**) The relative abundance of CD163-L1β compared with the total CD163-L1 mRNA. Absolute quantification of CD163-L1 splice variants was estimated through assay-specific copy-number control dilution series included in each run. Values are mean ± SD of three independently performed experiments.

immunostaining. In general, the tissue distribution of CD163-L1 in the lymphoid and intestinal tissues appeared similar to that of CD163. However, the number of positive macrophages and the signal intensity in the cells residing in the follicles of the spleen were more pronounced for CD163-L1. Well-defined immunostaining of CD163-L1 and CD163 was also observed in macrophages residing in the chorionic villi of the placenta, whereas weak staining was observed in these macrophages when stained for CD11c and CD68. A prominent difference in the localization of CD163-L1 and CD163 was observed in the liver and lung. Resident tissue macrophages such as Kupffer cells in the liver and alveolar macrophages in the lung showed pronounced immunostaining of CD163, whereas no or only weak expression of CD163-L1 was detected in these cells. The interstitial macrophages in the lung did, however, show moderate CD163-L1 immunostaining. Absence of CD163-L1$^+$ cells was also observed in the cerebellum and in the epidermis of the skin, suggesting that neither microglia nor Langerhans cells express CD163-L1.

Western blotting of human tissue extracts

Triton X-100 lysates prepared from tissue samples of human spleen, placenta, and small intestines were analyzed by Western blotting using the mAb HG-Hyb1-3 (Fig. 4A). The Ab identified a protein band with the approximate molecular mass of 160 kDa in the unreduced state. No cross-reaction with CD163 (130 kDa) was observed in any of the lysates. The identified protein showed the same molecular mass as recombinant full-length CD163-L1α expressed by HEK293 cells, suggesting that CD163-L1 exists as a 160-kDa protein in human tissues. The identity of CD163-L1 was confirmed by mass spectrometry after immunoprecipitation from placenta lysates using HG-Hyb1-1 or HG-Hyb1-3 (Fig. 4B). To study the glycosylation level of CD163-L1, cell lysates containing recombinant CD163-L1α or CD163-L1β were deglycosylated using PNGase F (Fig. 4C). The deglycosylation caused a reduction in the molecular mass from ~160 to 135 kDa, suggesting that several of the 16 potential N-linked glycosylation sites residing within both CD163-L1α and CD163-L1β are indeed glycosylated in vivo.

FACS analysis of peripheral blood leukocytes

The expression of CD163-L1 by freshly isolated human peripheral blood leukocytes was determined by FACS analysis using the mAbs HG-Hyb1-1 and HG-Hyb1-3 (Fig. 5). Both Abs clearly recognized CD163-L1α expressed on HEK293 cells (Fig. 5A). However, none of peripheral blood leukocytes showed a fluorescent signal above that of the isotype control, suggesting very low

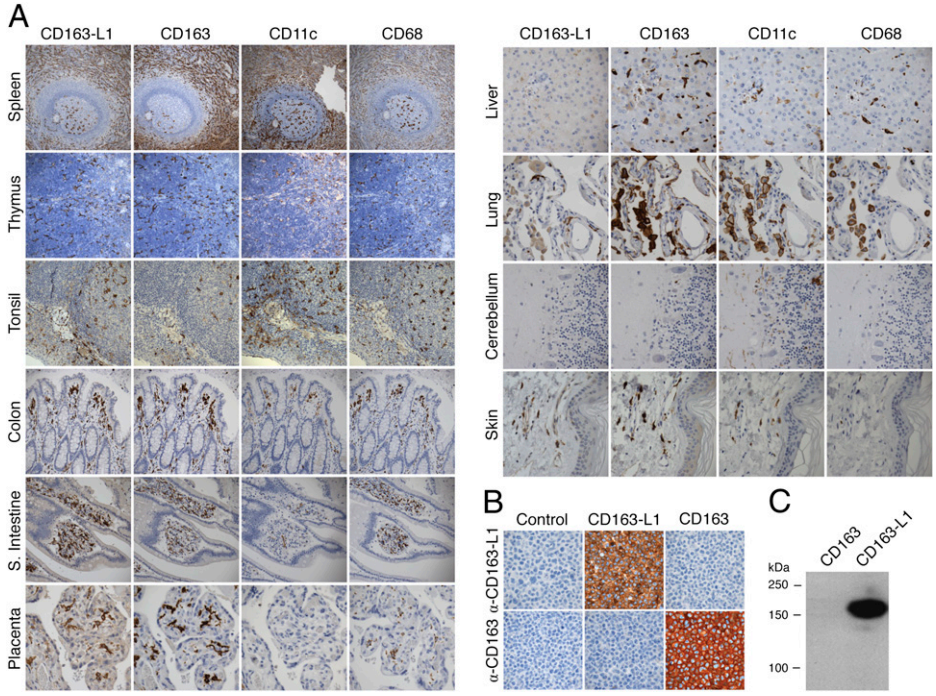

FIGURE 3. Immunohistochemical analysis of CD163-L1 in human tissues. (**A**) Human tissues immunostained with CD163-L1–specific affinity-purified polyclonal Ab (Protein Atlas ID: HPA015663) compared with immunostaining of CD163 (clone 10D6) and the myeloid cell markers CD11c (clone 5D11) and CD68 (clone 10D6). The tissue sections predominantly showed immunostaining of CD163-L1 in lymphoid tissues such as the spleen, thymus, and tonsils and in intestinal tissues such as the small intestines and colon. Original magnification ×100 (spleen); ×200 (thymus, tonsil, colon, and small intestine); ×400 (placenta, liver, lung, cerebellum, and skin). (**B**) Immunostaining of untransfected as well as CD163-L1– and CD163-transfected HEK293 cells. Original magnification ×400. (**C**) Western blot analysis of HEK293 cells transfected with CD163 or CD163-L1 cDNA using the CD163-L1–specific affinity-purified polyclonal Ab. No cross-reaction was observed between the CD163-L1– or CD163-specific Abs.

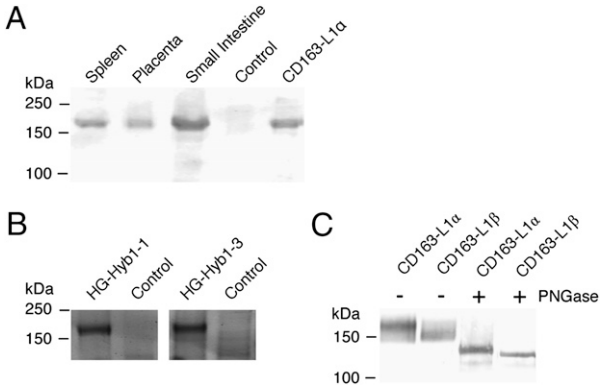

FIGURE 4. Identification of CD163-L1 as a 160-kDa glycoprotein in tissues. (**A**) Western blot analysis (unreduced) of Triton X-100 tissue lysates from spleen, placenta, and small intestine, together with cell lysates of untransfected and CD163-L1α transfected HEK293 cells. The blot was developed using the mAb HG-Hyb1-3. (**B**) Silver staining of CD163-L1 immunoprecipitated from Triton X-100 placenta lysate using immobilized HG-Hyb1-1 or HG-Hyb1-3. The identity of CD163-L1 was verified by mass spectrometry. (**C**) Western blot analysis (unreduced) of PNGase F-treated Triton X-100 lysates of CD163-L1α and CD163-L1β transfected HEK293 cells. The blot was developed using HG-Hyb1-3.

levels or absence of CD163-L1 on the surface of peripheral blood leukocytes (Fig. 5B–D).

Expression of CD163-L1 mRNA in cultured monocytes, macrophages, and dendritic cells

The immunohistochemical analysis of CD163-L1 showed a pronounced staining of myeloid cells residing in the lymphoid and intestinal tissues, whereas no expression of CD163-L1 was detected by FACS analysis of peripheral blood leukocytes. This discrepancy suggests that CD163-L1 expression is increased during monocyte-to-macrophage differentiation. To address this, we performed a qRT-PCR analysis of CD163-L1 mRNA expression from freshly isolated $CD14^+$ monocytes differentiated into macrophages or dendritic cells in vitro. The CD163 mRNA expression was assessed in parallel and used for comparison and as a control for macrophage differentiation. Monocyte-derived macrophages were obtained through culturing of freshly isolated peripheral blood monocytes in the presence of M-CSF for 7 d. During the 7-d time course, the expression of CD163-L1 mRNA was increased 35-fold ($p < 10^{-4}$), whereas the expression of CD163 mRNA was increased 8-fold ($p < 10^{-3}$) (Fig. 6A). Monocyte-derived dendritic cells were generated by culturing freshly isolated peripheral blood monocytes for 6 d in the presence of GM-CSF and IL-4. The monocyte-to-dendritic cell differentiation did not appear to influence the regulation of CD163-L1 mRNA expression, whereas CD163 mRNA expression was reduced approximately 20-fold ($p < 10^{-6}$) (Fig. 6B). It is noteworthy that only the CD163-L1α splice variant was detected in the freshly isolated and cultured $CD14^+$ monocytes (data not shown), suggesting that CD163-L1β is differentially expressed.

Regulation of CD163-L1 by inflammatory mediators

Myeloid markers of differentiation are often also regulated by pro- or anti-inflammatory stimuli. To analyze this further, we performed a qRT-PCR analysis of the monocyte-derived macrophages (Fig. 6A, day 7) cultured for an additional 24 h in the presence of various inflammatory mediators. Proinflammatory mediators such as LPS, IFN-γ, and TNF-α, associated with classical activation of macrophages, resulted in a significant downregulation of CD163-L1 (Fig. 6C). In particular, LPS combined with IFN-γ resulted in a pronounced decrease in CD163-L1 expression ($p < 10^{-6}$). A less prominent but significant downregulation of CD163-L1 was also

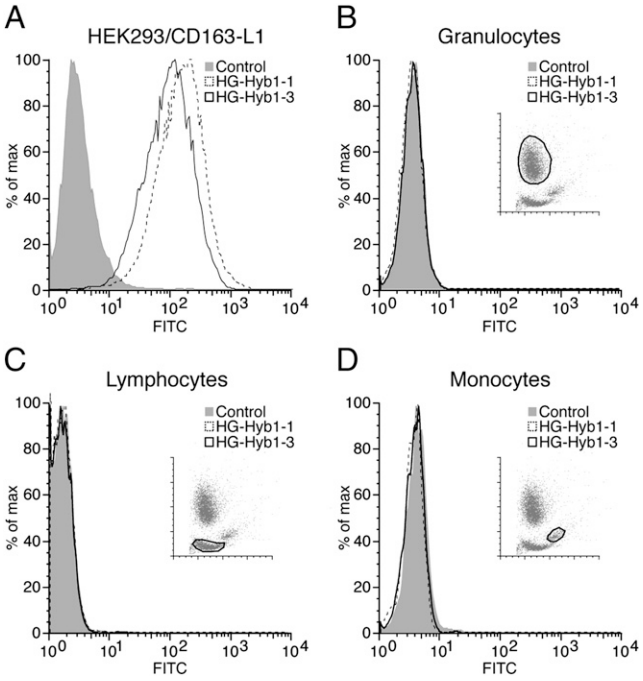

FIGURE 5. Flow cytometric analysis of freshly isolated peripheral blood leukocytes. (**A**) Histogram showing CD163-L1 staining of HEK293 cells transfected with CD163-L1α cDNA using the mAbs HG-Hyb1-1 and HG-Hyb1-3 versus an isotype control. (**B–D**) Histograms of isolated granulocyte, lymphocyte, and monocyte populations. No fluorescence signal above the isotype control was observed in any of the analyzed cell populations.

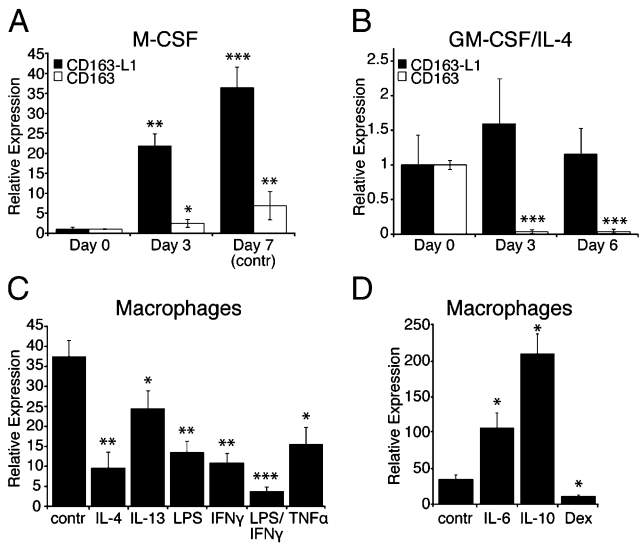

FIGURE 6. CD163-L1 mRNA expression in cultured monocytes, macrophages, and dendritic cells. The relative expression of CD163-L1 mRNA was determined by qRT-PCR and compared with the expression of CD163 mRNA. (**A**) $CD14^+$ peripheral blood monocytes cultured in vitro with M-CSF for 7 d, generating monocyte-derived macrophages. (**B**) $CD14^+$ peripheral blood monocytes cultured in vitro with GM-CSF and IL-4 for 6 d, generating monocyte-derived dendritic cells. (**C**) Monocyte-derived macrophages stimulated with the inflammatory mediators IL-4, IL-13, LPS, IFN-γ, LPS/IFN-γ, and TNF-α. (**D**) Monocyte-derived macrophages stimulated with the inflammatory mediators IL-6, IL-10, and dexamethasone. Measurements are mean ± SD of five independently performed experiments. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

observed after stimulation with the proinflammatory mediators IL-4 and IL-13 both associated with alternative activation of macrophages ($p < 10^{-3}$ and $p < 0.05$, respectively). Stimulation with the cytokines IL-6 and IL-10 both resulted in a significant upregulation of CD163-L1 (Fig. 6D). IL-6, a pleiotropic cytokine, resulted in a 3-fold upregulation of CD163-L1 ($p < 0.05$), and the anti-inflammatory cytokine IL-10 resulted in a 6-fold upregulation of CD163-L1 ($p < 0.05$). Surprisingly, we found that treatment with glucocorticoid, which is also known to have strong anti-inflammatory properties and to upregulate CD163, resulted in a more than 2-fold downregulation of CD163-L1 ($p < 0.05$).

Subcellular distribution of the CD163-L1 splice variants

Both CD163-L1α and CD163-L1β are predicted to be membrane proteins. To investigate this, we analyzed HEK293 cells transfected with full-length CD163-L1α or CD163-L1β cDNA by immunofluorescence confocal microscopy. Cells transfected with CD163-L1α cDNA showed a distinct cell surface staining, whereas cells transfected with CD163-L1β cDNA displayed a substantially lower degree of cell surface staining (Fig. 7A, *upper panel*). This difference in fluorescence intensity was not evident when the same cells were permeabilized (Fig. 7A, *lower panel*). Western blot analysis of crude cell lysates prepared from the transfected HEK293 cells showed similar expression levels of CD163-L1α and CD163-L1β (Fig. 7D), suggesting that the pronounced difference in cell surface staining observed between CD163-L1α and CD163-L1β was due to a difference in the cellular distribution of the two splice variants. To confirm and quantify this apparent difference in subcellular distribution, we performed a flow cytometric analysis of the HEK293 cells expressing the splice variants. A clear difference in fluorescence intensity was observed between fixed, surface-stained CD163-L1α– and CD163-L1β–expressing cells (Fig. 7B), which was diminished upon permeabilization of the cells (Fig. 7C). Gentle fixation did not significantly affect cell surface staining, suggesting that the epitope recognized by the mAb and the cell membrane integrity were intact (data not shown). The geometric means of the measured fluorescent signals from the surface-stained and permeabilized cells were used to quantify the cellular distribution of the splice variants (Fig. 7E). Approximately 25% of the total amount of CD163-L1α was estimated to be cell surface associated, whereas less than 3% of CD163-L1β was associated with the cell surface.

Internalization of CD163-L1

The endocytic properties of CD163-L1 were initially analyzed by confocal fluorescence microscopy after cross-linking with mAbs. HEK293 cells expressing CD163-L1α were cell surface stained with Alexa 488-conjugated HG-Hyb1-3 at 4°C, and unbound Ab was removed by washing. Incubation was then continued for 20 min at either 4°C or 37°C. Cells kept at 4°C showed a well-defined cell surface staining of CD163-L1, whereas cells incubated at 37°C showed a pronounced intracellular CD163-L1 staining (Fig. 8A). To assess whether the observed internalization of CD163-L1 resulted from the Ab-mediated cross-linking of CD163-L1, a parallel experiment was performed using the corresponding Fab fragment of HG-Hyb1-3 (Fig. 8B). CD163-L1 ligation using Alexa 488-conjugated Fab fragment resulted in the internalization of the Fab fragment, which was visually indistinguishable from the internalization of the intact Ab. To quantify the endocytic properties of CD163-L1, we performed a time-course experiment by flow cytometry. The cell surface distribution of CD163-L1α, visualized by intact or Fab HG-Hyb1-3, was found to decrease rapidly over a time course of 10 min reaching a steady plateau of

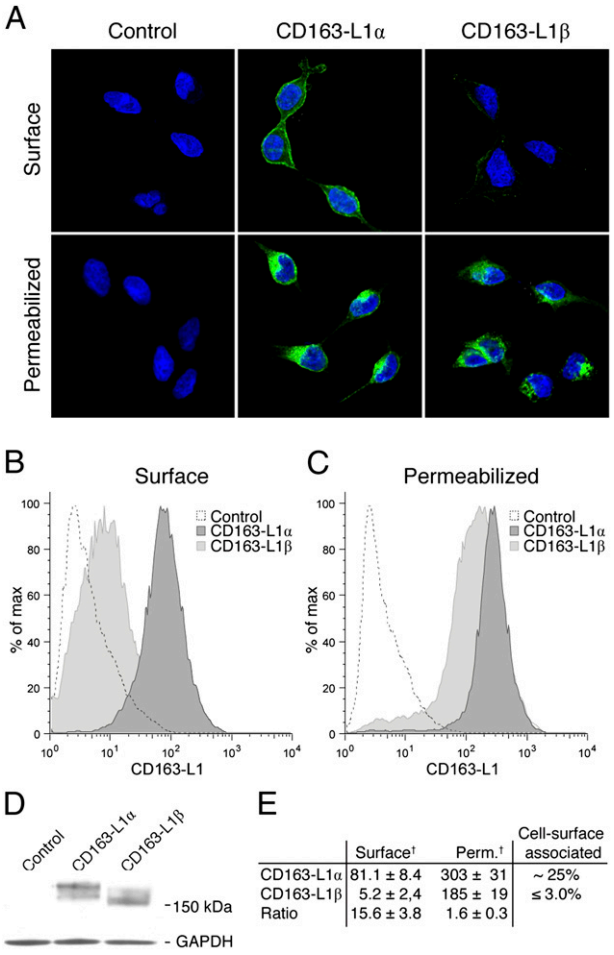

FIGURE 7. Subcellular distribution of the CD163-L1 splice variants. (**A**) Analysis of CD163-L1 splice variants by confocal microscopy (original magnification ×200). Cell surface-stained and permeabilized cells were analyzed using HG-Hyb1-1 and FITC-conjugated polyclonal $F(ab')_2$ goat anti-mouse Ig. (**B** and **C**) Flow cytometric analysis of cell surface-stained and permeabilized cells using HG-Hyb1-1 and FITC-conjugated polyclonal $F(ab')_2$ goat anti-mouse Ig. The experiments shown are representative of three independently performed experiments. (**D**) Semiquantitative Western blot analysis of the expression levels of CD163-L1 splice variants expressed in HEK293 cells. The blot was developed using HG-Hyb1-3 standardized to GAPDH. (**E**) Statistical analysis of the subcellular distribution of the CD163-L1 splice variants. Measurements are mean ± SD of three independently performed experiments.

~25% (Fig. 8C). A similar decrease was not observed using Alexa 488-conjugated Abs, demonstrating that the decrease in cell surface association was not due to Ab disassociation but due to internalization of both intact IgG and the corresponding Fab fragment. To investigate the possible mechanism behind the internalization of CD163-L1, we performed site-directed mutagenesis of the classical di-leucine internalization motif residing in the cytoplasmic tail of CD163-L1. However, substitution of both leucines with alanines did not appear to influence the internalization of CD163-L1 (Fig. 8D). We found, however, that the presence of sucrose or chlorpromazine, both inhibitors of clathrin-mediated endocytosis, resulted in an almost complete inhibition of wild-type CD163-L1α internalization, whereas no inhibition was observed in the presence of nystatin, an inhibitor of lipid raft/caveolae-mediated endocytosis (Fig. 8E).

CD163 has previously been demonstrated to mediate the endocytosis and degradation of ^{125}I-labeled Hp–Hb complexes (8, 23). Thus, to demonstrate directly the endocytic capacity of the

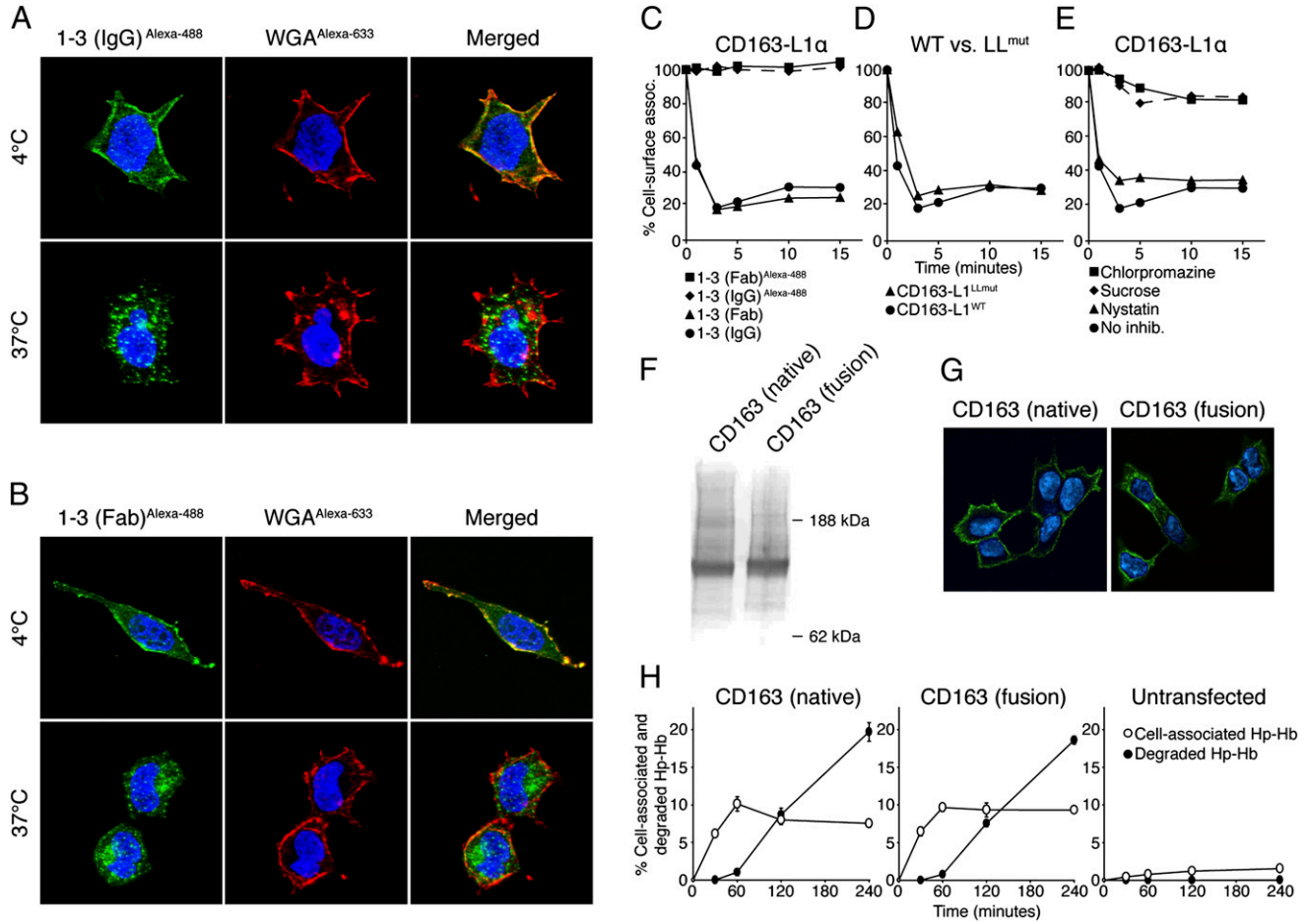

FIGURE 8. The endocytic properties of CD163-L1. (**A** and **B**) Confocal fluorescence microscopy of CD163-L1 internalization. HEK293 cells transfected with CD163-L1α were incubated with Alexa 488-conjugated HG-Hyb1-3 (IgG or Fab), then washed to remove unbound Ab before incubation was continued for 20 min at 4˚C or 37˚C. Cells were counterstained with Alexa 633-conjugated wheat germ agglutinin and stained with DAPI before analysis. Original magnification ×200. (**C**–**E**) Flow cytometric analysis of CD163-L1 internalization. HEK293 cells transfected with CD163-L1α cDNA were incubated with Alexa 488-conjugated HG-Hyb1-3 (IgG/Fab) or unlabeled HG-Hyb1-3 (IgG/Fab), then washed to remove unbound Ab before incubation was continued at 4˚C or 37˚C. Internalization was followed over a time course of 20 min. Cells were subsequently stained with FITC-conjugated polyclonal $F(ab')_2$ goat anti-mouse Ig before analysis. (C) Internalization of CD163-L1α using Alexa 488-conjugated and unlabeled Abs. (D) Internalization of CD163-L1α (wild-type) versus CD163-L1α (LLmut). (E) Internalization of CD163-L1α in the presence of chlorpromazine (100 μM), sucrose (0.4 M), or nystatin (40 μM), known inhibitors of internalization. (**F**) Western blot analysis of lysate from HEK293 cells transfected with CD163 or CD163–CD163-L1 fusion cDNA. The primary Ab used was a rabbit polyclonal anti-CD163 Ab. (**G**) Confocal microscopy of cell surface-stained HEK293 cells transfected with either CD163 wild-type or CD163–CD163-L1 fusion cDNA. Cells were stained using rabbit polyclonal Ab against CD163 and Alexa 488-labeled anti-rabbit Ab, in addition to staining with DAPI. Original magnification ×200. (**H**) Cell association and degradation of ^{125}I-labeled Hp–Hb complexes in untransfected HEK293 cells or cells transfected with either CD163 wild-type or CD163–CD163-L1 fusion cDNA.

CD163-L1 tail, we performed an equivalent experiment using a recombinant CD163–CD163-L1 hybrid protein comprising the extracellular and transmembrane regions of CD163 (aa 1–1067) linked to the cytoplasmic part of CD163-L1α (aa 1383–1453). The endocytic capacity and degradation of the Hp–Hb complexes, as well as the expression level and cell surface distribution of the CD163–CD163-L1 fusion protein, was found to be equivalent to that of wild-type CD163 (Fig. 8F–H).

CD163 lacks bacterial binding activity in vitro and is functionally distinct from CD163

Several members of the SRCR superfamily such as SR-AI, CD163, gp-340, MARCO, CD5, CD6, and Spα function as pattern recognition receptors with the ability to bind pathogen-associated molecular patterns (12–18). We investigated the ability of recombinant CD163-L1 ectodomain to bind to a range of clinical isolates of both Gram-positive and Gram-negative bacteria in a pull-down experiment (Fig. 9A). gp-340, known to bind directly to different bacterial strains (14, 25–27), was included as an experimental control. gp-340 bound to all tested bacterial strains as indicated by its presence in the bacterial pellets. In contrast, no recombinant CD163-L1 protein was detected in any of the bacterial pellets, showing that recombinant CD163-L1 does not bind to these bacteria.

The strong resemblance to the Hp–Hb receptor CD163 led us to investigate the possible interaction between CD163-L1 and the Hp–Hb complex. Using CD163 as a positive control, we performed an SPR analysis and, as expected, demonstrated a high-affinity binding between immobilized CD163 and both the Hp1-1 and Hp2-2 phenotypes of haptoglobin in complex with hemoglobin as reported by Kristiansen et al. (8). Similar high-affinity binding was not observed to recombinant CD163-L1 ectodomain regardless of the haptoglobin type or the presence of hemoglobin (Fig. 9B, 9C). In agreement with these findings, CD163-L1–transfected HEK293 cells were unable to mediate internalization of Hp–Hb (data not shown).

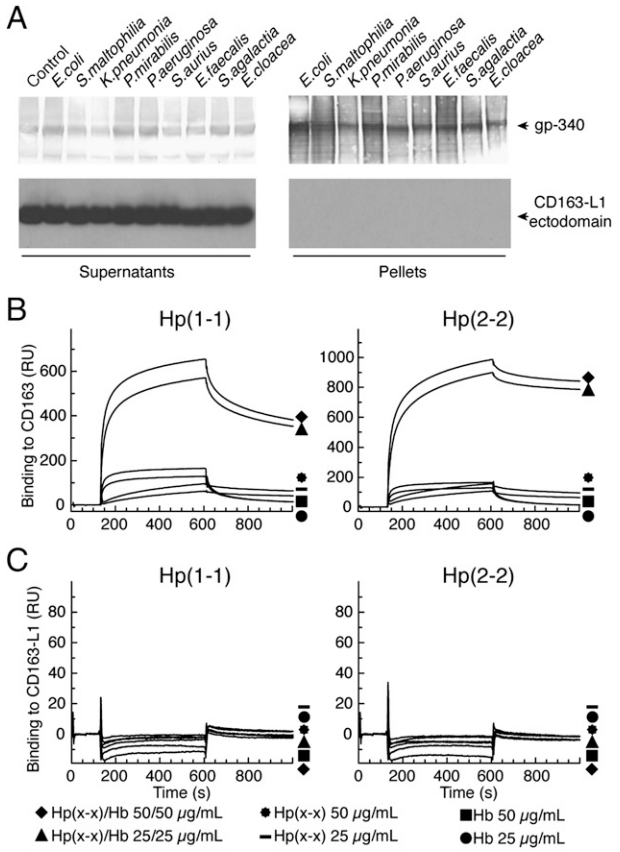

FIGURE 9. Binding of recombinant CD163-L1 ectodomain to whole bacteria and Hp–Hb. (**A**) Recombinant gp-340 and CD163-L1 binding to nine clinical isolates in the presence of 5 mM $CaCl_2$. Bacteria were incubated with recombinant protein, washed, and subjected to Western blot analysis. Recombinant protein was detected in supernatants and pellets with anti–gp-340 polyclonal Ab and HG-Hyb1-3, respectively. (**B** and **C**) SPR analysis of binding of Hp–Hb to CD163 and CD163-L1 ectodomain. The measurements were carried out at hemoglobin (Hb) concentrations of 0, 25, and 50 μg/ml in the absence or presence of 0, 25, or 50 μg/ml haptoglobin (Hp; phenotype 1-1 or 2-2).

Discussion

We have previously cloned and characterized CD163-L1 (M160) as a type I transmembrane SRCR molecule, expressed by cells of myeloid origin (1), yet little is known about the expression pattern and function of CD163-L1.

In the current report, we characterized the expression of CD163-L1 on peripheral blood leukocytes by flow cytometry. Through the use of two mAbs with different CD163-L1 epitopes, we showed that the expression of CD163-L1 was absent or negligible on the peripheral blood leukocytes. In contrast, the expression of CD163 on the monocyte population has been reported to vary from a few to 99% depending on the experimental conditions and the Abs used (28–32). MAC2-158, which recognizes the first SRCR domain of CD163, is a well-characterized Ab that stains more than 80% of the monocyte population positive for CD163 (32).

To further characterize the expression of CD163-L1, we isolated peripheral blood monocytes, and we could demonstrate that the expression of CD163-L1 mRNA was increased 35-fold during the in vitro-driven cytokine differentiation of monocytes to macrophages, whereas the mRNA levels appeared unchanged during the differentiation of monocytes to dendritic cells. Thus, altogether these results clearly suggest that CD163-L1 is a genuine monocyte-to-macrophage differentiation marker.

To extend these findings, we compared the expression pattern of CD163-L1 to that of CD163 and the myeloid markers CD11c and CD68 by immunohistochemical analysis of various human tissues. We observed a predominant immunostaining for CD163-L1 in myeloid cells residing in lymphoid tissues such as the spleen, thymus, and tonsils and in intestinal tissues such as the small intestine and colon. In the spleen, white- as well as red-pulp macrophages were CD163-L1$^+$, whereas marginal-zone macrophages were negative. To our surprise, we saw low or absence of staining in long-lived tissue-specific macrophages such as Kupffer cells, alveolar macrophages, and microglia, which stained positive for the other myeloid markers. Also, dendritic cells like Langerhans cells residing in the epidermis appeared to be negative. This staining pattern indicates that CD163-L1 is a marker for a special subset of macrophages and not a general macrophage marker.

Finally, we used Western blot analysis of solubilized tissues from placenta, spleen, and small intestine to verify the presence and the molecular mass of CD163-L1 in tissues. We identified a single band of 160 kDa in all tested tissues, which was identical to the band found in HEK293 cells expressing full-length CD163-Ll, and we verified the identity of CD163-L1 in the bands by mass spectrometry. The calculated molecular mass of full-length CD163-L1α is 154.5 kDa without the signal peptide, and we showed that deglycosylation of CD163-L1 reduced the molecular mass to approximately this level. Thus, we are confident that the tissue staining that we detected with our Abs indeed represents the full-length CD163-L1 gene product.

We next studied the regulation of CD163-L1 in monocyte-derived macrophages driven in different directions by cytokines and other inflammatory mediators. LPS, which is the canonical innate immune stimulus that drives macrophages in the direction of classically activated macrophages (M1-polarized macrophages) (33, 34), significantly downregulated the expression of CD163-L1, and there was an additive effect of IFN-γ, the key cytokine driving macrophages further down that lane. TNF-α, another inducer of classically activated macrophages, also reduced the level of CD163-L1. Stimulation with IL-13 and especially IL-4, both of which lead to alternatively activated macrophages (M2-polarized macrophages) (33–36), also reduced the CD163-L1 expression. In contrast to this, we observed a clear upregulation of CD163-L1 expression when the monocyte-derived macrophages were stimulated with IL-10, which leads to a regulatory/anti-inflammatory macrophage phenotype (37). We observed, however, that stimulation with dexamethasone, which also directs into a regulatory/anti-inflammatory macrophage phenotype, resulted in a downregulation of CD163-L1. This could indicate that CD163-L1 is a marker for a subset of regulatory macrophages. This is consistent with the view that the M1/M2 classification of macrophage phenotypes is not an exhaustive description of the many different macrophage phenotypes present in the different tissue milieus (34, 35, 37).

Similar regulation of expression during monocyte differentiation to macrophages and subsequent regulation by inflammatory mediators has been reported for CD163 (38, 39). The major difference between the regulations of expression between the two molecules is that dexamethasone is a strong inducer of CD163 (38, 40, 41). CD163 is well characterized as the Hp–Hb receptor (8–10) and has been proposed to function in the innate immune response and resolution of inflammation. Degradation of Hp–Hb has been shown to mediate the release of metabolites with strong anti-oxidative and anti-inflammatory effects (42), and binding of Hp–Hb complexes to CD163 has been shown to mediate the direct secretion of IL-10 (43). In contrast, other reports have shown that cross-linking of CD163 with mAbs, as well as interaction with

bacteria, mediates the release of proinflammatory cytokines (12, 41, 44). Thus, CD163 appears to function as an immune modulator with the ability to suppress as well as stimulate the innate immune response. The many similarities to CD163 with regard to tissue distribution and regulation, combined with induction of expression by the acute-phase inflammatory mediator IL-6, suggests that CD163-L1 in a similar way may function in the modulation of the innate immune response.

We have identified two cytoplasmic splice variants for CD163-L1: the full-length variant (CD163-L1α) with a cytoplasmic tail of 71 residues and the short-tail variant (CD163-L1β) of 39 residues (1). CD163-L1α contains three potential phosphorylation sites, which are lost in CD163-L1β due to alternative splicing of exon 18. However, both splice variants contain the di-leucine internalization motif. In agreement with our previous report where we used conventional endpoint RT-PCR, our current qRT-PCR analyses identified CD163-L1α as the predominant CD163-L1 mRNA species in various human tissues. When expressed in HEK293 cells, CD163-L1α was found to be cell surface associated, whereas CD163-L1β appeared mainly in the intracellular compartments. The functional implication of these pronounced quantitative differences in expression and subcellular distribution of the two splice variants are unknown. Similar differences in splice variant distribution have been reported for CD163 (23), underlining the many observed similarities between CD163 and CD163-L1.

The resemblance to CD163 and the presence of a putative di-leucine internalization motif in the cytoplasmic tail of CD163-L1 led us to examine the endocytic properties of CD163-L1. By use of confocal microscopy, flow cytometry, and endocytosis assays, we could demonstrate that CD163-L1 indeed functions as an endocytic receptor with an endocytic capacity equivalent to that of CD163. Moreover, the ability of both intact IgG and Fab fragment to mediate internalization of CD163-L1 suggests that CD163-L1 is constitutively internalized independently of ligand ligation.

Constitutive internalization has been described for CD163, whereas the internalization of CD5 appears to be somewhat dependent on ligand ligation (45, 46). Nevertheless, both CD163 and CD5 contain an internalization motif of the type YXXϕ (where ϕ represents a bulky hydrophobic residue), which has been shown to be associated with clathrin-mediated endocytosis (46, 47). The presence of sucrose or chlorpromazine, which are inhibitors of clathrin-mediated endocytosis (48, 49), almost completely abolished the internalization of CD163-L1, suggesting that internalization of CD163-L1 in a similar way is mediated through clathrin-coated pits. However, the mechanisms resulting in this internalization seem to differ from those of CD163 and CD5, as the cytoplasmic tails of CD163-L1 do not include the YXXϕ internalization motif. Surprisingly, removal of the putative di-leucine internalization motif in the cytoplasmic tail of CD163-L1 by site-directed mutagenesis did not influence the internalization of CD163-L1.

Many of the members of the SRCR group B family that show high homology to CD163-L1, including gp-340, CD5, CD6, and Spα, function as pattern recognition receptors by binding to intact microorganisms (16–18, 25). The peptide sequence involved in the microbial binding has been identified for several of these molecules, and CD163-L1 includes the consensus sequence in several of the SRCR domains. Nevertheless, we were unable to demonstrate binding to a series of clinical isolates of bacteria that in parallel experiments bound to gp-340. Likewise, we were not able to demonstrate binding between CD163-L1 and the Hp–Hb complex as described for CD163 (8). Other attempts to identify possible ligands for CD163-L1 including various coprecipitation experiments and screening of peptide libraries have to date been negative. Future attempts to elucidate the possible ligands for CD163-L1 include mass spectrometry combined with biotinylated recombinant extracellular CD163-L1 as bait for endogenous as well as microbial ligands. Furthermore, we will use immobilized anti–CD163-L1 Abs to study the possible signaling and/or modulation responses mediated through CD163-L1.

In the current report, we demonstrate that CD163-L1 is a monocyte-to-macrophage differentiation marker, which is absent from the main classes of long-lived resident tissue macrophages but present on the majority of other tissue macrophages. In vitro stimulation of monocyte-derived macrophages suggests that many of these cells may be of the regulatory macrophage phenotype. CD163 has been suggested as a diagnostic tool and as a potential target for immunotherapy in malignant diseases (50–55). Whether CD163-L1 in a similar way could function as a diagnostic tool or as a potential target for immunotherapy remains to be defined. However, one could speculate that the absence of expression of CD163-L1 on, for example, Kupffer cells and blood monocytes could prove beneficial in immunotherapy to minimize the unintended adverse drug delivery side effects often associated with chemotherapeutic agents.

Acknowledgments

We thank Prof. Hans J. Kolmos, Institute of Clinical Research, University of Southern Denmark for providing the clinical isolates.

Disclosures

The authors have no financial conflicts of interest.

References

1. Gronlund, J., L. Vitved, M. Lausen, K. Skjodt, and U. Holmskov. 2000. Cloning of a novel scavenger receptor cysteine-rich type I transmembrane molecule (M160) expressed by human macrophages. *J. Immunol.* 165: 6406–6415.
2. Wheeler, G. L., D. Miranda-Saavedra, and G. J. Barton. 2008. Genome analysis of the unicellular green alga *Chlamydomonas reinhardtii* indicates an ancient evolutionary origin for key pattern recognition and cell-signaling protein families. *Genetics* 179: 193–197.
3. Sarrias, M. R., J. Grønlund, O. Padilla, J. Madsen, U. Holmskov, and F. Lozano. 2004. The Scavenger Receptor Cysteine-Rich (SRCR) domain: an ancient and highly conserved protein module of the innate immune system. *Crit. Rev. Immunol.* 24: 1–37.
4. Martínez, V. G., S. K. Moestrup, U. Holmskov, J. Mollenhauer, and F. Lozano. 2011. The conserved scavenger receptor cysteine-rich superfamily in therapy and diagnosis. *Pharmacol. Rev.* 63: 967–1000.
5. Resnick, D., A. Pearson, and M. Krieger. 1994. The SRCR superfamily: a family reminiscent of the Ig superfamily. *Trends Biochem. Sci.* 19: 5–8.
6. Stover, C. M., J. Schleypen, J. Grønlund, M. R. Speicher, W. J. Schwaeble, and U. Holmskov. 2000. Assignment of CD163B, the gene encoding M160, a novel scavenger receptor, to human chromosome 12p13.3 by in situ hybridization and somatic cell hybrid analysis. *Cytogenet. Cell Genet.* 90: 246–247.
7. Law, S. K., K. J. Micklem, J. M. Shaw, X. P. Zhang, Y. Dong, A. C. Willis, and D. Y. Mason. 1993. A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. *Eur. J. Immunol.* 23: 2320–2325.
8. Kristiansen, M., J. H. Graversen, C. Jacobsen, O. Sonne, H. J. Hoffman, S. K. Law, and S. K. Moestrup. 2001. Identification of the haemoglobin scavenger receptor. *Nature* 409: 198–201.
9. Madsen, M., H. J. Møller, M. J. Nielsen, C. Jacobsen, J. H. Graversen, T. van den Berg, and S. K. Moestrup. 2004. Molecular characterization of the haptoglobin. hemoglobin receptor CD163. Ligand binding properties of the scavenger receptor cysteine-rich domain region. *J. Biol. Chem.* 279: 51561–51567.
10. Graversen, J. H., M. Madsen, and S. K. Moestrup. 2002. CD163: a signal receptor scavenging haptoglobin-hemoglobin complexes from plasma. *Int. J. Biochem. Cell Biol.* 34: 309–314.
11. Fabriek, B. O., M. M. Polfliet, R. P. Vloet, R. C. van der Schors, A. J. Ligtenberg, L. K. Weaver, C. Geest, K. Matsuno, S. K. Moestrup, C. D. Dijkstra, and T. K. van den Berg. 2007. The macrophage CD163 surface glycoprotein is an erythroblast adhesion receptor. *Blood* 109: 5223–5229.
12. Fabriek, B. O., R. van Bruggen, D. M. Deng, A. J. Ligtenberg, K. Nazmi, K. Schornagel, R. P. Vloet, C. D. Dijkstra, and T. K. van den Berg. 2009. The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria. *Blood* 113: 887–892.
13. Peiser, L., P. J. Gough, T. Kodama, and S. Gordon. 2000. Macrophage class A scavenger receptor-mediated phagocytosis of *Escherichia coli*: role of cell heterogeneity, microbial strain, and culture conditions in vitro. *Infect. Immun.* 68: 1953–1963.

14. Bikker, F. J., A. J. Ligtenberg, K. Nazmi, E. C. Veerman, W. van't Hof, J. G. Bolscher, A. Poustka, A. V. Nieuw Amerongen, and J. Mollenhauer. 2002. Identification of the bacteria-binding peptide domain on salivary agglutinin (gp-340/DMBT1), a member of the scavenger receptor cysteine-rich superfamily. *J. Biol. Chem.* 277: 32109–32115.
15. Elomaa, O., M. Kangas, C. Sahlberg, J. Tuukkanen, R. Sormunen, A. Liakka, I. Thesleff, G. Kraal, and K. Tryggvason. 1995. Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. *Cell* 80: 603–609.
16. Vera, J., R. Fenutría, O. Cañadas, M. Figueras, R. Mota, M. R. Sarrias, D. L. Williams, C. Casals, J. Yelamos, and F. Lozano. 2009. The CD5 ectodomain interacts with conserved fungal cell wall components and protects from zymosan-induced septic shock-like syndrome. *Proc. Natl. Acad. Sci. USA* 106: 1506–1511.
17. Sarrias, M. R., M. Farnós, R. Mota, F. Sánchez-Barbero, A. Ibáñez, I. Gimferrer, J. Vera, R. Fenutría, C. Casals, J. Yélamos, and F. Lozano. 2007. CD6 binds to pathogen-associated molecular patterns and protects from LPS-induced septic shock. *Proc. Natl. Acad. Sci. USA* 104: 11724–11729.
18. Sarrias, M. R., S. Roselló, F. Sánchez-Barbero, J. M. Sierra, J. Vila, J. Yélamos, J. Vives, C. Casals, and F. Lozano. 2005. A role for human Sp alpha as a pattern recognition receptor. *J. Biol. Chem.* 280: 35391–35398.
19. Etzerodt, A., M. B. Maniecki, K. Møller, H. J. Møller, and S. K. Moestrup. 2010. Tumor necrosis factor α-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163. *J. Leukoc. Biol.* 88: 1201–1205.
20. Schlosser, A., T. Thomsen, J. M. Shipley, P. W. Hein, F. Brasch, I. Tornøe, O. Nielsen, K. Skjødt, N. Palaniyar, W. Steinhilber, et al. 2006. Microfibril-associated protein 4 binds to surfactant protein A (SP-A) and colocalizes with SP-A in the extracellular matrix of the lung. *Scand. J. Immunol.* 64: 104–116.
21. Leth-Larsen, R., C. Nordenbaek, I. Tornoe, V. Moeller, A. Schlosser, C. Koch, B. Teisner, P. Junker, and U. Holmskov. 2003. Surfactant protein D (SP-D) serum levels in patients with community-acquired pneumonia small star, filled. *Clin. Immunol.* 108: 29–37.
22. Renner, M., G. Bergmann, I. Krebs, C. End, S. Lyer, F. Hilberg, B. Helmke, N. Gassler, F. Autschbach, F. Bikker, et al. 2007. DMBT1 confers mucosal protection in vivo and a deletion variant is associated with Crohn's disease. *Gastroenterology* 133: 1499–1509.
23. Nielsen, M. J., M. Madsen, H. J. Møller, and S. K. Moestrup. 2006. The macrophage scavenger receptor CD163: endocytic properties of cytoplasmic tail variants. *J. Leukoc. Biol.* 79: 837–845.
24. End, C., S. Lyer, M. Renner, C. Stahl, J. Ditzer, A. Holloschi, H. M. Kuhn, H. T. Flammann, A. Poustka, M. Hafner, et al. 2005. Generation of a vector system facilitating cloning of DMBT1 variants and recombinant expression of functional full-length DMBT1. *Protein Expr. Purif.* 41: 275–286.
25. Bikker, F. J., A. J. Ligtenberg, C. End, M. Renner, S. Blaich, S. Lyer, R. Wittig, W. van't Hof, E. C. Veerman, K. Nazmi, et al. 2004. Bacteria binding by DMBT1/SAG/gp-340 is confined to the VEVLXXXXW motif in its scavenger receptor cysteine-rich domains. *J. Biol. Chem.* 279: 47699–47703.
26. Leito, J. T., A. J. Ligtenberg, K. Nazmi, J. M. de Blieck-Hogervorst, E. C. Veerman, and A. V. Nieuw Amerongen. 2008. A common binding motif for various bacteria of the bacteria-binding peptide SRCRP2 of DMBT1/gp-340/salivary agglutinin. *Biol. Chem.* 389: 1193–1200.
27. Ericson, T., and J. Rundegren. 1983. Characterization of a salivary agglutinin reacting with a serotype c strain of *Streptococcus mutans*. *Eur. J. Biochem.* 133: 255–261.
28. Backé, E., R. Schwarting, J. Gerdes, M. Ernst, and H. Stein. 1991. Ber-MAC3: new monoclonal antibody that defines human monocyte/macrophage differentiation antigen. *J. Clin. Pathol.* 44: 936–945.
29. Pulford, K., K. Micklem, S. McCarthy, J. Cordell, M. Jones, and D. Y. Mason. 1992. A monocyte/macrophage antigen recognized by the four antibodies GHI/61, Ber-MAC3, Ki-M8 and SM4. *Immunology* 75: 588–595.
30. Högger, P., J. Dreier, A. Droste, F. Buck, and C. Sorg. 1998. Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). *J. Immunol.* 161: 1883–1890.
31. Maniecki, M. B., H. J. Møller, S. K. Moestrup, and B. K. Møller. 2006. CD163 positive subsets of blood dendritic cells: the scavenging macrophage receptors CD163 and CD91 are coexpressed on human dendritic cells and monocytes. *Immunobiology* 211: 407–417.
32. Maniecki, M. B., A. Etzerodt, S. K. Moestrup, H. J. Møller, and J. H. Graversen. 2011. Comparative assessment of the recognition of domain-specific CD163 monoclonal antibodies in human monocytes explains wide discrepancy in reported levels of cellular surface CD163 expression. *Immunobiology* 216: 882–890.
33. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3: 23–35.
34. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25: 677–686.
35. Gordon, S., and F. O. Martinez. 2010. Alternative activation of macrophages: mechanism and functions. *Immunity* 32: 593–604.
36. Martinez, F. O., L. Helming, and S. Gordon. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu. Rev. Immunol.* 27: 451–483.
37. Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8: 958–969.
38. Buechler, C., M. Ritter, E. Orsó, T. Langmann, J. Klucken, and G. Schmitz. 2000. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J. Leukoc. Biol.* 67: 97–103.
39. Sulahian, T. H., P. Högger, A. E. Wahner, K. Wardwell, N. J. Goulding, C. Sorg, A. Droste, M. Stehling, P. K. Wallace, P. M. Morganelli, and P. M. Guyre. 2000. Human monocytes express CD163, which is upregulated by IL-10 and identical to p155. *Cytokine* 12: 1312–1321.
40. Högger, P., U. Erpenstein, P. Rohdewald, and C. Sorg. 1998. Biochemical characterization of a glucocorticoid-induced membrane protein (RM3/1) in human monocytes and its application as model system for ranking glucocorticoid potency. *Pharm. Res.* 15: 296–302.
41. Van den Heuvel, M. M., C. P. Tensen, J. H. van As, T. K. Van den Berg, D. M. Fluitsma, C. D. Dijkstra, E. A. Döpp, A. Droste, F. A. Van Gaalen, C. Sorg, et al. 1999. Regulation of CD 163 on human macrophages: cross-linking of CD163 induces signaling and activation. *J. Leukoc. Biol.* 66: 858–866.
42. Otterbein, L. E., M. P. Soares, K. Yamashita, and F. H. Bach. 2003. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol.* 24: 449–455.
43. Philippidis, P., J. C. Mason, B. J. Evans, I. Nadra, K. M. Taylor, D. O. Haskard, and R. C. Landis. 2004. Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. *Circ. Res.* 94: 119–126.
44. Polfliet, M. M., B. O. Fabriek, W. P. Daniëls, C. D. Dijkstra, and T. K. van den Berg. 2006. The rat macrophage scavenger receptor CD163: expression, regulation and role in inflammatory mediator production. *Immunobiology* 211: 419–425.
45. Schaer, C. A., G. Schoedon, A. Imhof, M. O. Kurrer, and D. J. Schaer. 2006. Constitutive endocytosis of CD163 mediates hemoglobin-heme uptake and determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin. *Circ. Res.* 99: 943–950.
46. Lu, X., R. C. Axtell, J. F. Collawn, A. Gibson, L. B. Justement, and C. Raman. 2002. AP2 adaptor complex-dependent internalization of CD5: differential regulation in T and B cells. *J. Immunol.* 168: 5612–5620.
47. Ohno, H., J. Stewart, M. C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J. S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* 269: 1872–1875.
48. Inal, J., S. Miot, and J. A. Schifferli. 2005. The complement inhibitor, CRIT, undergoes clathrin-dependent endocytosis. *Exp. Cell Res.* 310: 54–65.
49. Tulapurkar, M. E., R. Schäfer, T. Hanck, R. V. Flores, G. A. Weisman, F. A. González, and G. Reiser. 2005. Endocytosis mechanism of P2Y2 nucleotide receptor tagged with green fluorescent protein: clathrin and actin cytoskeleton dependence. *Cell. Mol. Life Sci.* 62: 1388–1399.
50. Vos, J. A., S. L. Abbondanzo, C. L. Barekman, J. W. Andriko, M. Miettinen, and N. S. Aguilera. 2005. Histiocytic sarcoma: a study of five cases including the histiocyte marker CD163. *Mod. Pathol.* 18: 693–704.
51. Bächli, E. B., D. J. Schaer, R. B. Walter, J. Fehr, and G. Schoedon. 2006. Functional expression of the CD163 scavenger receptor on acute myeloid leukemia cells of monocytic lineage. *J. Leukoc. Biol.* 79: 312–318.
52. Walter, R. B., E. B. Bächli, D. J. Schaer, R. Rüegg, and G. Schoedon. 2003. Expression of the hemoglobin scavenger receptor (CD163/HbSR) as immunophenotypic marker of monocytic lineage in acute myeloid leukemia. *Blood* 101: 3755–3756.
53. Nguyen, T. T., E. J. Schwartz, R. B. West, R. A. Warnke, D. A. Arber, and Y. Natkunam. 2005. Expression of CD163 (hemoglobin scavenger receptor) in normal tissues, lymphomas, carcinomas, and sarcomas is largely restricted to the monocyte/macrophage lineage. *Am. J. Surg. Pathol.* 29: 617–624.
54. Pouryazdanparast, P., L. Yu, J. E. Cutlan, S. H. Olsen, D. R. Fullen, and L. Ma. 2009. Diagnostic value of CD163 in cutaneous spindle cell lesions. *J. Cutan. Pathol.* 36: 859–864.
55. Harms, P. W., B. Bandarchi, and L. Ma. 2010. CD163 expression in leukemia cutis. *J. Cutan. Pathol.* 37: 953–957.