Endocytosis by macrophages: interplay of macrophage scavenger receptor-1 and LDL receptor-related protein-1

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Supplementary data

Culture of MDMs and U87MG cells

Blood samples of healthy volunteers were used in accordance with Dutch regulations and after approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Written informed consent was given by all participants. Human monocytes were isolated from blood using MACS CD14-microbeads (Miltenyi Biotec) and were differentiated to macrophages using 50 ng/ml hrM-CSF (PeproTech) in RPMI-1640 (Lonza), supplemented with 10% FCS (Bodinco).[1] U87MG cells were obtained from ATCC (HTB-14) and cultured in phenol red-free DMEM-F12 with HEPES and L-glutamine (Thermo Fisher Scientific) supplemented with 10% FCS.

Expression and purification of recombinant proteins

HPC4 mouse hybridoma (HB9892) was obtained from ATCC and cultured in IMDM supplemented with 10% heat-inactivated fetal calf serum (FCS). HPC4 antibody was purified with protein G sepharose according to the manufacturer's protocol. The D'D3 and D'D3A1 fragments were designed in pcDNA3.1(+) and contained two point mutations at position C1099S and C1142S in order to prevent dimerization as described.[2] A HPC4 tag was fused to the C-terminus of both fragments which was used for purification and detection. Sequences were codon optimized to enhance expression in human cells. Coding regions of both constructs were verified by sequence analysis using the BigDye Terminator Sequencing kit (Applied Biosystem). Recombinant proteins were transiently expressed in HEK 293 Freestyle cells using Polyethylenimine (PEI) transfection. Cells were grown in Freestyle Expression medium (Thermo Fisher Scientific, Waltham, MA). Five days after transfection, proteins were purified from medium by immunoaffinity chromatography using CNBr-Sepharose 4B coupled with an anti-HPC4 antibody purified from mouse hybridoma HB9892. Fragments were loaded on the anti-HPC4 column in 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $CaCl_2$. After loading, the column was washed with 20 mM Tris-HCl (pH 7.4), 1 M NaCl, 10 mM $CaCl_2$. Proteins were eluted with 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA. Subsequently, protein-containing fractions were dialyzed against 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM $CaCl_2$, 50% (v/v) glycerol and stored at -20 °C. Concentration of the fragments was assessed using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Glutathione-S-transferase – Receptor Associated Protein (gst-RAP) and LRP1 Cluster-II expression and purification was performed as described.[3,4]

Uptake of VWF fragments

Cells were washed with HEPES buffer (10 mM HEPES (pH 7.4), 135 mM NaCl, 10 mM KCl, 5 mM $CaCl_2$, 2 mM $MgSO_4$) and incubated with VWF fragments for 30 minutes at 37 °C in the absence or presence of a 5x molar excess of RAP, LRP-cluster II or mouse monoclonal anti-human SR-A1/MSR (clone #351620, R&D systems). After incubation, cells were washed and fixed in 4% paraformaldehyde (Electron microscopy sciences) in PBS for 15 minutes.

Immunofluorescence labeling and confocal imaging

After fixation, cells were washed with TBS followed by blocking and permeabilization with staining buffer (1% BSA, 0.1% saponin, 5 mM $CaCl_2$ in TBS). Cells were sequentially incubated with primary and secondary antibodies diluted in staining buffer and counter stained with Hoechst 33342 and HCS CellMask™ deep red (Thermo Fisher Scientific) for quantification purposes. Samples were imaged on

a Leica SP8 confocal laser scanning microscope (Leica microsystems) equipped with a HC PL APO CS2 63x/1.40 oil immersion objective. Tile scans were collected for quantification and analyzed using ImageJ (version 1.52d, Wayne Rasband; National Institutes of Health) and Graphpad prism 7 (GraphPad Prism Software Inc.) software.

Antibodies for immunofluorescent labeling

Primary antibodies used for labeling were: mouse monoclonal EEA-1 IgG1(BD biosciences, San Jose, CA), mouse monoclonal anti-HPC4 IgG1 (see above, purified from mouse hybridoma HB9892), rabbit polyclonal HPC4 IgG (Cell Signaling technology, Beverly, MA) and mouse monoclonal IgG2b anti-MSR-1(Clone #351615, R&D Systems). Secondary antibodies were: goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 568 and F (ab')2-Goat anti-Rabbit IgG Alexa Fluor 488 (all from Thermo Fisher Scientific).

Flow cytometry

Adherent cells were detached using a citric saline solution of 1% (w/v) KCl and 0.25% (w/v) Sodium citrate. After restoring the osmotic balance with the HEPES buffer described above, cells were placed in a V-bottom 96 wells plate using 2 x 10^5 cells per condition. Cells were incubated with VWF fragments for 30 minutes at 37 °C, washed and fixed similarly as described above. To stain for the VWF fragments directly, we conjugated anti-HPC4 antibodies to Pacific blue using a Pacific Blue™ protein labeling kit (Thermo Fisher Scientific). Since the binding of the HPC4 antibody is calcium sensitive we used the same staining protocol as described for immunofluorescence labeling. After incubating cells with antibodies, cells were washed twice with staining buffer and were then placed in TBS 2.5 mM $CaCl_2$ for analysis on a BD FACSCanto or BD FACSCanto II (BD biosciences). Data was analyzed using FlowJo 10.4 (FlowJo, LLC, Ashland, OR) and Graphpad prism 7.

Cell surface labeling and mass spectrometry sample preparation

Cell surface proteins were labeled using a membrane-impermeable biotin label as described previously with minor modifications.[5] 10 cm Ø petri dishes of macrophages or U87MG cells were washed 3x with HEPES buffer and incubated for 30 min with 2 ml/dish 3 mM EZ-link Sulfo-NHS-LC-Biotin (Thermo Scientific) at 4 ºC. Excess label was quenched by washing 4x with ice-cold HEPES buffer with 100 mM glycine, and cells were lysed at RT with 100 µl 4% SDS, 100 mM Tris-HCl, 0.1 M DTT, 1x HALT protease inhibitor (Thermo Scientific). Lysates were processed using the FASP method.[6] 50 µg of the tryptic digests was subjected to strong-anion exchange using Empore Anion and Cation Exchange-SR Extraction disks (3M) as described[6], with elution buffer pHs of 11, 8, 6, 5, 4 and 3. The flow-through of fractions 8 and 11 were collected, acidified to pH <2.5 using trifluoroacetic acid and subjected to C18 desalting (named flow-through). Fractions were desalted using C18 StageTips.[7] The remainder of the tryptic digests was subjected to biotin pull-down by incubating peptides for 2 hours in 50 mM NH_4HCO_3, 150 mM NaCl pH 8.3 in 3 wells/sample of a SigmaScreen Streptavidin High Capacity Coated plates. Another round of pull-down was performed with the flow-through. Captured peptides were diluted using 70% acetonitrile, 5% formic acid. Samples were vacuum-dried to remove the acetonitrile and desalted in C18 StageTips.

Mass Spectrometry analysis and data processing

Peptides were separated on nanoscale C18 reverse chromatography coupled on line to an Orbitrap

Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanospray ion scource (Nanospray Flex Ion Scource, Thermo Scientific) as described previously previously.[8] For the fractionated proteome the elution gradient was adjusted to 5-25% buffer B (pH 11), 5-30% buffer B (pH 5, 6, 8, flow-through) and 5-35% buffer B (pH 3, 4). For the biotin pull-down samples, the MS acquisition settings were adjusted to perform the MS^2 analysis in the orbitrap analyzer, as has been described before for phosphopeptides.[8] All data were acquired with Xcalibur software. To identify proteins and peptides, raw files were analyzed with the MaxQuant (1.5.3.30) computational platform[9], using the Andromeda search engine by querying the human Uniprot database (release 3-2017, 70947 entries) using standard settings with the following adjustments. Protein quantification was based on unique peptides, the 'match between runs' option was enabled. In the cell surface samples, a variable modification comprising the biotin added mass (339.16166 Da), was added. Perseus 1.5.6.0 was used to estimate protein copy numbers with the proteomic ruler plugin.[10] These data, as well as the cell surface data, were loaded in Rstudio 1.1.383 (R version 3.4.2).[11] Reverse values, potential contaminants and 'only identified by site' values were filtered out, as well as peptides without at least 2 valid values in 1 of the groups. Peptide numbers per protein were counted for the surface data, and these were coupled to the copy number estimates obtained from the proteome data. ComplexHeatmap 1.14.0[12] was used to generate heatmaps. The .raw MS files and search/identification files obtained with MaxQuant are available via ProteomeXchange[13] with identifier PXD011490.

Immunosorbent assay of VWF fragment binding to MSR-1

Microtiter plates were coated over night at 4 °C with human recombinant soluble MSR-1 (R&D Systems) or BSA using 50 µl/well of a 2.5 µg/ml solution. Wells were rinsed with wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 0.1% Tween-20) and blocked with working buffer (50 mM Tris pH 7.4, 150mM NaCl, 2 mM $CaCl_2$, 0.1% Tween-20, 0.1% PolyVinylPyrollidone) for 1 h at 37 °C to prevent non-specific binding. A concentration series of the VWF fragments diluted in working buffer were incubated for 2 h at 37 °C. On wells coated with BSA only the lowest and highest concentration of each fragment was examined. After incubation, unbound proteins were washed away and bound protein was detected by incubating sequentially with anti-HPC4 (10 µg/ml) and goat-anti-mouse HRP (1:5000, Southern biotech), both for 1 h. Peroxidase activity was detected using a tetramethylbenzidine substrate solution (TMB). Absorbance was measured at 450 nm.

Surface plasmon resonance analysis

SPR analysis was performed using a BIAcoreT200 biosensor system (Biacore AB, Uppsala, Sweden). For assessment of VWF fragment binding to LRP-1, LRP-1 purified from placenta (Biomac, Leipzig) was immobilized at ~2 fmol/mm^2 to a CM5-sensor chip via primary amino groups, using the amine-coupling kit as prescribed by the supplier. Varying concentrations of D'D3 and D'D3A1 ranging from 0 to 250 nM were passed over the immobilized LRP-1 for 240 s. For assessment of sMSR-1 (R&D systems) and Cluster-II binding to RAP, gst-RAP was coupled directly to a CM5 sensor chip at 8 fmol/mm^2. Concentrations ranging from 0 to 40 nM of Cluster-II and sMSR-1 were passed over immobilized gat-RAP for 240 s in SPR buffer. For assessment of sMSR-1 binding to LRP-1 Cluster-II, recombinant LRP-1 Cluster-II was directly coupled to a CM5 sensor chip at a density of ~18 fmol/mm^2. A concentration range of 0-80 nM sMSR-1 was passed over immobilized Cluster-II for 240 s. For the competition experiment we used 40 nM sMSR-1 and titrated in a concentration series of 0, 20, 40, and 80 nM Cluster-II. In all experiments, the proteins were diluted in SPR buffer containing 20 mM Hepes

(pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$ and 0.05% (v/v) Tween-20 and binding was measured at 25 °C at a flow rate of 30 µl/min. After each binding experiment the sensor chip was regenerated by washing repeatedly with 1M NaCl ,50 mM EDTA. Binding curves were corrected for the binding response that was measured in the absence of an immobilized protein. Binding responses were plotted using Graphpad prism 7 software.

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Supplementary figures

Figure S1. Cell surface receptors of MDMs
Whole-cell proteome and cell surface proteome of MDMs were analyzed by mass spectrometry to map putative VWF clearance receptors (n=3). (A) Workflow of sample preparation and data processing. (B) The cell surface top 10 proteins. Numbers indicate the number of unique peptides identified per protein in each of the samples. The color coding represents log10 values of the estimated copy numbers per cell. (C) Identified VWF clearance receptors that were previously described, represented as in panel B.

Figure S2. Association of D'D3A1 to LRP-1 is dependent on the A1 domain
SPR analysis of D'D3A1 and D'D3 binding to immobilized LRP-1 (2 fmol/mm2). For each fragment a concentration series of 0, 31.25, 62.5, 125 and 250 nM was passed over the chip.

Figure S3. The ability of VWF to bind sMSR-1 resides in the D'D3 region
Immunosorbent assay on D'D3A1 and D'D3 binding (0-200 nM) to wells coated with sMSR-1 or bovine serum albumin (BSA, control). Bound fragments were detected via TMB-hydrolysis following labeling with an anti-HPC4 antibody and polyclonal peroxidase-labeled goat anti-mouse antibody. Control data points represents the binding of 0 and 200 nM D'D3A1 to BSA.

Figure S4. Anti-MSR-1 competes with VWF-fragment binding to sMSR-1
Immunosorbent assay on D'D3A1 and D'D3 binding (40 nM) to wells coated with sMSR-1. Bound fragments were detected via TMB-hydrolysis following labeling with a polyclonal rabbit anti-HPC4 antibody and polyclonal peroxidase-labeled swine anti-rabbit antibody.

Table S1. Protein copy number estimates and cell surface peptides of MDMs and U87MG cells
Cell surface peptides and estimated cell surface protein copy numbers per cell of MDMs (M1-3) and U87MG cells (U1-3). NA = not available.

Figure S1

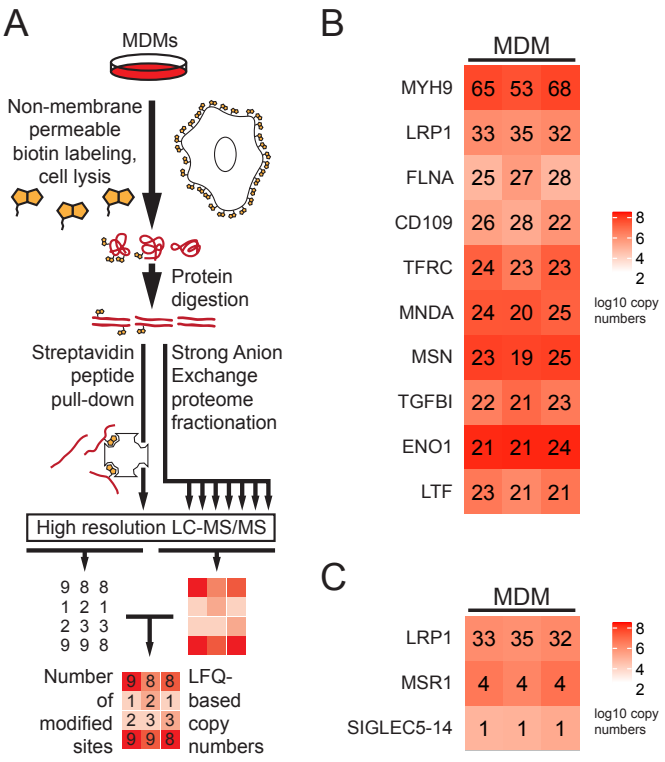

A
MDMs
Non-membrane permeable biotin labeling, cell lysis
Protein digestion
Streptavidin peptide pull-down
Strong Anion Exchange proteome fractionation
High resolution LC-MS/MS
9 8 8
1 2 1
2 3 3
9 9 8
Number of modified sites
LFQ-based copy numbers
B
MDM
MYH9 65 53 68
LRP1 33 35 32
FLNA 25 27 28
CD109 26 28 22
TFRC 24 23 23
MNDA 24 20 25
MSN 23 19 25
TGFBI 22 21 23
ENO1 21 21 24
LTF 23 21 21
8
6
4
2
log10 copy numbers
C
MDM
LRP1 33 35 32
MSR1 4 4 4
SIGLEC5-14 1 1 1
8
6
4
2
log10 copy numbers

Figure S2

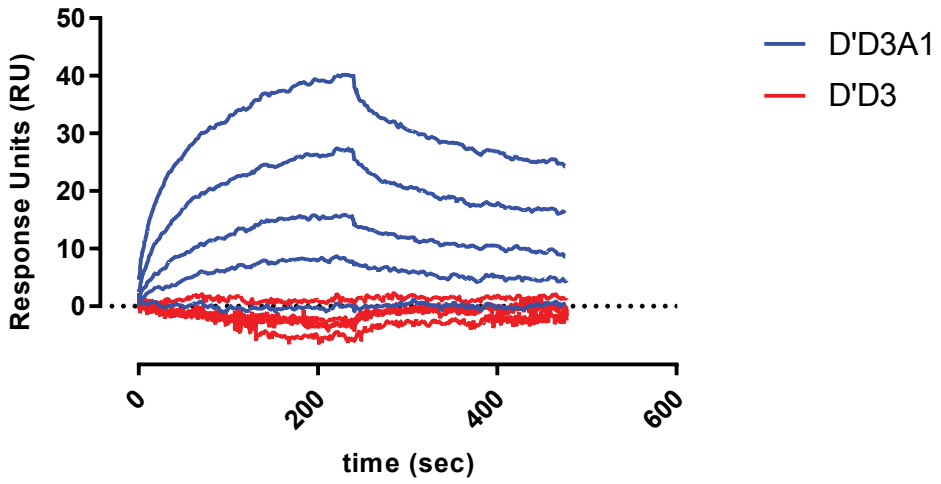

Response Units (RU)
50
40
30
20
10
0
0
200
400
600
time (sec)
D'D3A1
D'D3

Figure S3

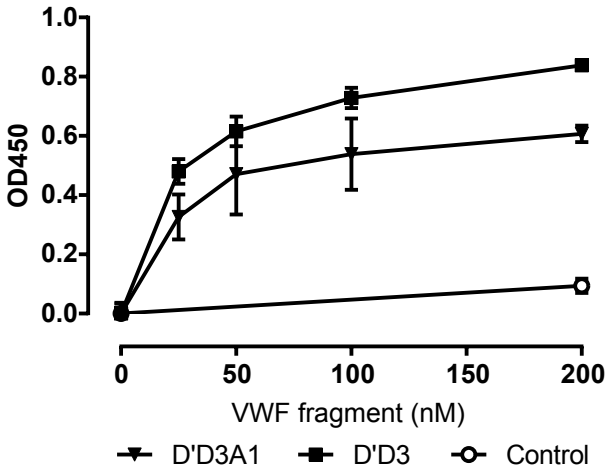
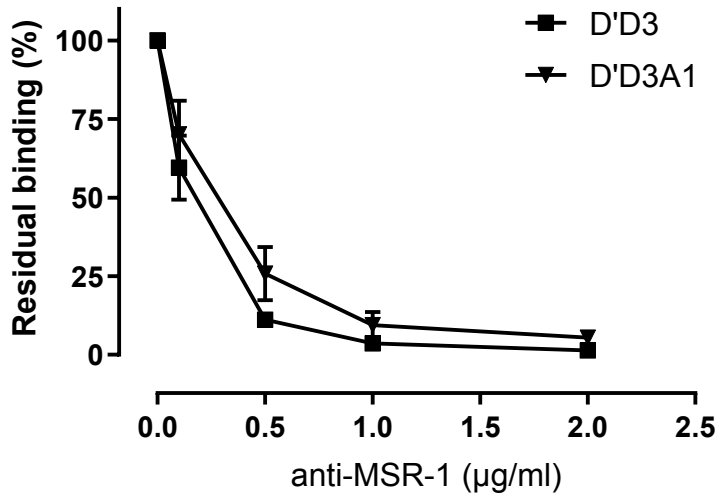

1.0
0.8
0.6
0.4
0.2
0.0
OD450
0
50
100
150
200
VWF fragment (nM)
D'D3A1
D'D3
Control

Figure S4


Residual binding (%)
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
2.5
anti-MSR-1 (µg/ml)
D'D3
D'D3A1