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¹ Scavenger receptor collectin placenta 1 is a novel receptor

2 involved in the uptake of myelin by phagocytes

Jeroen Bogie^{1,+}, Jo Mailleux^{1,+}, Elien Wouters¹, Winde Jorissen¹, Elien Grajchen¹, Jasmine
Vanmol¹, Kristiaan Wouters^{2,3}, Niels Hellings¹, Jack Van Horsen⁴, Tim Vanmierlo¹, Jerome
Hendriks^{1*}

- 6
- ⁷¹ Biomedical Research Institute, Hasselt University / Transnational University Limburg,
- 8 School of Life Sciences, Diepenbeek, Belgium
- 9 ²Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical
- 10 Centre (MUMC), Maastricht, The Netherlands
- ³ Department of Internal Medicine, Maastricht University Medical Centre (MUMC),
- 12 Maastricht, The Netherlands
- ⁴ Department of Molecular Cell Biology and Immunology, VU University Medical Center,
- 14 Amsterdam, The Netherlands
- 15
- ^{*}corresponding author (Jerome.hendriks@uhasselt.be)
- ⁺these authors contributed equally to this work
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- 19
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25 Abstract

Myelin-containing macrophages and microglia are the most abundant immune cells in active 26 multiple sclerosis (MS) lesions. Our recent transcriptomic analysis demonstrated that 27 collectin placenta 1 (CL-P1) is one of the most potently induced genes in macrophages after 28 29 uptake of myelin. CL-P1 is a type II transmembrane protein with both a collagen-like and 30 carbohydrate recognition domain, which plays a key role in host defense. In this study we sought to determine the dynamics of CL-P1 expression on myelin-containing phagocytes and 31 define the role that it plays in MS lesion development. We show that myelin uptake increases 32 the cell surface expression of CL-P1 by mouse and human macrophages, but not by primary 33 34 mouse microglia in vitro. In active demyelinating MS lesions, CL-P1 immunoreactivity was localized to perivascular and parenchymal myelin-laden phagocytes. Finally, we demonstrate 35 that CL-P1 is involved in myelin internalization as knockdown of CL-P1 markedly reduced 36 myelin uptake. Collectively, our data indicate that CL-P1 is a novel receptor involved in 37 myelin uptake by phagocytes and likely plays a role in MS lesion development. 38

39 Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, neurodegenerative disease of the central nervous system (CNS). Macrophage- and microglia-mediated myelin destruction is considered to be the primary effector mechanism in MS lesion development ¹. Previous studies defined that complement-receptor 3, scavenger receptors I/II, and Fcγ receptors, facilitate the clearance of myelin by macrophages and microglia ^{2,3}. However, considering the complexity of myelin, it is unlikely that solely these receptors are involved in the uptake of myelin by activated microglia and macrophages in MS lesions.

Using genome wide gene expression analysis, we previously found that internalization of 47 myelin alters the expression of 676 genes in rat peritoneal macrophages⁴. Collectin placenta 1 48 (CL-P1) was one of the most potently induced genes in macrophages upon uptake of myelin. 49 50 CL-P1 is structurally related to scavenger receptor class A (SRA) due to its collagen-like domain⁵. However, CL-P1 also contains a C-type lectin/carbohydrate recognition domain (C-51 type CRD)^{6,7}, typically found in C-type lectin receptors, such as dendritic cell-specific 52 ICAM3-grabbing non-integrin (DC-SIGN)⁸. Functionally, CL-P1 is associated with binding 53 and internalization of bacteria, yeast, and oxidized low-density lipoproteins ^{5-7,9}. Furthermore, 54 CL-P1 recognizes carcinoma-associated antigens, possibly via interaction with Lewis^x 55 trisaccharide on tumor cells ^{10,11}, hereby mediating tumor cell-endothelium interactions ^{12,13}. 56 Finally, a recent study showed that the collagen-like domain of CL-P1 facilitates amyloid beta 57 (A β) clearance by microglia and that uptake of A β increases the expression of CL-P1 ¹⁴. 58 These findings indicate that CL-P1 plays a role host defense and cellular uptake in different 59 diseases. 60

In this study, we sought to determine if myelin internalization increases surface expression of CL-P1 on peripheral and CNS-resident phagocytes, its involvement in internalization of myelin, and its cellular distribution in MS lesions. We show that myelin uptake increases the 64 cell surface expression of CL-P1 by mouse and human macrophages, but not by primary 65 mouse microglia *in vitro*. In active MS lesions CL-P1 immunoreactivity was localized to 66 parenchymal and pervivascular myelin-containing phagocytes. Finally, we show that silencing 67 of CL-P1 strongly reduces myelin uptake. Collectively, our data indicate that CL-P1 mediates 68 the uptake of myelin and likely plays a role in MS lesion development.

69 **Results**

70 Myelin increases the surface expression of CL-P1 on phagocytes

By using a transcriptomic approach, we previously demonstrated that myelin induces gene 71 expression of CL-P1 in peritoneal rat macrophages ⁴. Here, we validated this increase in CL-72 P1 mRNA expression on protein level in mouse and human primary phagocytes and 73 phagocyte cell lines. By using western blot (fig. 1a-b and s1), immunohistochemistry (fig. 1c), 74 and flow cytometry (fig. 1d and s2a), we show that human primary monocytes express CL-P1 75 and that myelin internalization increases the expression of CL-P1. For western blot analysis, 76 77 two separate antibodies were used to confirm the myelin-induced increase in CL-P1 expression. We further show that mouse primary microglia and bone-marrow derived 78 macrophages (BMDMs), as well as cell lines closely resembling these phagocytes (BV-2, 79 80 microglia; RAW264.7, macrophages), express CL-P1 and that myelin uptake results in an elevated expression of CL-P1 by these cells (fig 1e and s2b-e). Interestingly, CL-P1 81 expression was not increased on primary mouse microglia after myelin uptake. In addition, we 82 found increased expression of CL-P1 by high granular (SSC^{hi}) myelin-containing mouse 83 primary BMDMs compared to low granular (SSC^{lo}) cells that did not substantially 84 85 phagocytose myelin (fig. 1f and s2f). This finding indicates that the intensity of CL-P1 immunoreactivity correlates with the amount of internalized myelin. 86

In MS lesions, phagocytes are likely to encounter modified forms of myelin such as oxidized myelin ¹⁵⁻¹⁷. We demonstrate that oxidized myelin more prominently increases the surface expression of CL-P1 on macrophages compared to unmodified myelin (fig.1g and s2g). In addition, while CL-P1 surface expression gradually decreased on macrophages treated with unmodified myelin, macrophages treated with oxidized myelin retained a high expression of CL-P1 over time (fig. 1h and s2h-k).

Previously, we found that myelin-derived lipids, such as cholesterol metabolites and fatty 93 acids, partially account for the phenotype of phagocytes after myelin uptake ^{4,18}. Activation of 94 the liver X receptor (LXR) and peroxisome proliferator-activated receptor β/δ (PPAR β/δ) 95 underlies the impact of these lipids on the phenotype of phagocytes. By using synthetic 96 agonists for LXR and PPAR β/δ , we show that myelin increases the expression of CL-P1 in an 97 LXR- and PPARB/ô-independent manner (fig. 1i and s21). We further demonstrate that 98 inflammatory stimuli, such as IFNy and LPS, do not impact CL-P1 expression by both 99 100 untreated and myelin-treated macrophages (fig. 1j and s2m-n). Collectively, these data show that myelin uptake increases the surface expression of CL-P1 on phagocytes in vitro in an 101 LXR- and PPAR^{β/δ}-independent manner, and that inflammatory stimuli do not impact CL-P1 102 expression. 103

104

105 CL-P1 is expressed by phagocytes in MS lesions

106 The observed increase in the expression of CL-P1 on macrophages following myelin 107 internalization in vitro, prompted us to determine CL-P1 expression in active MS lesions. We 108 show that CL-P1 is predominantly expressed on brain endothelial cells in the normalappearing white matter (NAWM) (fig. 2a). In MS lesions, a profound increase in the 109 expression of CL-P1 was observed (fig. 2b-d). Immune-double labeling revealed that CD68⁺ 110 parenchymal and perivascular phagocytes expressed CL-P1 within MS lesions (fig. 3a-b). 111 Within the NAWM, CD68⁺ microglia and perivascular macrophages expressed CL-P1 (fig. 112 3c). Findings were validated using an alternative antibody directed against CL-P1 (fig. s3a-b). 113 Interestingly, within active MS lesions, GFAP⁺ astrocytes also expressed CL-P1 (fig. s3c). 114 Control staining did not show any immunoreactivity (data not shown). Oil Red O staining 115 116 further showed that lipid-containing phagocytes were abundantly present in both the parenchyma and perivascular spaces within these lesions (fig. 4a-b). These data indicate that 117

118 CL-P1 is expressed on astrocytes and myelin-laden perivascular and parenchymal phagocytes119 within active MS lesions.

120

121 CL-P1 mediates the uptake of myelin

Considering that CL-P1 is structurally related to SRA and that the uptake of myelin by 122 phagocytes is mediated by SRA 3,5, we determined whether CL-P1 is involved in the 123 internalization of myelin. For this purpose, plasmids expressing shRNA directed against CL-124 125 P1 were used. HEK293.1 cells were used as an easy transfectable human cell line with phagocytic properties. Importantly, HEK293.1 avidly endocytosed human myelin debris (fig. 126 5a and s4a) and expressed CL-P1 (fig. 5b-d). To define the knockdown efficacy and the role 127 that CL-P1 plays in the uptake of myelin, HEK293.1 cells were exposed to a pool of shRNAs 128 directed against CL-P1. We show that the pool of shRNAs (shRNA1-4) completely reduced 129 130 the cell surface expression of CL-P1 compared to scrambled shRNA (fig. 5e and s4b). Western blot and qPCR analysis demonstrated a ~60% reduction in CL-P1 expression when 131 132 cells were exposed to Cl-P1 shRNAs (fig. 5c-d and s5). Importantly, we show that silencing 133 of CL-P1 reduced the uptake of myelin by ~50% compared to scrambled shRNA (fig. 5e and s4c). These data indicate that CL-P1 is involved in the internalization of myelin. 134

135 **Discussion**

Foamy phagocytes containing myelin debris are the most abundant immune cells in active MS 136 lesions. Our recent transcriptomic analysis demonstrated that CL-P1 is one of the most 137 potently induced genes in macrophages after uptake of myelin. In this study we sought to 138 determine the dynamics of CL-P1 expression on myelin-phagocytosing phagocytes and 139 unravel what function CL-P1 has on these phagocytes. We show that CL-P1 is expressed by 140 phagocytes in inflammatory MS lesions and that myelin uptake induces cell surface 141 expression of CL-P1 in mouse and human phagocytes in vitro. Moreover, we demonstrate that 142 143 CL-P1 is involved in myelin internalization as knockdown of CL-P1 markedly reduced myelin uptake. These data indicate that CL-P1 is a novel receptor involved in the 144 internalization of myelin by macrophages and likely plays a role in the pathophysiology of 145 146 MS.

In this study, we show that both mouse macrophages and human monocytes express CL-P1 on 147 their cell surface and that myelin internalization increases the surface expression of CL-P1 on 148 BMDMs in a dose-dependent manner in vitro. However, whereas primary mouse microglia 149 expressed CL-P1, myelin internalization did not increase the expression of CL-P1 by these 150 151 phagocytes. This discrepancy may underline the fact that microglia and infiltrating macrophages react differently to environmental cues¹⁹⁻²¹. Ontogenic differences in signaling 152 pathways involved in the regulation of CL-P1 might explain the observed discrepancy 153 between the two phagocyte subsets ^{22,23}. In active MS lesions, HLA-DR⁺ phagocytes markedly 154 expressed CL-P1 suggesting that myelin internalization also enhances CL-P1 expression by 155 phagocytes in MS lesions. 156

157 Myelin is composed of a variety of lipids and proteins, many of which can alter the 158 physiology of phagocytes upon binding and internalization. Recently, we showed that myelin 159 uptake skews macrophages towards a less-inflammatory phenotype, at least in part, through

the activation of the lipid sensing LXR and PPAR ^{4,18}. Unlike SRAs, such as SPa, MARCO, 160 and CD36, which are well-known target genes of LXRs or PPARs^{24,25}, we found that the 161 expression of CL-P1 was not regulated by agonists for either of these nuclear receptors. 162 Likewise, inflammatory signaling pathways activated by IFNy and LPS did not significantly 163 impact the surface expression of CL-P1 on control and myelin-containing phagocytes in vitro. 164 Future studies are needed to elucidate how myelin uptake regulates the expression of CL-P1. 165 Interestingly, oxidized myelin more potently induced and maintained the expression of CL-P1 166 on phagocytes compared to unmodified myelin. Defining transcriptional differences between 167 phagocytes exposed to unmodified and oxidized myelin may lead to the identification of the 168 169 biological pathway controlling CL-P1 expression.

Several receptors, such as the complement-receptor 3, SRA I/II, and Fcy receptors, facilitate 170 the clearance of myelin by macrophages and microglia^{2,3}. Our data indicate that CL-P1 also 171 172 contributes to the internalization of myelin. The phagocytic capacity of SRA largely depends on its collagen-like domain ²⁶. Considering that CL-P1 and SRA share the same collagen-like 173 domain ⁵, this domain may underlie the role that CL-P1 plays in the internalization of myelin. 174 175 Future studies are warranted to determine if CL-P1 contributes to myelin uptake in vivo and how this impacts neuroinflammation and neurodegeneration. As uptake of myelin leads to 176 both demyelination and CNS repair, depending on whether it concerns intact myelin or 177 1,27-29 myelin debris, CL-P1-mediated myelin uptake can be both beneficial or detrimental 178 In our *in vitro* experiments, myelin debris is used to define the impact of CL-P1 on the uptake 179 of myelin. Hence, it is tempting to speculate that CL-P1 might play a role in myelin debris 180 clearance *in vivo*, thereby facilitating remyelination ²⁷⁻²⁹. 181

Aside from a collagen-like domain, CL-P1 contains a C-type CRD that binds with high affinity to glycans bearing Lewis^x and Lewis^a trisaccharides ^{10,11}. Interestingly, based on this glycan-specificity, parallels can be drawn between CL-P1 and both DC-SIGN and selectins ^{30,31}. This suggests that CL-P1 may also play a role in cell migration, cell differentiation, antigen-capture, and T cell priming ^{32,33}. Interestingly, we found that CL-P1 is markedly expressed on foamy-appearing phagocytes in and near perivascular cuffs in MS lesions. As perivascular cuffs accommodate lymphocytes during active MS, CL-P1 on phagocytes may play a role in T cell priming. Additionally, as myelin-containing phagocytes are located in CNS-draining lymphoid organs ³⁴⁻³⁶, future studies should determine whether CL-P1 may facilitate lymph node directed migration of these phagocytes.

Increasing evidence indicates that astrocytes actively participate in various processes underlying MS pathogenesis, including neuroinflammation, demyelination, and remyelination ³⁷. We show that astrocytes have increased expression of CL-P1 in MS lesions. Of interest, CL-P1 immunoreactivity is also increased on reactive astrocytes in AD ¹⁴. Follow-up studies should address whether this increased expression of CL-P1 on astrocytes in MS lesions plays a role in the phagocytic capacity of astrocytes, as well as their migration and differentiation.

Based on our findings, we propose a positive feedback model in which CL-P1 mediates the uptake of myelin by phagocytes and subsequently increases its own expression. Considering its role in the uptake of myelin, CL-P1 likely plays an important role in the pathophysiology of MS.

202 Methods

203 Cell isolation and culture

Bone marrow-derived macrophages were obtained as described previously ³⁸. Briefly, femoral 204 205 and tibial bone marrow suspensions from 12 week-old C57Bl/6J mice (Harlan, Horst, Netherlands) were cultured in 10 cm plates at a concentration of 10×10^6 cells/plate and 206 differentiated in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 207 10% fetal calf serum (FCS, Gibco, Merelbeke, Belgium), 50 U/ml penicillin (Invitrogen), 50 208 U/ml streptomycin (Invitrogen), and 15% L929-conditioned medium. Microglia cultures were 209 210 prepared from postnatal P3 C57BL/6J mouse pups. Isolated forebrains of mice pups were placed in L15 Leibovitz medium (Gibco) containing 1:10 Trypsin (Sigma-Aldrich, Diegem, 211 Belgium) (37°C, 15 min). Next, high glucose DMEM medium (Invitrogen) supplemented 212 213 with 10% FCS, 50 U/ml penicillin, 50 U/ml streptomycin, (DMEM 10:1 medium), and 100 µl/ml DNase I (Sigma-Aldrich) was added to the forebrain tissue. Nervous tissue was 214 dissociated by trituration with serum-coated Pasteur pipettes (Sigma-Aldrich). The dissociated 215 mix was passed through a 70 µm cell strainer, rinsed with 5 ml of DMEM 10:1 medium, and 216 centrifuged (170g, 10 min, RT). After a second centrifugation step, cell suspension was 217 seeded at 2 forebrains/75 cm² flask. After 2 days, DMEM 10:1 medium was changed and after 218 reaching confluence (± 6 days later), 2/3 DMEM 10:1 medium containing 1/3 L929-219 220 conditioned medium was added. Six days later, microglia isolation was performed using the 221 shake-off method (200 rpm, 2h, RT). Microglia were centrifuged (170g, 10min, RT), suspended in DMEM 10:1 medium containing B27 supplement (Invitrogen), and cultured at 222 250.000 cells/well in poly-L-lysine (Sigma-Aldrich)-coated 24-well plates. Animals were 223 224 housed in the animal facility of the Biomedical Research Institute of Hasselt University. All experimental protocols and methods involving animals within this study were conducted in 225

accordance with institutional guidelines and approved by the Ethical Committee for AnimalExperiments Hasselt University.

Peripheral blood mononuclear cells were isolated from whole blood by density gradient 228 229 centrifugation on lympholyte-H cell separation media (Cedarlane, Ontario, Canada). Blood samples were collected from healthy controls after obtaining informed written consent. 230 Subjects with signs of infection were excluded. All experimental protocols and methods were 231 conducted in accordance with institutional guidelines and approved by the Medical Ethical 232 Committee Hasselt University. CD14⁺ monocytes were collected using the EasySep human 233 CD14 positive selection kit (Stemcell Technologies, Grenoble, France) according to 234 manufacturer's instructions. After isolation, cells were cultured $(1 \times 10^6 \text{ cells/ml})$ in RPMI 235 1640 supplemented with 10% human serum (Sigma-Aldrich, Saint Louis, USA), 50 U/ml 236 penicillin and 50 U/ml streptomycin. 237

238 The immortalized mouse macrophage (RAW 264.7), mouse microglia (BV-2), and human embryonic kidney (HEK293.1) cell lines were cultured in DMEM (Invitrogen) with 50 U/ml 239 240 penicillin, 50 U/ml streptomycin), and 10% FCS. To determine the effect of myelin and LXR 241 and PPAR β/δ agonists for LXR and PPAR β/δ on the expression of CL-P1, cells were treated for 24 hours with 100 µg/ml of isolated myelin, 10 µM T0901317 (T09; LXR agonist; 242 Cayman Chemical, Huissen, The Netherlands), or 10 μ M GSK0660 (PPAR β/δ agonist; 243 Sigma-Aldrich). To determine the impact of inflammation on CL-P1 expression, cells 244 exposed to 100 ng/ml LPS (Sigma-Aldrich) and/or IFNy (Peprotech, Hamburg, Germany). 245

246

247 Myelin isolation, labelling, and phagocytosis

Myelin was purified from postmortem mouse and human brain tissue by means of density gradient centrifugation, as described previously ³⁹. Experimental protocols and methods were conducted in accordance with institutional guidelines and approved by the Medical Ethical Committee Hasselt University and the Ethical Committee for Animal Experiments Hasselt

University. Written informed consent was obtained from all donors. Myelin protein 252 concentration was determined by using the BCA protein assay kit (Thermo Fisher Scientific, 253 Erembodegem, Belgium), according to manufacturer's instructions. Endotoxin content was 254 255 determined using the Chromogenic Limulus Amebocyte Lysate assay kit (Genscript Incorperation, Aachen, Germany). Isolated myelin contained a negligible amount of 256 endotoxin ($\leq 1.8 \times 10^{-3}$ pg/µg myelin). To obtain oxidized myelin, myelin was exposed to 10 257 µM CuSO4 at 37°C for 20 hours. Myelin was fluorescently labelled, according to the method 258 of Van der Laan et al.⁴⁰. In short, 10 mg/ml myelin was incubated with 12.5 µg/ml 1,1"-259 diotadecyl-3,3,3',3',-tetramethylindocarbocyanide perchlorate (DiI; Sigma-Aldrich) for 30 min 260 at 37°C. To determine the capacity of cells to phagocytose myelin, cells were exposed to 100 261 µg/ml DiI-labeled myelin. The amount of myelin phagocytosed was determined using a 262 FACSCalibur (BD Biosciences, Erembodegem, Belgium). HEK293.1 were used to define the 263 264 impact of CL-P1 on myelin phagocytosis as BV-2 and RAW264.7 cells are not easily transfectable. Of note, HEK293.1 are often used as a model system to study phagocytic 265 receptors ^{41,42}. 266

267

268 Western blot

CL-P1 protein expression was determined via SDS-PAGE and western blot analysis. Briefly, 269 270 samples were denaturated and separated on a 8% polyacrylamide gel containing Tris-glycine and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, 271 Buckinghamshore, UK). Non-specific binding was blocked by incubating the membranes in 272 5% (w/v) nonfat powdered milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 273 (TBS-T) for 1 hour. Subsequently, membranes were incubated with primary antibodies goat 274 275 anti-human CL-P1 (R&D Systems, Abingdon, UK 1:1000), goat-anti-human CL-P1 (Novus Biologicals, Abingdon, UK, 1:1000), and rabbit anti-human B-actin (1:10000, Santa Cruz 276 Biotechnology, Heidelberg, Germany) in TBS-T overnight at 4°C. Membranes were 277

incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated rabbit-anti
goat and goat anti-rabbit antibodies (Dako, 1:2000) in 5% milk in TBS-T. For stripping and
reprobing, a mild stripping buffer was used (0.2M glycine, 0.1% SDS, 1% Tween-20, pH
2.2). An ECL Plus detection kit (Thermo Fisher Scientific) was used and the generated
chemiluminescent signal was detected by a luminescent image analyzer (ImageQuant LAS
4000 mini; GE Healthcare).

284

285 shRNA and transfection

The X-tremegene HP transfection kit (Roche Diagnostics, Mannheim, Germany) was used to 286 transfect HEK293.1 cells according to the manufacturer's instructions. In short, 287 0.25x10⁶ HEK293.1 cells were transfected with 1.5 µg of shRNA in 50 µl Opti-MEM® I 288 Reduced Serum Media (Thermo Fisher Scientific). Cells were then resuspended in complete 289 culture medium and incubated for 48 hours at 37°C. CL-P1 (shRNA-1); 290 291 AACATCTCGCCAAACCTATGA, CL-P1 (shRNA-2); CAGGCTATCCAGCGAATCAAGAA, CL-P1 (shRNA-3); 292 AAGAAATGAAGCTAGTAGACT, CL-P1 (shRNA-4); AACGATTTCCAATGTGA 293 AGAC, scrambled; CCTAAGGTTAAGTCGCCCTCG. 294

295

296 Flow cytometry

Flow cytometry was used to assess the expression of CL-P1 on all cell types. Cells were stained with goat-anti-mouse CL-P1 (R&D Systems), goat-anti-human CL-P1 (R&D Systems), or normal goat IgG (R&D Systems). Alexa fluor 488 F(ab')₂ fragment of rabbit-anti goat (Invitrogen) was used as a secondary antibody. The FACSCalibur was used to quantify cellular fluorescence.

302

303 Immunohistochemistry

304 Frozen brain material from active MS lesions was obtained from the Netherlands Brain Bank (NBB, Amsterdam, Netherlands). Human monocytes were cultured on glass cover slides 305 306 (Thermo Fisher Scientific) and fixed in 4% PFA for 30 minutes. Cryosections were fixed in acetone for 10 minutes. Cryosections and human monocytes were blocked for 20 minutes 307 with 10% normal serum from the same species as the secondary antibody (Dako, Heverlee, 308 Belgium). For 3, 3' diaminobenzidine (DAB) staining, slides were incubated with goat-anti-309 human CL-P1 (R&D Systems). After washing, HRP-conjugated rabbit-anti-goat (Dako) was 310 added. Subsequently, DAB substrate (Dako) was used to stain slides. Sections were 311 counterstained with hematoxylin (Merck, Darmstadt, Germany). For fluorescence staining, 312 cryosections were incubated with goat-anti-human CL-P1 (R&D Systems), goat-anti-human 313 CL-P1 (Novus Biologicals), mouse-anti-human CD68 (Ebioscience, Vienna, Austria), 314 mouse-anti-human Human Leucocyte Antigen DR/DP/DQ (HLA-DR/DP/DQ; Dako), or 315 rabbit-anti glial fibrillary acidic protein (GFAP; Dako). Cryosections were stained with Alexa 316 317 flour secondary antibodies (Invitrogen). Nuclei were visualized using 4,6'-diamidino-2-318 phenylindole (DAPI; Invitrogen). Analysis was carried out using a Nikon eclipse 80i microscope and NIS Elements BR 3.10 software (Nikon, Tokyo, Japan). Intracellular myelin 319 degradation products were defined with oil-red O (ORO), which stains neutral lipids, as 320 described previously ⁴³. 321

322

323 **Quantitative PCR**

Total RNA from cultures was prepared using the RNeasy mini kit (Qiagen, Venlo, The Netherlands), according to manufacturer's instructions. The RNA quality was determined with a NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). RNA was converted to cDNA using the reverse transcription system (Quanta Biosciences, Gaithersburg, USA) and quantitative PCR was performed on a StepOnePlus detection system

(Applied Biosystems, Gaasbeek, Belgium), as previously described ^{4,44}. Relative 329 quantification of gene expression was accomplished using the comparative C_t method. Data 330 were normalized to the most stable reference genes ^{45,46}. Primers: *CL-P1* (fw); 331 TGGTAGGGAGAGAGAGCCAC, CCCATCCAGCCACTTCCATT, 332 CL-P1 (rv); cyclophilin (fv); AGACTGAGTGGTTGGATGGC, 333 А (Cyca) Cyca (rv); TCGAGTTGTCCACAGTCAGC, ribosomal protein L13A (*Rpl13a*) (fv); 334 AAGTTGAAGTACCTGGCTTTCC, Rpl13a (rv); GCCGTCAAACACCTTGAGAC. 335

336

337 Statistical analysis

Data were statistically analyzed using GraphPad Prism for windows (version 4.03) and are reported as mean \pm SEM. D'Agostino and Pearson omnibus normality test was used to test normal distribution. An analysis of variances (ANOVA) or two-tailed unpaired student T-test (with Welch's correction if necessary) was used for normally distributed data sets. The Kruskal-Wallis or Mann-Whitney analysis was used for data sets which did not pass normality. *P \leq 0.05, **P \leq 0.01 and ***P \leq 0.001.

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477 Author contributions statement

JB, JM, EW, WJ, EG, JV, and TV performed the experiments and analyzed the data. JB wrote
the manuscript. KW and JvH provided experimental materials. JB, JM, EW, WJ, EG, JV,
KW, NH, JvH, TV, and JH revised the manuscript. JB, KW, NH, JvH, TV, and JH
participated in the design and coordination of the project.

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483 Additional information

484 The other authors declare that they have no competing interests.

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FIGURES AND FIGURE LEGEND



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Figure 1: Myelin uptake increases the surface expression of CL-P1 on myeloid cells. (a,b) 487 Human monocytes (hMono, n=5), were cultured with or without 100 µg/ml myelin for 24h. 488 Western blot analysis was used to define CL-P1 expression. Two antibodies were used to 489 expression. Western blots are displayed in cropped format. (c) define CL-P1 490 491 Immunohistochemistry (CL-P1, Novus Biologicals) was used to define the expression of CL-P1 by human monocytes cultured with or without 100 µg/ml myelin for 24h. (d) Human 492 monocytes (n=7) were cultured with or without 100 µg/ml myelin for 24h. CL-P1 expression 493 was determined with flow cytometry (CL-P1, R&D). Dotted line represents untreated cells 494 stained with an isotype antibody. (e) RAW264.7 (n=4), BV-2 (n=4), mouse BMDMs (n=4), 495 and mouse microglia (n=7), were cultured with or without 100 µg/ml myelin for 24h. CL-P1 496

expression was determined with flow cytometry (CL-P1, R&D). Dotted line represents 497 untreated cells stained with an isotype control antibody. (f) Mouse BMDMs cultured with 100 498 µg/ml myelin for 24h. CL-P1 expression was determined in high granular (SSC^{hi}), low 499 granular (SSC^{lo}), and all cells (SSC^{all}) using flow cytometry. Dotted line represents myelin-500 treated cells stained with the CL-P1 antibody (n=4). (g) RAW264.7 cells were exposed to 100 501 ug/ml unmodified and CU²-oxidized myelin for 24h, after which CL-P1 expression was 502 determined. Dotted line represents untreated cells stained with the CL-P1 antibody (n=4). (h) 503 RAW264.7 cells were cultured with 100 μ g/ml unmodified or CU²-oxidized myelin for 1.2.3. 504 and 8 days (n=3). CL-P1 expression was determined by using flow cytometry. (i) RAW264.7 505 cells were cultured with a T0901317 (LXR agonist), GW501516 (PPARβ/δ agonist), or 100 506 µg/ml myelin for 24h. CL-P1 expression was determined with flow cytometry. Dotted line 507 represents untreated cells stained with the CL-P1 antibody (n=6). (j) Untreated or myelin 508 509 treated RAW264.7 cells were exposed to 500 U/ml IFNy, 100 ng/ml LPS, a combination IFNy and LPS, or left untreated (n=4). CL-P1 expression was determined using flow cytometry. 510 511 Dotted line represents untreated cells stained with the CL-P1 antibody. Data are presented as 512 mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

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Figure 2: CL-P1 is highly expressed in MS lesions. (a) Image of normal-appearing matter
stained for CL-P1 (40x magnification). Arrows depict blood vessels. (b-d) Active MS lesion
stained for CL-P1 (b-c, 10x magnification; d, 40x magnification). Arrow depicts a
perivascular cuff filled with infiltrated myeloid cells.

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Figure 3: CL-P1 is expressed by phagocytes in MS lesions. (a-b) Representative images of
active MS lesion stained for CD68 and CL-P1 (Novus Biologicals; (a), 10x magnification;
(b), 40x magnification). (c) NAWM stained for CD68 and CL-P1 (Novus Biologicals, 40x
magnification). Perivascular macrophages and microglia are designated by an arrow and
arrowheads, respectively.



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527 Figure 4: Abundant lipid-containing phagocytes in perivascular space and parenchyma

528 of active MS lesion. (a,b) ORO staining of active MS lesion showing foamy phagocytes

529 containing neutral intracellular lipids (a, 10x magnification; b, 40x magnification).



Figure 5: CL-P1 is involved in the uptake of myelin. (a) HEK293.1 cells were exposed to 531 DiI-labeled myelin for 1.5h (n=4). Myelin uptake was assessed using flow cytometry. Cells 532 were exposed to myelin at 4°C (binding) or 37°C (binding and uptake). Dotted line represents 533 534 untreated cells. (b-d) HEK293.1 cells were exposed to scrambled shRNA or a pool of shRNA directed against CL-P1 (shRNA1-4) for 48h. The mRNA and protein expression of CL-P1 535 was determined using qPCR (b, n=4), western blot (c, CL-P1 I (R&D), CL-P1 II (Novus 536 Biologicals), n=3), and flow cytometry (d, n=6). Western blots are displayed in cropped 537 format. (e) HEK293.1 cells were exposed to scrambled shRNA or a pool of shRNA directed 538 against CL-P1 (shRNA1-4) for 48h. Next, DiI-labeled myelin was added for 1.5h (n=8). Flow 539 cytometry was used to define myelin uptake. Dotted line represents untreated cells. Data are 540 presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. 541