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1 **Scavenger receptor collectin placenta 1 is a novel receptor**  
2 **involved in the uptake of myelin by phagocytes**

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25 **Abstract**

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

## 39 **Introduction**

40 Multiple sclerosis (MS) is a chronic, inflammatory, neurodegenerative disease of the central  
41 nervous system (CNS). Macrophage- and microglia-mediated myelin destruction is  
42 considered to be the primary effector mechanism in MS lesion development <sup>1</sup>. Previous  
43 studies defined that complement-receptor 3, scavenger receptors I/II, and Fc $\gamma$  receptors,  
44 facilitate the clearance of myelin by macrophages and microglia <sup>2,3</sup>. However, considering the  
45 complexity of myelin, it is unlikely that solely these receptors are involved in the uptake of  
46 myelin by activated microglia and macrophages in MS lesions.

47 Using genome wide gene expression analysis, we previously found that internalization of  
48 myelin alters the expression of 676 genes in rat peritoneal macrophages <sup>4</sup>. Collectin placenta 1  
49 (CL-P1) was one of the most potently induced genes in macrophages upon uptake of myelin.  
50 CL-P1 is structurally related to scavenger receptor class A (SRA) due to its collagen-like  
51 domain <sup>5</sup>. However, CL-P1 also contains a C-type lectin/carbohydrate recognition domain (C-  
52 type CRD) <sup>6,7</sup>, typically found in C-type lectin receptors, such as dendritic cell-specific  
53 ICAM3-grabbing non-integrin (DC-SIGN) <sup>8</sup>. Functionally, CL-P1 is associated with binding  
54 and internalization of bacteria, yeast, and oxidized low-density lipoproteins <sup>5-7,9</sup>. Furthermore,  
55 CL-P1 recognizes carcinoma-associated antigens, possibly via interaction with Lewis<sup>x</sup>  
56 trisaccharide on tumor cells <sup>10,11</sup>, hereby mediating tumor cell-endothelium interactions <sup>12,13</sup>.  
57 Finally, a recent study showed that the collagen-like domain of CL-P1 facilitates amyloid beta  
58 (A $\beta$ ) clearance by microglia and that uptake of A $\beta$  increases the expression of CL-P1 <sup>14</sup>.  
59 These findings indicate that CL-P1 plays a role host defense and cellular uptake in different  
60 diseases.

61 In this study, we sought to determine if myelin internalization increases surface expression of  
62 CL-P1 on peripheral and CNS-resident phagocytes, its involvement in internalization of  
63 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 perivascular 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**

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

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

93 Previously, we found that myelin-derived lipids, such as cholesterol metabolites and fatty  
94 acids, partially account for the phenotype of phagocytes after myelin uptake<sup>4,18</sup>. Activation of  
95 the liver X receptor (LXR) and peroxisome proliferator-activated receptor  $\beta/\delta$  (PPAR $\beta/\delta$ )  
96 underlies the impact of these lipids on the phenotype of phagocytes. By using synthetic  
97 agonists for LXR and PPAR $\beta/\delta$ , we show that myelin increases the expression of CL-P1 in an  
98 LXR- and PPAR $\beta/\delta$ -independent manner (fig. 1i and s2l). We further demonstrate that  
99 inflammatory stimuli, such as IFN $\gamma$  and LPS, do not impact CL-P1 expression by both  
100 untreated and myelin-treated macrophages (fig. 1j and s2m-n). Collectively, these data show  
101 that myelin uptake increases the surface expression of CL-P1 on phagocytes *in vitro* in an  
102 LXR- and PPAR $\beta/\delta$ -independent manner, and that inflammatory stimuli do not impact CL-P1  
103 expression.

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 normal-  
109 appearing white matter (NAWM) (fig. 2a). In MS lesions, a profound increase in the  
110 expression of CL-P1 was observed (fig. 2b-d). Immune-double labeling revealed that CD68<sup>+</sup>  
111 parenchymal and perivascular phagocytes expressed CL-P1 within MS lesions (fig. 3a-b).  
112 Within the NAWM, CD68<sup>+</sup> microglia and perivascular macrophages expressed CL-P1 (fig.  
113 3c). Findings were validated using an alternative antibody directed against CL-P1 (fig. s3a-b).  
114 Interestingly, within active MS lesions, GFAP<sup>+</sup> astrocytes also expressed CL-P1 (fig. s3c).  
115 Control staining did not show any immunoreactivity (data not shown). Oil Red O staining  
116 further showed that lipid-containing phagocytes were abundantly present in both the  
117 parenchyma and perivascular spaces within these lesions (fig. 4a-b). These data indicate that

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

120

### 121 **CL-P1 mediates the uptake of myelin**

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



## 135 **Discussion**

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

147 In this study, we show that both mouse macrophages and human monocytes express CL-P1 on  
148 their cell surface and that myelin internalization increases the surface expression of CL-P1 on  
149 BMDMs in a dose-dependent manner *in vitro*. However, whereas primary mouse microglia  
150 expressed CL-P1, myelin internalization did not increase the expression of CL-P1 by these  
151 phagocytes. This discrepancy may underline the fact that microglia and infiltrating  
152 macrophages react differently to environmental cues<sup>19-21</sup>. Ontogenic differences in signaling  
153 pathways involved in the regulation of CL-P1 might explain the observed discrepancy  
154 between the two phagocyte subsets<sup>22,23</sup>. In active MS lesions, HLA-DR<sup>+</sup> phagocytes markedly  
155 expressed CL-P1 suggesting that myelin internalization also enhances CL-P1 expression by  
156 phagocytes in MS lesions.

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

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

170 Several receptors, such as the complement-receptor 3, SRA I/II, and Fc $\gamma$  receptors, facilitate  
171 the clearance of myelin by macrophages and microglia<sup>2,3</sup>. Our data indicate that CL-P1 also  
172 contributes to the internalization of myelin. The phagocytic capacity of SRA largely depends  
173 on its collagen-like domain<sup>26</sup>. Considering that CL-P1 and SRA share the same collagen-like  
174 domain<sup>5</sup>, this domain may underlie the role that CL-P1 plays in the internalization of myelin.  
175 Future studies are warranted to determine if CL-P1 contributes to myelin uptake *in vivo* and  
176 how this impacts neuroinflammation and neurodegeneration. As uptake of myelin leads to  
177 both demyelination and CNS repair, depending on whether it concerns intact myelin or  
178 myelin debris, CL-P1-mediated myelin uptake can be both beneficial or detrimental<sup>1,27-29</sup>.

179 In our *in vitro* experiments, myelin debris is used to define the impact of CL-P1 on the uptake  
180 of myelin. Hence, it is tempting to speculate that CL-P1 might play a role in myelin debris  
181 clearance *in vivo*, thereby facilitating remyelination<sup>27-29</sup>.

182 Aside from a collagen-like domain, CL-P1 contains a C-type CRD that binds with high  
183 affinity to glycans bearing Lewis<sup>x</sup> and Lewis<sup>a</sup> trisaccharides<sup>10,11</sup>. Interestingly, based on this  
184 glycan-specificity, parallels can be drawn between CL-P1 and both DC-SIGN and selectins  
185<sup>30,31</sup>. This suggests that CL-P1 may also play a role in cell migration, cell differentiation,

186 antigen-capture, and T cell priming <sup>32,33</sup>. Interestingly, we found that CL-P1 is markedly  
187 expressed on foamy-appearing phagocytes in and near perivascular cuffs in MS lesions. As  
188 perivascular cuffs accommodate lymphocytes during active MS, CL-P1 on phagocytes may  
189 play a role in T cell priming. Additionally, as myelin-containing phagocytes are located in  
190 CNS-draining lymphoid organs <sup>34-36</sup>, future studies should determine whether CL-P1 may  
191 facilitate lymph node directed migration of these phagocytes.

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

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

## 202 **Methods**

### 203 **Cell isolation and culture**

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

226 accordance with institutional guidelines and approved by the Ethical Committee for Animal  
227 Experiments Hasselt University.

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

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

246

#### 247 **Myelin isolation, labelling, and phagocytosis**

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

252 University. Written informed consent was obtained from all donors. Myelin protein  
253 concentration was determined by using the BCA protein assay kit (Thermo Fisher Scientific,  
254 Erembodegem, Belgium), according to manufacturer's instructions. Endotoxin content was  
255 determined using the Chromogenic Limulus Amebocyte Lysate assay kit (Genscript  
256 Incorporation, Aachen, Germany). Isolated myelin contained a negligible amount of  
257 endotoxin ( $\leq 1.8 \times 10^{-3}$  pg/ $\mu$ g myelin). To obtain oxidized myelin, myelin was exposed to 10  
258  $\mu$ M CuSO<sub>4</sub> at 37°C for 20 hours. Myelin was fluorescently labelled, according to the method  
259 of Van der Laan *et al.*<sup>40</sup>. In short, 10 mg/ml myelin was incubated with 12.5  $\mu$ g/ml 1,1'-  
260 diotadecyl-3,3,3',3',-tetramethylindocarbocyanide perchlorate (DiI; Sigma-Aldrich) for 30 min  
261 at 37°C. To determine the capacity of cells to phagocytose myelin, cells were exposed to 100  
262  $\mu$ g/ml DiI-labeled myelin. The amount of myelin phagocytosed was determined using a  
263 FACSCalibur (BD Biosciences, Erembodegem, Belgium). HEK293.1 were used to define the  
264 impact of CL-P1 on myelin phagocytosis as BV-2 and RAW264.7 cells are not easily  
265 transfectable. Of note, HEK293.1 are often used as a model system to study phagocytic  
266 receptors<sup>41,42</sup>.

267

## 268 **Western blot**

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

278 incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated rabbit-anti  
279 goat and goat anti-rabbit antibodies (Dako, 1:2000) in 5% milk in TBS-T. For stripping and  
280 reprobing, a mild stripping buffer was used (0.2M glycine, 0.1% SDS, 1% Tween-20, pH  
281 2.2). An ECL Plus detection kit (Thermo Fisher Scientific) was used and the generated  
282 chemiluminescent signal was detected by a luminescent image analyzer (ImageQuant LAS  
283 4000 mini; GE Healthcare).

284

### 285 **shRNA and transfection**

286 The X-tremegene HP transfection kit (Roche Diagnostics, Mannheim, Germany) was used to  
287 transfect HEK293.1 cells according to the manufacturer's instructions. In short,  
288  $0.25 \times 10^6$  HEK293.1 cells were transfected with 1.5  $\mu$ g of shRNA in 50  $\mu$ l Opti-MEM® I  
289 Reduced Serum Media (Thermo Fisher Scientific). Cells were then resuspended in complete  
290 culture medium and incubated for 48 hours at 37°C. CL-P1 (shRNA-1);  
291 AACATCTCGCCAAACCTATGA, CL-P1 (shRNA-2);  
292 CAGGCTATCCAGCGAATCAAGAA, CL-P1 (shRNA-3);  
293 AAGAAATGAAGCTAGTAGACT, CL-P1 (shRNA-4); AACGATTTCCAATGTGA  
294 AGAC, scrambled; CCTAAGGTTAAGTCGCCCTCG.

295

### 296 **Flow cytometry**

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

302

### 303 **Immunohistochemistry**

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

322

### 323 **Quantitative PCR**

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



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

336

### 337 **Statistical analysis**

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

344 **References**

- 345 1 Bogie, J. F., Stinissen, P. & Hendriks, J. J. Macrophage subsets and microglia in  
346 multiple sclerosis. *Acta neuropathologica* **128**, 191-213 (2014).
- 347 2 Smith, M. E. Phagocytic properties of microglia in vitro: implications for a role in  
348 multiple sclerosis and EAE. *Microscopy research and technique* **54**, 81-94 (2001).
- 349 3 Reichert, F. & Rotshenker, S. Complement-receptor-3 and scavenger-receptor-AI/II  
350 mediated myelin phagocytosis in microglia and macrophages. *Neurobiology of disease*  
351 **12**, 65-72 (2003).
- 352 4 Bogie, J. F. *et al.* Myelin-derived lipids modulate macrophage activity by liver X  
353 receptor activation. *PloS one* **7**, e44998 (2012).
- 354 5 Kodama, T. *et al.* Type I macrophage scavenger receptor contains alpha-helical and  
355 collagen-like coiled coils. *Nature* **343**, 531-535 (1990).
- 356 6 Nakamura, K., Funakoshi, H., Miyamoto, K., Tokunaga, F. & Nakamura, T.  
357 Molecular cloning and functional characterization of a human scavenger receptor with  
358 C-type lectin (SRCL), a novel member of a scavenger receptor family. *Biochemical  
359 and biophysical research communications* **280**, 1028-1035 (2001).
- 360 7 Nakamura, K., Funakoshi, H., Tokunaga, F. & Nakamura, T. Molecular cloning of a  
361 mouse scavenger receptor with C-type lectin (SRCL)(1), a novel member of the  
362 scavenger receptor family. *Biochimica et biophysica acta* **1522**, 53-58 (2001).
- 363 8 Drickamer, K. & Taylor, M. E. Recent insights into structures and functions of C-type  
364 lectins in the immune system. *Current opinion in structural biology* **34**, 26-34 (2015).
- 365 9 Ohtani, K. *et al.* The membrane-type collectin CL-P1 is a scavenger receptor on  
366 vascular endothelial cells. *The Journal of biological chemistry* **276**, 44222-44228  
367 (2001).

- 368 10 Feinberg, H., Taylor, M. E. & Weis, W. I. Scavenger receptor C-type lectin binds to  
369 the leukocyte cell surface glycan Lewis(x) by a novel mechanism. *The Journal of*  
370 *biological chemistry* **282**, 17250-17258 (2007).
- 371 11 Coombs, P. J., Graham, S. A., Drickamer, K. & Taylor, M. E. Selective binding of the  
372 scavenger receptor C-type lectin to Lewisx trisaccharide and related glycan ligands.  
373 *The Journal of biological chemistry* **280**, 22993-22999 (2005).
- 374 12 Yoshida, T. *et al.* SRCL/CL-P1 recognizes GalNAc and a carcinoma-associated  
375 antigen, Tn antigen. *Journal of biochemistry* **133**, 271-277 (2003).
- 376 13 Elola, M. T. *et al.* Lewis x antigen mediates adhesion of human breast carcinoma cells  
377 to activated endothelium. Possible involvement of the endothelial scavenger receptor  
378 C-type lectin. *Breast cancer research and treatment* **101**, 161-174 (2007).
- 379 14 Nakamura, K. *et al.* Possible role of scavenger receptor SRCL in the clearance of  
380 amyloid-beta in Alzheimer's disease. *Journal of neuroscience research* **84**, 874-890  
381 (2006).
- 382 15 Haider, L. *et al.* Oxidative damage in multiple sclerosis lesions. *Brain : a journal of*  
383 *neurology* **134**, 1914-1924 (2011).
- 384 16 Wallberg, M., Bergquist, J., Achour, A., Breij, E. & Harris, R. A. Malondialdehyde  
385 modification of myelin oligodendrocyte glycoprotein leads to increased  
386 immunogenicity and encephalitogenicity. *European journal of immunology* **37**, 1986-  
387 1995 (2007).
- 388 17 Wheeler, D., Bandaru, V. V., Calabresi, P. A., Nath, A. & Haughey, N. J. A defect of  
389 sphingolipid metabolism modifies the properties of normal appearing white matter in  
390 multiple sclerosis. *Brain : a journal of neurology* **131**, 3092-3102 (2008).
- 391 18 Bogie, J. F. *et al.* Myelin alters the inflammatory phenotype of macrophages by  
392 activating PPARs. *Acta neuropathologica communications* **1**, doi:0.1186/2051-5960-  
393 1-43 (2013).

- 394 19 Carson, M. J. *et al.* A rose by any other name? The potential consequences of  
395 microglial heterogeneity during CNS health and disease. *Neurotherapeutics : the*  
396 *journal of the American Society for Experimental NeuroTherapeutics* **4**, 571-579  
397 (2007).
- 398 20 Kuhlmann, T. *et al.* Differential regulation of myelin phagocytosis by  
399 macrophages/microglia, involvement of target myelin, Fc receptors and activation by  
400 intravenous immunoglobulins. *Journal of neuroscience research* **67**, 185-190 (2002).
- 401 21 Yamasaki, R. *et al.* Differential roles of microglia and monocytes in the inflamed  
402 central nervous system. *The Journal of experimental medicine* **211**, 1533-1549 (2014).
- 403 22 Ajami, B., Bennett, J. L., Krieger, C., McNagny, K. M. & Rossi, F. M. Infiltrating  
404 monocytes trigger EAE progression, but do not contribute to the resident microglia  
405 pool. *Nature neuroscience* **14**, 1142-1149 (2011).
- 406 23 Kierdorf, K. *et al.* Microglia emerge from erythromyeloid precursors via Pu.1- and  
407 Irf8-dependent pathways. *Nature neuroscience* **16**, 273-280 (2013).
- 408 24 Joseph, S. B. *et al.* LXR-dependent gene expression is important for macrophage  
409 survival and the innate immune response. *Cell* **119**, 299-309 (2004).
- 410 25 Tontonoz, P., Nagy, L., Alvarez, J. G., Thomazy, V. A. & Evans, R. M. PPARgamma  
411 promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* **93**,  
412 241-252 (1998).
- 413 26 Mori, K. *et al.* Scavenger receptor CL-P1 mainly utilizes a collagen-like domain to  
414 uptake microbes and modified LDL. *Biochimica et biophysica acta* **1840**, 3345-3356  
415 (2014).
- 416 27 Kotter, M. R., Li, W. W., Zhao, C. & Franklin, R. J. Myelin impairs CNS  
417 remyelination by inhibiting oligodendrocyte precursor cell differentiation. *The Journal*  
418 *of neuroscience : the official journal of the Society for Neuroscience* **26**, 328-332  
419 (2006).

- 420 28 Prinjha, R. *et al.* Inhibitor of neurite outgrowth in humans. *Nature* **403**, 383-384  
421 (2000).
- 422 29 GrandPre, T., Nakamura, F., Vartanian, T. & Strittmatter, S. M. Identification of the  
423 Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* **403**, 439-444  
424 (2000).
- 425 30 Appelmelk, B. J. *et al.* Cutting edge: carbohydrate profiling identifies new pathogens  
426 that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic  
427 cells. *Journal of immunology* **170**, 1635-1639 (2003).
- 428 31 Guo, Y. *et al.* Structural basis for distinct ligand-binding and targeting properties of  
429 the receptors DC-SIGN and DC-SIGNR. *Nature structural & molecular biology* **11**,  
430 591-598 (2004).
- 431 32 Svajger, U., Anderluh, M., Jeras, M. & Obermajer, N. C-type lectin DC-SIGN: an  
432 adhesion, signalling and antigen-uptake molecule that guides dendritic cells in  
433 immunity. *Cellular signalling* **22**, 1397-1405 (2010).
- 434 33 Ehrhardt, C., Kneuer, C. & Bakowsky, U. Selectins-an emerging target for drug  
435 delivery. *Advanced drug delivery reviews* **56**, 527-549 (2004).
- 436 34 de Vos, A. F. *et al.* Transfer of central nervous system autoantigens and presentation  
437 in secondary lymphoid organs. *Journal of immunology* **169**, 5415-5423 (2002).
- 438 35 Fabrick, B. O. *et al.* In vivo detection of myelin proteins in cervical lymph nodes of  
439 MS patients using ultrasound-guided fine-needle aspiration cytology. *Journal of*  
440 *neuroimmunology* **161**, 190-194 (2005).
- 441 36 van Zwam, M. *et al.* Brain antigens in functionally distinct antigen-presenting cell  
442 populations in cervical lymph nodes in MS and EAE. *Journal of molecular medicine*  
443 **87**, 273-286 (2009).
- 444 37 Nair, A., Frederick, T. J. & Miller, S. D. Astrocytes in multiple sclerosis: a product of  
445 their environment. *Cellular and molecular life sciences : CMLS* **65**, 2702-2720 (2008).

446 38 Wouters, K. *et al.* Bone marrow p16INK4a-deficiency does not modulate obesity,  
447 glucose homeostasis or atherosclerosis development. *PLoS one* **7**, e32440 (2012).

448 39 Norton, W. T. & Poduslo, S. E. Myelination in rat brain: changes in myelin  
449 composition during brain maturation. *Journal of neurochemistry* **21**, 759-773 (1973).

450 40 van der Laan, L. J. *et al.* Macrophage phagocytosis of myelin in vitro determined by  
451 flow cytometry: phagocytosis is mediated by CR3 and induces production of tumor  
452 necrosis factor-alpha and nitric oxide. *Journal of neuroimmunology* **70**, 145-152  
453 (1996).

454 41 Albert, M. L., Kim, J. I. & Birge, R. B. alpha5beta1 integrin recruits the CrkII-  
455 Dock180-rac1 complex for phagocytosis of apoptotic cells. *Nature cell biology* **2**, 899-  
456 905 (2000).

457 42 Underhill, D. M., Rossnagle, E., Lowell, C. A. & Simmons, R. M. Dectin-1 activates  
458 Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen  
459 production. *Blood* **106**, 2543-2550 (2005).

460 43 van Horssen, J., Bo, L., Vos, C. M., Virtanen, I. & de Vries, H. E. Basement  
461 membrane proteins in multiple sclerosis-associated inflammatory cuffs: potential role  
462 in influx and transport of leukocytes. *Journal of neuropathology and experimental*  
463 *neurology* **64**, 722-729 (2005).

464 44 Bogie, J. F. *et al.* Myelin alters the inflammatory phenotype of macrophages by  
465 activating PPARs. *Acta neuropathologica communications* **1**, 43 (2013).

466 45 Nelissen, K., Smeets, K., Mulder, M., Hendriks, J. J. & Ameloot, M. Selection of  
467 reference genes for gene expression studies in rat oligodendrocytes using quantitative  
468 real time PCR. *J.Neurosci.Methods* **187**, 78-83 (2010).

469 46 Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data  
470 by geometric averaging of multiple internal control genes. *Genome Biol.* **3**,  
471 RESEARCH0034 (2002).

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476

## 477 **Author contributions statement**

478 JB, JM, EW, WJ, EG, JV, and TV performed the experiments and analyzed the data. JB wrote

479 the manuscript. KW and JvH provided experimental materials. JB, JM, EW, WJ, EG, JV,

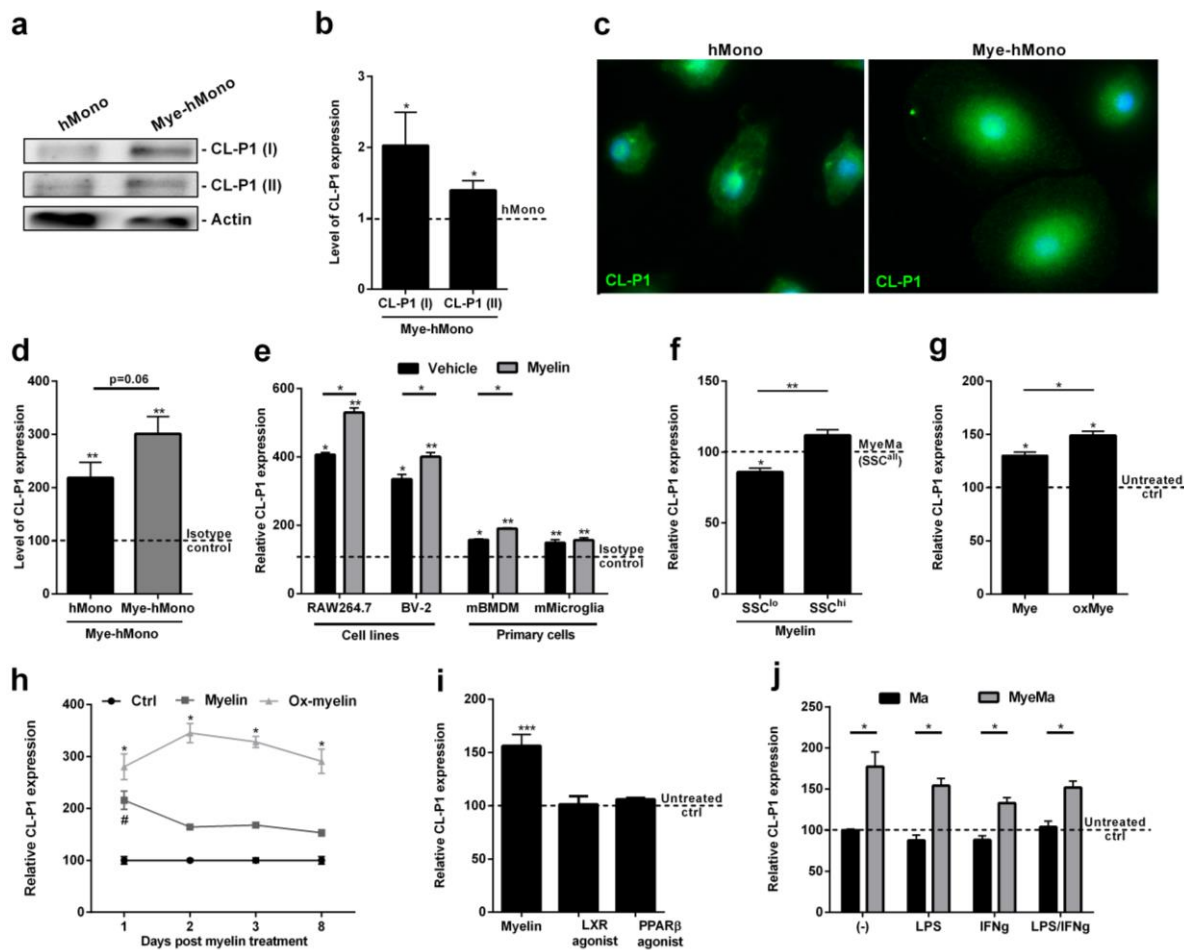
480 KW, NH, JvH, TV, and JH revised the manuscript. JB, KW, NH, JvH, TV, and JH

481 participated in the design and coordination of the project.

482

## 483 **Additional information**

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



486

487 **Figure 1: Myelin uptake increases the surface expression of CL-P1 on myeloid cells.** (a,b)

488 Human monocytes (hMono, n=5), were cultured with or without 100  $\mu$ g/ml myelin for 24h.

489 Western blot analysis was used to define CL-P1 expression. Two antibodies were used to

490 define CL-P1 expression. Western blots are displayed in cropped format. (c)

491 Immunohistochemistry (CL-P1, Novus Biologicals) was used to define the expression of CL-

492 P1 by human monocytes cultured with or without 100  $\mu$ g/ml myelin for 24h. (d) Human

493 monocytes (n=7) were cultured with or without 100  $\mu$ g/ml myelin for 24h. CL-P1 expression

494 was determined with flow cytometry (CL-P1, R&D). Dotted line represents untreated cells

495 stained with an isotype antibody. (e) RAW264.7 (n=4), BV-2 (n=4), mouse BMDMs (n=4),

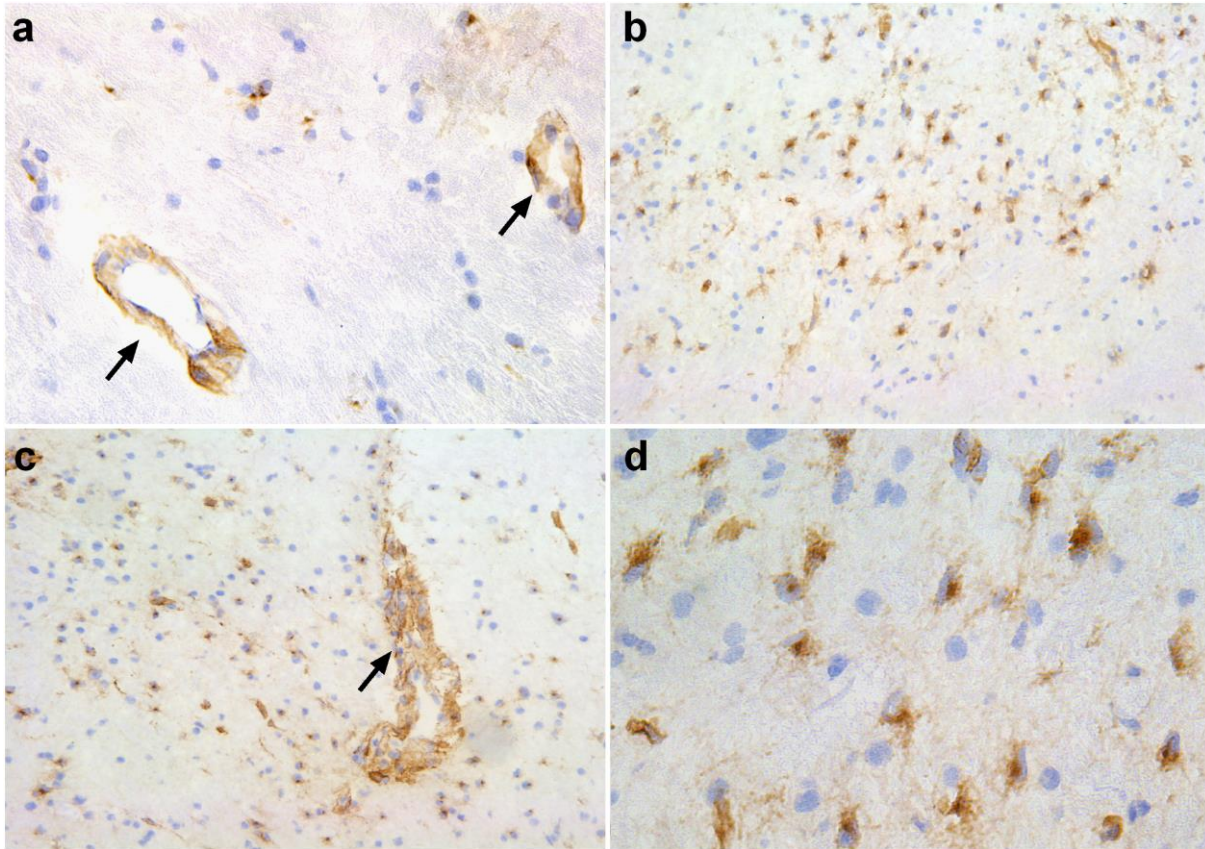
496 and mouse microglia (n=7), were cultured with or without 100  $\mu$ g/ml myelin for 24h. CL-P1



497 expression was determined with flow cytometry (CL-P1, R&D). Dotted line represents  
498 untreated cells stained with an isotype control antibody. (f) Mouse BMDMs cultured with 100  
499  $\mu\text{g/ml}$  myelin for 24h. CL-P1 expression was determined in high granular ( $\text{SSC}^{\text{hi}}$ ), low  
500 granular ( $\text{SSC}^{\text{lo}}$ ), and all cells ( $\text{SSC}^{\text{all}}$ ) using flow cytometry. Dotted line represents myelin-  
501 treated cells stained with the CL-P1 antibody (n=4). (g) RAW264.7 cells were exposed to 100  
502  $\mu\text{g/ml}$  unmodified and  $\text{Cu}^{2+}$ -oxidized myelin for 24h, after which CL-P1 expression was  
503 determined. Dotted line represents untreated cells stained with the CL-P1 antibody (n=4). (h)  
504 RAW264.7 cells were cultured with 100  $\mu\text{g/ml}$  unmodified or  $\text{Cu}^{2+}$ -oxidized myelin for 1,2,3,  
505 and 8 days (n=3). CL-P1 expression was determined by using flow cytometry. (i) RAW264.7  
506 cells were cultured with a T0901317 (LXR agonist), GW501516 (PPAR $\beta/\delta$  agonist), or 100  
507  $\mu\text{g/ml}$  myelin for 24h. CL-P1 expression was determined with flow cytometry. Dotted line  
508 represents untreated cells stained with the CL-P1 antibody (n=6). (j) Untreated or myelin  
509 treated RAW264.7 cells were exposed to 500 U/ml  $\text{IFN}\gamma$ , 100 ng/ml LPS, a combination  $\text{IFN}\gamma$   
510 and LPS, or left untreated (n=4). CL-P1 expression was determined using flow cytometry.  
511 Dotted line represents untreated cells stained with the CL-P1 antibody. Data are presented as  
512 mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

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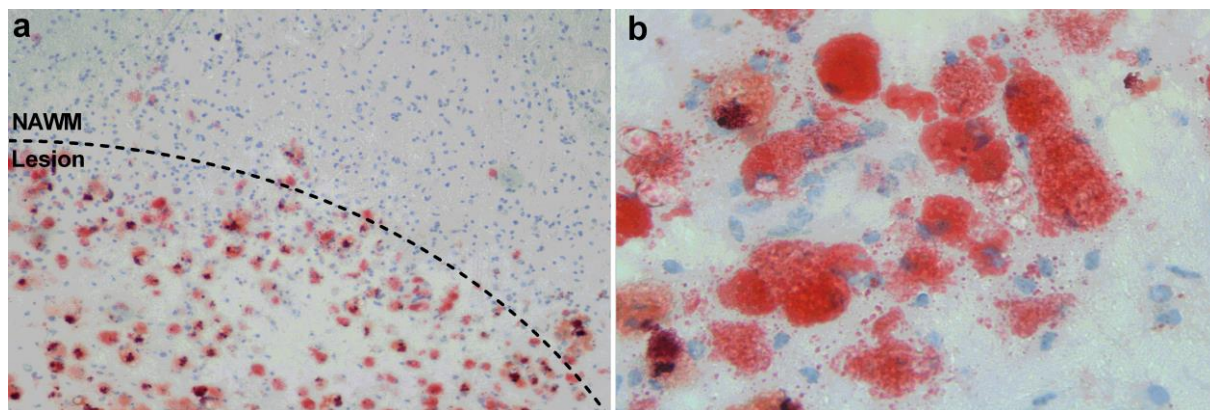


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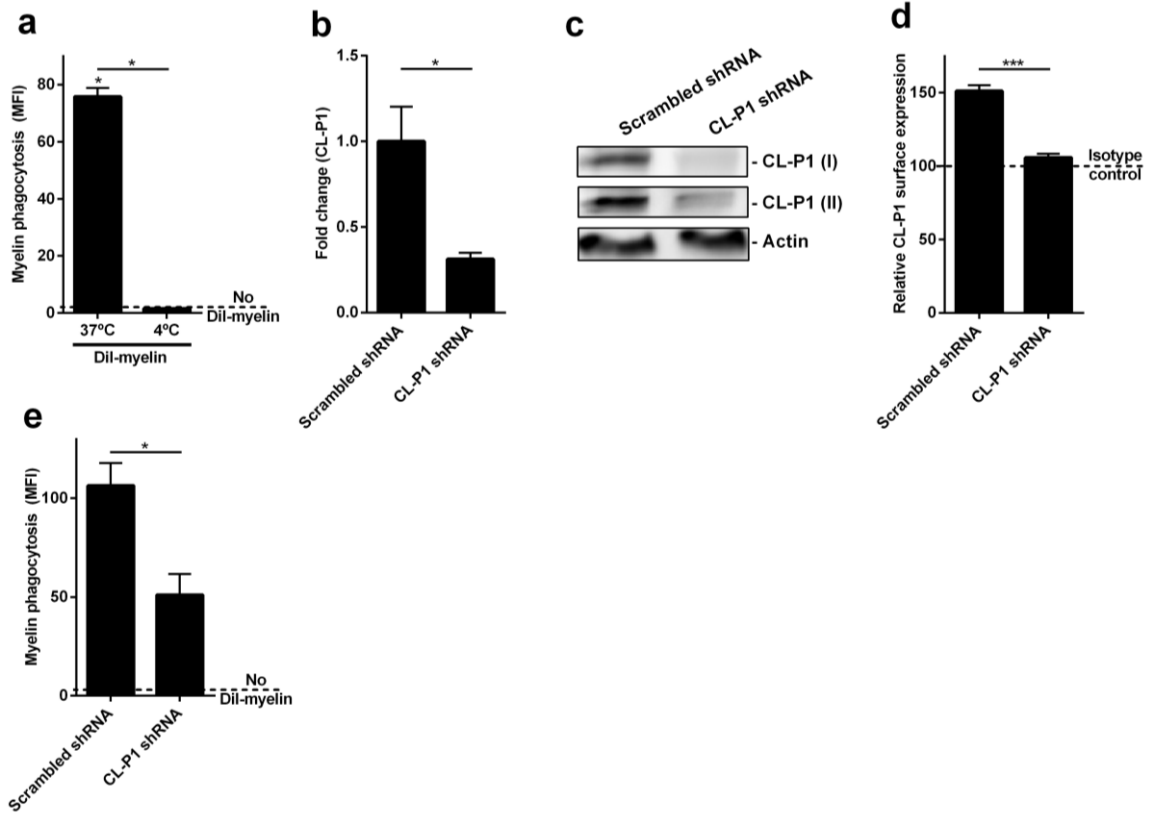
516 **Figure 2: CL-P1 is highly expressed in MS lesions.** (a) Image of normal-appearing matter  
517 stained for CL-P1 (40x magnification). Arrows depict blood vessels. (b-d) Active MS lesion  
518 stained for CL-P1 (b-c, 10x magnification; d, 40x magnification). Arrow depicts a  
519 perivascular cuff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

520

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



526  
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).



530

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