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This is a Peer Reviewed Accepted version of the following article, accepted for publication in *Science Immunology*.

2021-12-14

Microglial autophagy-associated phagocytosis is essential for recovery from neuroinflammation

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Sci Immunol. 2020 Oct 16;5(52):eabb5077.

American Association for the Advancement of Science

<http://doi.org/10.1126/sciimmunol.abb5077>

<http://hdl.handle.net/10616/47846>

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2 **Microglial autophagy-associated phagocytosis is essential for**
3 **recovery from neuroinflammation**

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20
21 **One sentence summary:** Degradation of tissue debris and recovery from neuroinflammatory
22 disease are impaired in *Atg7*-deficient microglia, a process mirrored by aging.

23 **Abstract**

24 Multiple Sclerosis (MS) is a leading cause of incurable progressive disability in young adults
25 caused by inflammation and neurodegeneration in the central nervous system (CNS). The capacity
26 of microglia to clear tissue debris is essential for maintaining and restoring CNS homeostasis. This
27 capacity diminishes with age, and age strongly associates with MS disease progression, although
28 the underlying mechanisms are still largely elusive. Herein, we demonstrate that the recovery from
29 CNS inflammation in a murine model of MS is dependent on the ability of microglia to clear tissue
30 debris. Microglia-specific deletion of the autophagy regulator *Atg7*, but not the canonical
31 macroautophagy protein *Ulk1*, led to increased intracellular accumulation of phagocytosed myelin
32 and progressive MS-like disease. This impairment correlated with a microglial phenotype
33 previously associated with neurodegenerative pathologies. Moreover, *Atg7*-deficient microglia
34 showed striking transcriptional and functional similarities to microglia from aged wild-type mice
35 that were also unable to clear myelin and recover from disease. In contrast, induction of autophagy
36 in aged mice using the disaccharide trehalose ubiquitously found in plant-based diets, led to
37 functional myelin clearance and disease remission. Our results demonstrate that a non-canonical
38 form of autophagy in microglia is responsible for myelin degradation and clearance leading to
39 recovery from MS-like disease, and that boosting this process has a therapeutic potential for age-
40 related neuroinflammatory conditions.

41 **Introduction**

42 Multiple Sclerosis (MS) is a chronic disease characterized by inflammation in the central nervous
43 system (CNS) that triggers demyelination, glial cell dysfunction and irreversible neuro-axonal
44 damage (1). While recent understanding of this immune dysfunction has led to the development of
45 effective disease-modulatory treatments for inflammatory-active relapsing-remitting phase of MS,
46 there is only one recently approved treatment for secondary progressive phase (2). Disease
47 progression is not only the most clinically challenging aspect of MS, but it is the least
48 mechanistically explored. Microglia activation, mitochondrial damage and ionic imbalance, among
49 other mechanisms, have been associated with progressive neurodegeneration during MS (3).

50 Progressive MS exhibits a degenerative disease phenotype with glial cell dysfunction rather
51 than infiltration of peripheral immune cells (4). In MS, microglia express an increased pro-
52 inflammatory profile with both age and disease-burden (4). Age is the strongest risk factor for
53 developing progressive MS (5). Many age-associated neurodegenerative pathologies such as
54 Alzheimer's disease (AD) (4, 6-8), as well as autoinflammatory diseases including Crohn's disease
55 and Systemic Lupus Erythematosus (SLE) (9, 10), are characterized by impaired autophagy, a
56 lysosomal degradation pathway used for removal of cellular constituents. Indeed, microglia
57 surrounding MS lesions exhibit enhanced autophagy (11) and have the ability to phagocytose
58 oligodendrocytes, as recently shown by single-nucleus RNA-sequencing (12). While among CNS
59 myeloid cells microglia have been ascribed the highest phagocytic activity (13), the underlying
60 mechanisms and their association with MS progression or tissue repair remain to be characterized.

61 Canonical autophagosome formation is highly dependent on the Unc-51-Like Kinase 1
62 (ULK1)-complex formation, as well as on Autophagy Related Protein 7 (ATG7) lipidation of LC3,
63 the key component of autophagosomes. In addition, LC3 can also be conjugated to membranes of

64 phagosomes and endosomes, thereby facilitating the degradation of their cargo during processes
65 termed LC3-associated phagocytosis and endocytosis, respectively (14-16), broadly referred to as
66 non-canonical autophagy hereafter. Non-canonical autophagy is dependent on the protein
67 RUBICON (17), which also inhibits the canonical autophagy pathway (18). Mutations in the
68 *Rubicon* gene are associated with a familial form of ataxia with impaired lysosomal degradation
69 (19, 20). Furthermore, inhibition of non-canonical autophagy in macrophages elicited by deletion
70 of either *Atg7* or *Rubicon* causes an SLE-like disease in mice due to defective degradation of
71 phagocytosed apoptotic cells (21). Similarly, specific impairment of non-canonical autophagy in
72 microglia has been associated with reduced clearance of β -amyloid and progressive
73 neurodegeneration in a murine model of AD (16), also evident in *Atg7* deficient mice (22). While
74 targeting microglial autophagy in these diseases has been proposed to have great therapeutic
75 potential (8), whether canonical or non-canonical autophagy impacts disease progression in MS is
76 currently unknown.

77 We previously established a link between *Atg7* and disease severity in a common animal
78 model of MS, experimental autoimmune encephalomyelitis (EAE) (23). Herein we pinpoint this
79 effect to microglia, and we reveal how processing of myelin debris by microglia is dependent on
80 ATG7 in a non-canonical form of autophagy. We further establish this process as a determinant of
81 microglial phenotype in disease and aging and demonstrate how therapeutically inducing this
82 pathway can restore CNS homeostasis.

83 **Results**

84 ***Microglial Atg7 deficiency prevents recovery from EAE***

85 To investigate the association between *Atg7* and EAE, we deleted *Atg7* from two compartments
86 highly relevant for disease pathogenesis, T cells and myeloid cells, using CRE recombinase
87 expressed under *Lck* and *Lyz2* promoters, respectively (fig. S1). Deletion of *Atg7* in T cells did not
88 affect clinical disease, although we did observe a previously reported reduction in CD8⁺ T cell
89 numbers (24) (fig. S1, A and B). In contrast, deletion of *Atg7* in *Lyz2*-expressing myeloid cells led
90 to a persistent disease state that lacked the recovery evident in wild-type control mice (fig. S1C).
91 *Lyz2^{Cre}* targets several myeloid cell types including microglia (fig. S1D) (22, 25, 26), and the effect
92 of *Atg7* deletion was restricted to the EAE recovery phase, suggesting CNS-intrinsic regulation.
93 We detected the highest expression and most prominent *Atg7* u-regulation during EAE in CD11b⁺
94 CD45^{Int} cells (microglia) compared to other *Lyz2*-expressing CNS myeloid populations (Fig. 1A).
95 Expression of the floxed *Atg7* exon 14 was dramatically reduced in microglia of *Atg7^{fl/fl} Lyz2^{Cre}*
96 mice after EAE induction (fig. S1E). In addition, microglia from these mice displayed reduced
97 lipidated membrane-bound LC3B (II) (fig. S1F), indicating less autophagosome formation and
98 lysosomal loading of myelin after *in vitro* exposure (fig. S1G).

99 To test the hypothesis that microglial ATG7 plays a role in EAE progression, we utilized
100 *Atg7* deletion under a tamoxifen-inducible CRE recombinase expressed under the *Cx3cr1*
101 promoter. Tamoxifen-induced deletion is sustained in the self-renewing microglia population while
102 other *Cx3cr1*-expressing cells essential for EAE such as monocytes and dendritic cells are derived
103 from bone marrow precursors and repopulated from the bone marrow after 2-4 weeks (fig. S2A)
104 (27). In all experiments, EAE was induced 4-8 weeks after tamoxifen treatment. Deletion of *Atg7*
105 exon 14 in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia was confirmed at both 2 and 42 weeks after tamoxifen

106 administration (fig. S2B), as was reduced ATG7 protein expression (fig. S2C). *Atg7^{fl/fl}*
107 *Cx3cr1^{CreERT2}* mice exhibited a dramatic loss of recovery from EAE, confirming our hypothesis of
108 a microglia-dependent phenotype (Fig. 1B).

109

110 ***Atg7 deficiency impacts microglial tissue debris clearance through non-canonical autophagy***

111 As ATG7 is essential for both canonical and non-canonical autophagy (14, 17), we compared
112 phenotypes of *Atg7^{fl/fl} Cx3cr1^{CreERT2}* mice with mice in which microglia were deficient in *Ulk1*, a
113 protein only required for canonical autophagy (21). The *Ulk1^{fl/fl} Cx3cr1^{CreERT2}* mice did not exhibit
114 lack of recovery from EAE, suggesting that impaired non-canonical autophagy drives the disease
115 phenotype in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* mice (Fig. 1C). This notion was further supported by the finding
116 that microglia from both strains displayed reduced but comparable levels of starvation-induced
117 canonical autophagy, as evident by decreased membrane-bound LC3B (II) (Fig. 1D) and increased
118 IL-1 β secretion and mitochondrial membrane potential (fig. S2, D and E). This reflects an
119 impairment in typical canonical autophagy functions in securing mitophagy and inflammasome
120 stability (22, 28-31). In addition, we determined the key regulator of non-canonical autophagy,
121 *Rubicon* (17), to accompany elevated *Atg7* expression in wild-type microglia after disease
122 induction (Fig. 1E and table S1).

123 Deficiency in non-canonical autophagy has been associated with impaired degradation of
124 bacterial proteins during infection as well as with impaired phagocytosis of apoptotic cells and β -
125 amyloid, leading to SLE-like disease and AD, respectively (16, 20-22, 32). Indeed, *Atg7^{fl/fl}*
126 *Cx3cr1^{CreERT2}* microglia displayed a dramatically increased load of intracellular myelin detected *ex*
127 *vivo* during EAE (Fig. 1F). The *ex vivo* EAE microglia also exhibited reduced autophagosome
128 formation detected as membrane-bound intracellular LC3B (II) after selective digitonin

129 permeabilization (Fig. 1G). Immunofluorescence of microglia sorted 5 days after EAE induction,
130 when microglia were activated but had not yet accumulated endogenous myelin load, confirmed
131 the increased load of fluorescently-labeled myelin and decreased LC3 co-localization to myelin-
132 containing vesicles in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* compared to *Ulk1^{fl/fl} Cx3cr1^{CreERT2}* and wild-type
133 control mice (Fig. 1H).

134 To further validate the impaired degradation of the phagocytosed debris in *Atg7* deficient
135 microglia, we pulsed microglia with myelin stained with both a lipophilic dye (CellVue™) and a
136 pH-sensitive dye (pHrodo™). Microglia were sorted *ex vivo* after immunization, circumventing
137 the need for further stimulations, and we limited all incubations to ≤ 48 h in order to minimize *in*
138 *vitro* culture-induced changes of the microglial phenotype (33). We observed a decreased
139 pHrodo™ signal in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia accompanied by increased CellVue™ (Fig.
140 1I), indicating impaired degradation in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia. This finding was further
141 demonstrated using time-lapse imaging of microglia pulsed with labeled myelin, revealing an
142 accumulation of phagocytosed myelin in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia (Movie S1).

143 The impaired loading to degradation vesicles was also observed when *Atg7^{fl/fl} Cx3cr1^{CreERT}*
144 microglia were provided irradiated apoptotic CD171⁺ (neurons), GLAST⁺ (astrocytes) or O4⁺
145 (oligodendrocytes) cells (fig. S2F). Further analysis demonstrated a dramatically reduced clearance
146 capacity of myelin debris from medium by microglia from *Atg7^{fl/fl} Cx3cr1^{CreERT2}* compared to
147 *Ulk1^{fl/fl} Cx3cr1^{CreERT2}* and wild-type control mice following pulsed exposure to fluorescently-
148 labeled myelin (Fig. 1J). Taken together, our data suggest that an impairment in microglial
149 autophagy-associated degradation of phagocytosed myelin compromises the clearance of myelin
150 debris leading to an inability to recover from MS-like disease.

151

152 ***Microglial Atg7 deficiency drives an altered transcriptional phenotype***

153 To obtain a comprehensive overview of the consequences of *Atg7* deletion during EAE, we
154 performed RNA sequencing of microglia sorted from naïve mice at 21- and 35-days post-induction
155 of disease (p.i.). We detected 467 and 147 differentially expressed genes (DEG) (adj. p-value <
156 0.05, fold change > 1.5) between *Atg7^{fl/fl} Cx3cr1^{CreERT2}* and littermate controls on days 21 and 35
157 p.i., respectively (table S1). Only 13 DEGs were detected (adj. p-value < 0.05, fold change > 1.5)
158 between the genotypes in naïve microglia, suggesting that differences predominantly arise during
159 EAE (table S1). We clustered DEGs (p < 0.01, fold change > 1.5) and then grouped the clusters
160 based on their expression patterns (Fig. 2A), performing functional annotation of the groups using
161 IPA, ORA and REViGO (34, 35) (table S2). The first group (orange) represented changes
162 considerably more pronounced in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia that occurred early in disease
163 (day 21 p.i.) and returned to levels in the naïve state by day 35 p.i. (Fig. 2A). These changes
164 associated with pathways typical for activation of immune cells during EAE, such as IFN- γ -,
165 STAT3- and GM-CSF-signaling, cell activation, expansion and migration (Fig. 2B). The second
166 group (blue) represented genes that remained downregulated during disease, with *Atg7^{fl/fl}*
167 *Cx3cr1^{CreERT2}* microglia showing modest changes (Fig. 2A). Functionally, this group was enriched
168 in pathways involved in myeloid cell function such as quantity, movement, and degranulation of
169 myeloid cells, as well as activation of GATA2, which is important for the development of myeloid
170 lineage cells (Fig. 2B). Genes in the third group (yellow) had a similar pattern to the first group in
171 wild-type but not in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia, which demonstrated the opposite pattern (Fig.
172 2A). These changes are predominantly associated with energy-related functions such as glycolysis
173 and mitochondrial function, oxidative stress and cell adhesion (Fig. 2B and fig. S3). The fourth
174 group (purple) represented genes that gradually increased their expression during disease

175 progression specifically in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia (Fig. 2A). These genes are associated
176 with cellular growth, including protein synthesis, EIF2- and mTOR-signaling, and were closely
177 related to microglial development and function in CNS pathology (36) (Fig. 2B and fig. S3). The
178 functional differences between the groups translated into differential disease enrichment, with early
179 changes associating with inflammatory and infectious diseases, whereas changes in the progressive
180 stage of EAE demonstrated strong enrichment in neurodegenerative diseases (Fig. 2C). These
181 patterns resemble the course of MS, with the initial phase being dominated by inflammatory
182 processes followed by neurodegeneration that, in later disease stages, becomes decoupled from the
183 initial inflammation (1).

184 *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia exhibited pronounced but transient changes during early
185 disease (Fig. 2, A and B, orange and yellow) that translated into altered microglia function and
186 EAE development. These transcriptional profiles associated with immune cell activation and
187 migration, reflected in increased microglia numbers and a more robust infiltration of bone marrow-
188 derived macrophages (BMDMs), neutrophils, and T and B cells into the CNS parenchyma during
189 early EAE (Fig. 3A). In accordance with IFN- γ and TNF predicted to be activated upstream
190 regulators (Fig. 2B and table S2), a larger proportion of infiltrating T cells in *Atg7^{fl/fl} Cx3cr1^{CreERT2}*
191 mice were IFN- γ ⁺ (fig. S4A). *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia themselves secreted dramatically
192 larger amounts of IFN- γ , TNF and IL-1 β (Fig. 3B), the latter suggesting activation of the
193 inflammasome as previously reported to occur in response to myelin accumulation (37). Moreover,
194 *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia were capable of stimulating T cell proliferation and expansion of
195 pathogenic IFN- γ -producing cells *in vitro* (fig. S4, B-D). *Atg7* deficiency promoted a microglial
196 phenotype that can augment inflammatory responses during EAE (fig. S4).

197 In MS patients, an increased inflammatory activity during the first 2 years after diagnosis
198 has been associated with the risk of clinical progression during early disease (38). Long-term
199 disability, nevertheless, better correlates with brain atrophy, reflecting neuro-axonal loss (39, 40).
200 Interestingly, *Atg7* deletion resulted in a microglial phenotype that associated with neuronal
201 function and neurodegenerative diseases (4), suggesting sustained transcriptional changes that are
202 critical for disease progression (Fig. 2, A and C, purple). Indeed, a late stage transcriptional profile
203 of *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia demonstrated a remarkable enrichment in microglial genes
204 associated with neurodegenerative diseases (4, 41) and MS-associated microglia identified in
205 recent single-cell RNA-sequencing studies (42, 43) (Fig. 3C, fig. S5 and table S3). We confirmed
206 differences in protein levels of CLEC7A and CD11c/ITGAX (fig. S6A). In contrast, the
207 homeostatic and tolerogenic state genes (4, 44) were depleted in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia
208 (Fig. 3C and fig. S5).

209

210 ***Microglia deficient in Atg7 have impaired signs of debris uptake and degradation***

211 The *Clec7a* gene encodes the C-type lectin Dectin-1, which is known to induce LC3-associated
212 phagocytosis and recognizes ligands released upon CNS damage (45-48). *Clec7a* expression
213 characterizes a microglia population associated with neurodegenerative conditions (4). *Atg7^{fl/fl}*
214 *Cx3cr1^{CreERT2}* showed dramatically increased number of CLEC7A⁺ IBA1⁺ microglia compared to
215 control mice (Fig. 3D and fig. S6B).

216 We observed that *Atg7* deletion did not affect the frequency of the CLEC7A^{int} population,
217 but it did lead to a near loss of CLEC7A^{low} and a robust increase in CLEC7A^{high} cells (Fig. 3, E
218 and F). CLEC7A^{high}, and in particular CLEC7A^{int}, microglial populations displayed dramatically
219 lower surface expression of the scavenging receptors MSR1 (SR-A, CD204) (Fig. 3G), CD36 and

220 other receptors (e.g. CD200R, IA/IE) that are implicated in microglial function during
221 inflammation (49-51) (fig. S6C). We further demonstrated that myelin phagocytosis relies, at least
222 in part, on uptake mediated by MSR1, because the uptake of pHrodo-labelled myelin by microglia
223 *in vitro* could be blocked using an MSR1 antibody (Fig. 3H), and myelin induced robust surface
224 expression of MSR1 (Fig. 3I). Interestingly, whereas RNA-sequencing implicated an increased
225 *Msr1* expression in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia (fig. S6D), flow cytometry analysis revealed
226 reduced surface receptor quantity (fig. S6E). A similar pattern was observed for CD36 (fig. S6, D
227 and F). However, a pool of intracellular MSR1 was detected upon permeabilization (Fig. 3J),
228 suggesting a blocking of retrograde trafficking of phagosomal receptors that has been described in
229 the context of disrupted non-canonical autophagy (16). To confirm this we blocked the fusion of
230 phagosomes and lysosomes in microglia *ex vivo* using Bafilomycin A1, which resulted in an
231 increase in intracellular MSR1 and CD36 levels (Fig. 3I and fig. S6G). These data indicate that
232 stalling myelin-loaded autophagosomes can lead to MSR1 retention that could further sustain
233 microglial failure to clear myelin debris, as observed after repeated myelin pulsing (Fig. 1J). This
234 pattern of intracellular accumulation in relation to myelin exposure and genotype was not detected
235 upon targeting other potential myelin scavenger receptors such as CR3, CD16, CD64 or MARCO
236 (fig. S6H).

237 Transcriptomic alterations of late stage microglia further supported the notion of impaired
238 phagosomal degradation in *Atg7*-deficient microglia. A marked upregulation of apolipoprotein E
239 (*ApoE*) was observed in late-stage *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia by RNA-sequencing (Fig. 3K),
240 together with other lipoproteins (*Apoc1*, *Apoc4*) and lipoprotein lipase (*Lpl*) (fig. S6I).
241 Upregulation of APOE occurs in response to the binding of myelin-derived cholesterol to the
242 endogenous liver X receptor (LXR), which is transcriptionally activated in *Atg7^{fl/fl} Cx3cr1^{CreERT2}*

243 microglia (Fig. 2B) and is crucial for the export of cholesterol from microglia to the extracellular
244 space (37). Moreover, we observed increased expression of *ApoE* when phagosome-lysosome
245 fusion was impaired using bafilomycin A treatment (Fig. 3K), again suggesting that *ApoE*
246 upregulation in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia constitutes a response to intracellular myelin
247 accumulation. We also observed that TREM2, which can bind APOE and other lipids (52) and is
248 an inducer of the APOE pathway (4) and plays a major role in recovery from EAE (53), was
249 upregulated during late EAE stages (fig. S6J), and that the pathway of TREM2 was highly activated
250 (fig. S5). Finally, levels of TGF- β 1, a marker of homeostatic microglia as opposed to LXR-APOE-
251 TREM2 disease-associated microglia (4, 54), was dramatically decreased in *Atg7^{fl/fl} Cx3cr1^{CreERT2}*
252 microglia (Fig. 3L). *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia thus appear to cope with increase myelin load
253 by engaging the LXR-APOE-TREM2 pathway and by upregulating the expression of the MSR1
254 and other scavenging receptors to compensate for reduced retrograde transport and failed myelin
255 degradation.

256 Accumulation of myelin debris is a strong inhibitor of oligodendrocyte differentiation and
257 remyelination, suggested to hinder recovery from inflammatory insults during MS and EAE and
258 leads to neuro-axonal loss (55, 56). Indeed, staining of *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia *ex vivo* with
259 an antibody against degraded myelin basic protein (dMBP) revealed an accumulation of this protein
260 in late stage disease compared to microglia from control animals (Fig. 3M). Immunostaining of
261 spinal cords further demonstrated the accumulation of dMBP in tissue, which overlapped with areas
262 of activated microglia/infiltrating BMDMs (MAC3 bright) (Fig. 3N and fig. S7A). This was
263 accompanied by unresolved inflammation evident as increased representation of immune cells
264 during late stage EAE (fig. S7, B and C). Moreover, pulsing late stage *Atg7^{fl/fl} Cx3cr1^{CreERT2}*
265 microglia with myelin *ex vivo* led to lower uptake as compared to microglia from control animals

266 (fig. S7D). We addressed whether the increased pool of tissue infiltrating BMDMs (fig. S7C) could
267 compensate for the reduced phagocytic capacity in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* mice, but BMDM cells
268 underperformed microglia in phagocytosis of myelin as assessed by both *ex vivo* myelin load in
269 cells isolated from late disease stage as well as by an *in vitro* myelin clearance assay (fig. S7, D
270 and E). Finally, flow cytometry assessment of oligodendrocytes isolated from the spinal cord at
271 this time point confirmed a lower frequency of CD45⁻GALC⁺MOG⁺ myelinating cells in *Atg7^{fl/fl}*
272 *Cx3cr1^{CreERT2}* animals as compared to controls (fig. S7F). Accordingly, we recorded reduced
273 myelination in the *Atg7^{fl/fl} Cx3cr1^{CreERT}* late stage EAE spinal cords (fig. S7G). *Atg7*-dependent
274 impairment of microglia needed to clear myelin upon an inflammatory demyelinating insult could
275 not be compensated for by infiltrating phagocytes and was associated with reduced CNS
276 myelination during late stage disease.

277

278 ***Aged microglia recapitulate the phenotype of young Atg7-deficient microglia***

279 Since age is the strongest risk factor for progressive MS (5, 57) and as many age-associated
280 neurodegenerative pathologies are characterized by impaired autophagy (4, 6-8), we compared the
281 impact of *Atg7* deficiency in the context of aging. We observed a remarkable similarity between
282 the transcriptomes of *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia and microglia from aged mice (> 80 weeks)
283 compared to control young microglia (Fig. 4A and table S4). Both late disease stage microglia from
284 *Atg7^{fl/fl} Cx3cr1^{CreERT2}* mice and microglia from aged (> 80 weeks) wild-type mice revealed
285 enrichment of genes associated with microglia during neurodegenerative diseases (4, 41), as well
286 as MS-associated microglia (42, 43) (fig. S5). Accordingly, aged mice developed aggravated EAE
287 with a clinical course similar to that of *Atg7^{fl/fl} Cx3cr1^{CreERT2}* mice (Fig. 4B) and exhibited signs of
288 accumulated myelin load (Fig. 4C). Similar to *Atg7^{fl/fl} Cx3cr1^{CreERT2}*, *in vitro* time-lapse imaging

289 demonstrated that microglia from aged mice had reduced lysosomal loading of myelin as assessed
290 by co-localization with low pH-sensing dye (pHrodoTM) and the pH-indifferent dye (PKH26TM),
291 the latter detecting accumulation of myelin-containing phagosomes (Movie S2).

292

293 *Trehalose boosts autophagy in aged microglia and promotes recovery from EAE*

294 While increased understanding of the immune dysfunction during early relapsing-remitting phases
295 of MS has led to the recent development of effective disease-modulatory treatments, progressive
296 stages still largely lack treatment options. In accordance with our data, boosting microglial
297 autophagy in neurodegenerative diseases has been proposed to have considerable therapeutic
298 potential (58-60). However, whether this could have beneficial effects in MS, and whether
299 canonical or non-canonical autophagy is involved in disease progression, is currently unknown. To
300 target phagocytosed myelin through autophagy we administered trehalose, a disaccharide known
301 to induce autophagy and ameliorate age-associated diseases (61-66). Daily trehalose administration
302 starting 1 week before EAE induction led to a reduction in the clinical severity of EAE, and more
303 than 40% of aged control mice recovered as well as aged *Ulk1^{fl/fl} Cx3cr1^{CreERT2}* mice that have
304 compromised canonical autophagy (Fig. 1D, Fig. 4B and fig. S8A). We did not observe any
305 trehalose effect on clinical EAE in younger mice, regardless of the genotype, nor in aged *Atg7^{fl/fl}*
306 *Cx3cr1^{CreERT2}* mice (Fig. 4B and fig. S8A), suggesting that trehalose acts by boosting non-canonical
307 autophagy. Indeed, we observed that trehalose increased nuclear density of TFEB, a key
308 transcription factor for autophagy and lysosome-associated genes (61), and an increased formation
309 of lysosomes defined by LAMP1, a major lysosome membrane component (Fig. 4D). We also
310 detected increased expression of multiple autophagy and lysosome genes upon trehalose treatment
311 of aged microglia (Fig. 4E).

312 Following trehalose treatment, we detected a reduction of intracellular microglial
313 myelin load in aged EAE mice *ex vivo* (Fig. 4C), and increased myelin clearance and degradation
314 through lysosomes *in vitro* (Fig. 4, F and G). Trehalose treatment also reduced the frequency of
315 disease-associated microglia defined by expression of CLEC7A and APOE (Fig. 4H) as well as the
316 infiltration of BMDMs (fig. S8B). Furthermore, microglia from trehalose-treated aged mice
317 displayed reduced secretion of the disease-associated cytokines IFN- γ , TNF and IL-1 β , and
318 increased TGF- β 1 secretion that reflected a normalization of the profile evident in microglia from
319 young mice (Fig. 4I). Taken together we validated an age effect on autophagy-associated vesicular
320 biogenesis, with a decline in lysosome loading of phagocytosed myelin debris, similar to that in
321 *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia. In aged mice this impairment was successfully mitigated with
322 trehalose treatment, with consequent effects on transcription, lysosome biogenesis, cytokine
323 secretion and clinical disease outcome.

324 **Discussion**

325 We herein demonstrate that a non-canonical form of autophagy in microglia is responsible for
326 myelin degradation and clearance and that impairment of this pathway, which occurs during aging,
327 contributes to the progression of MS-like disease. Importantly, we show that we can modulate this
328 process therapeutically, with implications in other age-related neuroinflammatory disease.

329 The cellular events underlying inflammatory bouts typical of MS and EAE are well
330 characterized, while the events promoting resolution of inflammation and limiting progression are
331 much less understood (67). However, accumulation of myelin and inflammatory debris in the target
332 tissue are known factors with inhibitory effects on remyelination (55, 56). We now demonstrate
333 that the impaired myelin clearance capacity of microglia leads to increased tissue deposits of
334 myelin debris accompanied by reduced myelination and oligodendrocyte differentiation. The
335 deletion of *Atg7* in microglia caused persistent neuroinflammation and, by comparing *Atg7^{fl/fl}*
336 *Cx3cr1^{CreERT2}* mice with *Ulk1^{fl/fl} Cx3cr1^{CreERT2}*, we demonstrated that the phenotype was largely
337 independent from canonical autophagy. In several *in vivo* and *in vitro* experimental settings, we
338 observed that the lack of *Atg7* drives microglial dysfunction in clearance and processing of myelin
339 debris and apoptotic CNS cells. The reduced clearance capacity of the microglia is most likely a
340 consequence of internalization of scavenger receptors due to impaired *Atg7*-dependent lysosomal
341 degradation.

342 Our model presented an opportunity to study impaired degradation of phagocytosed
343 components as a regulator of microglial phenotype, which is relevant for a broad range of myeloid
344 cell-associated pathologies. The elevated infiltration of peripheral immune cells 21 days after EAE
345 induction was associated with an altered *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglial cytokine profile, likely
346 in synergy with increased myelin tissue deposits and local CNS expansion of immune cell

347 populations. Interestingly, the infiltrating macrophage population did not compensate for the
348 reduced myelin clearance of *Atg7^{fl/fl} Cx3cr1^{CreERT2}* mice. In fact, the capacity for myelin clearance
349 of this population at a late disease stage was dramatically lower than that of microglia, which
350 corroborates previous findings (13) and supports the established idea of microglia being promoters
351 of homeostasis, in contrast to the monocyte-derived macrophages which exhibit a more
352 inflammatory phenotype (68).

353 Although the day 21 EAE microglia of *Atg7^{fl/fl} Cx3cr1^{CreERT2}* mice reflect an acute
354 inflammatory state, the day 35 microglia represent a more unique phenotype relevant for evaluating
355 the challenges of chronic inflammation and tissue degeneration. The microglial transcriptome from
356 persistent EAE at day 35 in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* mice was similar to other reported disease-
357 associated microglial transcriptomes, indicating shared microglial pathology (4, 41). Potentially
358 pathogenic microglia are dependent on the TREM2-APOE axis (4, 69). In our *Atg7^{fl/fl} Cx3cr1^{CreERT2}*
359 day 35 EAE microglia, we observed an enriched TREM2 pathway accompanied by elevated *ApoE*
360 expression *ex vivo* and *in vitro* as a consequence of increased intracellular myelin load. Intracellular
361 lipids are sensed by LXR which acts as a transcription factor inducing *ApoE* expression. We
362 propose an LXR-mediated pathogenic feed-forward mechanism through APOE in which
363 intracellular myelin is not functionally degraded. The enriched LXR pathway in *Atg7^{fl/fl}*
364 *Cx3cr1^{CreERT2}* microglia resembled the macrophage phenotype characteristic of atherosclerosis, a
365 disease state in which there is pathogenic accumulation of intracellular lipid compounds (64).

366 Phagocytosis of tissue debris has been reported to be essential for the maintenance of tissue
367 homeostasis (70), and we demonstrate that upon inflammation the re-establishment of an anti-
368 inflammatory response and subsequent tissue recovery are curbed when phagocytosis is decoupled
369 from downstream cargo degradation through non-canonical autophagy. Expression of molecules

370 associated with disease states (e.g. CLEC7A, CD11c) was increased in *Atg7^{fl/fl} Cx3cr1^{CreERT2}*
371 microglia while the expression of genes that characterize homeostatic microglia was reduced (e.g.
372 P2Ry12, CSF1R, CD200R, MSR1, TGF- β 1) (54). Among these, CLEC7A is of great interest as it
373 is a strong inducer of LC3-associated phagocytosis (47, 48). Elevated expression of CLEC7A
374 reported here for EAE and previously associated with pathology-associated microglia in other
375 models (4) is therefore directly linked to an important functional outcome.

376 Increased age leads to a decline in autophagy and is a risk factor for progressive MS and
377 neurodegeneration (57, 71). Dysfunctional aged myeloid cells have been reported in other settings
378 to be a consequence of inefficient autophagy (72). We thus explored the potential of induced
379 autophagy in ameliorating age-associated aggravated EAE disease. Treatment with the autophagy-
380 inducing disaccharide trehalose led to a robust recovery rate and decline in clinical symptoms in
381 aged mice, reminiscent of the recovery characteristic of untreated young wild-type mice (65, 73).
382 However, trehalose treatment did not affect recovery of young wild-type mice or *Atg7^{fl/fl}*
383 *Cx3cr1^{CreERT2}* mice, suggesting that non-canonical autophagy in microglia from young mice is
384 already at sufficient capacity, and that the effect of trehalose treatment in aged mice is dependent
385 on microglial ATG7, regardless of whether trehalose exerts its effect upstream or downstream of
386 ATG7. The trehalose treatment induced vesicle biogenesis through transition of transcription factor
387 TFEB to the nucleus, increased lysosome density and myelin clearance and degradation. Trehalose
388 treatment *in vivo* also reduced the density of CNS infiltrating bone marrow-derived macrophages
389 during EAE and a reduced pro-inflammatory cytokine profile evident in microglia from aged mice.

390 The tools for defining and studying microglia have developed vastly over the past years,
391 allowing for more accurate *ex vivo* and *in vitro* experiments, which however have an impact on the
392 microglial phenotype, especially regarding contextual and dynamic processes such as autophagy.

393 Another challenge for future work is the characterization of autophagy-associated phagocytosis in
394 humans, especially in disease context as in progressive human neuroinflammation. Additional
395 work on molecules of the microglial autophagy-associated phagocytosis including RUBICON
396 would also add support to our findings and could unveil interesting pharmacological targets.
397 Finally, the phenotypes observed on autophagy-deficient microglia could partially stem from
398 secondary effects such as increased phagosome load which also warrants further exploration.

399 Taken together our findings demonstrate that degradation of inflammatory myelin debris
400 by microglia is dependent on the non-canonical arm of autophagy, a function necessary for cell and
401 CNS tissue homeostasis. We thus provide a functional link between age, autophagy and myeloid
402 dysfunction. We associate our phenotype with the newly described microglial transcriptomes
403 primarily described in neurodegenerative diseases, suggesting a shared pathology and providing a
404 functional characterization. Finally, we propose this pathway to be as a promising treatment target
405 for age-associated CNS pathology.

406

407 **Materials and Methods**

408

409 **Study design**

410 Previous work by our lab established a link between *Atg7* and disease severity in EAE, an animal
411 model for MS (23). In this study, we set out to understand whether this effect was intrinsic to the
412 immune system by broadly targeting the deletion of *Atg7* in mice to either T cells or myeloid cells
413 by crossing *Atg7*-floxed mice to *Lck^{CRE}* or *Lyz2^{CRE}* expressing strains, respectively. Since the effect
414 of the deletion was observed during the recovery phase of EAE, which pointed to effects within
415 the CNS, we further restricted *Atg7* deletion to microglia and CNS-resident macrophage
416 populations by crossing *Atg7*-floxed mice to a *Cx3cr1^{CreERT2}* expressing strain (see Experimental
417 subjects section). Experiments were performed with littermate controls, using both males and
418 females. Animals were randomized and the majority of analysis was done in a blinded fashion.
419 Sample sizes varied depending on the goal of each experiments (i.e. dissection at one or multiple
420 timepoints, *in vitro* cultures, etc.) and expected effect sizes, and numbers of animals as well as
421 statistical analysis methods are thus given in each figure for every experimental setup. No animals
422 were excluded from analyses apart from two samples in RNA-sequencing that did not have correct
423 genotype. Catalogue numbers and the description of different primers, antibodies and kits used
424 throughout the study can be found as an additional technical sheet (table S5).

425

426 **Ethics Statement**

427 Animal experiments were approved and performed in accordance with the guidelines from the
428 Swedish National Board for Laboratory Animals and the European Community Council Directive
429 (86/609/EEC) under the ethical permits N284/07 (N332/06), N338/, N138/14, N1387/14 and 9328-

430 2019 which were approved by the North Stockholm Animal Ethics Committee (Stockholms Norra
431 djurförsöksetiska nämnd). Mice were tested according to a health-monitoring program at the
432 National Veterinary Institute (Statens Veterinärmedicinska Anstalt, SVA) in Uppsala, Sweden.

433

434 **Experimental Subjects**

435 Gene-deleted mice on the C57BL/6 background were generated by cross-breeding of *Atg7^{fl/fl}* or
436 *Ulk1^{fl/fl}* to *Lck^{CRE}*, *Lyz2^{CRE}* or *Cx3cr1^{CreERT2}* transgenic mice. All strains were purchased from The
437 Jackson Laboratory except *Atg7^{fl/fl}* that was a gift from Dr. Klas Blomgren. All experimental Cre
438 mice had a hemizygote genotype. *Cx3cr1^{CreERT2}* mice were treated with 4mg tamoxifen (TAM;
439 Sigma), dissolved in corn oil and administrated subcutaneously three times, at 48-hour intervals.
440 Experiments were initiated at earliest 4 weeks after the first tamoxifen administration to allow for
441 repopulation of peripheral bone marrow-derived *Cx3cr1^{CreERT2}* expressing cells such as monocytes,
442 while the gene deletion effect is preserved in the self-renewing CNS resident microglial population.
443 While border associated macrophages in meninges and perivascular spaces also express *Cx3cr1*,
444 self-renew and are targeted by TAM administration, they have been shown not to contribute to T
445 cell activation and CNS damage during EAE and are absent in the parenchyma where the processes
446 of myelin uptake and degradation described in this paper occur(74, 75). No dramatic influence
447 from the *Atg7^{fl/fl}* allele or Cre^{+/-} toxicity was observed. In EAE experiments, 10-18 weeks old
448 littermate mice were used. Aged mice were > 80 weeks old.

449

450 **Induction and Clinical Evaluation of EAE**

451 Recombinant mouse myelin oligodendrocyte glycoprotein (rmMOG) aa1-125 from the N-
452 terminus, was expressed in *Escherichia coli* and purified to homogeneity using chelate

453 chromatography, as previously described(76, 77). The purified protein, dissolved in 6M urea, was
454 dialyzed against PBS. For EAE induction mice were immunized with a single subcutaneous
455 injection at the dorsal tail base with 100µl of inoculum containing rmMOG, 18-30µg/mouse in
456 saline solution emulsified in a 1:1 ratio with Complete Freund's Adjuvant (CFA, Chondrex)
457 (100µg *Mycobacterium tuberculosis*/mouse), all under isoflurane (Baxter) anesthesia.
458 Additionally, all experimental animals received an i.p. injection of 200ng/mouse pertussis toxin
459 (PTX, Calbiochem) at days 0 and 2 p.i.

460 The clinical score was graded as follows: 0, no clinical signs of EAE; 1 - tail weakness or tail
461 paralysis; 2 - hind leg paraparesis or hemiparesis; 3 - hind leg paralysis or hemiparalysis; 4 -
462 tetraplegia or moribund; 5 - death. EAE remission was calculated as number of mice with full
463 recovery (score 0) divided by total EAE incidence within the subgroup.

464

465 **Single cell suspensions from CNS**

466 CNS cells were extracted using the Neural Tissue dissociation kit T (Miltenyi Biotech). Mice were
467 anesthetized with isoflurane and transcardially perfused with ice-cold PBS. Brains and spinal cords
468 were mechanically minced and resuspended in enzyme mix according to the manufacturer's
469 protocol. The CNS homogenates were then passed through a 40µm cell strainer and washed with
470 PBS containing 5mM Ethylenediaminetetraacetic acid (EDTA). The pellet was resuspended in a
471 38% Percoll (Sigma) solution and centrifuged at 800g for 15 min (no brake). The myelin gradient
472 layer was extracted and cells resuspended in PBS.

473

474 **Cell cultures**

475 Cells were cultured in Dulbecco's modified Eagles medium (DMEM, Sigma) conditioned with
476 Fetal bovine serum 10% (vol/vol) (FBS, Sigma) and Penicillin/Streptomycin 1% (vol/vol) (Sigma)
477 and M-CSF 20ng/ml (R&D). For the starvation-induced autophagy experiment cells were kept in
478 Earle's balanced salt solution (EBSS, Sigma) for 5h before analysis. Bafilomycin A1 (Sigma) was
479 used in concentration of 1 μ M.

480

481 **Flow cytometry**

482 CNS cells were analyzed at several time points from naive to day 35 p.i. EAE. Single-cell
483 suspensions were plated and stained with conjugated antibodies and LIVE/DEAD™ Fixable Near-
484 IR Dead Cell Stain (Invitrogen; L34976). Intracellular/Intranuclear staining was performed after
485 permeabilization using a Fixation/Permeabilization kit (BD biosciences/eBioscience). LC3 was
486 detected using a digitonin kit causing mild permeabilization, leaving mainly membrane-bound LC3
487 in the cell for analysis. Mitochondrial membrane potential was quantified using the Mitotracker
488 deep red™ probe. Cells were acquired using a Gallios flow cytometer (Beckman Coulter) and
489 analyzed using Kaluza software (Beckman Coulter). All antibodies and reagents are specified in
490 the enclosed technical data file.

491 **Cell sorting**

492 Cells from mouse CNS were sorted using a BD Influx cell sorter. Microglia were sorted as Live,
493 CD11b⁺ CD45^{Intermediate(Int)} Ly6G⁻ and/or eYFP⁺ (fig. S9). Bone marrow-derived macrophages were
494 sorted as Live CD11b⁺ CD45^{High} Ly6G⁻ and/or eYFP⁻. Neutrophils were sorted as Live CD11b⁺
495 Ly6G⁺ and/or eYFP⁻. Cells for some *in vitro* experiments were sorted from naive or day 5 p.i.
496 mouse CNS using CD11b magnetic beads and columns (MACS, Miltenyi Biotech). Purity was
497 determined using flow cytometry to be > 90% YFP⁺ cells. The purpose of sorting cells from day 5

498 p.i. was to acquire activated microglia without accumulated intracellular myelin phagosomes.
499 Blood monocytes were sorted using Ly6C magnetic beads (MACS, Miltenyi Biotech).

500

501 **Mouse cell RNA, cDNA preparation and Expression Analysis**

502 Cell pellets from sorted mouse CNS cells were lysed in RLT buffer and RNA extracted using a
503 RNeasy mini kit (Qiagen). Reverse transcription of total RNA was performed using random
504 hexamer primers (Invitrogen) and Superscript Reverse Transcriptase (Invitrogen). cDNA was
505 stored at -20°C until use. qPCR was performed in triplicates using a CFX384™ Real-Time PCR
506 Detection Systems with SYBR green as fluorophore (Bio-Rad). C(t) values with inter-duplicate
507 differences more than one cycle were excluded. Target expression was calculated using the Bio-
508 Rad CFX Manager V1.6. software. *Hprt* or the geometrical mean of *Gapdh* and *Hprt* was used as
509 housekeeping gene reference.

510

511 **Next-generation sequencing**

512 Sorted microglia were pooled 1:1 female and male. RNA was prepared using a RNeasy Mini Kit
513 (Qiagen) followed by quality control assessed with a Bioanalyzer 2100 (Agilent). All samples
514 included had high quality RNA (RIN = 8.5–10). RNA was amplified with a SMARTer Stranded
515 Total RNA-Seq Kit–Pico Input Mammalian (Clontech). Next-generation sequencing and
516 generation of bioinformatic data was performed by the National Genomics Infrastructure (NGI) at
517 the Science for Life Laboratory using a HiSeq 2500 System with a HiSeq Rapid SBS Kit v2
518 (Illumina). Data normalization and analysis of differential gene expression were performed using
519 the DESeq2 R package with a negative binomial test(78). The false-discovery-rate-adjusted P value
520 was estimated using the Benjamini–Hochberg correction(79). Data was further analyzed using

521 Ingenuity pathway analysis (IPA, Qiagen) and Gene set enrichment analysis (GSEA, Broad
522 institute). GSEA analysis performed with standard settings – Classic scoring scheme for the
523 enrichment score signal2noise metrics for the ranked gene list. Heat maps show in different figures
524 show the range of expression values in red – blue (denoting high - low) calculated from normalized
525 counts.

526

527 **Myelin isolation and staining**

528 Pure myelin was obtained using a protocol adapted from Norton and Poduslo (80). Briefly, myelin
529 was isolated through mechanical homogenization of perfused brains in homogenization buffer with
530 PBS containing 0.32M sucrose. After two washes in homogenization buffer, an 0.85M sucrose
531 solution in PBS underlay was added to the CNS homogenate. The CNS gradient was centrifuged
532 at 4500g for 50 min. The interphase containing myelin was then washed twice in water. The
533 purified myelin was then incubated with pHrodo dye and/or CellVue™ Plum and /or
534 Fluoromyelin™ (all from Thermo Fischer Scientific) and/or PKH26™ (Sigma) in PBS/Hepes
535 according to the manufacturer's instructions, followed by washing.

536

537 **Immunofluorescence**

538 CNS cells were sorted and plated into poly-L-lysine coated plates and incubated for 36h before
539 adding purified myelin for an additional 12h. After washing, cells were fixed with 4% PFA and
540 permeabilized with 0.2% Tween-20. Non-specific binding was blocked by adding 10% BSA and
541 serum from secondary antibody producing species. Cells were then incubated overnight with
542 primary antibodies diluted in PBS containing 1% BSA and 0.2% Tween-20. After washing,
543 secondary antibodies diluted in host serum were added and incubated at 37°C for 1 h. Finally,

544 DAPI solution (4',6-Diamidino-2-Phenylindole, Dihydrochloride, 0.2µg/ml, BD Biosciences) was
545 added to the wells for 3 min before final washing. Samples were analyzed using a Leica Confocal
546 microscope and Leica LAS-X software. CellprofilerTM (Broad institute) software was used for
547 quantitative analysis (analysis pipeline enclosed). Images were acquired using the same settings
548 for all samples.

549

550 **Phagocytosis assays**

551 Phagocytic uptake and loading to low pH lysosomes were quantified by flow cytometry analysis
552 of *ex vivo* CNS cells or sorted *in vitro* myeloid populations. For apoptotic cell phagocytosis panels
553 CNS cells were sorted using MACSTM kits for either CD171, O4 or GLAST (Miltenyi biotech).
554 Apoptosis was induced by radiation using a Precision X-rad (1Gy/min at 320KV, 12.5mA) twice
555 with 8 h incubation pause (Li et al., 1996). pHrodoTM labeled cells were pulsed with stained myelin
556 or apoptotic cells for 30 min followed by washing and flow cytometry.

557

558 **Incucyte time-lapse imaging**

559 Sorted microglia were pulsed with purified myelin stained with pHrodo Green and PKH26.
560 Incubation plates were immediately transferred to an incubator connected to an Incucyte ZOOMTM
561 instrument in which wells were analyzed using 20x ocular magnification every hour for 16-28h,
562 generating time-lapse movies. (Green: Excitation Wavelength: 460 nm, Emission Wavelength: 524
563 nm. Red: Excitation Wavelength: 585 nm, Emission Wavelength: 635 nm)

564

565 **LC3B-II detection and Digitonin permeabilization protocol**

566 Membrane-bound lipidated LC3 (II) was detected after mild digitonin permeabilization extracting
567 cytosolic LC3 according to a previously described protocol(81). Cells seeded in 96 well plates were
568 treated with the non-ionic detergent Digitonin (Sigma) at a concentration of 50µg/mL in PBS for
569 5 min at room temperature. This permeabilizes the membrane for extracellular diffusion of non-
570 bound LC3 while membrane-bound LC3 remains in the autophagosomal membrane. Cells were
571 then fixed in 4% (w/vol) paraformaldehyde/PBS for 10 min at room temperature. After two washes
572 in PBS, cells were incubated with an anti-LC3B antibody for detecting membrane-bound LC3B.

573

574 **Acid wash stripping of surface molecules**

575 Analysis of receptor internalization was achieved using a protocol for acid-wash stripping of
576 surface receptors. Cultured cells were detached with EDTA and incubated with PBS containing
577 glycine (100mM) and NaCl (150mM) (pH 2.5) for 5 min on ice. Cells were then stained and
578 analyzed by flow cytometry after fixation with or without permeabilization.

579

580 **Myelin clearance assay**

581 Sorted *ex vivo* microglia from immunized mice (day 5 or 21 p.i.) were seeded at 5×10^4 cells per
582 well in 96 well plates and incubated for 12 h before PKH26- conjugated myelin was added. At the
583 indicated timepoints, supernatant was removed and analyzed using a SpectraMax 384 microplate
584 reader for fluorescence at 560nm. The remaining myelin concentration was determined in relation
585 to a standard dilution series.

586

587 **Microglia-CD4 T-cell co-culture**

588 Sorted *ex vivo* microglia from immunized mice (day 5 p.i.) were seeded at 2×10^4 cells per well in

589 96 well plates coated with poly-L-lysine. After 24 h, 2×10^4 MACS-sorted CD4⁺ T cells (Miltenyi
590 Bioscience) from EAE mice (day 21 p.i.) were added per well. After 36 h cells, cells were analyzed
591 by flow cytometry after intranuclear Ki67 labeling as a marker of proliferation.

592

593 **ELISA**

594 Sorted *ex vivo* microglia from naive mice and mice during EAE were incubated in DMEM, (Sigma)
595 conditioned with Fetal bovine serum 10% (vol/vol) (FBS, Sigma) and penicillin/streptomycin 1%
596 (vol/vol) for 24h in 96 well plates. Supernatants were collected and cytokine production was
597 quantified using ready-set-go ELISA kits (eBioscience, Invitrogen) and a SpectraMax 384
598 microplate reader plate reader according to the manufacturer's instructions.

599

600 **Trehalose treatment of EAE**

601 For studies of the clinical effects of trehalose, mice were treated with water supplemented with
602 either 5% (w/vol) D-(+)-trehalose dihydrate or 5% (w/vol) sucrose (both from Sigma) starting at
603 the day of immunization. Sucrose was used as control given its similarity to trehalose since both
604 are disaccharides, thus excluding elevated calorie availability as a determinant of EAE recovery.
605 I.p. injections of 20% (w/vol) trehalose, 20% (w/vol) sucrose, 20% (w/vol) or PBS supplemented
606 the treatment every third day starting from EAE onset until end of experiment. During severe EAE,
607 mice were fed with trehalose, sucrose and water at a final concentration of 20% (w/vol). The i.p.
608 doses were equal to previously reported clinical experiments while the drinking water was enriched
609 to 5% (w/vol) trehalose (compared to 3% (w/vol) (62). This was a result of a titration experiment
610 using 0, 3, and 5% (w/vol) with a stronger impact evident with the higher dose.

611

612 **Trehalose and TFEB *in vitro* assays**

613 *Ex vivo* microglia were cultured in medium with or without D-(+)-trehalose dihydrate at a final
614 concentration of 3% (w/vol). ICC experiments were performed as described above using
615 antibodies against TFEB (Rabbit, Bethyl, diluted 1:1000) and LAMP1 (Rat, Sigma, diluted
616 1:1000), which labels lysosomes, and secondary antibodies Alexa fluor 647 goat anti-rat (Thermo
617 fisher) and goat-anti rabbit Alexa fluor 546 (Thermo fisher), respectively. TFEB and LAMP1⁺
618 lysosomes were quantified in relation to DAPI-defined nuclei (0.2 µg/ml) using Cellprofiler™
619 software.

620

621 **Histopathology and immunofluorescence (IF)**

622 Histopathological and IF analyses were performed on 3–5 µm thick paraffin-embedded spinal cord
623 cross-sections. Luxol fast blue (Kluever; Sigma) was used to assess tissue
624 demyelination. Quantitative evaluation of demyelination presented as the demyelination score
625 (DM) was performed on an average of 7 complete cross-sections of the spinal cord per mouse, as
626 previously described by Storch *et al.* (Storch et al., 1998). All images were captured using a Leica
627 Polyvar 2 microscope.

628 For IF analyses, the paraffin-embedded spinal cord cross-sections were treated as previously
629 described(82). After deparaffinization in xylol, sections were transferred to 90% (vol/vol) ethanol.
630 Endogenous peroxidase was blocked by incubation in methanol with 0.02% H₂O₂ for 30 min at RT
631 and rehydration to distilled water followed via a 90% (vol/vol), 70% (vol/vol), and 50% (vol/vol)
632 ethanol series. Antigen retrieval was performed with Dako target retrieval solution (Dako) for 1 h
633 in a steamer device at 98°C. Sections were subsequently incubated in 10% FCS in PBS for 30 min
634 at RT before incubation with the primary antibody on 4°C, overnight. Primary antibodies used in

635 costainings were Mac3 (Rat, BD Biosciences, diluted 1:200), dMBP (Rabbit, Millipore diluted,
636 1:200), CLEC7A (Rabbit, Abcam, diluted 1:500) and YFP (Chicken, Abcam, diluted 1:200), CD45
637 (Rat, BD Biosciences, diluted 1:200), CLEC7A (Rabbit, Abcam diluted 1:200). After washing in
638 PBS, sections were incubated with a secondary antibody for 1 h at RT. Secondary antibodies were
639 used in the following combinations: Alexa Fluor 555 donkey anti-rat IgG (Abcam), Alexa Fluor
640 488 donkey anti-rabbit IgG (Abcam) or rabbit anti-chicken IgY FITC (Thermo fischer),
641 respectively. DAPI (0.2µg/ml) was included in the last washing step to visualize the nuclei. All
642 images were acquired using Zeiss LSM700 confocal microscope and the ZEN 2009 software.
643 Representative images shown are maximum intensity projections of 3µm thick z-stacks.
644 Quantifications of the specific immunoreactivity was performed on five whole spinal cord cross-
645 sections per mouse using ImageJ64, based on the number of pixels above an estimated threshold.

646

647 **Statistical Analysis**

648 GraphPad Prism 8 (<http://www.graphpad.com/>) was used for all the statistical analysis. In graphs
649 with several comparisons, a dotted line separates the datasets that were compared. All figure
650 legends include information regarding statistical tests used and sample size.

651 **Supplementary materials**

652 Fig. S1. Recovery from experimental autoimmune encephalomyelitis (EAE) requires functional
653 autophagy in the myeloid but not in the T cell compartment.

654 Fig. S2. *Atg7* and *Ulk1* deficiency in microglia impact non-canonical and canonical autophagy,
655 respectively.

656 Fig. S3. *Atg7* deficiency induces alterations in microglial transcriptome during experimental
657 autoimmune encephalomyelitis (EAE).

658 Fig. S4. *Atg7* deficiency in microglia increases T cell proliferation and polarization to an
659 inflammatory phenotype.

660 Fig. S5. Gene-set enrichment analysis.

661 Fig. S6. *Atg7* deficient microglia have impaired scavenger receptor recirculation associated with
662 increased inflammation and a reduced myelinating oligodendrocyte population in experimental
663 autoimmune encephalomyelitis (EAE).

664 Fig. S7. Late stage experimental autoimmune encephalomyelitis (EAE) is characterized by
665 extensive tissue destruction and signs of increased inflammation in mice with *Atg7*-deficient
666 microglia.

667 Fig. S8. Trehalose boosts experimental autoimmune encephalomyelitis (EAE) recovery and
668 decreases immune infiltration in aged mice.

669 Fig. S9. Gating strategy for defining cell populations by flow cytometry.

670

671 Table S1. RNAseq data (Excel)

672 Table S2. IPA, ORA and REVIGO analysis (Excel)

673 Table S3. Genes shared among homeostatic or pathogenic gene sets (Excel)

- 674 Table S4. RNAseq data (Excel)
- 675 Table S5. Technical data file (Excel)
- 676 Table S6. Raw data (Excel)
- 677 Movie S1. Accumulation of phagocytosed myelin in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia
- 678 Movie S2. Accumulation of myelin-containing phagosomes in microglia of aged mice

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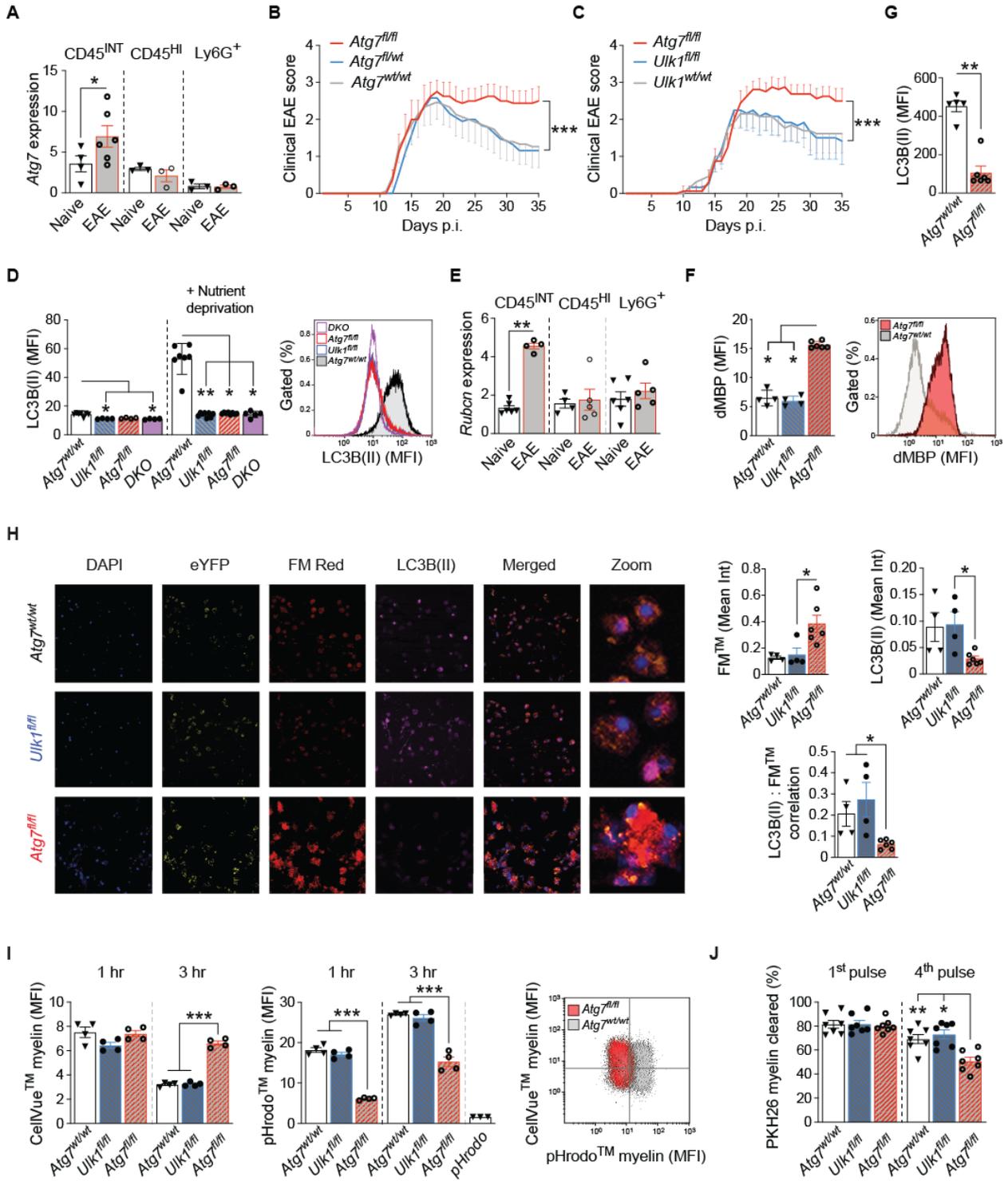
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- 867

868 **Acknowledgments:** The authors would like to thank Dr. Masaaki Komatsu at the Tokyo
869 Metropolitan Institute of Medical Science for providing the *Atg7^{fl/fl}* mice. The authors acknowledge
870 Dr. Klas Blomgren at the University of Gothenburg for providing breeding couples of *Atg7^{fl/fl}* mice.
871 We thank the staff at the animal facility at Karolinska University Hospital and in particular Helen
872 Kungsmark for animal care taking. We also thank Dr. Annika van Vollenhoven for assisting with
873 flow cytometry sorting. We would like to acknowledge support from Science for Life Laboratory,
874 the National Genomics Infrastructure (NGI). **Funding:** This work was supported by grants from
875 the Swedish Research Council, the Swedish Brain Foundation, the Swedish Association for
876 Persons with Neurological Disabilities, the Stockholm County Council (ALF project), AstraZeneca
877 (AstraZeneca-Science for Life Laboratory collaboration), European Union Horizon 2020/European
878 Research Council Consolidator Grant (Epi4MS), the Knut and Alice Wallenbergs Foundation,
879 Margeretha af Ugglas Foundation, Alltid Litt Sterkere, Foundation of Swedish MS research,
880 NEURO Sweden and Karolinska Institutet. **Author contributions:** The study was conceived by
881 RB, AOGC, MTH, TO and MJ. Most experiments were conducted by RB with assistance from
882 AOGC, HL and EN. MTH and RP assisted in EAE characterization. Histopathology and
883 immunofluorescence of CNS were assessed by MZ, EN, SA and MZA. RNA extraction,
884 purification and quality control were performed by RB and SR. RNA-sequencing data preparation
885 and analysis was done by EE and functional annotation analysis by MJ. RB, AOGC, MJ wrote the
886 manuscript with input from other authors. Statistical analysis were done by RB and EE. The project
887 was supervised by AOGC, RAH, TO and MJ. **Competing interests:** The authors report no
888 competing interests. **Data and materials availability:** The RNA-sequencing data has been
889 deposited in the Gene Expression Omnibus (GEO) and the accession number is GSE154920. The
890 mouse strains can be purchased at RikenBRC (B6.Cg-Atg7^{tm1Tchi}) and Jackson laboratory

891 (B6.129-Ulk1tm1Thsn/J), (B6.129P2(Cg)-Cx3cr1tm1Litt/J, B6.129P2-Lyz2tm1(cre)Ifo/J, B6.Cg-
892 Tg(Lck-cre)548Jxm/J). All reagents are listed as Technical data with company details and order
893 number (table S5).

895 **Figure legends**



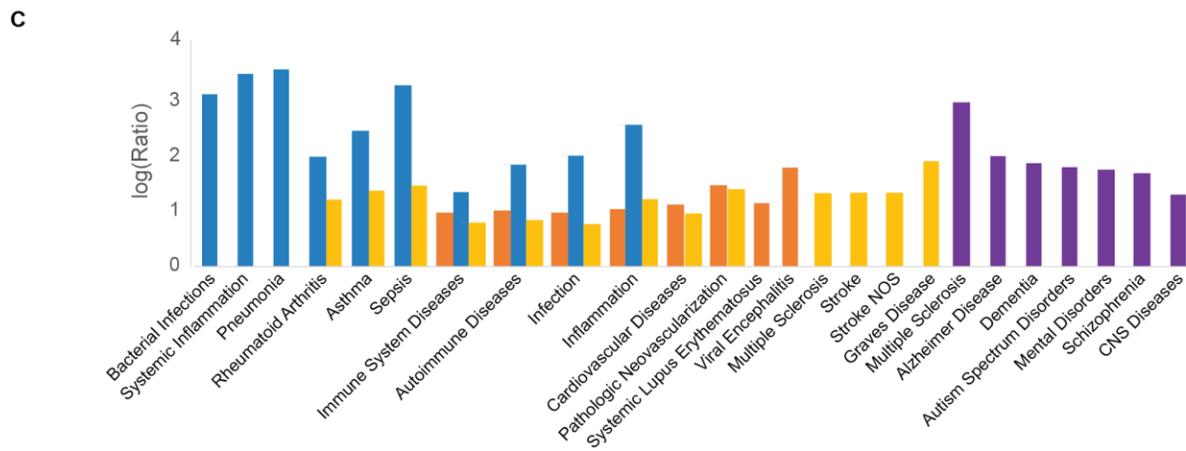
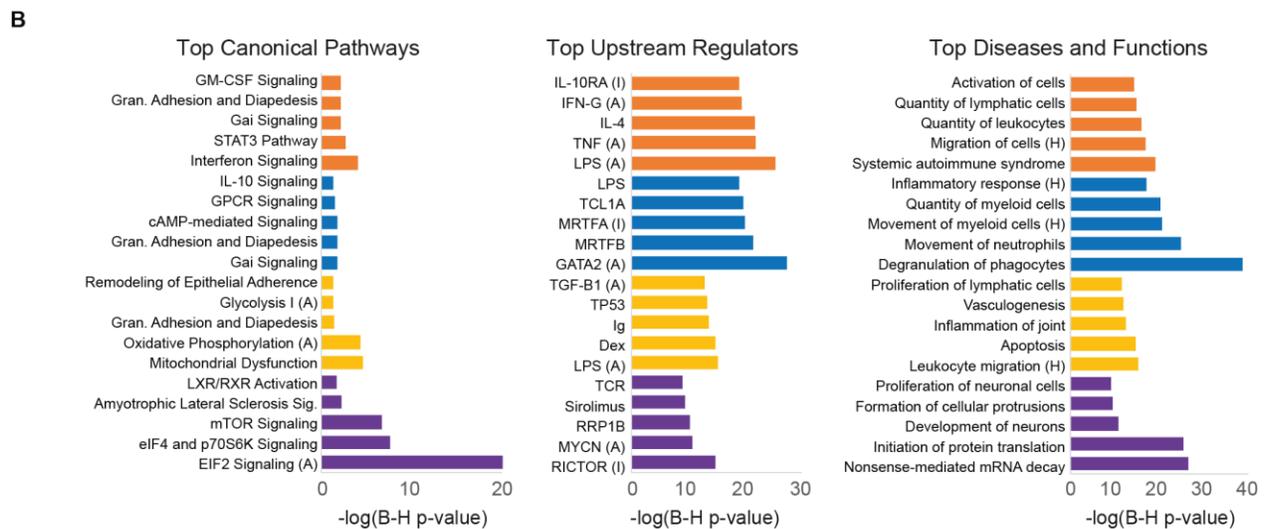
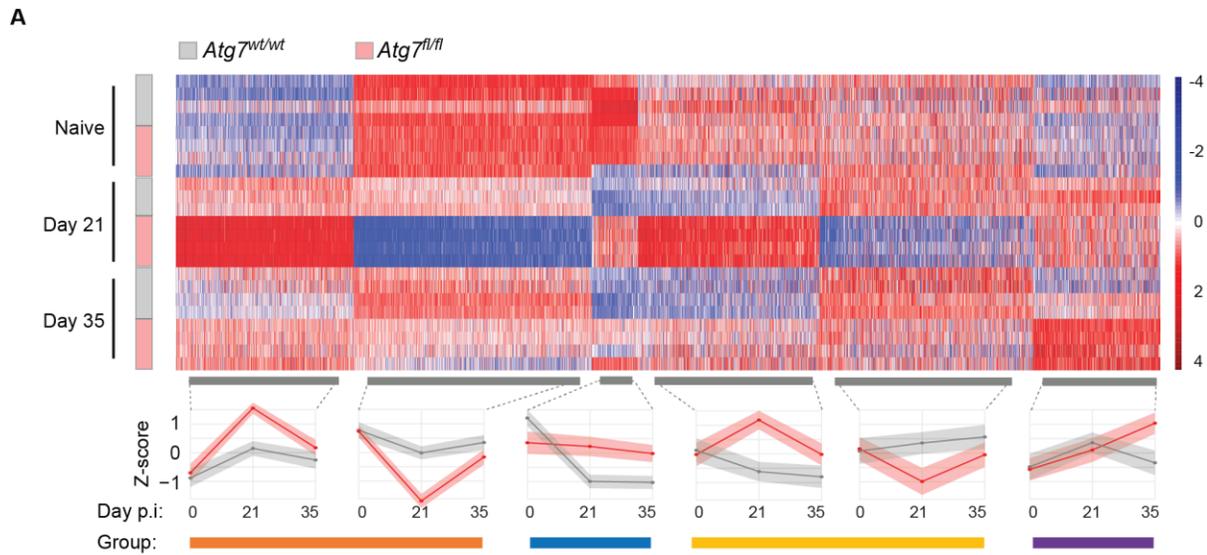
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898 **Fig. 1. Recovery from Experimental Autoimmune Encephalomyelitis (EAE) requires**
899 **microglial autophagy.**

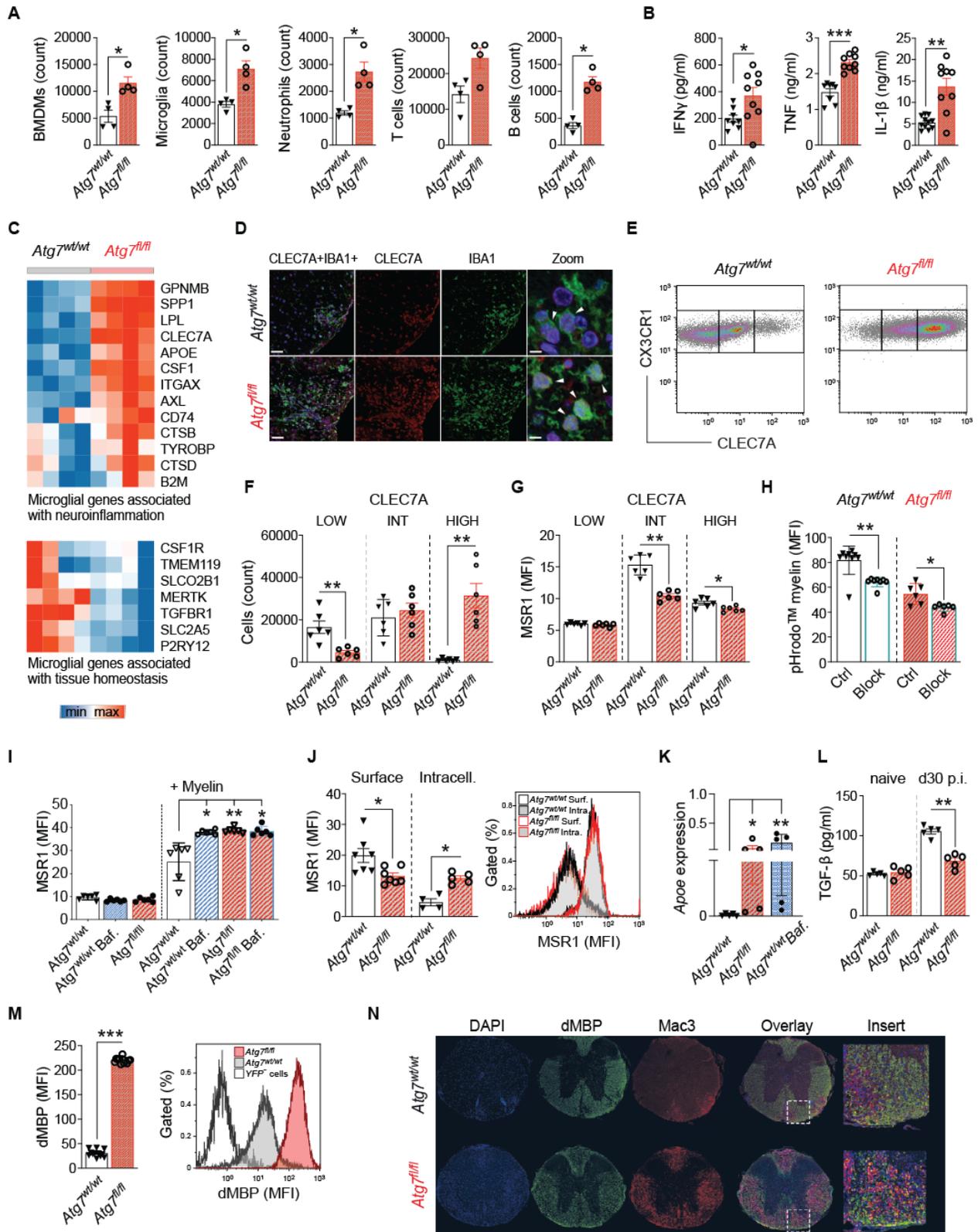
900 (A) Relative expression of *Atg7* in microglia (CD45^{INT} naïve; *n* = 4 and EAE; *n* = 6), bone marrow-
901 derived monocytes/macrophages (CD45^{INT} naïve; *n* = 3 and EAE; *n* = 3) and neutrophils (Ly6G⁺
902 naïve; *n* = 3 and EAE; *n* = 3) from naïve and day 15 EAE animals, detected by qPCR and
903 normalized to the geometric mean of two endogenous control genes, *Gapdh* and *Hprt*. (B) Disease
904 course in *Atg7*^{wt/wt} (*n* = 16), *Atg7*^{wt/fl} (*n* = 20) and *Atg7*^{fl/fl} (*n* = 19) mice and (C) in *Ulk1*^{wt/wt} (*n* =
905 13), *Ulk1*^{fl/fl} (*n* = 12) and *Atg7*^{fl/fl} (*n* = 16) mice. Clinical course was compared using one-way
906 ANOVA with Tukey's post-hoc test on Area Under Curve. Error bars indicate confidence intervals.
907 (D) Membrane bound LC3B (II) detected by flow cytometry in naïve *Atg7*^{wt/wt} (untreated; *n* = 7,
908 starved; *n* = 7), *Ulk1*^{fl/fl} (untreated; *n* = 4, starved; *n* = 8), *Atg7*^{fl/fl} (untreated; *n* = 4, starved; *n* = 8)
909 and *Atg7*^{fl/fl} *Ulk1*^{fl/fl} ("double knock-out"- DKO, untreated; *n* = 4, starved; *n* = 5) microglia after
910 starvation *in vitro*. (E) Relative expression of *Rubicon* in microglia (CD45^{INT} naïve; *n* = 6 and
911 EAE, *n* = 4), bone marrow-derived monocytes/macrophages (CD45^{HI} naïve; *n* = 4 and EAE; *n* =
912 5) and neutrophils (Ly6G⁺ naïve; *n* = 6 and EAE; *n* = 5) from naïve and day 15 EAE animals,
913 detected by qPCR and normalized to the geometric mean of two endogenous control genes, *Gapdh*
914 and *B-actin*. (F) Phagocytosed myelin debris (dMBP) assessed in microglia from *Atg7*^{wt/wt},
915 *Ulk1*^{fl/fl} and *Atg7*^{fl/fl} mice 21 days p.i. by flow cytometry (all conditions; *n* = 5). (G) Membrane
916 bound LC3B (II) detected by flow cytometry after antibody labeling of *ex vivo* microglia 21 days
917 p.i. in *Atg7*^{wt/wt} (*n* = 5) and *Atg7*^{fl/fl} (*n* = 6) mice. (H) Example images of immunofluorescence and
918 image analysis of FluoromyelinTM stained myelin (FM Red), LC3B (II) and LC3B (II):Myelin
919 (overlapping pixels) of *ex vivo* microglia 5 days p.i. from *Atg7*^{wt/wt} (*n* = 4), *Ulk1*^{fl/fl} (*n* = 4)
920 and *Atg7*^{fl/fl} (*n* = 6) mice. (I) *In vitro* pulsing of microglia 5 days p.i. with CellVueTM- and

921 pHrodoTM-stained myelin, assessed by flow cytometry (all conditions: $n = 4$ except “pHrodo” $n =$
922 3). (**J**) *In vitro* clearance of PKH26-stained myelin from medium by microglia 5 days p.i. from
923 *Atg7^{wt/wt}* ($n = 7$) and *Atg7^{fl/fl}* ($n = 7$) mice. Statistics: (**A**, **E** and **G**) Mann-Whitney U-test, (**D**, **F**, **H**
924 and **J**) Kruskal–Wallis test followed by Dunn's post-hoc test, (**I**) ANOVA followed by Dunnet's
925 post-hoc test (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Error bars indicate SEM. Experiments (**A**
926 to **D** and **E**, **H**) were performed twice and (**B**, **C**, **F**, **G**, **I**, **J**) three times.



928 **Fig. 2. *Atg7* deficiency induces pronounced and sustained alterations in microglial**
929 **transcriptome during Experimental Autoimmune Encephalomyelitis (EAE).**

930 Transcriptome analysis was performed using RNA-sequencing of microglia sorted from the
931 following groups of mice: naive *Atg7^{fl/fl}* ($n = 4$) and *Atg7^{wt/wt}* ($n = 3$), day 21 p.i. *Atg7^{fl/fl}* ($n = 4$,
932 average score 3) and *Atg7^{wt/wt}* ($n = 3$, average score 3) and day 35 p.i. *Atg7^{fl/fl}* ($n = 3$, average score
933 1.5) and *Atg7^{wt/wt}* ($n = 4$, average score 2.5) (table S1). (A) Heat map depicting gene clusters
934 associated with genotype and EAE disease stage based on transcripts that displayed a p-value <
935 0.01 and fold-change > 1.5. The scale represents Z-score transformed expression values (with red
936 and blue indicating upregulated and downregulated genes, respectively, compared to the mean
937 value of a gene from all samples). These gene clusters were further grouped according to their
938 pattern of expression into the four groups that were analyzed using Ingenuity pathway analysisTM
939 (IPA) to annotate significance: (B) Canonical pathways, Upstream regulators, and Diseases and
940 Functions (Benjamini-Hochberg adjusted p-value < 0.05) and (C) Over-representation analysis
941 (ORA) for Diseases using the GLAD4U database (FDR < 0.05). Details regarding the differential
942 expression analysis are presented in table S1 and a full list of significant functional annotations is
943 provided in table S2. (A), (I) and (H) indicate activated, inhibited and high, respectively.



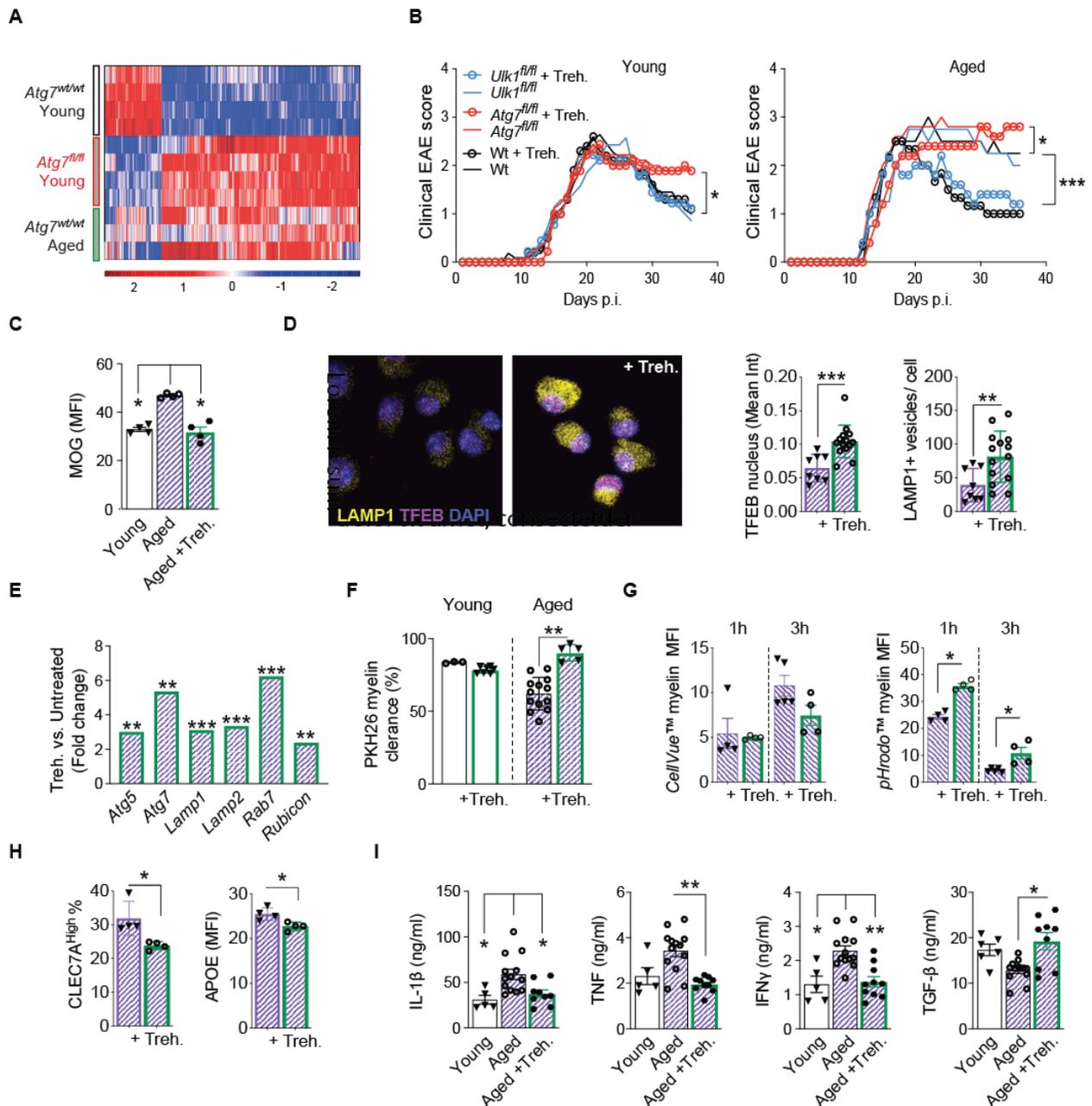
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946 **Fig. 3. *Atg7*-deficient microglia have impaired myelin degradation and scavenger receptor**
947 **recirculation associated to a pathogenic phenotype and increased inflammation in**
948 **Experimental Autoimmune Encephalomyelitis (EAE).**

949 (A) Spinal cord microglia and infiltrating immune cell counts from *Atg7^{wt/wt}* ($n = 4$) and *Atg7^{fl/fl}* (n
950 $= 4$) mice at day 21 p.i. analyzed by flow cytometry. (B) ELISA of supernatants from microglia
951 sorted 21 days p.i. and incubated for 24h *in vitro*; *Atg7^{wt/wt}* ($n = 8$) and *Atg7^{fl/fl}* ($n = 9$). (C)
952 Transcriptome of microglia sorted from *Atg7^{wt/wt}* ($n = 4$) and *Atg7^{fl/fl}* ($n = 4$) mice 35 days p.i.
953 analyzed by RNA-sequencing and compared to microglia gene sets associated with disease and
954 tissue homeostasis. (D) Representative images of immunofluorescence of CLEC7A and IBA1-
955 expressing microglia at day 32-37 p.i. in spinal cord from *Atg7^{wt/wt}* and *Atg7^{fl/fl}* mice. DAPI defines
956 nuclei. Size bars correspond to 500 μ m and 50 μ m in the left and right panels, respectively. (E and
957 F) Flow cytometry analysis of CLEC7A-expressing subpopulations in *Atg7^{wt/wt}* ($n = 6$) and *Atg7^{fl/fl}*
958 ($n = 6$) microglia at day 35 p.i. (G) Flow cytometry analysis of the density of surface MSR1 staining
959 in microglia from *Atg7^{wt/wt}* ($n = 6$) and *Atg7^{fl/fl}* ($n = 6$) mice in different subpopulations defined by
960 levels of CLEC7A expression as shown in (E and F). (H) Uptake of labeled myelin by sorted
961 *Atg7^{wt/wt}* (control; $n = 9$, block; $n = 8$) and *Atg7^{fl/fl}* (control; $n = 6$, block; $n = 6$) microglia after
962 blocking of MSR1. (I) Flow cytometry quantification of intracellular MSR1 from naïve
963 *Atg7^{wt/wt}* and *Atg7^{fl/fl}* microglia exposed to myelin 12h *in vitro* w/wo 6h Bafilomycin A1 treatment.
964 All conditions; $n = 6$. (J) Surface and intracellular MSR1 detection by flow cytometry in
965 *Atg7^{wt/wt}* (surface; $n = 7$, intracellular; $n = 4$) and *Atg7^{fl/fl}* (surface; $n = 7$, intracellular; $n = 5$)
966 microglia day 5 p.i. (K) Expression of *ApoE* in *Atg7^{wt/wt}* ($n = 4$), *Atg7^{fl/fl}* ($n = 5$) and Bafilomycin
967 A1 treated *Atg7^{wt/wt}* ($n = 5$) microglia exposed to myelin for 7 days *in vitro*. (L) ELISA of TGF- β 1
968 secretion from microglia from naïve and day 35 p.i. mice (*Atg7^{wt/wt}*; $n = 5$, *Ulk^{fl/fl}*; $n = 5$ and *Atg7^{fl/fl}*;

969 $n = 5$) after 24h *in vitro* culture. **(M)** Intracellular myelin debris (dMBP) assessed in microglia from
970 $Atg7^{wt/wt}$ ($n = 12$) and $Atg7^{fl/fl}$ ($n = 12$) mice 35 days p.i. by flow cytometry. **(N)** Representative
971 images of immunofluorescence of tissue deposits of myelin debris (dMBP) and density of Mac3⁺
972 macrophages at 37 p.i. in spinal cord from $Atg7^{wt/wt}$ and $Atg7^{fl/fl}$ mice. DAPI defines nuclei.
973 Statistics: **(A, B, F to H, J, L, M)** Mann-Whitney U-test, **(I and K)** Kruskal–Wallis test followed
974 by Dunn's post-hoc test (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Error bars indicate SEM.
975 Experiment **(N)** is representative of three independent experiments. Experiments **(B, H to M)** were
976 performed twice and **(A, F, G)** three times.



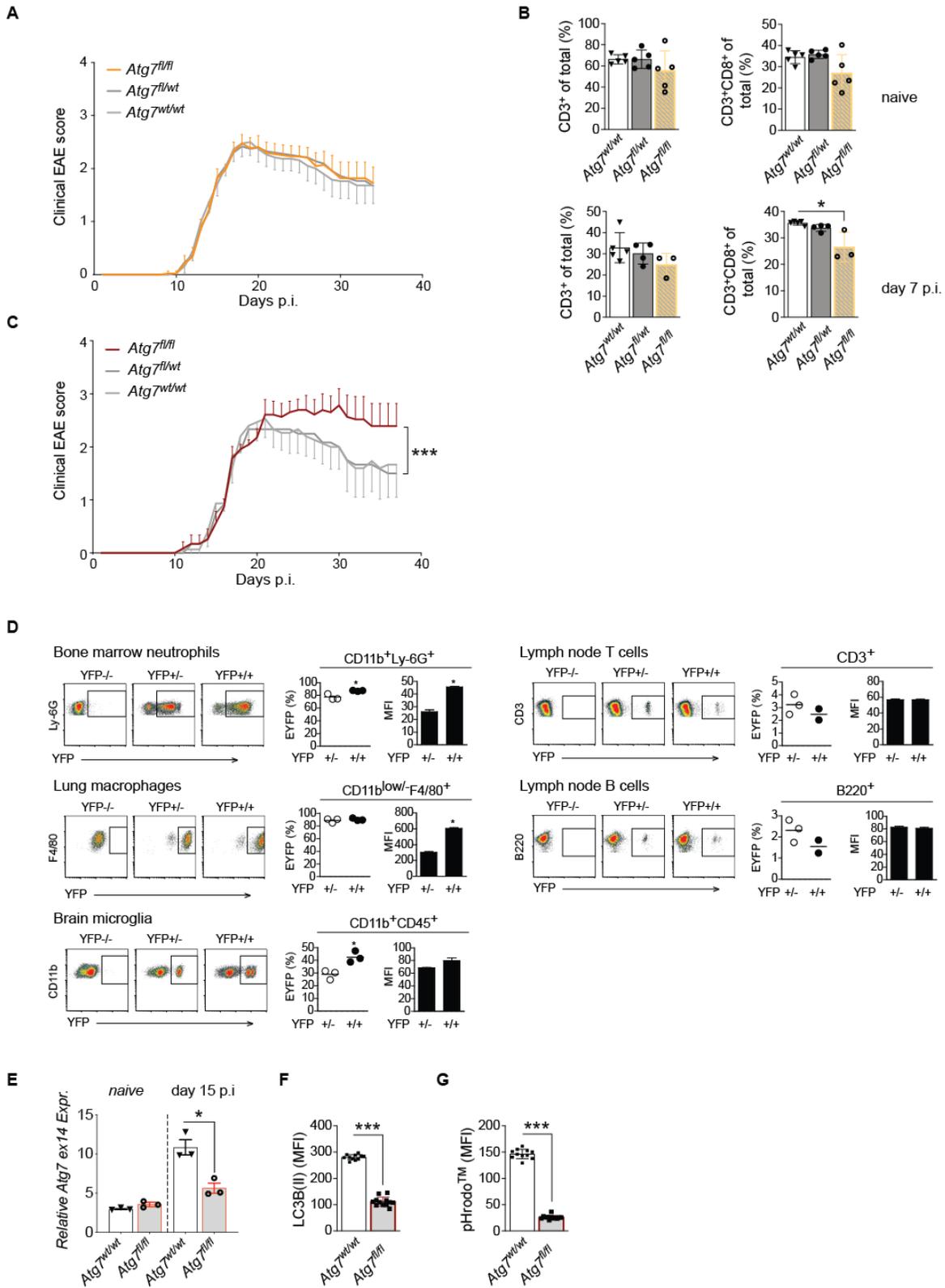
977
 978 **Fig. 4. Trehalose treatment boosts myelin clearance and ameliorates Experimental**
 979 **Autoimmune Encephalomyelitis (EAE) in aged mice.**

980 (A) Transcriptome heat map of microglia sorted from naïve aged (> 80 weeks) wild type mice (*n*
 981 = 3, average score 2.5) and *Atg7^{wt/wt}* (*n* = 4, average EAE score 1.5) and *Atg7^{fl/fl}* (*n* = 4, average
 982 EAE score 2.5) mice 35 days p.i. analyzed by RNA-sequencing (table S4). The scale represents Z-

983 score transformed expression values (with red and blue indicating upregulated and downregulated
984 genes, respectively, compared to the mean value of a gene from all samples). **(B)** Clinical course
985 of EAE and full recovery rate in young (12-22 weeks old) *Atg7^{wt/wt}* (control; *n* = 11, treated; *n* =
986 11), *Ulk1^{fl/fl}* (control; *n* = 7, treated; *n* = 9) and *Atg7^{fl/fl}* (control; *n* = 9, treated; *n* = 9) mice (left)
987 and aged *Atg7^{wt/wt}* (control; *n* = 4, treated; *n* = 6), *Ulk1^{fl/fl}* (control; *n* = 4, treated; *n* = 5) and *Atg7^{fl/fl}*
988 (control; *n* = 5, treated; *n* = 5) mice (right). Mice were fed with Trehalose in water or water as
989 control. Clinical course was compared using one-way ANOVA with Tukey's post-hoc test on Area
990 Under Curve. **(C)** Flow cytometry quantification of intracellular myelin debris in microglia from
991 young (*n* = 3), aged (*n* = 4) and Trehalose-treated aged (*n* = 4) mice at day 21 p.i. **(D)**
992 Immunofluorescence image and quantification showing TFEB translocation from cytosol to
993 nucleus upon 48h *in vitro* Trehalose-treated (*n* = 14) and untreated (*n* = 8) microglia from aged
994 mice at day 5 p.i. LAMP1 detects lysosomal structures. Data pooled from two experiments. **(E)**
995 Expression of selected key autophagosome, lysosome and phagosome vesicle biogenesis genes in
996 microglia of aged mice (*n* = 9) after 48h of *ex vivo* Trehalose treatment as fold change of untreated
997 control. **(F)** *In vitro* myelin clearance assay of microglia sorted 5 days p.i. from young (*n* = 3), aged
998 (*n* = 13) and Trehalose-treated young (*n* = 7) and aged (*n* = 5) mice pulsed 4 times with PKH26-
999 labeled myelin. **(G)** *In vitro* pulsing of microglia 5 days p.i. with CellVueTM- and pHrodoTM-stained
1000 myelin, assessed by flow cytometry (all conditions: *n* = 4 except aged 3h; *n* = 5). **(H)** CLEC7A
1001 and APOE detected by Flow cytometry on *ex vivo* day 21 EAE microglia from Aged (*n* = 4) w/wo
1002 Trehalose treatment (*n* = 4). **(I)** ELISA of cytokine production by CD11b⁺ cells isolated from the
1003 CNS of young (*n* = 5), aged (*n* = 13) and Trehalose-treated aged (*n* = 10) mice at day 21 p.i. cultured
1004 *ex vivo* for 24h. Statistics: **(D, F to H)** Mann-Whitney U-test, **(C and I)** Kruskal–Wallis test

1005 followed by Dunn's post-hoc test (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Error bars indicate SEM.
1006 Experiments (**B** to **D** and **F** to **I**) were performed twice.

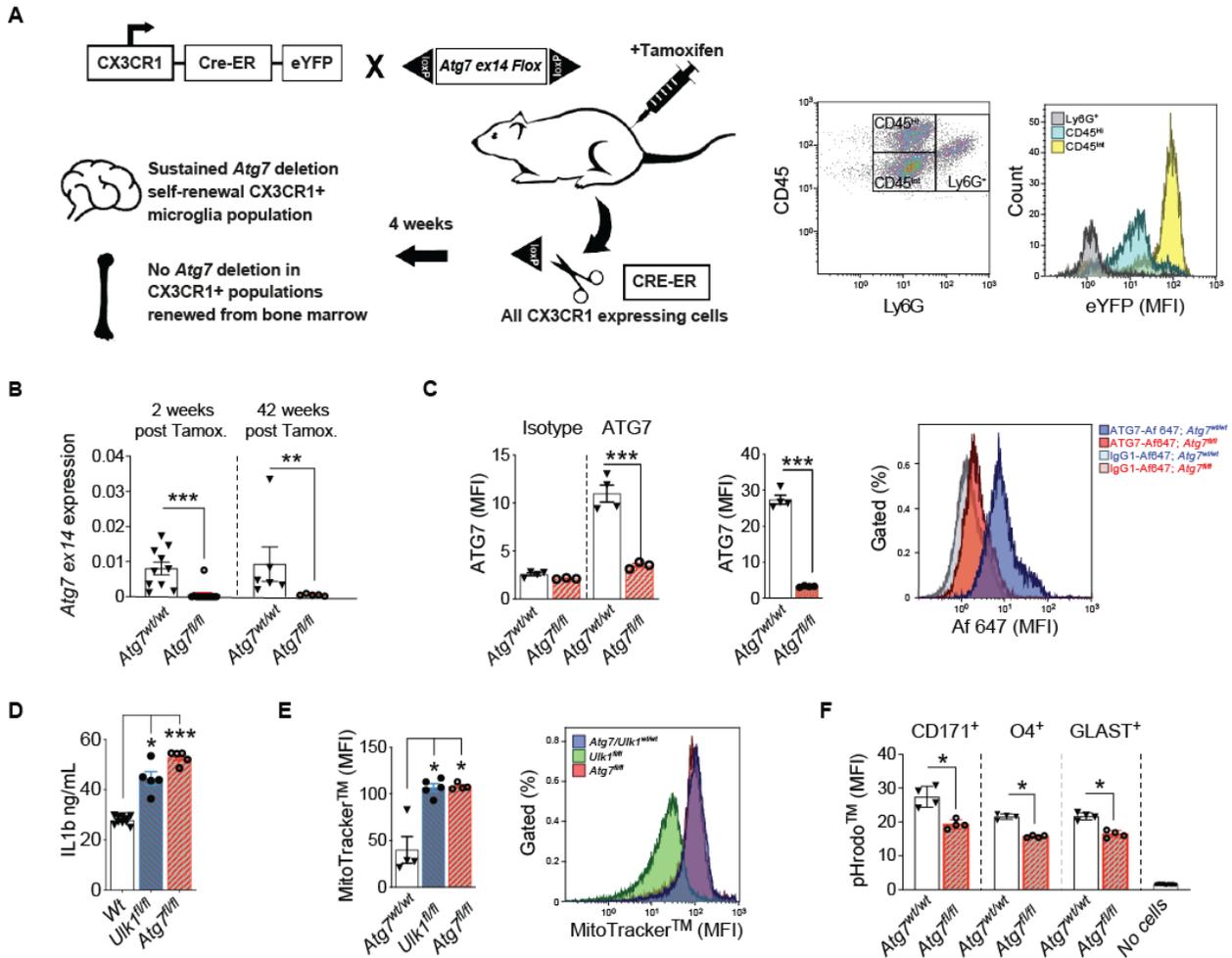
1007 **Supplementary Figure 1**



1009 **Fig. S1. Recovery from Experimental Autoimmune Encephalomyelitis (EAE) requires**
1010 **functional autophagy in the myeloid but not in the T cell compartment.**

1011 (A) Myelin Oligodendrocyte Glycoprotein (MOG)-induced EAE in *Atg7^{fl/fl}Lck^{Cre}* (*n* = 44),
1012 *Atg7^{fl/wt}Lck^{Cre}* (*n* = 43) and *Atg7^{wt/wt}Lck^{Cre}* (*n* = 38) mice (three pooled experiments). (B)
1013 Frequencies of cells from inguinal lymph nodes in *Atg7^{fl/fl}Lck^{Cre}* (*n* = 3-5), *Atg7^{fl/wt}Lck^{Cre}* (*n* = 4-5),
1014 and *Atg7^{wt/wt}Lck^{Cre}* (*n* = 5) mice as detected by flow cytometry. (C) MOG-induced EAE in
1015 *Atg7^{fl/fl}Lyz2^{Cre}* (*n* = 15), *Atg7^{fl/wt}Lyz2^{Cre}* (*n* = 10) and *Atg7^{wt/wt}Lyz2^{Cre}* (*n* = 23) mice. (D) LysM
1016 (*Lyz2*) expression was screened using a reporter system (*Lyz2*-Cre x Rosa26-STOP-YFP). Cells
1017 from different tissues were extracted, stained with fluorescently labeled antibodies, acquired by
1018 flow cytometry and analyzed by gating into different subsets according to the strategy defined
1019 above in the graphs. YFP positivity is shown for control, heterozygous or homozygous animals in
1020 representative plots, as well as frequencies of positive cells (shown as percent of each given
1021 population) and mean fluorescence intensity (MFI) for YFP expression. Bone marrow neutrophils
1022 and lung macrophages show near complete labelling, as expected. In microglia, 30-40% of cells in
1023 the naïve state exhibit *Lyz2* expression, while T and B cells show minimal targeting. (E) mRNA
1024 expression of *Atg7* loxP-flanked exon 14 normalized to *Gapdh* and *β-actin* in microglia from
1025 *Atg7^{fl/fl}Lyz2^{Cre}* (naïve; *n* = 3, day 15 p.i.; *n* = 3) and *Atg7^{wt/wt}Lyz2^{Cre}* controls (naïve; *n* = 3, day 15
1026 p.i.; *n* = 3). (F) Membrane-bound LC3B (II) and (G) intracellular pHrodo™-labeled myelin
1027 detected in *ex vivo* day 30 p.i. microglia from *Atg7^{fl/fl}Lyz2^{Cre}* (*n* = 12) and *Atg7^{wt/wt}Lyz2^{Cre}* (*n* = 11)
1028 mice by flow cytometry after 48h of myelin and LPS exposure *in vitro*. Statistics: (A and C) One-
1029 way ANOVA with Tukey's post-hoc test on Area Under Curve. Error bars indicate confidence
1030 interval; (B) Kruskal–Wallis test followed by Dunn's post-hoc test, (D, F to G) Mann-Whitney U-
1031 test, (E) Unpaired T-test (*** *p* < 0.001, ** *p* < 0.01, * *p* < 0.05). Error bars indicate SEM.
1032 Experiments (A to C) were performed three times and (E to G) were performed twice.

1033 **Supplementary Figure 2**



1034

1035 **Fig. S2. *Atg7* and *Ulk1* deficiency in microglia impact non-canonical and canonical**

1036 **autophagy, respectively.**

1037 (A) Scheme over the Tamoxifen inducible *Cx3cr1*^{CreERT2} gene deletion model. *Cx3cr1*^{CreERT2} is co-

1038 expressed with fluorescent eYFP. Expression of eYFP in infiltrating CD11b⁺CD45^{hi} (monocytes),

1039 CD11b⁺CD45^{hi/int} Ly6G⁺ (neutrophils) and CD11b^{int}CD45^{int} (microglia) is shown in the flow

1040 cytometry panels to the right. (B) mRNA expression of loxP-flanked *Atg7* exon 14 normalized to

1041 *Gapdh* and *Hprt* in microglia from *Atg7*^{fl/fl}*Cx3cr1*^{CreERT2} (2 weeks; n = 12, 42 weeks; n = 5) and

1042 *Atg7*^{wt/wt}*Cx3cr1*^{CreERT2} (2 weeks; n = 10, 42 weeks; n = 6) mice. (C) Flow cytometry detection of

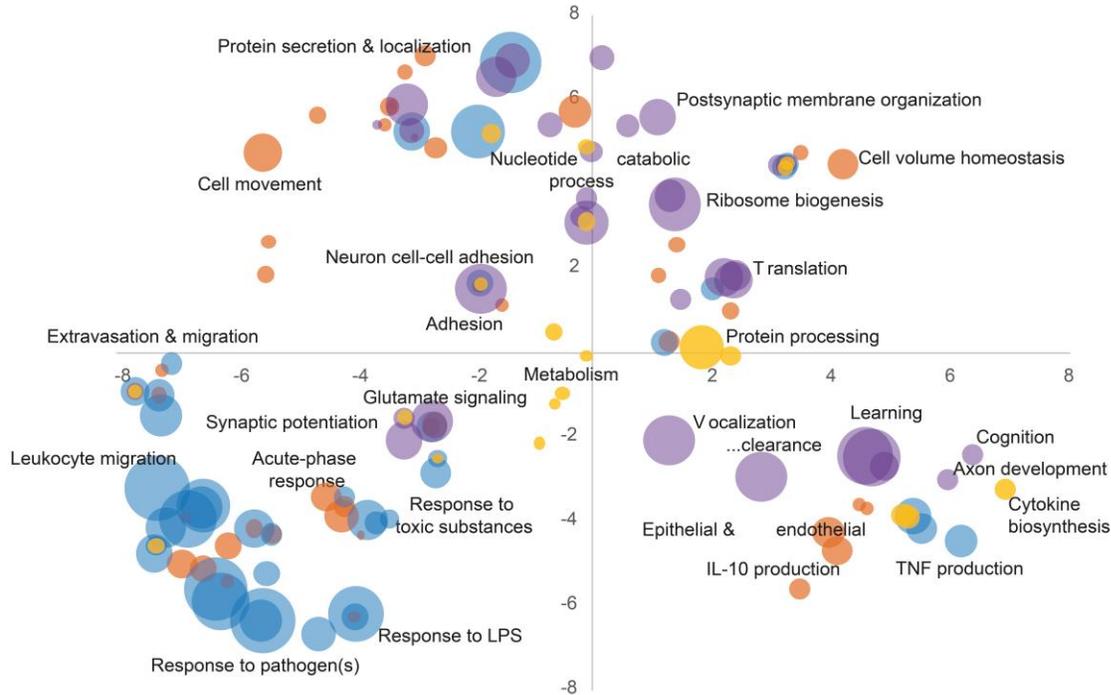
1043 ATG7 in microglia after *in vitro* (left) and *in vivo* (right) Tamoxifen-induced *Atg7* deletion in

1044 *Atg7*^{fl/fl}*Cx3cr1*^{CreERT2} (n = 3) and *Atg7*^{wt/wt}*Cx3cr1*^{CreERT2} (n = 4) mice. (D) IL-1β secretion by day

1045 15 p.i. microglia from *Atg7*^{wt/wt}*Cx3cr1*^{CreERT2} (n = 10), *Ulk1*^{fl/fl}*Cx3cr1*^{CreERT2} (n = 5) and *Atg7*^{fl/fl}

1046 *Cx3cr1^{CreERT2}* (*n* = 5) mice. **(E)** Mitochondrial membrane potential detected in *in vitro* LPS treated
1047 and nutrient deprived *Atg7^{wt/wt} Cx3cr1^{CreERT2}* (*n* = 4), *Ulk1^{fl/fl} Cx3cr1^{CreERT2}* (*n* = 5) and *Atg7^{fl/fl}*
1048 *Cx3cr1^{CreERT2}* (*n* = 4) microglia. **(F)** Phagocytosis of pHrodoTM-labeled apoptotic cells by *Atg7^{fl/fl}*-
1049 *Cx3cr1^{CreERT2}* (*n* = 4) and *Atg7^{wt/wt} Cx3cr1^{CreERT2}* (*n* = 4) microglia quantified by flow cytometry.
1050 Statistics: **(B and F)** Mann-Whitney U-test, **(C)** Unpaired T-test, **(D and E)** Kruskal–Wallis test
1051 followed by Dunn's post-hoc test (*** *p* < 0.001, ** *p* < 0.01, * *p* < 0.05). Error bars indicate SEM.
1052 Experiments **(D and E)** were performed twice.

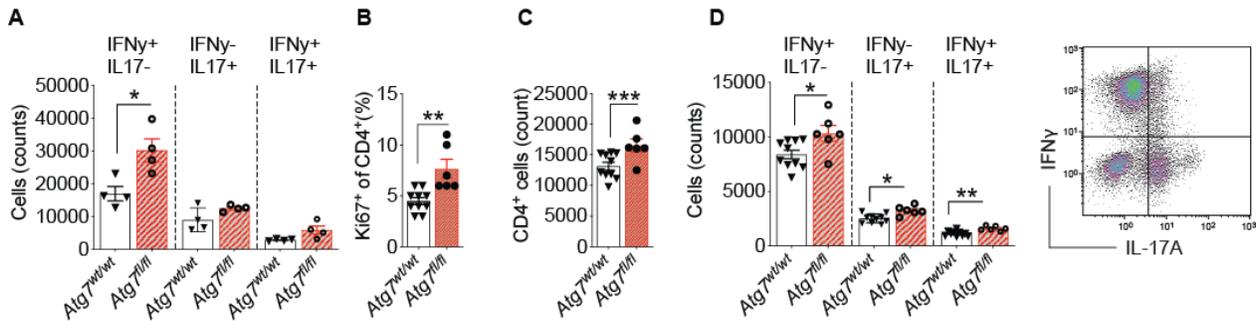
1053 **Supplementary Figure 3**



1054
 1055 **Fig. S3. *Atg7* deficiency induces alterations in microglial transcriptome during Experimental**
 1056 **Autoimmune Encephalomyelitis (EAE).**

1057 Transcriptomic analysis was performed using RNA-sequencing of microglia sorted from naive
 1058 *Atg7^{fl/fl} Cx3cr1^{CreERT2}* (*n* = 4) and *Atg7^{wt/wt} Cx3cr1^{CreERT2}* (*n* = 3) mice, at 21 days p.i. from *Atg7^{fl/fl}*
 1059 *Cx3cr1^{CreERT2}* (*n* = 4, average score 3) and *Atg7^{wt/wt} Cx3cr1^{CreERT2}* (*n* = 3, average score 3) mice,
 1060 and at day 35 p.i. from *Atg7^{fl/fl} Cx3cr1^{CreERT2}* (*n* = 3, average score 1.5) and *Atg7^{wt/wt} Cx3cr1^{CreERT2}*
 1061 (*n* = 4, average score 2.5) mice. REVIGO visualization of Gene Ontology terms for Biological
 1062 Processes for four different patterns of changes: orange - represents changes, considerably more
 1063 pronounced in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia that occurred early in disease (day 21 p.i.) and
 1064 returned to levels in the naïve state by day 35 p.i. (Fig. 2A); blue - represents genes that remained
 1065 downregulated during disease with *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia showing modest changes (Fig.
 1066 2A); yellow - had similar pattern to orange in wild type but *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia
 1067 demonstrated the opposite pattern (Fig. 2A); purple - represents genes that gradually increased their
 1068 expression during disease progression specifically in *Atg7^{fl/fl} Cx3cr1^{CreERT2}* microglia (Fig. 2A).
 1069 Details regarding the differential expression analysis are presented in table S1 and a full list of
 1070 significant functional annotations is provided in table S2.

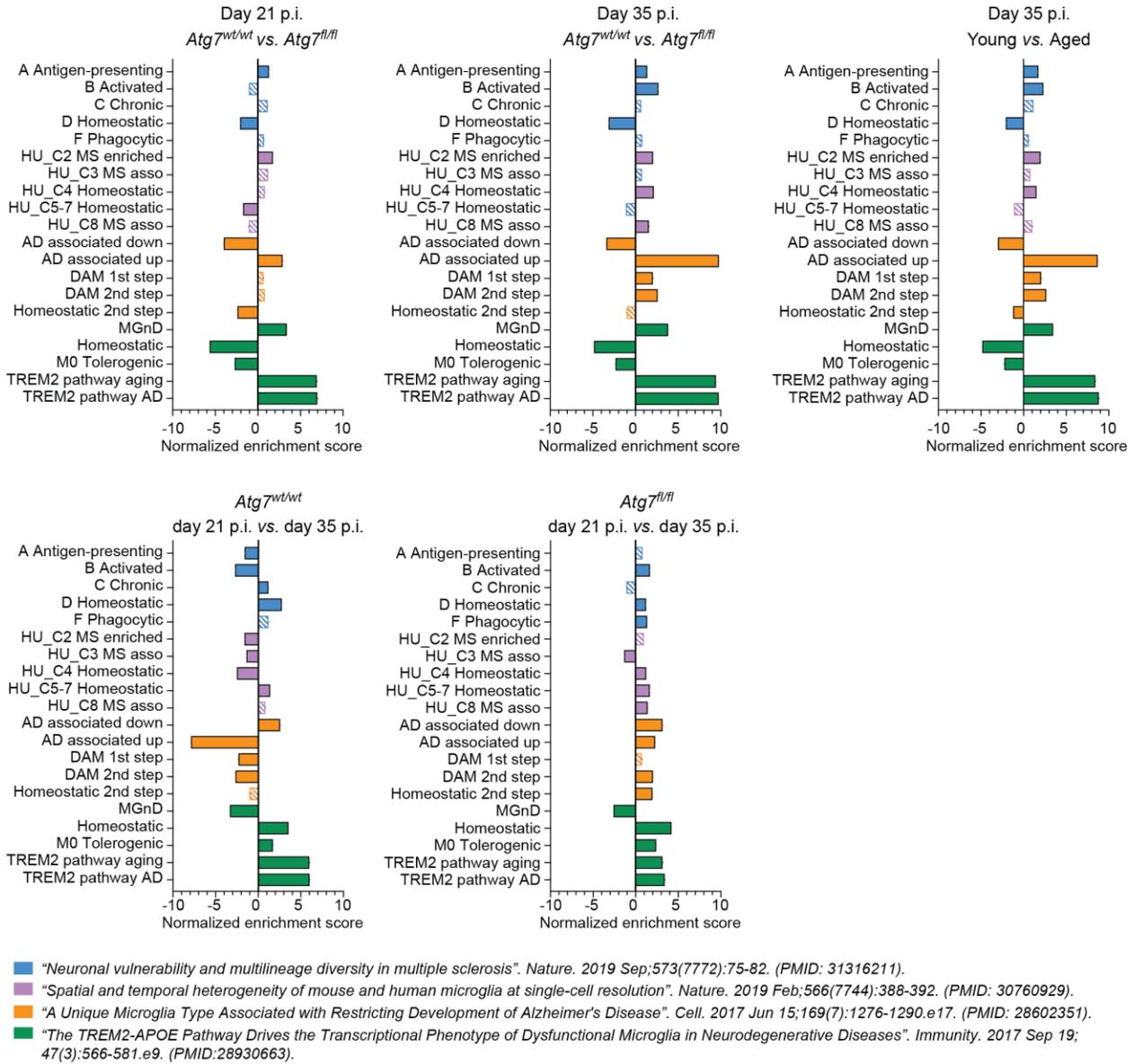
1072 **Supplementary Figure 4**



1073
1074 **Fig. S4. *Atg7* deficiency in microglia increases T cell proliferation and polarization to an**
1075 **inflammatory phenotype.**

1076 (A) Cytokine secretion profile of CD3⁺ T cell infiltrating the spinal cord of mice day 21 p.i. Data
1077 shows cell counts assessed by flow cytometry in *Atg7*^{wt/wt}*Cx3cr1*^{CreERT2} (*n* = 4) and
1078 *Atg7*^{fl/fl}*Cx3cr1*^{CreERT2} (*n* = 4) mice. (B) Flow cytometry detection of Ki67⁺ expression in T cells
1079 sorted from mouse central nervous system day 21 p.i. and co-cultured *in vitro* with
1080 *Atg7*^{wt/wt}*Cx3cr1*^{CreERT2} (*n* = 10) and *Atg7*^{fl/fl}*Cx3cr1*^{CreERT2} (*n* = 6) microglia for 36h. (C) Total
1081 counts of CD4⁺ T cells after co-culture with *Atg7*^{wt/wt}*Cx3cr1*^{CreERT2} and *Atg7*^{fl/fl}*Cx3cr1*^{CreERT2}
1082 microglia as described in (B). (D) Cytokine secretion profile from CD4⁺ T cells in the setup
1083 described in (B), as assessed by flow cytometry. Statistics: Mann-Whitney U-test for all
1084 comparisons (****p* < 0.001, ***p* < 0.01, **p* < 0.05). Error bars indicate SEM. Experiments (A to
1085 D) were performed twice.

1086



1088

1089 **Fig. S5. Gene-set enrichment analysis.**

1090 Gene-set enrichment analysis of transcriptomes from sorted microglia at 21 days p.i.

1091 *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* = 4, average EAE score 3) and *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 3, average EAE

1092 score 3) and at day 35 p.i. *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* = 3, average EAE score 1.5),

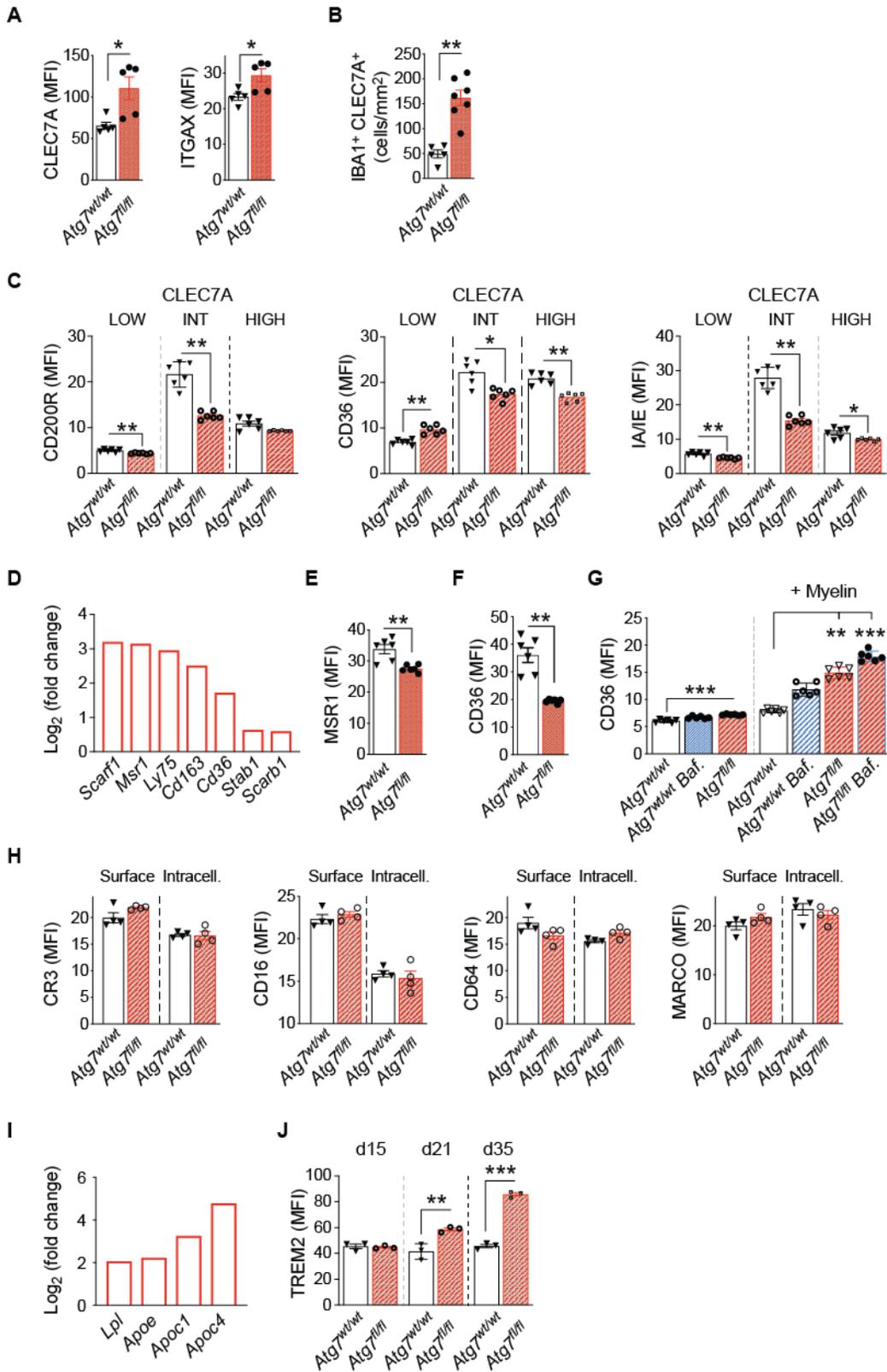
1093 *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 4, average EAE score 2.5) and aged *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 3,

1094 average EAE score 2.5). Filled bars indicate significant enrichment (FDR *q*-value < 0.25)

1095 Microglial reference gene sets from human and mouse models of central nervous system disease

1096 were extracted from publications as listed in the figure. Analysis was performed using whole gene
1097 signatures and the most significant genes defined by publications. All gene sets and references can
1098 be found in table S3. Detailed information on transcriptome analysis can be found in table S1 and
1099 S3.

1100 **Supplementary Figure 6**

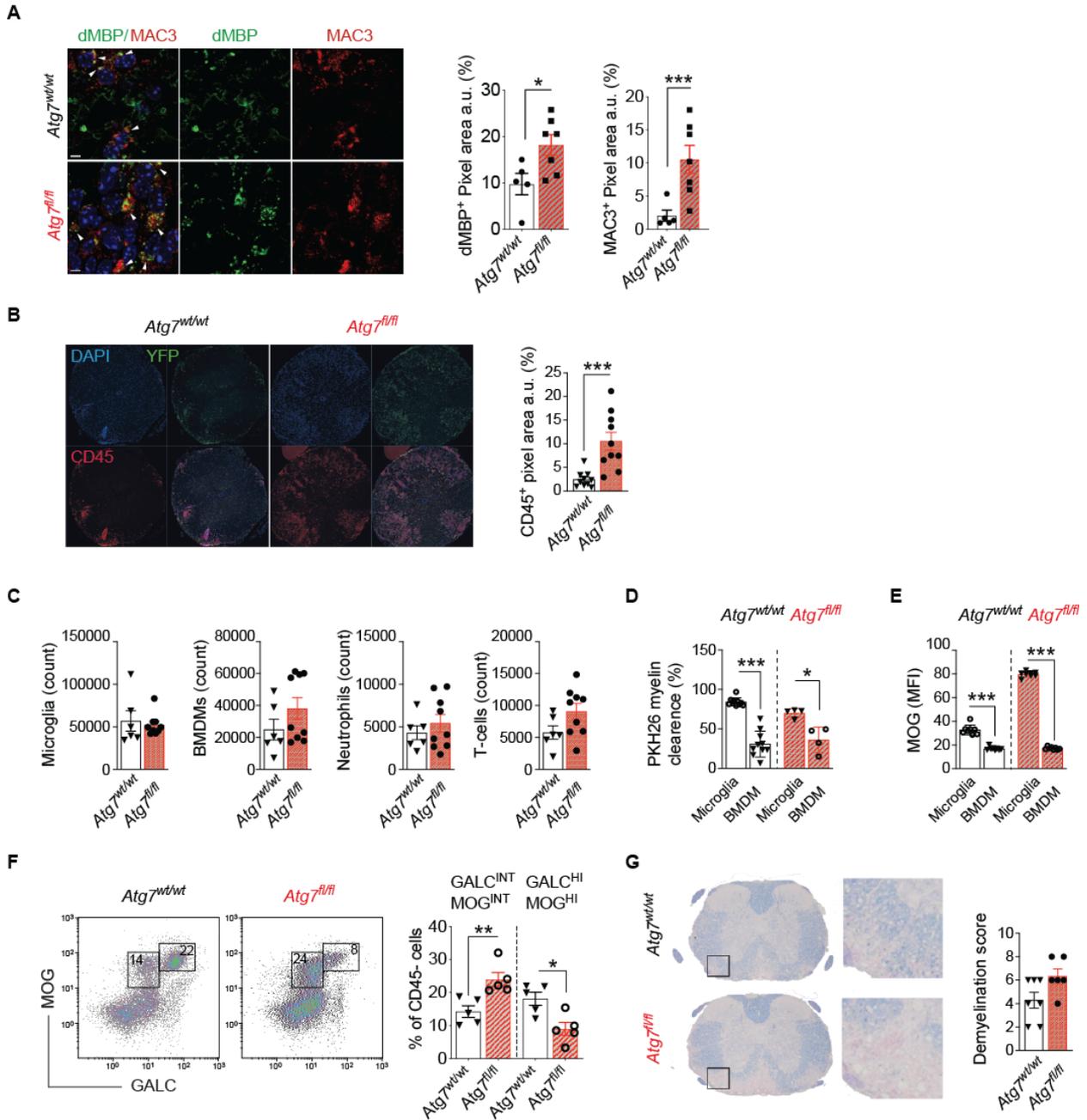


1102 **Fig. S6. *Atg7* deficient microglia have impaired scavenger receptor recirculation associated**
1103 **with increased inflammation and a reduced myelinating oligodendrocyte population in**
1104 **Experimental Autoimmune Encephalomyelitis (EAE).**

1105 (A) Flow cytometry quantification of surface CLEC7A and ITGAX (Cd11c) on
1106 *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 5) and *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* = 5) day 35 EAE microglia. (B)
1107 IBA1⁺CLEC7A⁺ positive cells per mm² in lesion area of central nervous system tissue day 32-37
1108 after EAE induction of *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 5) and *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* = 7). (C) Flow
1109 cytometry assessment of surface CD200R, CD36 and IA/IE in *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 6) and
1110 *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* = 6) microglia populations defined by levels of CLEC7A expression as
1111 defined in Fig. 3, E and F. (D) Fold change increase of scavenger receptor mRNA expression in
1112 *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* = 4) compared to *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 3) microglia (RNA-
1113 sequencing, day 21 p.i., as in Fig. 2). Flow cytometry quantification of surface (E) MSR1 and (F)
1114 CD36 on *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 6) and *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* = 6) day 35 EAE microglia.
1115 (G) Flow cytometry quantification of intracellular CD36 from naïve *Atg7^{wt/wt}* and *Atg7^{fl/fl}*
1116 microglia exposed to myelin 12h *in vitro* w/wo 6h Bafilomycin A1 treatment. All conditions; *n* =
1117 6. (H) Flow cytometry quantification of surface and intracellular density of scavenger receptors
1118 after 24h *in vitro* myelin exposure in *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 4) and *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* =
1119 4) microglia sorted at day 5 p.i. (I) Fold change increase of lipoprotein mRNA expression in
1120 *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (*n* = 4) compared to *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (*n* = 3) microglia (RNA-
1121 sequencing, day 21 p.i., as in Fig. 2). (J) Surface density of TREM2 assessed with flow cytometry
1122 (*n* = 3 for all conditions). Statistics: Mann-Whitney U-test for all comparisons except (G) Kruskal-
1123 Wallis test followed by Dunn's post-hoc test and (J) Unpaired T-test (***) *p* < 0.001, ** *p* < 0.01,
1124 * *p* < 0.05). Error bars indicate SEM. Experiments (A) were repeated three times and experiments
1125 (C, E to H, and J) were repeated twice.

1126

1127 **Supplementary Figure 7**



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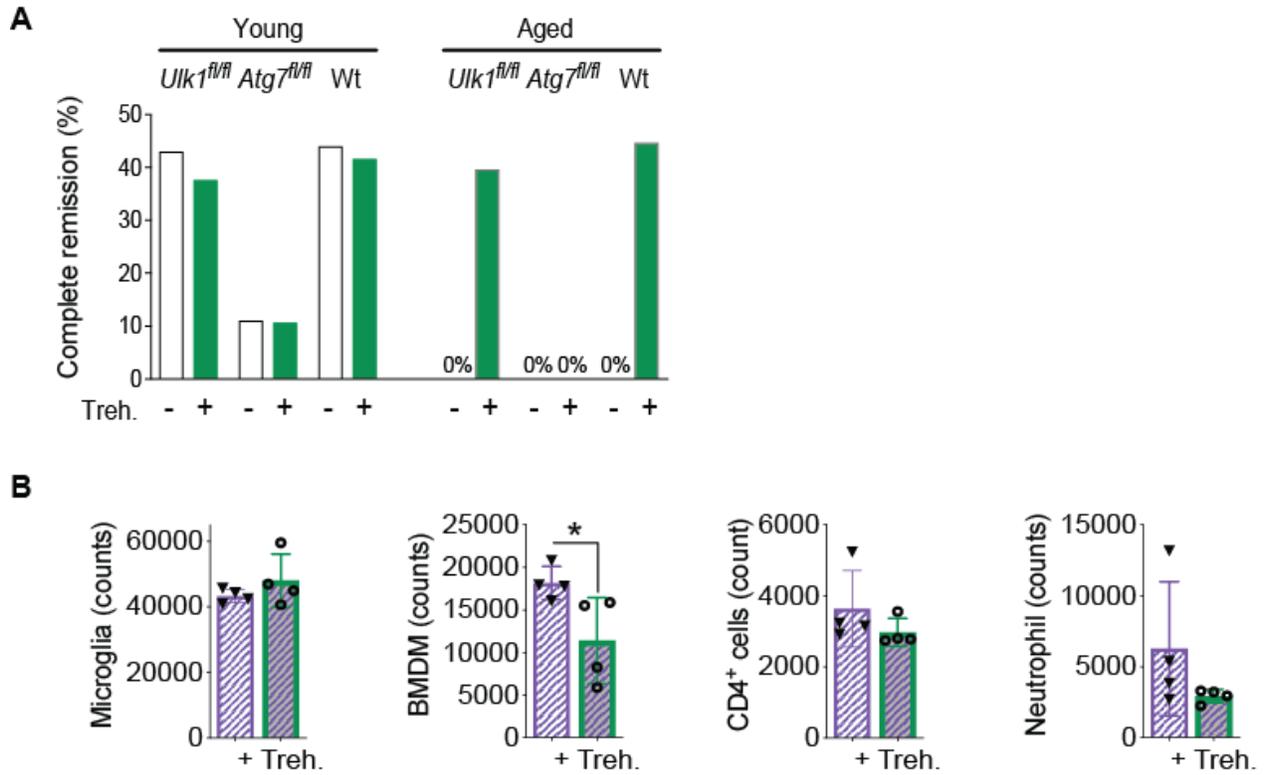
1130 **Fig. S7. Late stage Experimental Autoimmune Encephalomyelitis (EAE) is characterized by**
 1131 **extensive tissue destruction and signs of increased inflammation in mice with *Atg7*-deficient**
 1132 **microglia.**

1133 (A) Zoomed images and immunofluorescence quantification (as in Fig. 3N) of tissue deposits of
1134 myelin debris (dMBP) and density of MAC3⁺ macrophages at day 32-40 p.i. in spinal cord from
1135 *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (dMBP, n = 5; MAC3, n = 10) and *Atg7^{fl/fl}Cx3cr1^{CreERT2}* mice (dMBP, n =
1136 7; MAC3, n = 10). DAPI defines nuclei. (B) Immunofluorescence depicting CD45⁺ cells and YFP⁺
1137 microglia in spinal cord from mice day 32-40 p.i. CD45⁺ pixel area quantified from three pooled
1138 experiments (n = 10 per group). (C) Infiltrating immune cells and microglia from spinal cord of
1139 *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (n = 6) and *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (n = 9) mice at day 35 p.i. assessed by flow
1140 cytometry. Counts indicate numbers of microglia and infiltrating immune cells (bone marrow
1141 derived macrophages (BMDM), Ly6G⁺ neutrophils and CD3⁺ T-cells). (D) *In vitro* myelin
1142 clearance assay of day 35 sorted central nervous system myeloid cells pulsed 3 times with PKH26
1143 labeled myelin (*Atg7^{wt/wt}Cx3cr1^{CreERT2}*; n = 10, *Atg7^{fl/fl}Cx3cr1^{CreERT2}*; n = 4). (E) Flow cytometry
1144 detection of intracellular MOG in *ex vivo* stained microglia and BMDM at day 35 p.i. (n = 9 per
1145 group). (F) Flow cytometry detection of cells from the oligodendrocyte lineage 30 days p.i.
1146 Percentage of CD45⁻GALC⁺MOG⁺, reflecting a myelinating population, in *Atg7^{fl/fl}Cx3cr1^{CreERT2}*
1147 (n = 5) and *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (n = 5) mice. (G) Representative images of the spinal cord stained
1148 with Luxol fast blue showing reduced myelination in the *Atg7^{fl/fl}Cx3cr1^{CreERT2}* spinal cord during
1149 days 27-40 p.i. Graph showing demyelination scores in *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (n = 6) compared to
1150 *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (n = 7) mice. Statistics: Mann-Whitney U-test for all comparisons (*** p <
1151 0.001, ** p < 0.01, * p < 0.05). Error bars indicate SEM. Experiments (D and E) were performed
1152 twice and (C and F) three times.

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1154

1155 **Supplementary Figure 8**



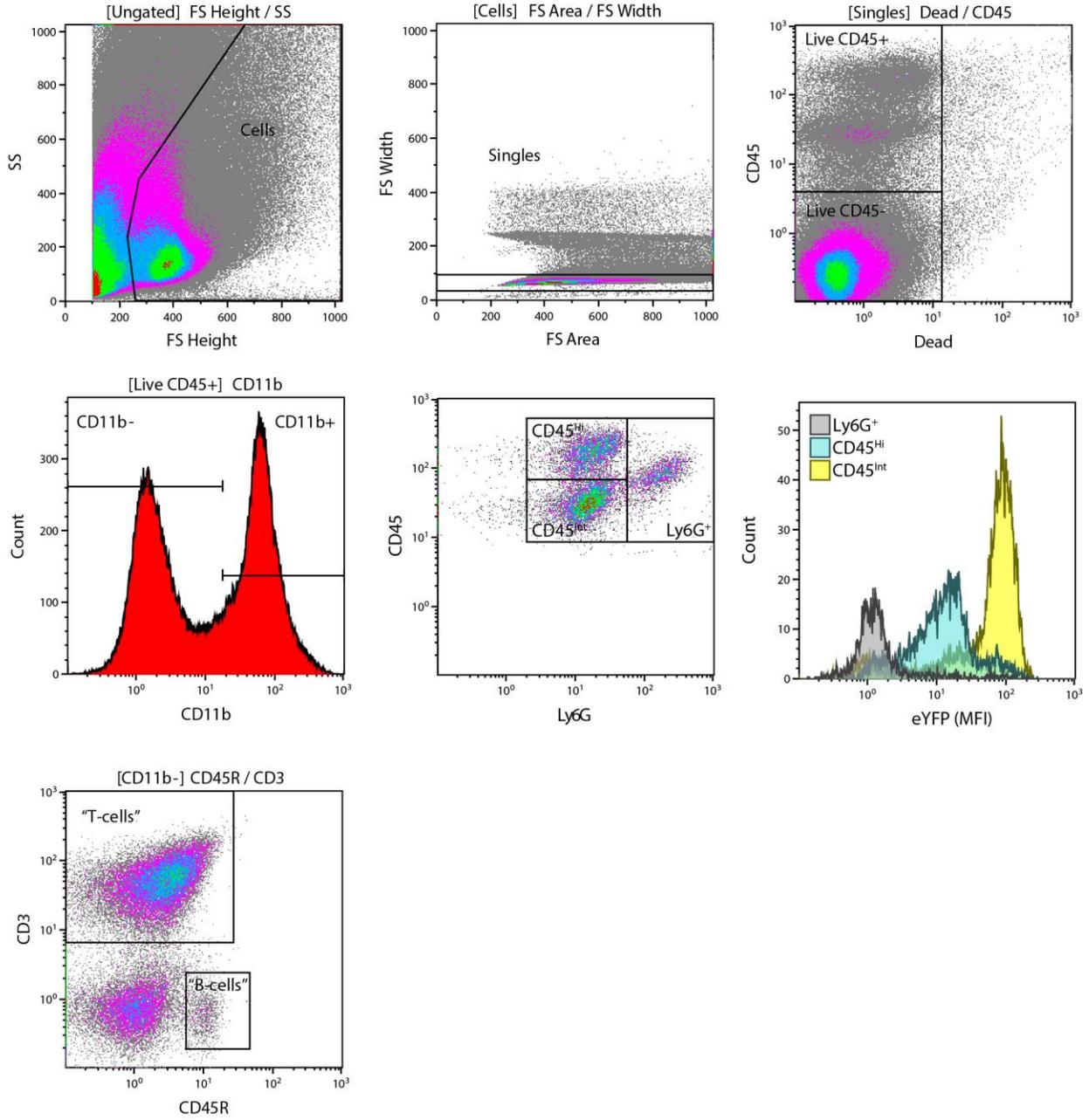
1156
1157 **Fig. S8. Trehalose boosts Experimental Autoimmune Encephalomyelitis (EAE) recovery and**
1158 **decreases immune infiltration in aged mice.**

1159 (A) EAE recovery rate in young (12-22 weeks old), *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (control; *n* = 11, treated;
1160 *n* = 11), *Ulk1^{fl/fl}Cx3cr1^{CreERT2}* (control; *n* = 7, treated; *n* = 9) and *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (control; *n*
1161 = 9, treated; *n* = 9) mice and aged *Atg7^{wt/wt}Cx3cr1^{CreERT2}* (control; *n* = 4, treated; *n* = 6),
1162 *Ulk1^{fl/fl}Cx3cr1^{CreERT2}* (control; *n* = 4, treated; *n* = 5) and *Atg7^{fl/fl}Cx3cr1^{CreERT2}* (control; *n* = 5,
1163 treated; *n* = 5) mice. Mice were treated with Trehalose or water control. (B) Infiltrating immune
1164 cells and microglia from spinal cord of aged mice (*n* = 4) and Trehalose treated aged mice (*n* = 4)
1165 at day 21 p.i. assessed by flow cytometry. Statistics: Mann-Whitney U-test for all comparisons (*
1166 *p* < 0.05). Error bars indicate SEM. Experiments (A and B) were performed twice.

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1171 **Supplementary Figure 9**

Gating strategy for defining cell populations by Flow cytometry



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