Ageing impairs the regenerative capacity of regulatory T cells in central nervous system remyelination

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Abstract

Myelin regeneration (remyelination) is essential to prevent neurodegeneration in demyelinating diseases such as Multiple Sclerosis but its efficiency declines with age. Regulatory T cells (Treg) recently emerged as critical players in tissue regeneration, including remyelination. However, the effect of ageing on Treg-mediated regenerative processes is poorly understood. Here, we show that expansion of aged Treg does not rescue age-associated remyelination impairment due to an intrinsically diminished capacity of aged Treg to promote oligodendrocyte differentiation and myelination. This decline in regenerative Treg functions can be rescued by a young environment. We identified Melanoma Cell Adhesion Molecule 1 (MCAM1) and Integrin alpha 2 (ITGA2) as novel candidates of Treg-mediated oligodendrocyte differentiation that decrease with age. Our findings demonstrate that ageing limits the neuroregenerative capacity of Treg, likely limiting their remyelinating therapeutic potential in aged patients and describe two novel mechanisms implicated in Treg-driven remyelination that may be targetable to overcome this limitation.

Introduction

Myelin is critical to the metabolic support and function of axons in the central nervous system (CNS)[1]. CNS myelin is produced by oligodendrocytes and damage to oligodendrocytes or myelin causes neurological impairment[2–4]. Myelin regeneration (remyelination) is highly neuroprotective and holds potential to restore lost function for patients with demyelinating diseases such as Multiple Sclerosis (MS)[5–7]. Like most regenerative processes, remyelination efficiency declines with age[8,9], contributing to age-associated disease progression and the accumulation of irreversible disability[10]. Despite the critical neuroprotective role of remyelination, there are currently no approved remyelination-enhancing therapies, a key unmet medical need. Thus, understanding how ageing alters the different cellular mechanisms governing myelin regeneration is key to overcoming age-associated

53 remyelination failure. This holds potential to prevent neuronal loss and the accumulation of
54 irreversible cognitive, sensory and motor disabilities.

55 Regulatory T cells (Treg) have recently emerged as key cellular players in tissue regeneration
56 due to both anti-inflammatory functions and capacity to modulate tissue-resident stem cell
57 differentiation[11,12]. Similar crosstalk has also been described in the CNS, where we and others showed
58 that Treg promote oligodendrocyte progenitor cell (OPC) differentiation and CNS remyelination[13–15].
59 The discovery of pro-remyelinating functions of Treg opened a new therapeutic avenue to prevent
60 remyelination decline and associated neurodegeneration. However, whether this important
61 neuroregenerative function of Treg is impaired with age remains unknown. Understanding how ageing
62 affects myelin-regenerative functions of Treg is essential to determine the relevance and suitability of
63 potential Treg-based pro-remyelination therapies in patients of advanced age in MS and other
64 demyelinating diseases.

65 Here, we show that ageing impairs the capacity of natural Treg to drive OPC differentiation *in*
66 *vitro* and myelination *ex vivo*. Surprisingly, this defect was reversible in a young remyelinating
67 environment *in vivo*. Transcriptomic comparison of young and aged Treg identified Melanoma Cell
68 Adhesion Molecule 1 (MCAM1) and Integrin alpha 2 (ITGA2) as novel candidates of Treg-mediated
69 OPC differentiation that decrease with age. This study identifies Treg-driven OPC differentiation as a
70 novel remyelination mechanism that is impaired by ageing and identifies potential therapeutic targets
71 to promote CNS remyelination.

72

73 **Results**

74 **Expansion of Treg does not rescue impaired OPC differentiation in aged mice**

75 We first examined murine Treg frequency with age, and in agreement with previous
76 studies[16,17], found increased numbers and proportions of Treg in aged mice compared to young mice
77 (**Fig. 1 A-D, Sup. Fig. 1 A**). We next verified that OPC differentiation into $CC1^+$ oligodendrocytes is
78 impaired with ageing *in vivo* as previously reported[9,18] (**Fig. 1 E**), despite the endogenous increase in

79 Treg proportions with age. The impairment in oligodendrocyte differentiation with age was not
80 rescued by further expanding Treg numbers in aged mice *in vivo* using IL-2/anti-IL-2 administration
81 (**Fig 1 F-I**). Hence, we hypothesised that the myelin-regenerative capacity of Treg may be intrinsically
82 impaired in aged Treg, irrespective of Treg frequency.
83
84 **Aged Treg demonstrate an impaired intrinsic myelin-regenerative capacity**
85 To compare the capacity of young and aged Treg to support the process of myelin
86 regeneration, we first investigated effects on oligodendrocyte development. To do this, we isolated
87 natural Treg from the spleens and lymph nodes of young (3-4 months) and aged (14-18 months) mice
88 (**Sup. Fig. 1 A,B**) and co-cultured these Treg with neonatal murine OPCs. While neither young nor aged
89 Treg modified OPC proliferation *in vitro* (**Fig. 2 A, B**), young Treg significantly increased OPC
90 differentiation into myelin basic protein- (MBP) expressing oligodendrocytes, in agreement with our
91 previous findings of Treg secretome[13,19]. Aged Treg however, did not enhance OPC differentiation
92 demonstrating an impairment in their capacity to support this vital step in the process of myelin
93 regeneration (**Fig. 2 C-E**).
94 To determine if the inability of aged Treg to support OPC differentiation was functionally
95 relevant to the process of myelin production, we compared the capacity of young and aged Treg to
96 drive myelin ensheathment of axons *ex vivo.* To do this, we co-cultured murine organotypic brain slices
97 with young or aged Treg for 7 days. Young Treg significantly enhanced axonal myelination compared
98 to control slices as described before[13]. However, aged Treg failed to significantly increase myelination
99 in brain slices compared to controls, demonstrating an impairment in the capacity of aged Treg to
100 drive myelin production (**Fig. 2 F, G**).
101
102 **Myelin-regenerative capacity of aged Treg is restored in a young environment**
103 We previously reported that Treg depletion using diphtheria toxin (DT) in B6.129(Cg)-
104 *Foxp3*$^{tm3(DTR/GFP)Ayr}$/J (FoxP3-DTR)[20] mice impaired OPC differentiation and remyelination, which was

105 rescued by the administration of wild type Treg[13]. Thus, we next compared the regenerative capacity
106 of young and aged Treg in a model of lysolecithin-induced spinal cord myelin damage *in vivo.* Following
107 endogenous GFP$^+$ Treg depletion (**Sup. Fig. 1 C, D**), we administered wild type (GFP$^-$) young or aged
108 Treg intraperitoneally and confirmed comparable detection of adoptively transferred Treg in each
109 group (**Sup. Fig. 1 E, F**). We next compared the neuroregenerative capacity of adoptively transferred
110 young and aged wild type Treg to enhance OPC differentiation during remyelination (**Fig. 3 A**). We did
111 not observe any differences in the burden of damage for each experimental group, indicated by the
112 lesion area (**Sup. Fig. 1 H**). There was also no difference in the number of oligodendrocyte lineage
113 cells, quantified by Olig2 immunostaining (**Fig. 3 B-D**), or proliferating OPCs (Olig2$^+$Ki67$^+$) at 14 days
114 post-lesion (dpl) (**Sup. Fig. 1 G, I, J**). As before[13], Treg depletion impaired OPC differentiation into CC1$^+$
115 and ASPA$^+$ oligodendrocytes, which was rescued by adoptive transfer of wild type young Treg.
116 Unexpectedly however, aged Treg injected into young Treg-depleted mice also rescued impaired OPC
117 differentiation, comparable to young Treg, (**Fig. 3 B, C, E, F**). To determine if aged Treg not only rescued
118 OPC differentiation but also restored remyelination, we examined axonal wrapping through the
119 quantification of circular myelin basic protein (MBP$^+$) patterns around neurofilament-H$^+$ (NFH$^+$) axons
120 as an early indication of axonal remyelination. Both, young and aged Treg administered to Treg-
121 depleted mice, restored axonal wrapping to levels comparable to non-depleted controls (**Fig. 3 G-J**).
122 These findings suggest that the age-induced defect of neuroregenerative Treg functions observed *in*
123 *vitro/ex vivo* is reversible in a young systemic environment *in vivo*, and therefore, potentially
124 therapeutically targetable.
125
126 **Ageing significantly alters Treg transcriptome**
127 We next sought to identify how ageing impairs the myelin regenerative functions of Treg by
128 examining gene expression differences in natural Treg purified from young and aged female mice. RNA
129 sequencing (RNAseq) analysis showed that young and aged Treg clustered separately (**Fig. 4 A, C**) as
130 shown in the literature[21,22] and identified 1423 upregulated and 302 downregulated mRNA transcripts

131 in aged Treg compared to young Treg (**Fig. 4 B**). However, there was no difference in the expression
132 of classic Treg markers such as *Foxp3*, *Cd4* or *Il2ra* (**Sup. Fig. 2 B**). Gene Ontology (GO) analysis
133 identified that the differentially expressed genes were associated with pathways linked to the
134 adaptive immune response, lymphocyte-mediated immunity, phagocytosis, cell-surface receptor
135 signalling and membrane invagination (**Fig. 4 D)**. Genes that were upregulated with ageing were
136 enriched for the following GO biological processes: extracellular matrix organisation, cell migration,
137 synapse assembly, axonal guidance, cell adhesion and inflammatory and immune responses (**Fig. 4 E**).
138 On the other hand, mRNA transcripts that were downregulated in aged Treg were associated with
139 defence responses to virus, B cell proliferation and protein folding GO biological processes (**Fig. 4 E)**
140 (GO cellular component, GO Molecular function and Kyoto Encyclopedia of Genes and Genome (KEGG)
141 database pathways analysis is shown in **Sup. Fig. 2**). To identify potential mechanisms underlying how
142 ageing may impair myelin regenerative functions of Treg, we examined expression of genes associated
143 with the Treg tissue repair programme described recently for skin, colon, lung and adipose tisse[23].
144 Unexpectedly, most of the 31 Treg tissue repair programme hallmark/signature genes[23] were in fact
145 upregulated with ageing, except for *Klrb1, Ccr5, Selplg, Bach2, Rad50, Tfrc, Cd84 and Cd164* (**Fig. 4 F,**
146 **G**). No difference in expression was detected in these hallmark genes between male and female
147 natural Treg, showing that gender does not alter the expression of genes associated with the tissue
148 repair programme (**Sup. Fig. 2 C, D**). These data suggest that the mechanism(s) involved in Treg-driven
149 myelin regeneration capacity that are lost with ageing, are likely different to mechanisms from the
150 Treg tissue repair programme described by Delacher et al[23].
151
152 **Mcam and Itga2 are associated with Treg capacity to drive oligodendrocyte formation**
153 To narrow down age-induced changes in Treg that diminish their capacity to induce OPC
154 differentiation, we first investigated whether the putative mechanisms are soluble or contact-
155 mediated; the latter being suggested by the close physical association of Treg with OPCs observed in
156 co-cultures (**Fig. 5A**). Young natural Treg were co-cultured either directly in contact with OPC, or in

157 the same well but separated using a transwell, which allowed for the exchange of secreted molecules
158 but not direct OPC-Treg contact. While young Treg co-cultured directly with OPCs enhanced OPC
159 differentiation into MBP$^+$ oligodendrocytes, this pro-differentiating effect was lost when Treg were
160 physically separated from OPCs (**Fig. 5 B, C**). To identify potential cell-to-cell contact mechanisms
161 responsible for Treg-driven OPC differentiation that may be lost with ageing, we combined our aged
162 Treg RNA sequencing data with a publicly available RNAseq dataset that compares between OPCs,
163 newly differentiated and differentiated oligodendrocytes[24]. We hypothesised that ligand-receptor
164 interactions between Treg and plasma membrane receptors enriched in OPCs underlie the pro-
165 differentiation effect of Treg on OPC. We prioritised ligand-receptor partnerships between Treg and
166 OPCs that were impacted by ageing, to identify mechanisms that could explain the lost myelin
167 regenerative capacity of aged Treg. We then performed a protein-protein interaction analysis using
168 String[25] (**Fig. 5 D**) and prioritised plasma membrane proteins that had the highest decrease in
169 expression for aged Treg and the highest number of potential binding partners amongst OPC-enriched
170 receptors compared to oligodendrocytes (**Fig. 5 E**). We identified the following key candidates: *Ccr7,*
171 *Itga2* (CD49b), *Klrb1c* (NK1.1), *Ly6c1, Mcam* (CD146) and *Sell* (CD62L) (**Fig. 5 D-F**). Next, we used a loss-
172 of-function approach to investigate whether these candidates may confer the lost regenerative
173 capacity of aged Treg, namely, CD62L, NK1.1, LY6C, MCAM (CD146) and ITGA2 (CD49b). Of these five
174 targets, antibody-mediated blocking of MCAM (CD146) and ITGA2 (CD49b) on Treg prior to Treg-OPC
175 co-culture inhibited the capacity of young Treg to drive OPC differentiation *in vitro* as indicated by the
176 fold change in the percentage of MBP+ cells and the MBP+ area per well (**Fig. 5 G-I, Sup. Fig. 2 H, I**).
177 This suggests that MCAM and ITGA2 are previously unidentified mediators of pro-remyelinating
178 functions of Treg and are downregulated with age.
179
180 **Discussion**
181

182 Ageing is one of the strongest risk factors associated with the transition between relapsing-
183 remitting to secondary progressive MS[10], a phase that is characterised by the accumulation of
184 neurodegeneration and irreversible disability. This is partly due to impaired remyelination with age[8,9].
185 Several factors have been linked to age-associated myelin regeneration failure, such as impaired
186 myelin debris phagocytosis by microglia and macrophages[26–28] and reduced capacity of OPCs to
187 differentiate into myelin-forming oligodendrocytes[18,29]. Therapies that enhance remyelination and
188 prevent age-related neurological decline are an urgent unmet need. Beyond their classic immune-
189 modulatory and anti-inflammatory role, Treg have recently emerged as key regulators of tissue
190 homeostasis and regeneration in several tissues[27], including the CNS, where we have previously
191 identified Treg as key promoters of remyelination[13]. However, how ageing affects the capacity of Treg
192 to enhance myelin regeneration had not been addressed.

193 Peripheral Treg increase with age[16,21,30], a somewhat paradoxical finding given the tissue-
194 regenerative functions of Treg and the impairment of tissue regeneration with age. Here, we further
195 expanded the murine Treg population *in vivo* and showed that despite this increased cell number,
196 aged-associated remyelination failure was not rescued. These data suggest that with ageing, Treg have
197 an intrinsically impaired myelin regenerative capacity. Ageing is known to enhance Treg senescence,
198 limit Treg proliferation[16,21] and there are conflicting reports on the effect of ageing on Treg
199 immunosuppressive capacity[16,21]. Additionally, previous work on muscle regeneration showed that
200 aged Treg have an impaired migratory capacity that hinders muscle regeneration[31]. In a model of
201 influenza infection, aged Treg demonstrated an impaired capacity to support lung repair, which was
202 associated with a cell autonomous impairment in their regenerative programme[22]. Here, we sought
203 to determine whether aged Treg have an intrinsically impaired regenerative capacity to drive CNS
204 myelin regeneration combining *in vitro, ex vivo* and *in vivo* approaches.

205 Purified aged Treg failed to significantly enhance OPC differentiation into MBP-expressing
206 oligodendrocytes or the myelination of cerebellar slices, which is in striking contrast to young Treg,
207 which robustly drove both biological processes. These data suggest that ageing is associated with a

208 cell-intrinsic impairment of the regenerative capacity of Treg in CNS myelin regeneration. Given that
209 IL-2-mediated Treg expansion *in vivo* is in clinical trial for MS (clinical trial number NCT02424396), our
210 findings are of translational importance as they suggest that Treg expansion as a potential pro-
211 remyelination therapy may be limited in older populations.

212 We next investigated whether the regenerative capacity of aged Treg could be restored, and
213 therefore therapeutically targetable. Surprisingly, aged Treg rescued OPC differentiation and myelin
214 wrapping similarly to that of young Treg in an *in vivo* model of young spinal cord remyelination. In
215 contrast to findings in the lung[22], the CNS remyelinating capacity of Treg can be partially restored by
216 a young environment. Previous studies identified a tissue repair transcriptomic signature in the skin,
217 adipose tissue, and the lung[23]. Unexpectedly, very few of these hallmark Treg tissue repair genes were
218 downregulated in aged Treg in our transcriptomic profiling studies. These data indicate that although
219 some Treg regenerative mechanisms may be common across tissues, other mechanisms may be
220 tissue-specific or process- specific[32].

221 We recognised that the impairment of myelin regenerative Treg functions with age presented
222 a new avenue to discover novel mechanisms of how Tregs support CNS remyelination and the
223 potential to identify new therapeutic targets to boost remyelination. Indeed, using a combined
224 approach of Treg/OPC transcriptomic dataset cross-refencing with *in vitro* antibody blocking assays,
225 we identified MCAM and ITGA2 as novel candidate mediators of Treg-driven OPC differentiation.
226 These two candidates are downregulated in aged compared to young Treg, likely interact directly with
227 OPCs and inhibit young Treg-driven OPC differentiation when blocked *in vitro*.

228 MCAM has previously been described as a cell-adhesion molecule contributing to brain
229 inflammation by facilitating pathogenic T-cell extravasation into the CNS[33–35]. However, our study is,
230 to our knowledge, the first report showing that MCAM signalling can promote OPC differentiation.
231 Together with a recently published abstract examining Treg in MS patients (Poster 4[36]) these data also
232 represent the first evidence that Treg express MCAM. We have also identified ITGA2 as a novel
233 mediator of Treg-driven OPC differentiation that is decreased with age. This aligns with previous

234 reports showing that other integrins are involved in oligodendrocyte development[37] and shows that
235 Treg are additional cellular contributors to integrin-mediated OPC differentiation.
236 Collectively, these studies have identified that ageing is associated with a cell-intrinsic
237 impairment of the myelin-regenerative functions of Treg. This discovery increases the range of
238 remyelination-associated mechanisms that are impaired with age[8,9,18,26–29,38]. Encouragingly, we have
239 shown that this impairment can be rescued in a young environment *in vivo*. We have also identified
240 two novel mediators of Treg-driven OPC differentiation, MCAM and ITGA2. The findings from this
241 study identify novel mechanisms involved in CNS regeneration which may hold therapeutic potential
242 for patients with demyelinating diseases.
243
244 **Methods**
245
246 **Animals**
247 All mice were on a C57BL/6J background and were either bred in-house or purchased from Charles
248 River Laboratories, UK. Foxp3-DTR mice were a kindly provided by Prof. Alexander Rudensky
249 (Memorial Sloan Kettering Institute, New York). Neonatal C57BL6/J P3-P7 pups were used for OPC and
250 brain slice preparations, which were combined with natural Treg were isolated exclusively from young
251 (3-4m) and aged (15-18m) C57BL6/J male mice for use with neonatal mixed sex pups. For *in vivo* Treg
252 adoptive transfer studies, natural Treg were isolated from young (3-4m) and aged (15m) C57BL6/J
253 female mice, and were injected into both, female and male recipient Foxp3-DTR mice (2-4m). To
254 deplete Foxp3^{+} cells in Foxp3-DTR mice, diphtheria toxin was administered as described below. All
255 animal maintenance and experiments were done in accordance with the UK Home Office regulation
256 (Project Licences 2789 and 2894) and were approved by the Queen's University Belfast's Animal
257 Welfare and Ethical Review Committee.
258
259 **Lysolecithin-induced spinal cord demyelination *in vivo***

260 Spinal cord demyelination was induced as described previously[13]. In brief, demyelination was induced
261 in the ventral white matter funiculus of the thoracic spinal cord (between vertebrae T11-12 or T12-
262 13) by the injection of 1.2 µL of 1% (w/v) L-α-Lysophosphatidylcholine (Lysolecithin; Sigma-Aldrich)
263 under general anaesthesia. At 10- or 14-days post lesion (dpl), mice were terminally anaesthetised
264 with intraperitoneal (i.p.) pentobarbital injections and transcardially perfused with ice-cold phosphate
265 buffered saline (PBS) followed by 4% paraformaldehyde (PFA) (Sigma-Aldrich). Spinal cords were
266 dissected and immersed overnight in 4% PFA at 4 °C. Next, spinal cords were cryoprotected with 30%
267 sucrose (Sigma-Aldrich) in PBS for 72h and snap-frozen in OCT (Tissue-Tek). Frozen spinal cords were
268 cryosectioned at 12 µm thickness and immunostained as described below.
269
270 ***In vivo* Treg expansion**
271 Aged mice (15m) were intraperitoneally injected with IL-2/anti-IL-2 antibody complexes to expand
272 endogenous Treg[39]. In brief, murine IL-2 (1 µg; Peprotech) was mixed with anti-mouse IL-2 (5 µg;
273 Bioxcell, Clone JES6-1A12) in PBS at 37 °C for 30 min. Each mouse was injected daily for 3 consecutive
274 days with IL-2 (1 µg) and anti-IL-2 (5 µg) in 200µl of PBS. Adequate Treg expansion was assessed by
275 flow cytometry as described below. Spinal cord demyelination was induced on the day after the third
276 injection of IL-2/anti-IL-2, by injecting lysolecithin into the ventrolateral white matter as described
277 above. Treg expansion was examined by flow cytometry as described below on day 10dpl, while OPC
278 differentiation was examined at 10 dpl.
279
280 ***In vivo* Treg depletion and adoptive transfer of wild type natural Treg**
281 Young (2-4m) male and female Foxp3-DTR mice were injected daily i.p. with diphtheria toxin (DT; 0.04
282 µg/g of body weight; Sigma, Cat. No. D0564) for 3 days prior to demyelination. To maintain
283 endogenous Treg depletion during remyelination, DT (0.04 µg/g of body weight) was injected i.p. every
284 fourth day. Control animals received 200 µl of saline i.p. Depletion was confirmed at the endpoint by
285 flow cytometric analysis of endogenous GFP^+ natural Treg in blood, spleen, and lymph nodes (see

286 below). To adoptively transfer young (2-4m) and aged (15m) natural Treg to Treg-depleted mice, we
287 immunomagnetically isolated natural Treg from wild type female mice using a $CD4^+$ and $CD25^+$ Treg
288 isolation kit (STEMCELL Technologies) as described below. In the 24h prior to lysolecithin-induced
289 demyelination, mice were injected i.p. with 10^6 wild type natural Treg which are resistant to DT.
290 Adequate reconstitution was evaluated by flow cytometry for Treg in lymph nodes at the endpoint
291 (see below).
292
293 **OPC isolation and culture**
294 OPCs were isolated from P3-P7 mice based on A2B5 expression. Briefly, pups were culled by an
295 overdose of pentobarbital (200 mg/mL, 20µl per mouse) and whole brain was dissected and placed in
296 ice-cold Hibernate-A (ThermoFisher Scientific). Dissected brain was minced using scalpels, transferred
297 to a 15mL tube, and centrifuged for 1 min at 100 g and 4 °C. Hibernate-A medium was aspirated, and
298 the pellet was resuspended in 5 mL dissociation media containing 165U of Papain (Worthington) and
299 DNAse type I (40 µg/mL; Worthington) in hibernate A for 30 min at 37 °C. Upon digestion papain was
300 washed off with Hanks Buffered Salt Solution (HBSS) (ThermoFisher Scientific) and cells were
301 centrifuged for 5 min at 300 g and 4 °C. The pellet was resuspended in 4 mL trituration buffer
302 (Hibernate-A with 2% B27; ThermoFisher Scientific) and 2 mM sodium pyruvate (ThermoFisher
303 Scientific) and gently triturated ten times using a 5 mL pipette. Tissue was left to settle for 2 min at
304 room temperature (RT), and supernatant was transferred to a clean 50 mL tube through a 70 µm
305 strainer (Corning). Remaining tissue was exposed to another 2 mL of trituration buffer and gently
306 triturated using a glass polished pipette. After trituration, tissue was left to settle for 2 min at RT and
307 supernatant was transferred to the tube through the 70 µm strainer. This step was repeated another
308 time with a glass fire-polished pipette of decreased diameter and then with a 1 mL pipette. To further
309 remove debris from the cell suspension, 11.5 mL of 90% Percoll (GE Healthcare) diluted in 10X PBS
310 (ThermoFisher Scientific) was added and topped up to a final volume of 45 mL with Dulbecco's
311 Modified Eagle's medium (DMEM) (ThermoFisher Scientific). The cell suspension was then centrifuged

312 at 800 g for 20 min at 4 °C. Upon centrifugation, the pellet was washed with HBSS and then
313 resuspended in 10 mL magnetic-activated cell sorting (MACS) buffer (Hibernate-A with 2 mM sodium
314 pyruvate, 2% B27, 0.5% BSA; ThermoFisher Scientific), 2 mM EDTA (Sigma-Aldrich) and 10 µg/mL
315 insulin (Sigma-Aldrich), and incubated for 30 min at 37 °C in a 10 cm petri dish coated with BSC1
316 Griffonia Simplicifolia Lectin (BSL1, 5 µg/mL)(Vektor Labs) to remove microglia. Then, the supernatant
317 was collected, and cells were counted and centrifuged at 300 g for 5 min at 4 °C. The pellet was
318 resuspended and incubated with 2 µg of A2B5 antibody (Millipore, Clone A2B5-105) in 500 µL of MACS
319 buffer per 10 million cells for 25 min on ice, with gentle resuspensions every 10 min. Cells were washed
320 with HBSS and spun at 300 g for 5min at 4 °C (Miltenyi Biotec). The pellet was resuspended and
321 incubated with 80 µL of MACS buffer and 20 µL of anti-IgM microbeads (Miltenyi Biotec) for 15 min.
322 Then, cell suspensions were centrifuged at 300 g for 5 min at 4 °C, resuspended in 0.5 ml MACS buffer
323 and placed in a MACS mini column (Miltenyi Biotec) on a MiniMACS Separator (Miltenyi Biotec) and
324 washed with 1.5mL MACS buffer. Once the liquid passed completely through the column, the column
325 was removed from the stand and placed in a new 15 mL tube, where 1 mL of OPC media was added
326 to the column and plunged through to elute the OPCs. OPC media contained DMEM, 2% B27, sodium
327 pyruvate (2 mM), insulin (5 µg/mL), Trace Elements B (0.01%; Corning), Forskolin (5 µM; Sigma-
328 Aldrich), Biotin (10 ng/mL; Sigma-Aldrich), Penicillin-streptomycin-glutamine (1%; ThermoFisher
329 Scientific), N-acetyl cysteine (60 µg/mL; Sigma-Aldrich) and 1% SATO stock solution. SATO stock
330 solution contained bovine serum albumin (BSA) fraction V (0.1 mg/mL; ThermoFisher Scientific),
331 sodium selenite (4 µg/mL; Sigma-Aldrich), putrescine (1.61 mg/mL; Sigma-Aldrich), apo-transferrin
332 (0.1 mg/mL; Sigma-Aldrich) and progesterone (4 µg/mL; Sigma-Aldrich). The purified population was
333 counted and diluted in OPC media for plating. OPCs were plated at a density of 3,000 cells per well in
334 96-well plates (Falcon) previously coated with poly-L-lysine (10 µg/mL; Sigma-Aldrich) and laminin (10
335 µg/mL; Sigma-Aldrich) diluted in DMEM. OPCs were plated with PDGFaa (20 ng/mL; Peprotech) and
336 NT-3 (10 ng/mL; Peprotech). The following day two thirds of the media was removed (100 µl) and 100
337 µl of fresh OPC media with PDGFaa (20 ng/mL) and NT3 (10 ng/mL) were added per well. Cells were

338 incubated at 37°C and 5% CO_2 for two days. On the third day, media was completely removed and
339 20,000 Treg in fresh media were added as indicated below.
340 For transwell assays, OPCs were selected with anti-PDGFRα panning. After microglia depletion in BSL1-
341 coated petri dishes, the supernatant was transferred to PDGFRα panning plates and incubated at RT
342 for 1 h. PDGFRα panning plates were coated with goat-anti-rat IgG (H+L) (7.5 µg/mL; ThermoFisher
343 Scientific) in PBS with 0.2% BSA and rat anti-PDGFRα (1.5 µg/mL in PBS; BD Biosciences) with 0.2% BSA
344 for 3 h. Supernatants were removed, cells were washed 5-8 times with PBS and the attached OPCs
345 were scraped, counted, and replated in 24-well plates (Sarstedt) at 10,000 cells per well. On the third
346 day *in vitro* 50,000 Treg were added either directly into the well with OPCs or in a transwell (Millipore)
347 above the OPCs.
348 After 6 days of OPC-Treg co-culture cells were fixed for 15 min with 4% PFA and washed twice with
349 PBS prior to immunostaining. Cells were blocked with 5% donkey serum (Sigma-Aldrich) with 0.1%
350 Triton-X-100 (Sigma-Aldrich) in PBS for 1h at RT. OPCs were then stained overnight at 4 °C with primary
351 antibodies diluted in blocking solution against Olig2 (1:500, Bio-techne), CNPase (1:500, Sigma-Aldrich,
352 clone 11-5B), MBP (1:500, Millipore, clone 12), Ki67 (1:300, Abcam, clone SP6), NG2 (1:200, Millipore),
353 and CD3 (1:500, eBioscience). Cells were then washed three times with PBS and incubated for 1 h at
354 room temperature with secondary antibodies diluted in blocking buffer (Alexa fluor (AF) 488 donkey
355 anti- rabbit (1:500; ThermoFisher Scientific), AF568 donkey-anti- rat (1:500; Abcam), AF647 donkey
356 anti-mouse (1:500; Abcam), AF647 donkey anti-rabbit (1:500; ThermoFisher Scientific) and AF755
357 donkey anti-goat (1:500; ThermoFisher Scientific) and Höechst (1:10,000; Sigma-Aldrich). Cells were
358 then washed with PBS three times and stored in 150 µL PBS/well for cell imaging.
359 Cell imaging was performed using the CellInsight CX5 high content imaging system (ThermoFisher
360 Scientific) for 96-plates and EVOS for 24-well plates. Twenty-five separate fields of view of each well
361 in 96-well plates and the cell populations of interest were quantified using CellInsight CX5 analysis
362 software and the SpotCounts programme. For 24-well plates we imaged randomly 5 fields of view per
363 well at 20X magnification and images were analysed manually with Fiji.

364

365 **Natural Treg isolation**

366 Young (2-4 m) and aged (15-18 m) male mice were culled by CO_2 overdose. Spleens and lymph nodes
367 were removed and mashed through a 70 µm strainer with a 2 ml syringe plunger. $CD4^+$ T cells were
368 immunomagnetically purified by negative selection according to manufacturer's instructions
369 (STEMCELL Technologies). Isolated $CD4^+$ T cells were then subjected to a $CD25^+$ cell isolation (STEMCELl
370 Technologies) using releasable magnetic spheres (STEMCELL Technologies) according to the
371 manufacturer's protocol. Purity was confirmed by flow cytometry for CD4, CD25 and Foxp3 (**Sup. Fig.**
372 **2A**). For i.p. injections in adoptive transfer experiments, cells were resuspended at 10^6 cells per 200
373 µL in saline. For OPC-natural Treg co-cultures 20,000 natural Treg were resuspended in 150 µl of OPC
374 Brainphys-based media and added to OPCs in 96-well plates. For 24-well transwell plate experiments
375 50,000 natural Treg were resuspended in 250 µl OPC-BrainPhys media and added to transwells above
376 cultured OPCs. OPC BrainPhys-based media contains BrainPhys (STEMCELL Technologies), B27 (2%;
377 ThermoFisher Scientific), Glutamax (0.5 mM; ThermoFisher Scientific), N-acetyl-cysteine (60 µg/mL),
378 SATO (1:100, as described above) and insulin (5 µg/mL). In the case of brain slices, 50,000 Treg were
379 diluted in 5 µL of brain slice media and dropped directly onto brain slices.

380 For natural Treg mechanistic studies, OPCs and Treg were isolated as described above. Prior to OPC-
381 natural Treg co-culture, 120,000 Treg were transferred to a 1.5 mL microcentrifuge tube and
382 centrifuged at 400 g for 5 min at 4 °C. Treg were resuspended in 100 µL of PBS with 2% FCS and a
383 blocking antibody (20 µg/mL) or corresponding isotype were added per tube. Natural Treg were
384 incubated with the blocking antibody for 45 min on ice and then cells were washed with PBS and
385 centrifuged for 10 min at 400 g and 4 °C. Cells were resuspended in 900 µl of OPC BrainPhys-based
386 media (see above) and 20,000 Treg in 150 µL of media were added per well in 96-well plates. As
387 previously, Treg were co-cultured with OPCs for 7 days *in* vitro (DIV) and then fixed and stained as
388 described above. Blocking antibodies used were anti-NK1.1 PE (eBioscience, clone PK136), anti-CD62L-
389 APC (eBioscience, clone Mel14), anti-Ly6c1-APC (eBioscience, clone RB6-BC5), rat-isotype-APC

390 (eBioscience), anti-Itga2 (Abcam, clone EPR5788), anti-MCAM (Abcam, clone EPR3208) and rabbit-
391 isotype (Vektor labs).
392
393 **Organotypic brain slice cultures**
394 Brainstem slices from male and female P3 C57BL/6J mice were prepared at 300 µm thickness using a
395 McIlwain Tissue Chopper as described previously[13]. Slices were separated and placed on Millicell
396 inserts in individual wells of a 24-well plate (Millipore) with 250 µl brain slice medium containing 46.6%
397 minimum essential medium (ThermoFisher Scientific), 25% Earls balanced salt solution (Sigma-
398 Aldrich), 25% heat-inactivated horse serum (Thermofisher Scientific), 1% penicillin-streptomycin
399 (Thermofisher Scientific), 1% glutamax (Thermofisher Scientific) and 1.4% D-glucose (Sigma). Brain
400 slices were incubated at 37 °C and 5% CO_2 overnight and then media was fully replaced. On day 3,
401 media was changed again and 50000 young or aged Treg were added directly on top of each slice in a
402 5 µl droplet. Brain slices were cultured for a further 7 DIV, with media changes every other day. On
403 day 10 (7 days post-treatment), brain slices were fixed with 4% PFA for 45 min and immunostained. In
404 brief, brain slices were blocked for 2 h in 10% normal donkey serum (Sigma-Aldrich), 1 mM Hepes
405 (Thermofisher Scientific), 1% BSA (ThermoFisher Scientific) and 0.5% Triton X-100 (Sigma-Aldrich) in
406 PBS. Slices were incubated with primary antibodies for rat anti-MBP (1:500; Millipore, clone 12) and
407 chicken anti-NFH (1:500; EncorBiotech, polyclonal) in blocking buffer at 4 °C for two overnights. Brain
408 slices were then washed with PBS-0.01% triton three times for 30 min. Slices were incubated with
409 secondary antibodies (AF488 donkey anti-chicken IgY (1:500; Abcam, polyclonal) and AF568 donkey
410 anti-rat IgG (1:500; Abcam, polyclonal) in blocking buffer overnight at 4 °C. Brain slices were washed
411 twice with PBS-0.01% triton and then incubated with Höechst stain (1:20000; Sigma-Aldrich) for 10
412 min. Slices were washed with PBS and mounted using fluoromount G (Thermofisher Scientific).
413 Imaging was performed using a Leica SP8 confocal microscope, using a 63X oil objective and the Leica
414 Navigator at 0.5 µm intervals over 10 µm. Four fields of view per brain slice were selected for imaging
415 based on NFH and Höechst staining but blinded to MBP. To quantify myelination index, the area

416 stained by NFH, MBP and the colocalising area of NFH^+MBP^+ were measured per stack in Fiji[40] using a
417 Fiji plug-in developed and kindly provided by the Williams laboratory at the University of Edinburgh[41].
418 Then, the ratio between total NFH^+ and NFH^+MBP^+ areas (myelination index) was calculated per z-
419 stack and the average of the myelination index per slice was calculated.
420
421 **Flow cytometry**
422 Spleens and lymph nodes were mashed through a 70 µm strainer. For splenocytes, cells were exposed
423 to red blood cell lysis buffer (STEMCELL Technologies) for 2 min at room temperature. Both, lymph
424 nodes and splenocytes were then washed with PBS and centrifuged at 300 g for 5 min at 4°C. Cells
425 were resuspended in 200 µL PBS and stained with a cell viability dye with eFluor 455-UV viability dye
426 (1:2000; ThermoFisher Scientific) and cell surface stained with antibodies for CD4 (1:500; eBioscience,
427 clone RM4.5) and CD25 (1:500; eBioscience, clone PC61.5) for 15 min at RT. Cells were washed with
428 flow cytometry staining buffer (FCSB) (2% FCS in PBS) and centrifuged at 300 g for 5 min at 4 °C. Cells
429 were then fixed with Fix & Perm A (ThermoFisher Scientific) for 10 min at RT. Fixative was washed off
430 with FCSB and centrifuged at 300 g for 5 min at 4 °C. The pellet was resuspended in PBS and data were
431 acquired on a FACSCanto II. In the natural Treg depletion experiment, presence of endogenous natural
432 Treg was determined by the expression of GFP. To determine the purity of natural Treg isolations or
433 the extent of natural Treg reconstitution by adoptive transfer, cells were washed and centrifuged for
434 5 min at 300 g and 4 °C. Cells were then resuspended in 100 µL Fix & Perm B (ThermoFisher Scientific)
435 with an anti-Foxp3 antibody (1:100; eBioscience, clone FJK-16S) overnight at 4 °C. Cells were then
436 washed with FCSB and centrifuged at 300 g and 4 °C for 5 min. Cells were then resuspended, data
437 were acquired on a FACSCanto II and analysed using FlowJo software version 9.0 (BD). To calculate
438 cell numbers, singlets were identified by FSC-H versus FSC-A and viable cells gated for CD3 and CD4,
439 and subsequently CD25 and $Foxp3^+GFP^+$ and $Foxp3^+GFP^-$ cells. To evaluate young and aged Treg
440 numbers in blood, 30 µL were incubated with 100 µL of the viability and cell surface antibody mix for
441 30 min at room temperature. Cells were then washed with FCSB and centrifuged at 300 g for 5 min at

442 4 °C. After resuspension, cells were fixed and lysed in 100 µL of OptiLyse B (Beckman Coulter) for 10
443 min at RT, washed with distilled water and then after 15 min, acquired on a FACSCanto II. After initial
444 data acquisition, cells were centrifuged at 300 g for 5 min at 4 °C and incubated overnight with anti-
445 Foxp3 antibody in Fix & Perm B, as described above. Cells were washed and data were acquired on a
446 FACSCanto II.
447
448 **Immunofluorescence staining of CNS tissue**
449 Spinal cord sections were dried for 30 min at RT and washed for 10 min in PBS. For Olig2, Ki67, CC1
450 and ASPA staining, spinal cord sections underwent antigen retrieval at 85 °C for 10 min with 1X citrate
451 buffer pH 6.0 (Sigma-Aldrich). Tissue sections were washed with PBS and permeabilised with 1%
452 Triton-X-100 in PBS for 30 min at RT. Sections were washed with PBS and incubated with blocking
453 solution (5% donkey serum; Sigma-Aldrich) diluted in TBS with 0.25% tween (Sigma-Aldrich) for 1h at
454 RT. If spinal cord sections were incubated with a primary antibody raised in mouse (e.g. anti-CC1)
455 sections underwent an additional 1 h blocking step at RT using Mouse on Mouse (M.O.M) blocking
456 reagent (Vector labs) in 5% donkey serum. Then spinal cord sections were incubated with primary
457 antibodies against Olig2 (1:500; Bio-Techne, polyclonal), anti-APC (1:400, Abcam, clone CC1), anti-
458 ASPA (1:300, Millipore, polyclonal), anti-NFH (1:500; Abcam, polyclonal), anti-MBP (1:500; Millipore,
459 clone 12) or anti-Ki67 (1:300; Abcam, clone SP6) overnight at 4 °C. Sections were washed with Tris-
460 Buffered-Saline (TBS) (ThermoFischer Scientific) with 0.25% Tween (Sigma-Aldrich). Secondary
461 antibodies including donkey anti-goat AF488, donkey anti-mouse AF568, donkey anti-rabbit AF647 (all
462 1:500; ThermoFisher Scientific), donkey anti-rat AF568 (1:500; Abcam), donkey anti-rabbit AF647
463 (1:500, Abcam) and Höechst (1:10000; Sigma-Aldrich) were added for 1 h at room temperature. Spinal
464 cord sections were then washed with TBS-0.25% Tween twice and mounted with fluoromount G.
465 Image acquisition was performed using the Leica TIRF and Leica DM5500 widefield fluorescent
466 microscopes. Further image processing was performed in Fiji[40] software and analysis was undertaken

467 by blinded manual counting. For NFH and MBP wrapping analysis, images were quantified using Cell
468 Profiler and Cell Profiler Analyst softwares[42,43].
469
470 **RNA sequencing and analysis**
471 Young (2-4m) and aged (16m-20m) natural Treg were isolated from spleen and lymph nodes by
472 magnetic-activated cell sorting and purity was checked by flow cytometry as described above (**Sup.**
473 **Fig. 3A**). Natural Treg were lysed in 0.5 mL of Trizol (Sigma-Aldrich) by vortexing and pipetting and
474 frozen at -80 °C. RNA was extracted combining Trizol and Chloform isolations with RNAeasy micro kit
475 columns (Qiagen). RNA concentration was determined using a Nanodrop, and RNA quality was
476 assessed by Qubit measurement and in an RNA nanochip Bioanalyzer. Sequencing libraries were
477 prepared using the Kapa Hyper Preparation kit with riboerase (Roche) following manufacturer's
478 instructions. Sequencing was performed on the NovaSeq 6000 in a pair-end 75 base pair format and
479 80 million reads.
480 The raw sequencing data was checked for lower quality bases and adaptor sequences with FastQC and
481 quality trimming was performed using CutAdapt[44]. The high-quality trimmed data were mapped on
482 mm10 mouse reference genome using STAR aligner (v 2.7.0a)[45]. The quantification of each gene
483 transcripts was performed with ensemble transcript annotation gtf file (GRCm38) using featureCounts
484 tool (v 2.0.0)[46]. The resultant read counts matrix was analyse using DESeq2 (v1.38.1)[47] to identify
485 differentially expressed genes between the young and aged natural Treg samples using Wald tests
486 with the Bonferroni correction for multiple testing. The differentially expressed genes (DEG) with a
487 multiple testing adjusted $p<0.05$ and fold change >2 were considered for further downstream analysis.
488 These genes were subjected to gene ontology enrichment analysis using enrichGO function in
489 clusterProfiler (v4.2) R package and DAVID (https://david.ncifcrf.gov). Over and underrepresented
490 ontology terms were identified (p value <= 0.05 and Bonferroni correction p adjustment method).
491 Next, enriched activated and suppressed functional pathways were identified using gseKEGG function
492 in clusterProfiler with p value<0.05.

493 Raw data files from RNAseq are available at the GEO database (accession number GSE218804).

494

495 **Bioinformatic analysis to identify putative Treg mechanisms**

496 To determine the molecular mechanisms associated with Treg-driven OPC differentiation that are
497 impaired with ageing, we combined our young and aged natural Treg RNA sequencing with data
498 obtained from Zhang *et al*. 2014 (GSE: 52564)[24]. We first extracted genes that were downregulated in
499 aged natural Treg and associated with the GO cellular component term "plasma membrane". We then
500 extracted the FPKM matrix and selected genes that were upregulated in OPC when compared with
501 newly myelinating oligodendrocytes and oligodendrocytes in the Zhang *et al*. database. We performed
502 GO cellular component analysis on those OPC-enriched genes and selected genes associated with the
503 GO term Apical Plasma Membrane. Then, both gene lists were subjected to a protein-protein
504 interaction analysis using the String open-source tool (www.string-db.org)[25]. Protein-protein
505 interactions within genes that were associated only with the natural Treg or only the OPC databases
506 were eliminated, and the remaining downregulated natural Treg membrane proteins were prioritised
507 considering their significance, fold change and the number of potential interactors enriched in OPCs.
508 Candidate genes were then selected based on their p-adjusted value, fold enrichment and the number
509 of interactions with OPC receptors.

510

511 **Statistical analysis**

512 All statistical analyses were performed in GraphPad Prism (GraphPad Software, Inc. version 9) or R.
513 First normality of datasets was assessed using Shapiro-Wilk and Kolmogorov-Smirnov tests. For
514 comparisons between 2 groups, such as young and aged Treg and OPC differentiation (Sup. Fig. 1),
515 unpaired two-tailed Student's t tests were used if data were normally distributed, and Mann-Whitney
516 U tests for non-parametric datasets. When more than two groups were analysed and datasets were
517 normally distributed, a one-way analysis of variance (ANOVA) was performed assuming equal
518 variances, followed by Sidak's multiple comparison test. When two factors were controlling the

519 outcome, such as replicates and treatments, 2-way ANOVA followed by Dunnet's comparison was
520 used. When datasets were was not normally distributed, a Kruskal-Wallis followed by Dunn's multiple
521 comparison test was used. For percentage data, arcsin conversion was performed to analyse the data
522 using parametric tests. For all statistical tests, differences were considered significant at $p<0.05$.
523
524 **Data and code availability**
525 Young and aged natural Treg RNA sequencing data will also be deposited at GEO (NCBI) with the
526 revised version.
527
528 **Figure legends**
529
530 **Figure 1: Expansion of Treg does not rescue impaired OPC differentiation in aged mice. A**) Flow
531 cytometric plot and **B**) quantification of natural Treg proportion, identified by CD4 and Foxp3
532 expression in young and aged mouse spleens (n=15-32, unpaired two-tailed Student's t test after
533 *arcsin* conversion). **C**) Flow cytometric plot and **D**) quantification showing the proportions of natural
534 Treg (endogenous GFP reporting Foxp3 expression in Foxp3-DTR mice) in aged blood compared to
535 young circulation (n=30-38 mice, unpaired two-tailed Student's t test after *arcsin* conversion). **E**) Bar
536 graph showing the density of $OLIG2^{+}CC1^{+}$ oligodendrocytes in young and aged lysolecithin-induced
537 demyelinating lesions at 14dpl (n=4 mice, unpaired Student's t test). **F**) Flow cytometric plot showing
538 CD25 and Foxp3 expression and the quantification of $CD25^{+}Foxp3^{+}$ natural Treg proportion in a $CD4^{+}$
539 T cell population from the spleen (**G**) and blood (**H**) of control aged mice and aged mice treated with
540 intraperitoneal injection of IL-2/anti-IL-2 complexes (n=3, unpaired two-tailed Student's t test after
541 *arcsin* conversion). **I**) Bar graph showing the quantification of $OLIG2^{+}CC1^{+}$ oligodendrocyte density at
542 10dpl in aged control and Treg-expanded mice (n=6-7, unpaired Student's t test).
543

544 **Figure 2: Aged natural Treg demonstrate impaired capacity to drive OPC differentiation and**
545 **myelination *in vitro*.** Representative images of OPCs co-cultured with young and aged natural Treg
546 and immunostained for OLIG2 (cyan) as a pan oligodendrocyte lineage marker, the proliferation
547 marker Ki67 (magenta, **A**) and differentiation markers CNP (grey) and MBP (magenta) (**C**) (Scale bar =
548 100 µm). **B**) Quantification of OPC proliferation when co-cultured with young and aged natural Treg
549 (n=6, 1-way ANOVA after *arcsin* conversion) **D**) Quantification of the proportion of OPCs reaching early
550 stage-differentiation when exposed to young and aged natural Treg, as indicated by CNPase staining
551 (n=9, 1-way ANOVA after *arcsin* conversion). **E**) Quantification of the proportion of OPCs expressing
552 late-stage differentiation marker MBP in control and OPCs treated with young and aged natural Treg
553 (n=9, 1-way ANOVA, Sidak's multiple comparisons test). **F**) Immunohistochemistry of control and
554 young and aged Treg treated cerebellar slices (MBP, magenta and NFH, green, Scale bar = 100 µm).
555 (**G**) Quantification of myelination index (ratio between MBP and NFH colocalization area and NFH
556 area) in neonatal cerebellar slices (n=7, 1-way ANOVA after *arcsin* conversion, Sidak's multiple
557 comparison tests).
558
559 **Figure 3: Myelin-regenerative capacity of aged Treg is restored in a young environment. A)** Diagram
560 explaining the experimental design of *in vivo* Treg depletion, Treg adoptive transfer and spinal cord
561 demyelination. Representative images of immunostaining identifying oligodendrocytes by the co-
562 localisation of the pan-oligodendrocyte lineage marker OLIG2 (green) with CC1 (magenta, **B**) or ASPA
563 (magenta, **C**) at 14 dpl (scale bar = 100 µm, demyelination area is highlighted by the white line). Bar
564 graphs show the quantification of total number of oligodendrocyte lineage cells (**D**), as well as CC1-
565 expressing (**E**) or ASPA-expressing (**F**) oligodendrocytes in the demyelinated lesions of PBS control
566 mice, natural Treg-depleted mice and mice depleted of endogenous Treg that received young or aged
567 natural Treg by adoptive transfer (n=7-9, 1-way ANOVA, Sidak's multiple comparisons test). **G**)
568 Representative images of immunostaining for neurofilament-H (NFH, green) and MBP (magenta) to
569 quantify myelin wrapping as an early marker of remyelination at 14 dpl (scale bar = 100 µm,

570 demyelination area is highlighted by the white line). Quantification shows the total number of axons
571 (**H**), the density of MBP wrapped axons (**I**) and the percentage of MBP-wrapped axons from the total
572 number of axons (**J**) (n=6-9, 1-way ANOVA, Sidak's multiple comparisons test).
573
574 **Figure 4: Ageing significantly alters natural Treg transcriptome. A**) Principal component analysis
575 demonstrating the clustering differences between young and aged natural Treg. **B**) Volcano plot
576 demonstrating 1456 genes upregulated and 302 genes downregulated genes in aged natural Treg
577 relative to young Treg. **C**) Heatmap demonstrating hierarchical clustering of the top 50 differentially
578 expressed genes between young and aged Treg. **D**) Graph showing the pathways enriched amongst
579 differentially expressed genes. **E**) Bar graph highlighting the GO biological processes associated with
580 genes that are upregulated or downregulated in aged Treg. **F**) Heatmap showing the normalised count
581 values for the Treg tissue repair programme identified by Delacher *et al*.[23] **G**) Graph showing the Treg
582 tissue repair programme signature genes score in aged and young natural Treg.
583
584 **Figure 5: *Mcam* and *Itga2* contribute to Treg-driven OPC differentiation and are downregulated in**
585 **aged Treg. A**) Representative images of immunostaining showing cell-to-cell contact between OPCs
586 and Treg in OPC-Treg co-cultures *in vitro*. OPCs are identified by the co-staining of OLIG2 (cyan) and
587 NG2 (grey), while Treg are identified by CD3 (red) (scale bar = 50 µm). **B**) Representative images of
588 immunostaining and **C**) quantification of MBP-expressing oligodendrocytes in control OPCs, OPCs
589 directly co-cultured with young Treg and OPCs cultured with young Treg in a transwell (n=6, 1-way
590 ANOVA after arcsin conversion, Sidak's multiple comparison's test). **D**) Diagram summarising
591 bioinformatic approaches to identify protein-protein interactions between OPCs and Treg. **E**) Graph
592 showing 21 protein candidates expressed in the Treg plasma membrane, that are downregulated in
593 aged Treg and have potential bindings partners enriched in OPCs vs oligodendrocytes. Log_2 Change, -
594 Log_{10} (Padj) and the number of OPC binding partners are indicated (see legend). **F**) Bar graphs showing
595 RNAseq normalised count values for the top 6 candidates. **G**) Representative images of

596 immunostaining showing OPC differentiation in co-culture with young Treg in the presence or absence
597 of neutralising antibodies against candidate cell surface mediators (scale bar = 100µm). Bar graphs
598 showing the quantification of OPC differentiation measured by the fold change in percentage of MBP^+
599 cells (**H**) and MBP^+ area per well (**I**) (n=7, 2-way ANOVA, Dunnett's multiple comparison tests).
600
601 **Supplementary information**
602
603 **Supplementary Figure 1: Endogenous Treg depletion and reconstitution during lysolecithin- induced**
604 **demyelination *in vivo*. A**) Gating strategy followed to identify natural Treg. **B**) Bar graph showing
605 natural Treg isolation purity for *in vitro* experiments and *in vivo* adoptive transfer studies. Natural Treg
606 are identified by CD4 and CD25 expression (average 91% of the isolated cells for both young and aged
607 are $CD4^+CD25^+$) and by the expression of CD4 and Foxp3 (average 87% of the isolated cells are positive
608 for CD4 and Foxp3 in both young and aged mouse isolations) (n=14 independent isolations for
609 different adoptive transfer rounds, each pool comprises 2-8 different mice, Student's t test, after
610 *arcsin* conversion). **C**) Flow cytometric plot showing CD4 and GFP expression to identify endogenous
611 natural Treg in lymph nodes. **D**) Bar graph showing quantification for the proportion of endogenous
612 GFP^+ Treg within the $CD4^+$ T cell population in the lymph nodes of young Foxp3-DTR mice at 14dpl
613 (n=7-9, 1-way ANOVA, after *arcsin* conversion, Sidak's multiple comparisons test). **E**) Flow cytometric
614 plots showing CD25 and Foxp3 expression to detect Treg reconstitution in lymph nodes after adoptive
615 transfer. **F**) Bar graph quantification shows the proportion of $CD25^+Foxp3^+$ cells within the $CD4^+$ T cell
616 population present in the lymph nodes of young Foxp3-DTR mice at 14dpl (n=7-12, Kruskal Wallis and
617 Dunn's multiple comparisons test). **G**) Representative images of immunostaining showing proliferating
618 OPCs at 14 dpl (OLIG2 (green) and Ki67 (magenta)) (scale bar = 100 µm, demyelinated area is indicated
619 by the white line). Bar graph showing the areas of demyelination (**H**), the density of proliferating OPCs
620 in the lesion (**I**) and the proportion of oligodendrocyte lineage cells proliferating in response to
621 damage (**J**) (n=7-8 mice, 1 way ANOVA).

622

623 **Supplementary Figure 2: Downstream analysis of differentially expressed genes between young and**
624 **aged natural Treg. A**) Bar graphs showing the purity of young and aged natural Treg subjected to RNA
625 sequencing (on average 89% of $CD4^+$ cells were $Foxp3^+$, while 94% of $CD4^+$ cells were $CD25^+$) (n=4-6
626 mice, Mann-Whitney U test). **B**) Bar graph showing normalised counts of natural Treg marker
627 transcripts *Foxp3*, *Cd4* and *IL2ra*. **C**) Heatmap and **D**) quantification of normalised count values of the
628 Treg tissue repair programme in male and female Treg. Bar graphs showing the GO cellular component
629 (**E**), molecular function (**F**) and KEGG pathways (**G**) enriched within the differentially expressed genes
630 upregulated (magenta) and downregulated (purple) in aged Treg. Bar graphs showing the
631 quantification of OPC differentiation in OPC and young Treg co-cultures in the presence or absence of
632 neutralising antibodies against candidate cell surface mediators, measured by the fold change in the
633 percentage of MBP^+ cells (**H**) and the fold change in MBP^+ area per well (**I**) (n=5-7, 2-way ANOVA,
634 Dunnet's multiple comparisons test).

635

636

637

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648

649 **Author contributions**

650 Experiments were designed by A.G.F., D.C.F., Y.D. and V.T. Experiments were performed and/or
651 analysed by A.G.F., M.D., E.H., N.V.G., J.W., A.Y., K.M., J.F., C.E.M., M.I., R.P., Y.D. and D.C.F.
652 R.J.I. and C.E.M. provided advice on experimental design and interpretation. Manuscript was written
653 by A.G.F. and D.C.F. with contributions from all authors. D.C.F and A.G.F. oversaw the study.

654

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656

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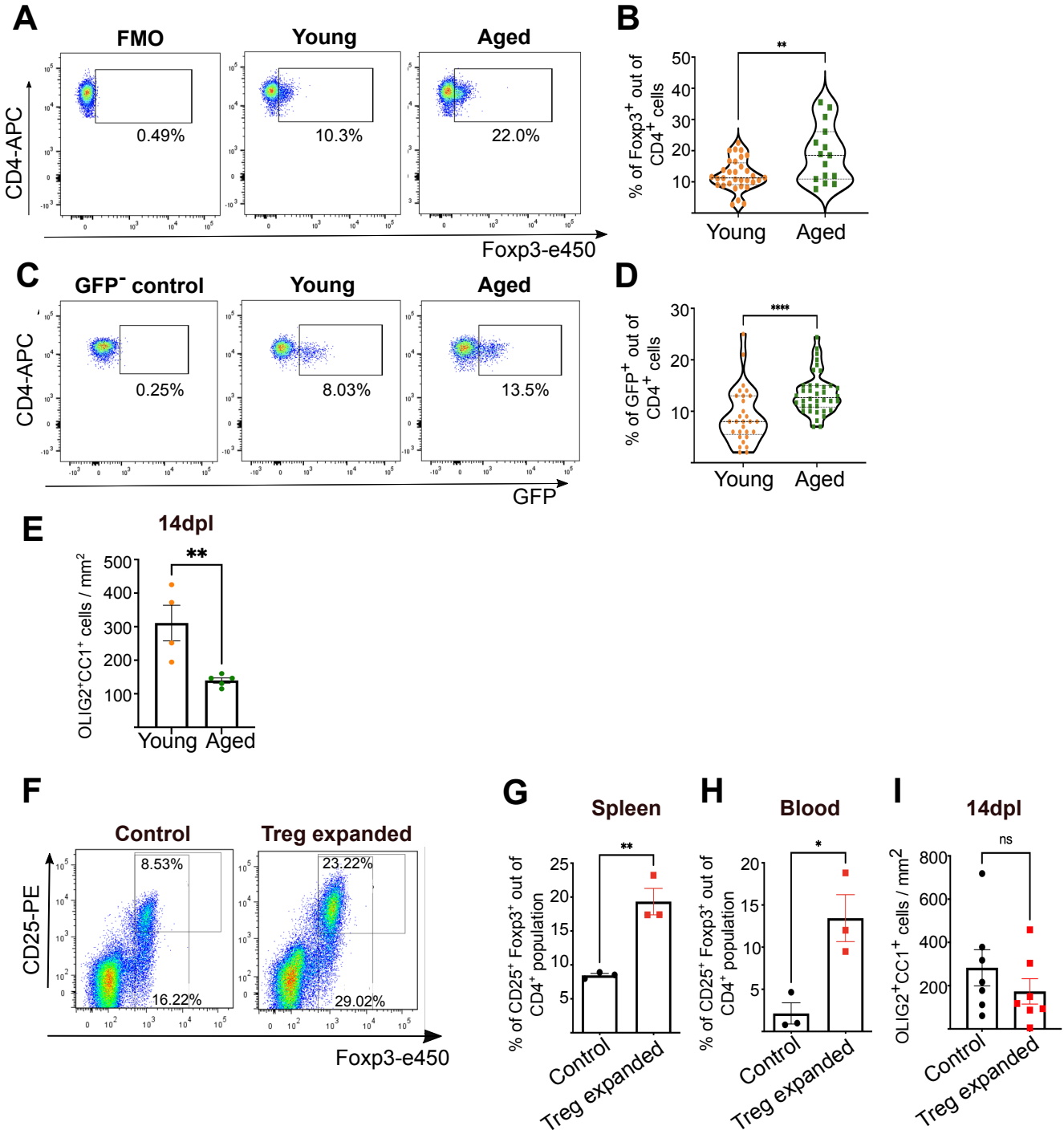

Figure 1: Expansion of Treg does not rescue impaired OPC differentiation in aged mice. **A**) Flow cytometric plot and **B**) quantification of natural Treg proportion, identified by CD4 and Foxp3 expression in young and aged mouse spleens (n=15-32, unpaired two-tailed Student's t test after *arcsin* conversion). **C**) Flow cytometric plot and **D**) quantification showing the proportions of natural Treg (endogenous GFP reporting Foxp3 expresion in Foxp3-DTR mice) in aged blood compared to young circulation (n=30-38, unpaired two-tailed Student's t test). **E**) Bar graph showing the density of OLIG2$^+$CC1$^+$ oligodendrocytes in young and aged lysolecithin-induced demyelinating lesions at 14dpl (n=4 mice, unpaired Student's t test). **F**) Flow cytometric plot showing CD25 and Foxp3 expression and the quantification of CD25$^+$Foxp3$^+$ natural Treg proportion in a CD4$^+$ T cell population from the spleen (**G**) and blood (**H**) of control aged mice and aged mice treated with intraperitoneal injection of IL-2/anti-IL-2 complexes (n=3, unpaired two-tailed Student's t test after arcsin c conversion). **I**) Bar graph showing the quantification of OLIG2$^+$CC1$^+$ oligodendrocyte density at 10dpl in aged control and Treg expanded mice (n=6-7, unpaired Student's t test).

Fig. 1

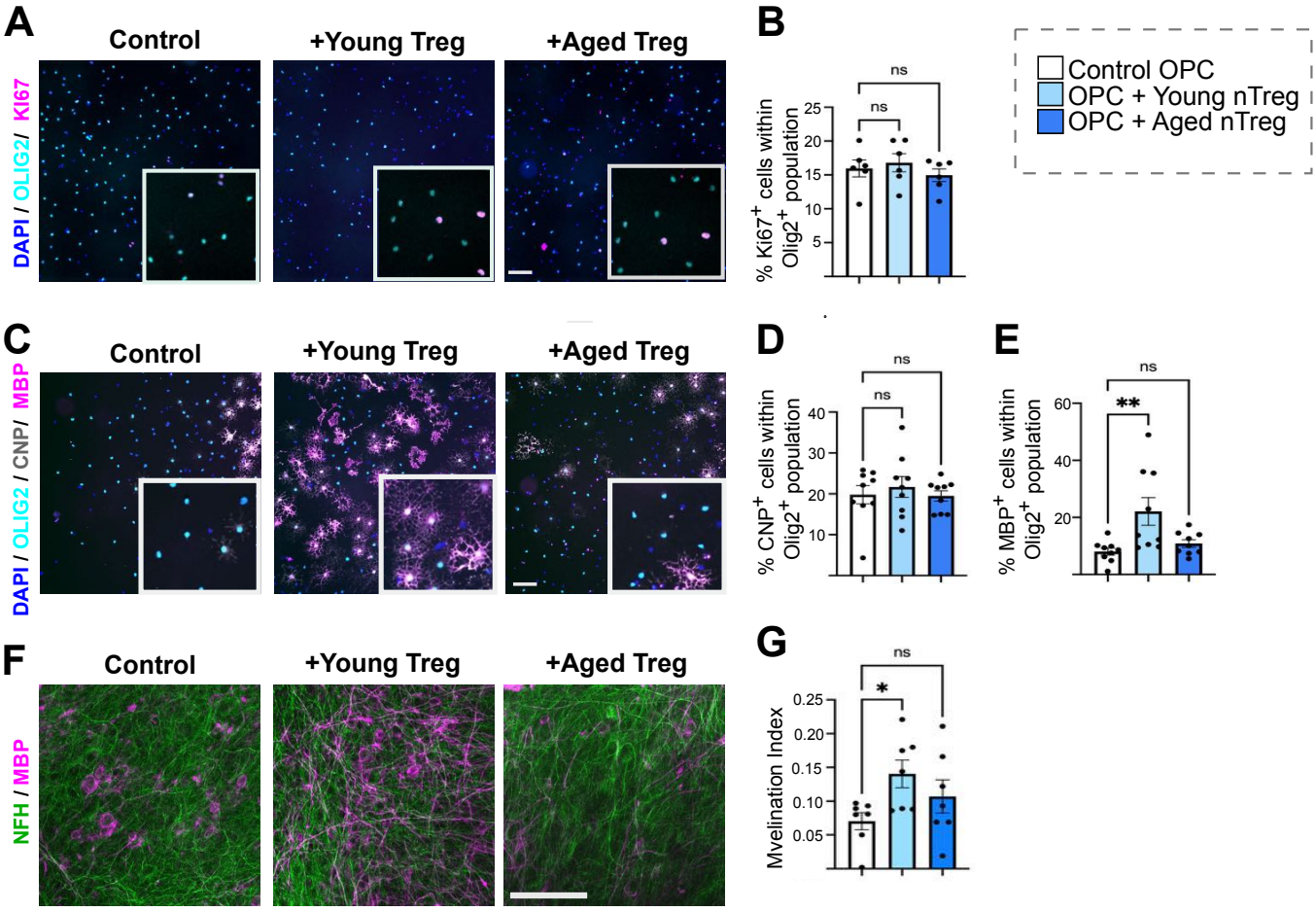

Figure 2: Aged natural Treg demonstrate impaired capacity to drive OPC differentiation and myelination *in vitro.* Representative images of OPCs co-cultured with young and aged nTreg and immunostained for OLIG2 (cyan) as a pan oligodendrocyte lineage marker, the proliferation marker Ki67 (magenta, **A**) and differentiation markers CNP (grey) and MBP (magenta) (**C**) (Scale bar = 100 µm). **B**) Quantification of OPC proliferation when co-cultured with young and aged nTreg (n=6, 1-way ANOVA after arcsin conversion) **D**) Quantification of the proportion of OPCs reaching early stage-differentiation when exposed to young and aged nTreg, as indicated by CNPase staining (n=9, 1-way ANOVA after arcsin conversion). **E**) Quantification of the proportion of OPCs expressing late-stage differentiation marker MBP in control and OPCs treated with young and aged nTreg (n=9, 1-way ANOVA, Sidak's multiple comparisons test). **F**) Immunohistochemistry of control and young and aged nTreg treated cerebellar slices (MBP, magenta and NFH, green, Scale bar = 100 µm). (**G**) Quantification of myelination index (ratio between MBP and NFH colocalization area and NFH area) in neonatal cerebellar slices (n=7, 1-way ANOVA after arcsin conversion, Sidak's multiple comparison tests). .

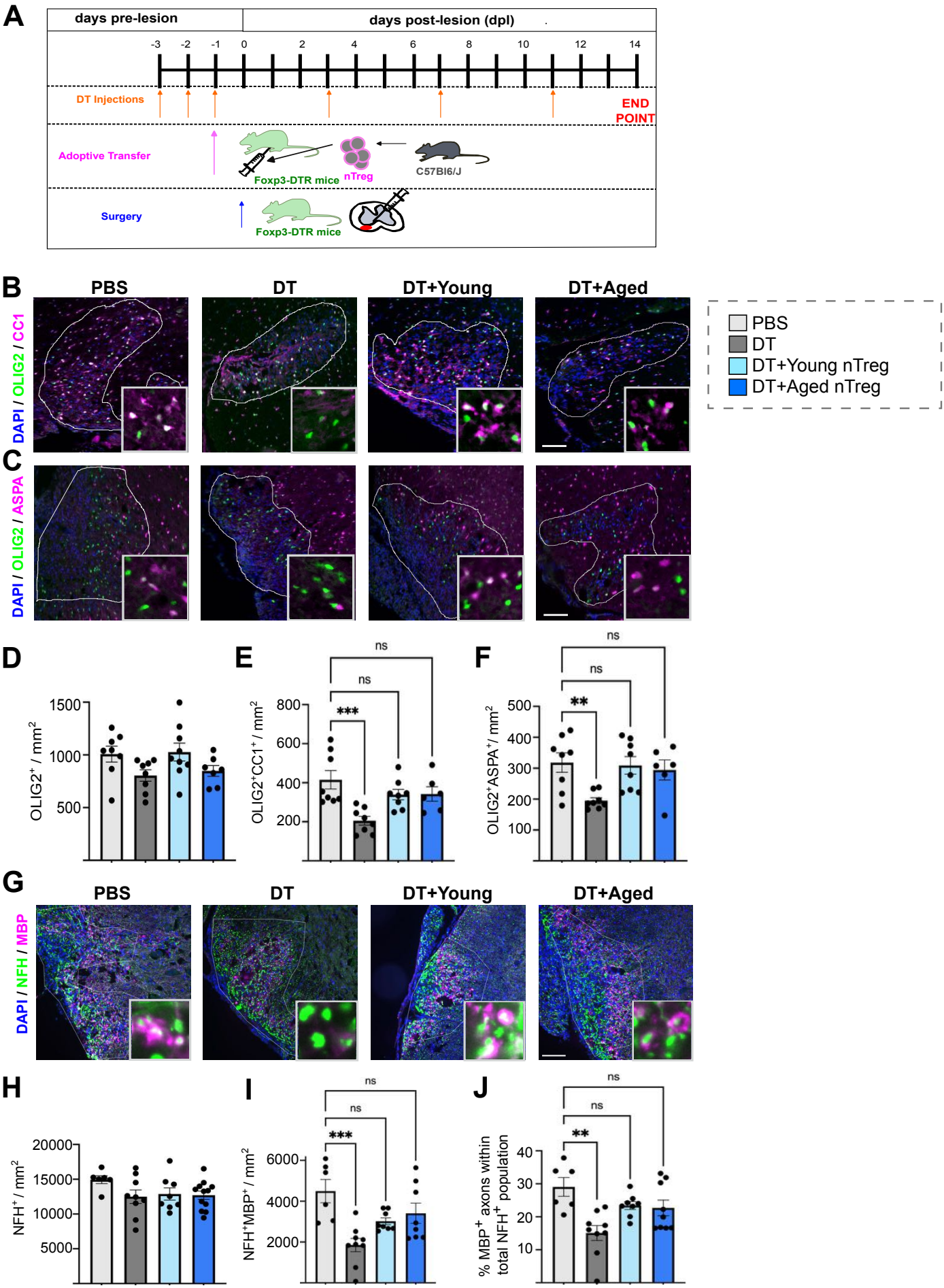
A
days pre-lesion
days post-lesion (dpl)
-3 -2 -1 0 2 4 6 8 10 12 14
DT Injections
END POINT
Adoptive Transfer
Foxp3-DTR mice
nTreg
C57Bl6/J
Surgery
Foxp3-DTR mice
B
PBS
DT
DT+Young
DT+Aged
DAPI / OLIG2 / CC1
PBS
DT
DT+Young nTreg
DT+Aged nTreg
C
DAPI / OLIG2 / ASPA
D
OLIG2+ / mm2
1500
1000
500
E
OLIG2+CC1+ / mm2
800
600
400
200
ns
ns
***
F
OLIG2+ASPA+ / mm2
500
400
300
200
100
ns
ns
**
G
PBS
DT
DT+Young
DT+Aged
DAPI / NFH / MBP
H
NFH+ / mm2
20000
15000
10000
5000
I
NFH+MBP+ / mm2
6000
4000
2000
ns
ns
***
J
% MBP+ axons within total NFH+ population
40
30
20
10
ns
ns
**

Fig. 3

Figure 3: Myelin-regenerative capacity of aged Treg is restored in a young environment. A) Diagram explaining the experimenta design of *in vivo* Treg depletion, Treg adoptive transfer and spinal cord demyelination. Representative iamges of immunostaining identifying oligodendrocytes by the co-localisation of the pan-oligodendrocyte lineage marker OLIG2 (green) with CC1 (magenta, **B**) or ASPA (magenta, **C**) at 14dpl (scale bar=100µm, demyelinated area is highlighted by the white line). Bar graphs show the quantification of total number of oligodendrocyte lineage cells (**D**), as well as CC1-expressing (**E**) or ASPA-expressing (**F**) oligodendrocytes in the demyelinated lesions of PBS control mice, natural Treg-depleted mice and mice depleted of endogenous Treg that received young or aged natural Treg by adoptive transfer (n=7-9, 1-way ANOVA, Sidak's multiple comparisons test). **G**) Representative images of immunostaining for neurofilament-H (NFH, green) and MBP (magenta) to quantifiy myelin wrapping as an early marker of remyelinationg at 14dpl (scale bar = 100µm, demyelination area is highlighted by the white line). Quantification shows the total number of axons (**H**), the density of MBP wrapped axons (**I**) and the percentage of MBP-wrapped axons from the total number of axons (**J**) (n=6-9, 1-way ANOVA, Sidak's multiple comparisons test).

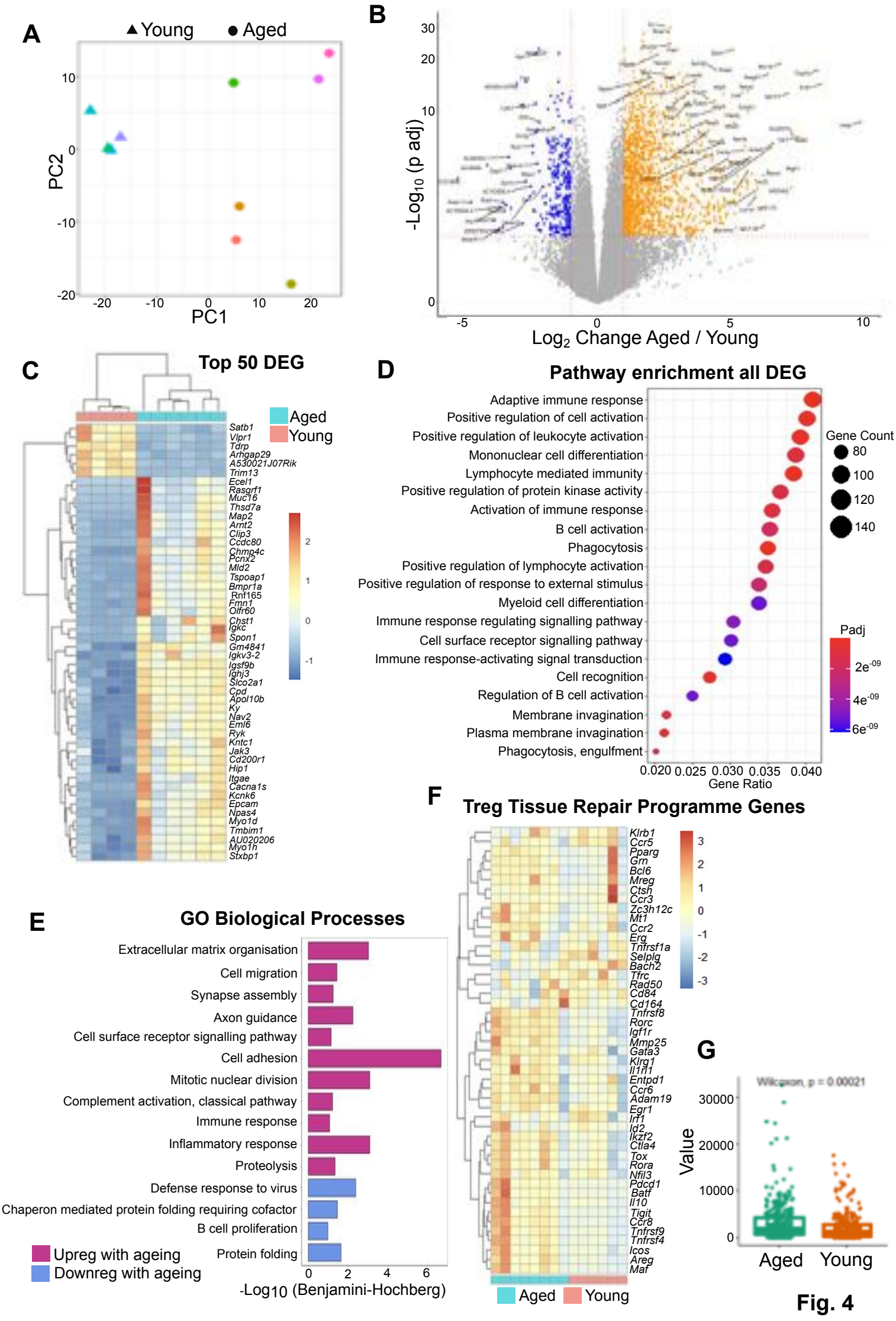
A
Young
Aged
PC2
PC1
B
-Log10 (p adj)
Log2 Change Aged / Young
C
Top 50 DEG
Aged
Young
Satb1
Vlpr1
Tdrp
Arhgap29
A530021J07Rik
Trim13
Ecel1
Rasgrf1
Muc16
Thsd7a
Map2
Arnt2
Clip3
Ccdc80
Chmp4c
Pcnx2
Mld2
Tspoap1
Bmpr1a
Rnf165
Fmn1
Olfr60
Chst1
Igkc
Spon1
Gm4841
Igkv3-2
Igsf9b
Ighj3
Slco2a1
Cpd
Apol10b
Ky
Nav2
Eml6
Ryk
Kntc1
Jak3
Cd200r1
Hip1
Itgae
Cacna1s
Kcnk6
Epcam
Npas4
Myo1d
Tmbim1
AU020206
Myo1h
Stxbp1
D
Pathway enrichment all DEG
Adaptive immune response
Positive regulation of cell activation
Positive regulation of leukocyte activation
Mononuclear cell differentiation
Lymphocyte mediated immunity
Positive regulation of protein kinase activity
Activation of immune response
B cell activation
Phagocytosis
Positive regulation of lymphocyte activation
Positive regulation of response to external stimulus
Myeloid cell differentiation
Immune response regulating signalling pathway
Cell surface receptor signalling pathway
Immune response-activating signal transduction
Cell recognition
Regulation of B cell activation
Membrane invagination
Plasma membrane invagination
Phagocytosis, engulfment
Gene Count
Padj
Gene Ratio
E
GO Biological Processes
Extracellular matrix organisation
Cell migration
Synapse assembly
Axon guidance
Cell surface receptor signalling pathway
Cell adhesion
Mitotic nuclear division
Complement activation, classical pathway
Immune response
Inflammatory response
Proteolysis
Defense response to virus
Chaperon mediated protein folding requiring cofactor
B cell proliferation
Protein folding
Upreg with ageing
Downreg with ageing
-Log10 (Benjamini-Hochberg)
F
Treg Tissue Repair Programme Genes
Klrb1
Ccr5
Pparg
Grn
Bcl6
Mreg
Ctsh
Ccr3
Zc3h12c
Mt1
Ccr2
Erg
Tnfrsf1a
Selplg
Bach2
Tfrc
Rad50
Cd84
Cd164
Tnfrsf8
Rorc
Igf1r
Mmp25
Gata3
Klrg1
Il1rl1
Entpd1
Ccr6
Adam19
Egr1
Irf1
Id2
Ikzf2
Ctla4
Tox
Rora
Nfil3
Pdcd1
Batf
Il10
Tigit
Ccr8
Tnfrsf9
Tnfrsf4
Icos
Areg
Maf
Aged
Young
G
Wilcoxon, p = 0.00021
Value
Aged
Young

Fig. 4

Figure 4: Ageing significantly alters natural Treg transcriptome. A) Principal component analysis demostrating the clustering differences bedtween young and aged natural Treg. **B**) Volcano plot demostrating 1456 genes upregulated and 302 genes genes downregulated genes natural Treg relative to young Treg. **C**) Heatmap demonstrating hierarchical clustering of the top 50 differentially expressed genes between young and aged Treg. **D**) Graph showing the pathways enriched amongst differntially expressed genes. **E**) Bar graph highlighting the GO biological processes associated with genes that are upregulated or downregulated in aged Treg. **F**) Heatmap showing the normalised count values for the Treg tissue repair programme identified by Delacher *et al*.[23]. **G**) Graph showing the Treg tissue repair programme signature genes score in aged and young natural Treg.

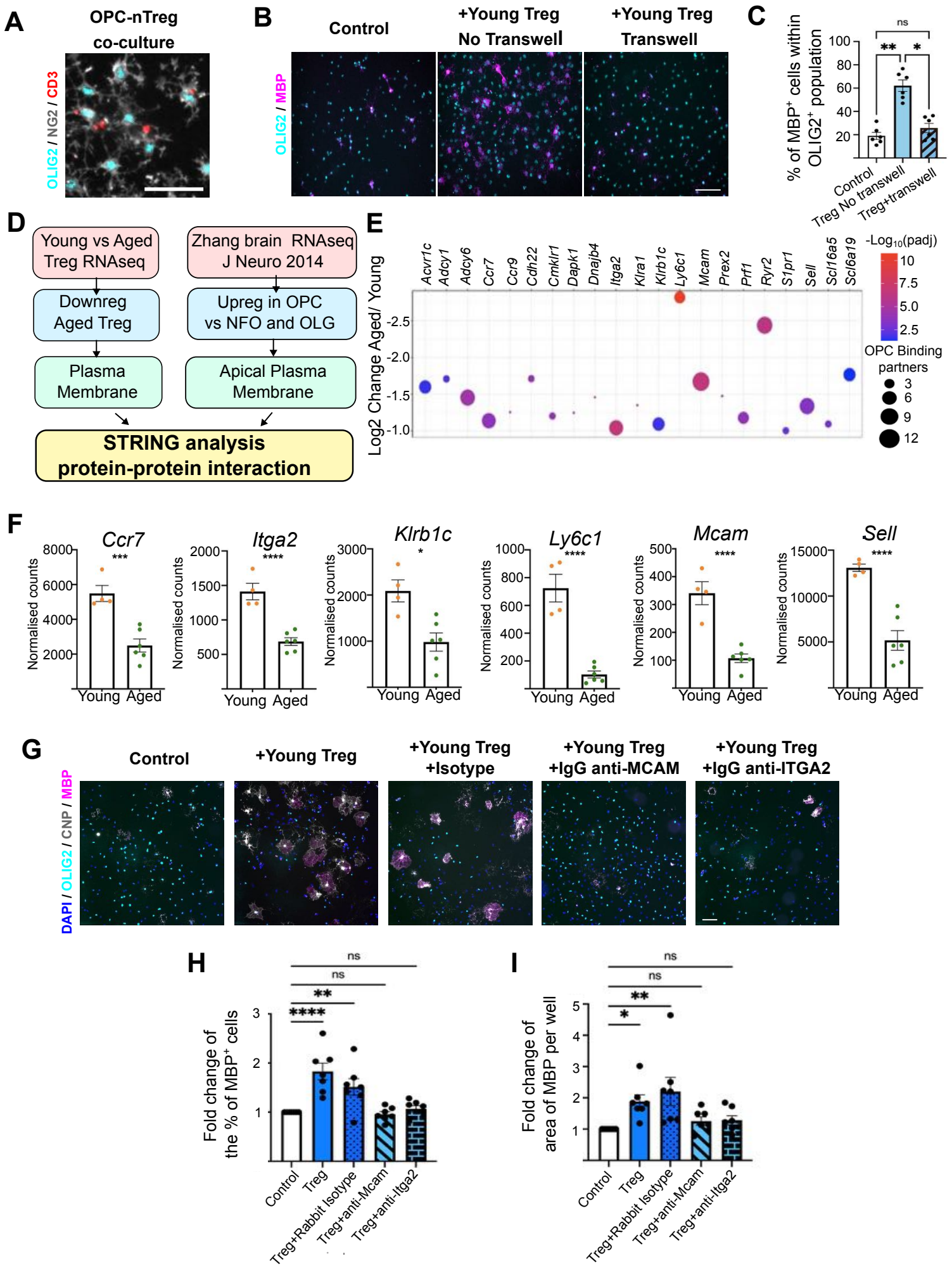
A
OPC-nTreg co-culture
OLIG2 / NG2 / CD3
B
Control
+Young Treg No Transwell
+Young Treg Transwell
OLIG2 / MBP
C
% of MBP+ cells within OLIG2+ population
ns
**
*
Control
Treg No transwell
Treg+transwell
D
Young vs Aged Treg RNAseq
Downreg Aged Treg
Plasma Membrane
Zhang brain RNAseq J Neuro 2014
Upreg in OPC vs NFO and OLG
Apical Plasma Membrane
STRING analysis protein-protein interaction
E
Acvr1c
Adcy1
Adcy6
Ccr7
Ccr9
Cdh22
Cmklr1
Dapk1
Dnajb4
Itga2
Klra1
Klrb1c
Ly6c1
Mcam
Prex2
Prf1
Ryr2
S1pr1
Sell
Scl16a5
Scl6a19
Log2 Change Aged/ Young
-Log10(padj)
OPC Binding partners
F
Ccr7
Itga2
Klrb1c
Ly6c1
Mcam
Sell
Normalised counts
Young
Aged
G
Control
+Young Treg
+Young Treg +Isotype
+Young Treg +IgG anti-MCAM
+Young Treg +IgG anti-ITGA2
DAPI / OLIG2 / CNP / MBP
H
Fold change of the % of MBP+ cells
Control
Treg
Treg+Rabbit Isotype
Treg+anti-Mcam
Treg+anti-Itga2
I
Fold change of area of MBP per well

Fig. 5

Figure 5: Mcam and Itga2 contribute to Treg-driven OPC differentiation and are downregulated in aged Treg. **A**) Representative images of immunostaining showing cell-to-cell contact between OPCs and Treg in OPC-Treg co-cultures *in vitro*. OPCs are identified by the co-staining of OLIG2 (cyan) and NG2 (grey), while Treg are identified by CD3 (red) (scale bar = 50 µm). **B**) Representative images of immunostaining and **C**) quantification of MBP-expressing oligodendrocytes in control OPCs, OPCs directly co-cultured with young Treg and OPCs cultured with young Treg in a transwell (n=6, 1-way ANOVA after arcsin conversion, Sidak's multiple comparison's test). **D**) Diagram summarising bioinformatic approaches to identify protein-protein interactions between OPCs and Treg. **E**) Graph showing 21 protein candidates expressed in the nTreg plasma membrane, that are downregulated in aged nTreg and have potential bindings partners enriched in OPCs vs oligodendrocytes. Log_2 Change, $-Log_{10}$ (Padj) and the number of OPC binding partners are indicated (see legend). **F**) Bar graphs showing RNAseq normalised count values for the top 6 candidates. **G**) Representative images of immunostaining showing OPC differentiation in co-culture with young Treg in the presence ofr absence of neutralising antibodies against candidate cell surface mediators (scale bar=100µm). Bar graphs showing the quantification of OPC differentiation measured by the fold change in percentage of MBP^+ cells (**H**) and MBP^+ area per well (**I**) (n=7, 2-way ANOVA, Dunnett{s multiple comparsion tests).