Supplementary Information – Copray et al.

Supplementary Materials and Methods

Generation of hiPSCs

iPSCs cells were generated from human dermal fibroblasts (from a skin biopsy taken from the inside of the upper arm, because of least exposure to UV and eventually less visible scar formation) using lentiviral transduction. Lentivirus was produced in HEK293T cells in a 100mm cell culture dish following transfection with EF1α-STEMCCA-RedLight-LoxP plasmid containing *OCT4, KLF4, SOX2* and mCherry genes, together with pMD2-VSV-G and pCMV-D8.91 plasmids. Lentivirus containing medium was collected 48h post-transfection, filtered through an 0,45µm filter and concentrated with Amicon Ultra 100,000 MWCO centrifugal filters (Millipore). The concentrated supernatant was diluted with 1ml of fresh fibroblast medium containing 8µg/ml polybrene (Sigma-Aldrich) and used to transduce circa 100,000 human fibroblasts. Lentivirus containing medium was discarded the following day and cells were cultured in fibroblast medium for another 3 days. Transduced cells were then trypsinized, seeded onto an irradiated mouse embryonic fibroblast (MEF) feeder layer and cultured in hES medium (DMEM/F12, 20% KSR, 1% NEAA, P/S, 0,1mM β-mercaptoethanol, 5ng/ml bFGF) until hiPSC colonies appeared. Around 28 days post-transduction, large hiPSC colonies were picked, cut into pieces and plated on Matrigel-coated cell culture plates in mTESR medium (StemCell Technologies). Subsequent hiPSC passages were performed mechanically and propagated in mTESR medium.

Pluripotency assays for hiPSCs

Subconfluent undifferentiated hiPSCs were harvested by cutting the colonies into small pieces and scraping them off the cell culture dish. Colony fragments were transferred into non-adherent cell culture plates and cultured in hEB medium (DMEM/F12, 20% KSR, 1% NEAA, 1:1000 MycoZap+) for 8 days (medium was changed every other day). At day 9, developing embryoid bodies (EBs) were plated onto gelatin (0,1%) or Matrigel-coated coverslips and cultured for another 2 - 4 weeks. At the end of the differentiation period cells were fixed with 4% PFA and examined for the presence of cells of all three germ layers with immunocytochemistry.

For the teratoma formation assay, undifferentiated hiPSCs were injected subcutaneously in the flank of NOD-SCID mice. For that, undifferentiated hiPSC colonies were harvested using dispase, gently dissociated into single cells and resuspended in 70% DMEM/F12 and 30% Matrigel solution. A volume of 300µl containing ~$1x10^6$ cells was used for each injection. The animals were sacrificed 5-7 weeks post-injection when the teratomas were clearly visible. Tumors were explanted, fixed with 4%

PFA and subjected to immunohistochemical analysis for the presence of tissues of all three germ layers.

Differentiation of hiPSCs towards oligodendrocytes

For the differentiation of hiPSCs into oligodendrocytes, a 5-stage protocol was developed:

Stage 1 – Generation of primitive neuroepithelial cells

hiPSCs were cultured on matrigel in mTESR1 medium, refreshed every other day. hiPSCs were passaged when they reached ~60% confluence and allowed to form EBs as described above. On day 8, EBs were collected and plated onto laminin-coated dishes in Neuronal Differentiation (ND) medium (DMEM/F12, 1% N2 supplement (PAA), 1% NEAA, 1mg/ml heparin, 1:1000 MycoZap+). Medium was changed every other day. Cells were kept in these conditions for a variable period of time (typically around 10-15 days) until clear neural rosette structures appeared.

Stage 2 – Specification of Olig2 progenitor cells

Medium was changed to ND+ medium (ND medium with 2% B27) supplemented with retinoic acid (RA, 100nM) for 5-7 days. “Mature” neural rosettes were collected, gently dissociated into small fragments and transferred into non-adherent cell culture dishes in ND+ medium supplemented with RA and 100nm Sonic Hedgehog (SHH) for 10 days (from now on cells were grown as spheres in suspension). After that period, cell aggregates were dissociated into single cells using accutase and cultured for another 10 days in ND+ medium supplemented with 10ng/ml bFGF and SHH (RA was removed).

Stage 3 – Generation of pre-OPCs

Next, hiPSC-derived spheres were cultured in GLIA medium (DMEM/F12, 1% N1 supplement, 2% B27 supplement, 1% NEAA, 60ng/ml T3, 100 ng/ml Biotin, 1µm cAMP) supplemented with PDGF-AA, IGF-1 and NT3 (all at 10ng/ml) and 100ng/ml SHH but without bFGF. Half of the cell culture medium was changed every 2 days and cells were kept in these conditions for 21 days. At the end of that period spheres were dissociated once again with accutase and kept in medium described above without SHH. Spheres were allowed to grow in these conditions for another 42 days (half of the medium was changed every other day)

Stage 4 – Maturation of pre-OPCs to OPCs

After 42 days, hiPSC-derived spheres grew big enough to be disaggregated one more time with accutase. After dissociation cells were cultured in GLIA medium except that concentrations of PDGF-AA, IGF-1 and NT3 were reduced to 5ng/ml.

Stage 5 – Differentiation of OPCs on substrate

At defined time points (e.g. every 2 weeks) some of the floating hiPSC-derived spheres were seeded onto polyornithine/laminin (20µg/ml) coated coverslips to check for the identity of the cells

migrating out of the cell clusters. At this stage, cells were cultured in the medium described for Stage 4. The number of OPCs migrating out typically increased over time; on average it took around 140-150 days to complete the protocol.

Cuprizone experiments

For cuprizone experiments, a total of twelve 8 – 10 weeks old female C57BL/6 mice were fed with powder food diet containing 0,2% cuprizone (Sigma-Aldrich) for 7 – 8 weeks. One week before cell implantation, one of the animals was sacrificed and its brain was assessed for the extent of demyelination of the corpus callosum using Luxol Fast Blue (LFB) and cresyl violet (CV) staining. The animals were implanted stereotactically with ~50.000 of either hiPSC-derived OPCs (5 mice) or human fibroblasts (control group, 5 mice) in 3µl of PBS. Stereotaxic coordinates (in relation to Bregma): anterior-posterior +1; lateral -1,75; vertical -2,25 (Paxinos atlas, 2002). After the operations, animals were kept on standard ad-libitum diet and were sacrificed at specific time points (perfusion with 4% PFA under anesthesia). The brains of the mice were collected, sectioned (20µm coronal cryosections) and subjected to immunohistochemical and histological analysis.

Experimental Autoimmune Encephalomyelitis (EAE) induction in mouse

11-13 week old female C57BL/6 inbred mice (C57BL/6 OlaHsd, Harlan Laboratories, The Netherlands) were used in this study. Animals were housed under standard conditions in a quiet environment with ad-libitum access to water and food. EAE was induced with an EAE kit (Hooke Laboratories, USA, Cat # EK-0114). Briefly, mice were subcutaneously injected at the lower and upper back with a total of 200µg MOG35-55 emulsified in complete Freund's Adjuvant followed by an intraperitoneal injection of 375ng of pertussis toxin (day 0). The pertussis toxin injection was repeated 24 hours after post immunization. Mice were daily monitored for their weight and EAE score. The following scoring system was used: 0) no obvious changes, 1) limp tail, 2) limp tail and impaired righting reflex, 3) limp tail and partial paralysis of hind legs, 4) limp tail and complete paralysis of hind legs, 5) moribund, 6) death due to EAE.

Transplantation of hiPSC-derived OPCs was performed on day 11 after EAE induction when the first symptoms of EAE could be detected, according to a procedure tested and described before [20]. hiPSC-derived-OPCs were gently detached from the substrate using accutase, spun down and resuspended in PBS at a concentration of 12.500 cells/µl. 4µl of cell suspension was stereotactically injected into the right ventricle of experimental group (n=10) using the following coordinates in relation to Bregma: anterior-posterior -0,5; lateral -1; vertical -2,3 (Paxinos atlas, 2002). Injection

speed was kept at 1µl of cell suspension per minute followed by 5 minutes of deposition time before needle retraction. Control EAE mice (n=8) received an injection with PBS. To prevent immune-rejection of the grafts, both animal groups received daily cyclosporine A (Novartis) injections (subcutaneous; 10mg/kg of body weight) starting from 1 day before operation. To evaluate a potential effect of cyclosporine on EAE progression, an extra control group (n=4) was included in which control animals received a daily subcutaneous injection of saline instead of cyclosporine.

In-vivo cell imaging

Two weeks before implantation, the cells were lentivirally labeled with luciferase to enable tracking using the IVIS-camera (Caliper LifeSciences IVIS 200). At specific time points post-implantation, mice were i.p. injected with 300mg/kg D-luciferine (Caliper LifeSciences) according to manufacturer's recommendations. Mice were anesthetized with isoflurane and imaged at 4 minutes intervals until the peak of bioluminescence signal was reached. The intensity of the signal (photons per second) was analyzed using Living image software. 35 days after EAE induction, animals were perfused with 4% PFA under anesthesia; brain and spinal cord were explanted, sectioned (20µm cryosections) and prepared for immunohistochemical and histological analysis.

RT-PCR and qRT-PCR

RNA was isolated using the standard Trizol-based procedure. Following cDNA synthesis and PCR reaction, DNA was visualized in an 1% agarose gel (RT-PCR). For qRT-PCR iTaq Supermix with ROX (Biorad, 172-5855) was used. Primer sequences used in this study are listed in Figure .S3

Supplementary Figures

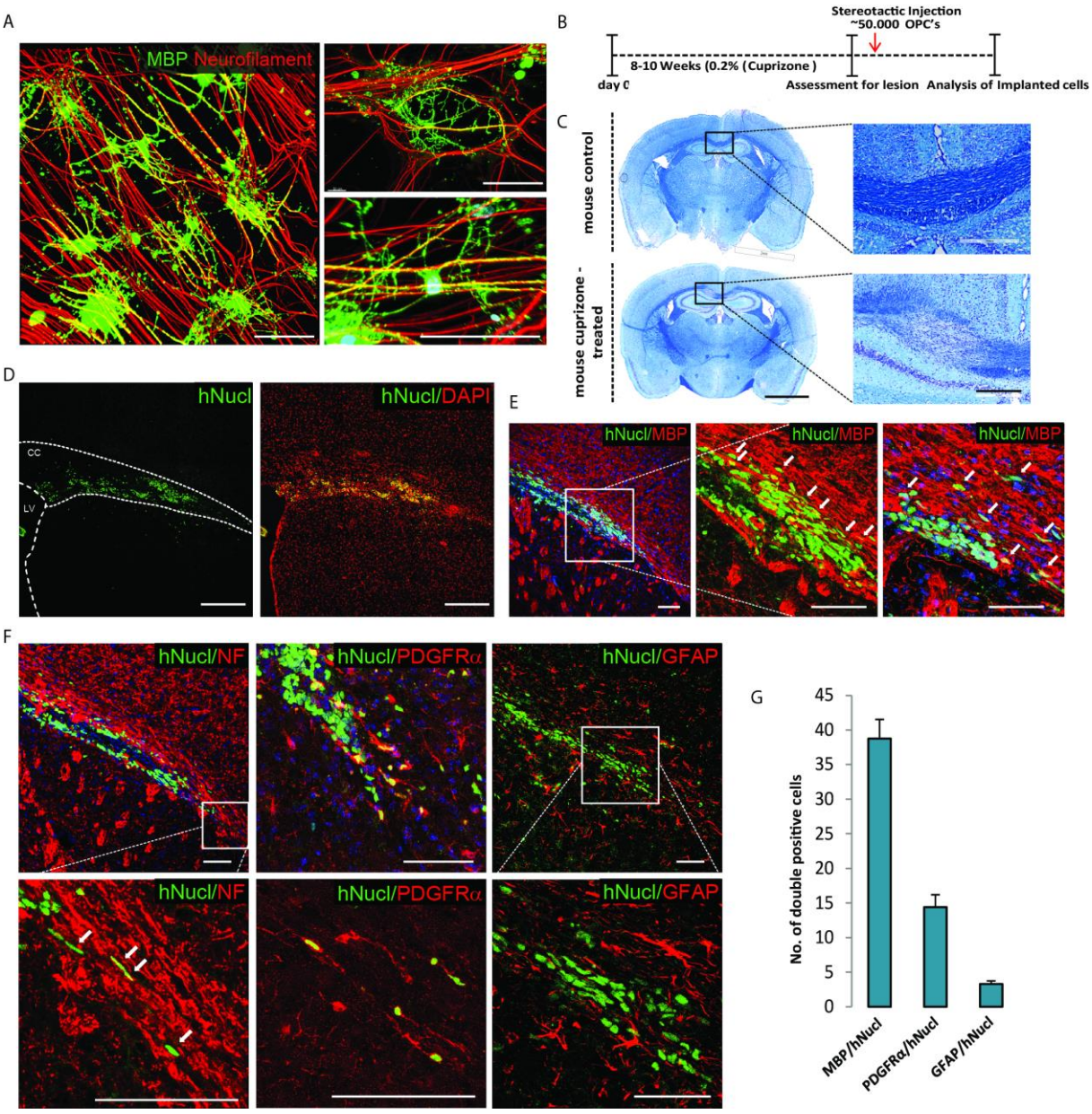


Figure S1. *In-vitro* and *in-vivo* myelination of human iPSC-derived OPCs. (A) 4-week old co-culture of hiPSC-derived OPCs (MBP-green) and rat DRG neurons (Neurofilament-red); High magnification images show the wrapping of green MBP-positive OPC extensions along and around red Neurofilament-stained axons. Scale bars: 50µm. **(B)** Set-up of implantation experiments with hiPSC-derived OPCs in the cuprizone mouse model. **(C)** Luxol fast blue (LFB) staining demonstrates a demyelinated lesion within the corpus callosum (CC). **(D)** Human nuclei (hNucl) immunostaining of implanted cells shows their distribution along the CC (LV=lateral ventricle). Scale bars: 200µm. **(E)** Human nuclei/MBP double immunostaining shows the contribution of implanted cells to remyelination (arrows). **(F)** Human nuclei/NF double immunostaining reveals implanted human cells located amongst CC axons (arrows); most of these cells appear to be immature OPCs (i.e. hNucl/PDGFRα-positive); hNucl/GFAP double immunostaining reveals that none of the implanted cells differentiated into astrocytes. **(G)** Quantification of MBP-, PDGFRα-, and GFAP-positive cells as percentage of implanted (hNucl-positive) cells in one standard area within the corpus callosum at 40 days after implantation, indicating that the majority of implanted cells became MBP-positive oligodendrocytes (n=5, mean ± SEM). Scale bars: 50µm.

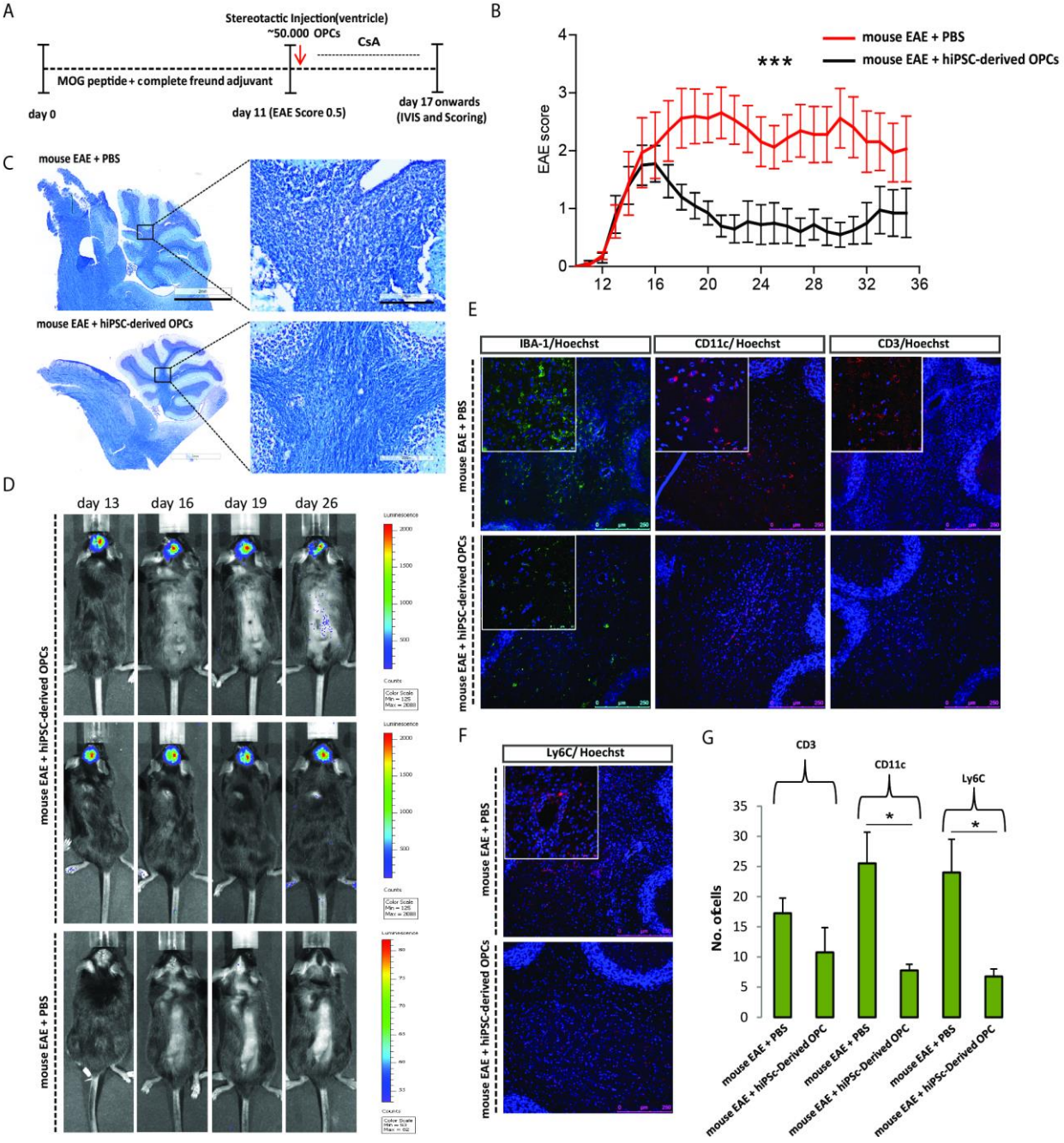

A
Stereotactic Injection(ventricle)
~50.000 OPCs
CsA
MOG peptide + complete freund adjuvant
day 0
day 11 (EAE Score 0.5)
day 17 onwards
(IVIS and Scoring)
B
mouse EAE + PBS
mouse EAE + hiPSC-derived OPCs
***
EAE score
C
mouse EAE + PBS
mouse EAE + hiPSC-derived OPCs
D
day 13
day 16
day 19
day 26
mouse EAE + hiPSC-derived OPCs
mouse EAE + PBS
E
IBA-1/Hoechst
CD11c/ Hoechst
CD3/Hoechst
mouse EAE + PBS
mouse EAE + hiPSC-derived OPCs
F
Ly6C/ Hoechst
mouse EAE + PBS
mouse EAE + hiPSC-derived OPCs
G
CD3
CD11c
Ly6C
No. of cells
mouse EAE + PBS
mouse EAE + hiPSC-Derived OPC

Figure S2 Implantation of hiPSC-derived OPCs in EAE mice. **(A)** Set-up of hiPSC-derived OPCs implantation experiments in mouse EAE model. **(B)** Average EAE score of experimental (with hiPSC-derived OPC grafts) (n=10**)** and control (PBS-injected) (n=8) mice demonstrates significant reduction of EAE symptoms in OPC-implanted animals. Statistics: One-way ANOVA; ***P<0,001; PBS**. (C)** LFB-staining demonstrates demyelination within the cerebellum in the EAE-PBS mice and not in EAE-hiPSC-derived OPCs injected mice. Scale bars: 2mm. **(D)** IVIS bioluminescence imaging of PBS (control) and human iPSC-derived OPCs implanted animals shows intracerebral localization of implanted cells but not in control group (13 days post implantation). (**E**) Immunostaining for IBA, mouse CD11c, CD3 and Ly6c within the cerebellum in the EAE-PBS mice and the EAE-hiPSC-derived OPCs injected mice shows differences in cell infiltrates. (**F&G**) Quantification of mouse CD11c-, CD3- and Ly6c-positive cells within the cerebellum in the EAE-PBS group and the EAE-hiPSC-derived OPCs injected group (n=4, mean ± SEM, Statistics: t-test ; *P<0,05). Scale bars: 250 µm (for magnification: 50µm).

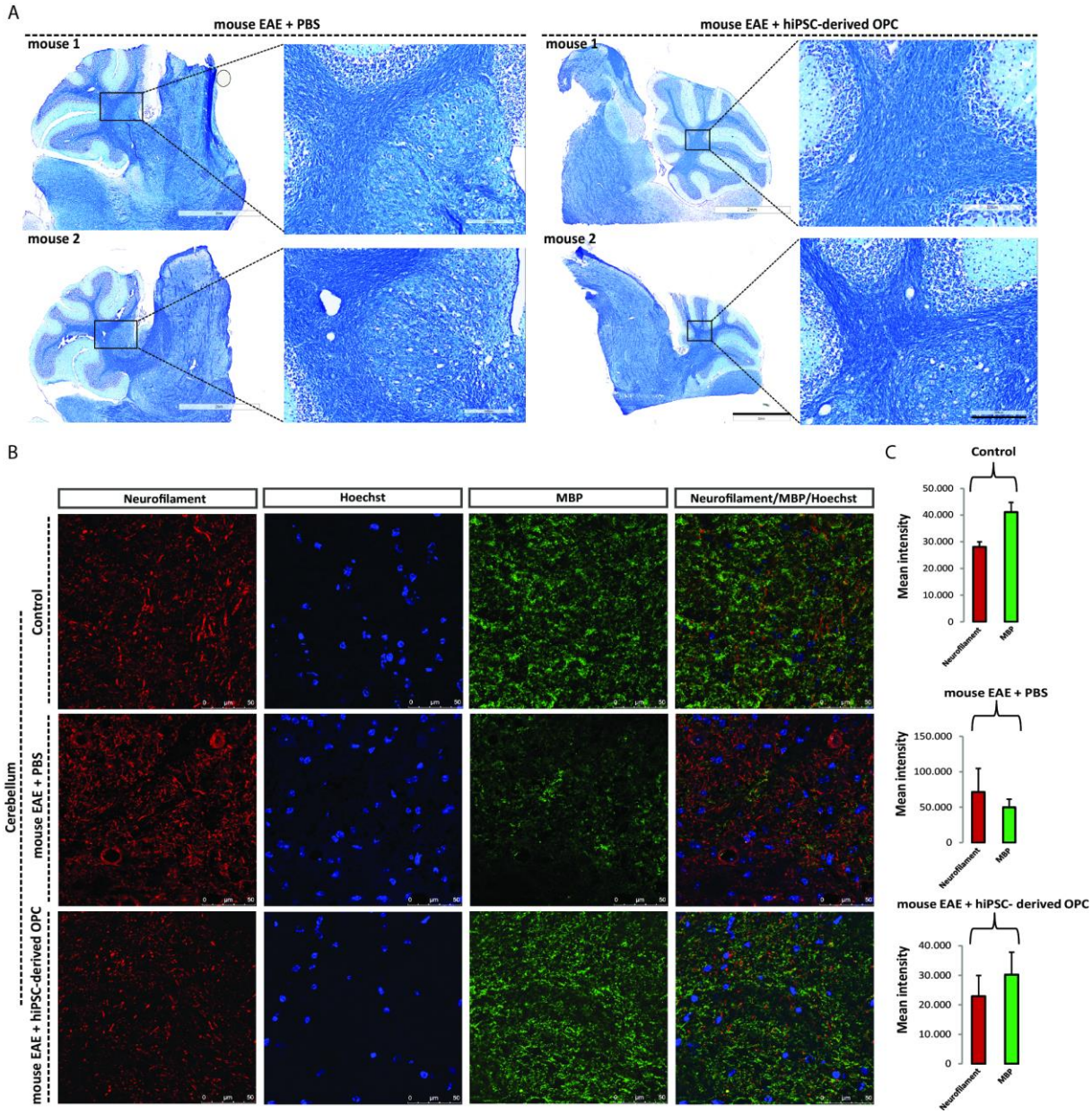
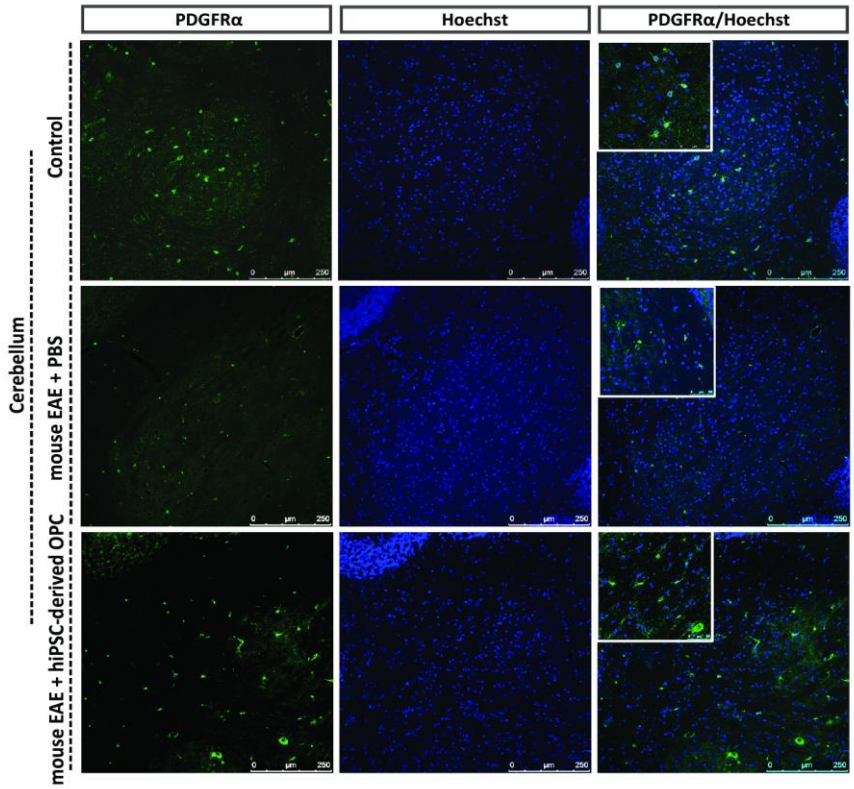
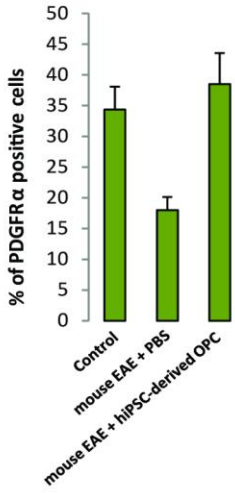


Figure S3. Implantation of hiPSC-derived OPCs in EAE mice. (A) LFB staining reveals demyelination within the cerebellum in the EAE-PBS mice and not in the EAE-hiPSC-derived OPCs injected mice. Scale bars: 2mm. **(B)** Neurofilament/MBP double immunostaining within the cerebellum in the control, EAE-PBS group and EAE-hiPSC-derived OPCs injected mice. **(C)** Quantification of Neurofilament and MBP within the cerebellum in the control, the EAE-PBS and EAE-hiPSC-derived OPCs injected mice (n=4, mean ± SEM). Scale bars: 50µm.

A


PDGFRα
Hoechst
PDGFRα/Hoechst
Cerebellum
Control
mouse EAE + PBS
mouse EAE + hiPSC-derived OPC
0 μm 250

B


% of PDGFRα positive cells
50
45
40
35
30
25
20
15
10
5
0
Control
mouse EAE + PBS
mouse EAE + hiPSC-derived OPC

C

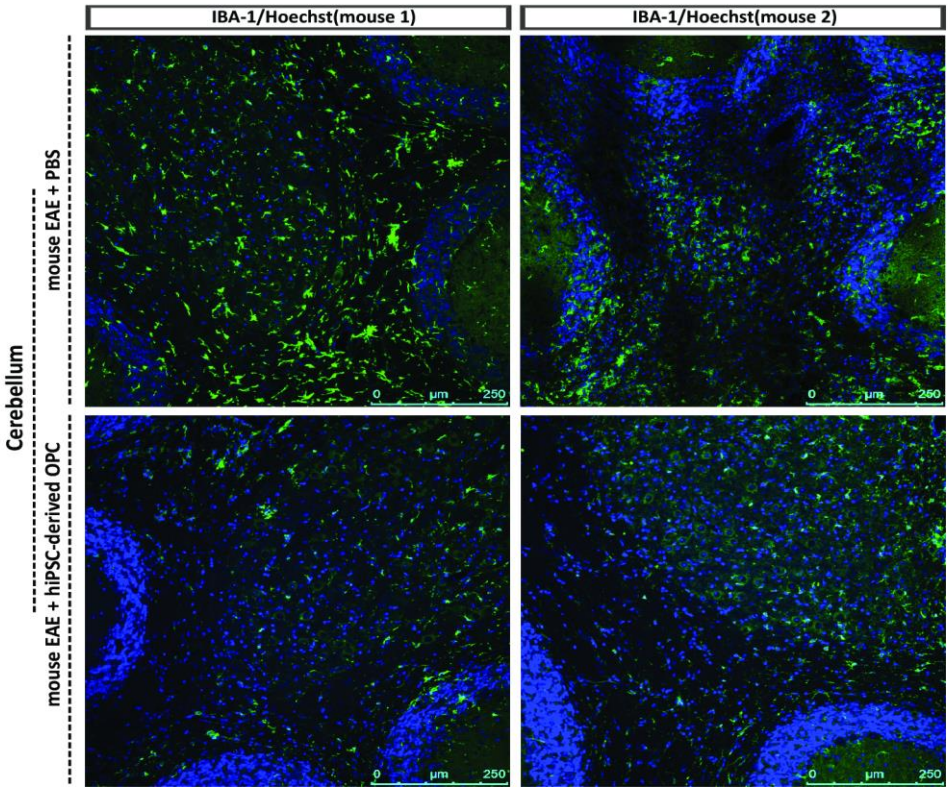
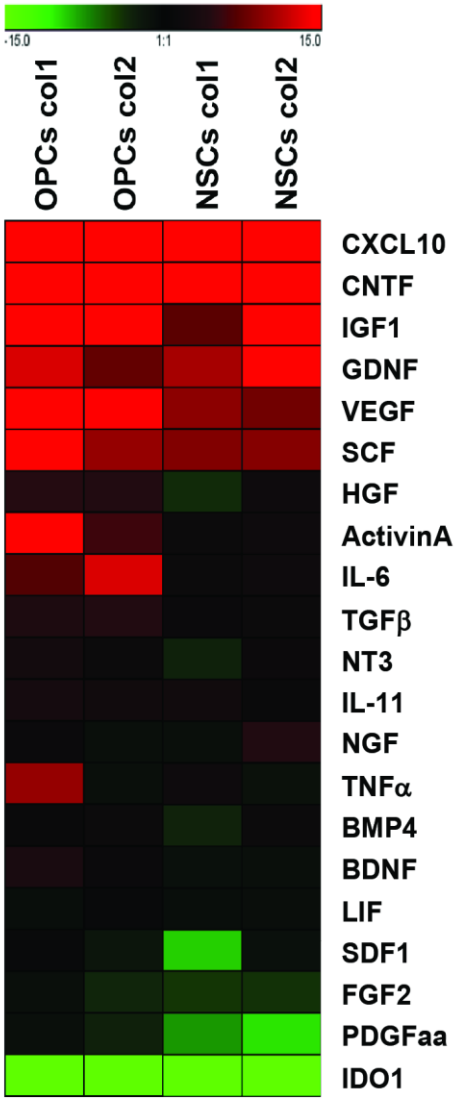

IBA-1/Hoechst(mouse 1)
IBA-1/Hoechst(mouse 2)
Cerebellum
mouse EAE + PBS
mouse EAE + hiPSC-derived OPC
0 μm 250

Figure S4. Implantation of hiPSC-derived OPCs in EAE mice. (A) PDGFRα immunostaining within the cerebellum in the control, the EAE-PBS and the EAE-hiPSC-derived OPCs injected mice. Scale bars: 250 µm (for magnifications 50µm). **(B)** Quantification of PDGFRα positive cells within the cerebellum in the control, the EAE-PBS and the EAE-hiPSC-derived OPCs injected mice (n=4, mean ± SEM). **(C)** IBA-1 immunostaining within the cerebellum in the EAE-PBS and the EAE-hiPSC-derived OPCs injected mice. Scale bars: 250µm.

A



B

Gene	*Forward primer*	*Reverse primer*
Oct4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
Sox2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
Klf4	ACGATCGTGGCCCCGGAAAAGGACC	TGATTGTAGTGCTTTCTGGCTGGGCTCC
Nanog	CAGCCCCGATTCTTCCACCAGTCCC	CGGAAGATTCCCAGTCGGGTTCACC
cMyc	GCGTCCTGGGAAGGGAGATCCGGAGC	TTGAGGGGCATCGTCGCGGGAGGCTG
CXCL10	AGCAGAGGAACCTCCAGTCT	ATGCAGGTACAGCGTACAGT
CNTF	TCGTTCAGACCTGACTGCTC	TTCTCTTGGAGTCGCTCTGC
IGF1	TTTCAACAAGCCCACAGGGT	TTGAGGGGTGCGCAATACAT
GDNF	GGGTCTGGGCTATGAAACCA	TGCCTGCCCTACTTTGTCAC
VEGF	CTTGCCTTGCTGCTCTACCT	GCAGTAGCTGCGCTGATAGA
SCF	GGAAGGCCAAAAATCCCCCT	GACTTGGCTGTCTCTTCTTCCA
HGF	TTTGCCTTCGAGCTATCGGG	TGTCACAGACTTCGTAGCGT
ActivinA	TTATGGAGCAGACCTCGGAGA	TGGCCTTGGGGACTTTTAGG
IL-6	ACCCCCAGGAGAAGATTCCA	TTACATGTCTCCTTTCTCAGGGC
TGFβ	TTCGCCTTAGCGCCCACTGC	GGCCGGTAGTGAACCCGTTGATG
NT3	CTGCGACAACAGAGACGCTA	CGTGATGTTCTGTTCGCCAC
IL-11	GGCTGGATGCTTGGGTAACT	CACAGAGCTGTCTGGGTGAC
NGF	GTGTGCTGTTTAGCACCCAG	GGATGGGATGATGACCGCTT
TNFα	CAGGCGCCACCACGCTCTTC	CTGGGGAACTCTTCCCTCTGGGG
BMP4	CCACAGCACTGGTCTTGAGT	CAGGGATGCTGCTGAGGTTA
BDNF	GCGCCACTCTGACCCTGCC	TCTGCTGCCGTTACCCACTCACT
LIF	AGAAGCTGGGCTGTCAACTC	CCACATCTGGACCCAACTCC
SDF1	GAACGCCAAGGTCGTGGT	TGGCTTTCGAAGAATCGGCA
FGF2	CACTTCAAGGACCCCAAGCG	TGCTTGAAGTTGTAGCTTGATGTG
PDGFaa	GACACGGATGTGAGGTGAGG	GGAGGAGAACAAAGACCGCA
IDO1	CACTTTGCTAAAGGCGCTGT	TGCCTTTCCAGCCAGACAAAT

Figure S5. Analysis of cytokine production by hiPSC-derived OPCs and hiPSC-derived NSCs. (A) Heatmap presents the expression of the major immunomodulatory and neurotrophic factors expressed by hiPSC-derived OPCs and NSCs based on quantitative-PCR analysis. Data normalized to undifferentiated pluripotent cells. **(B)** Primer sequences used in the study**.**

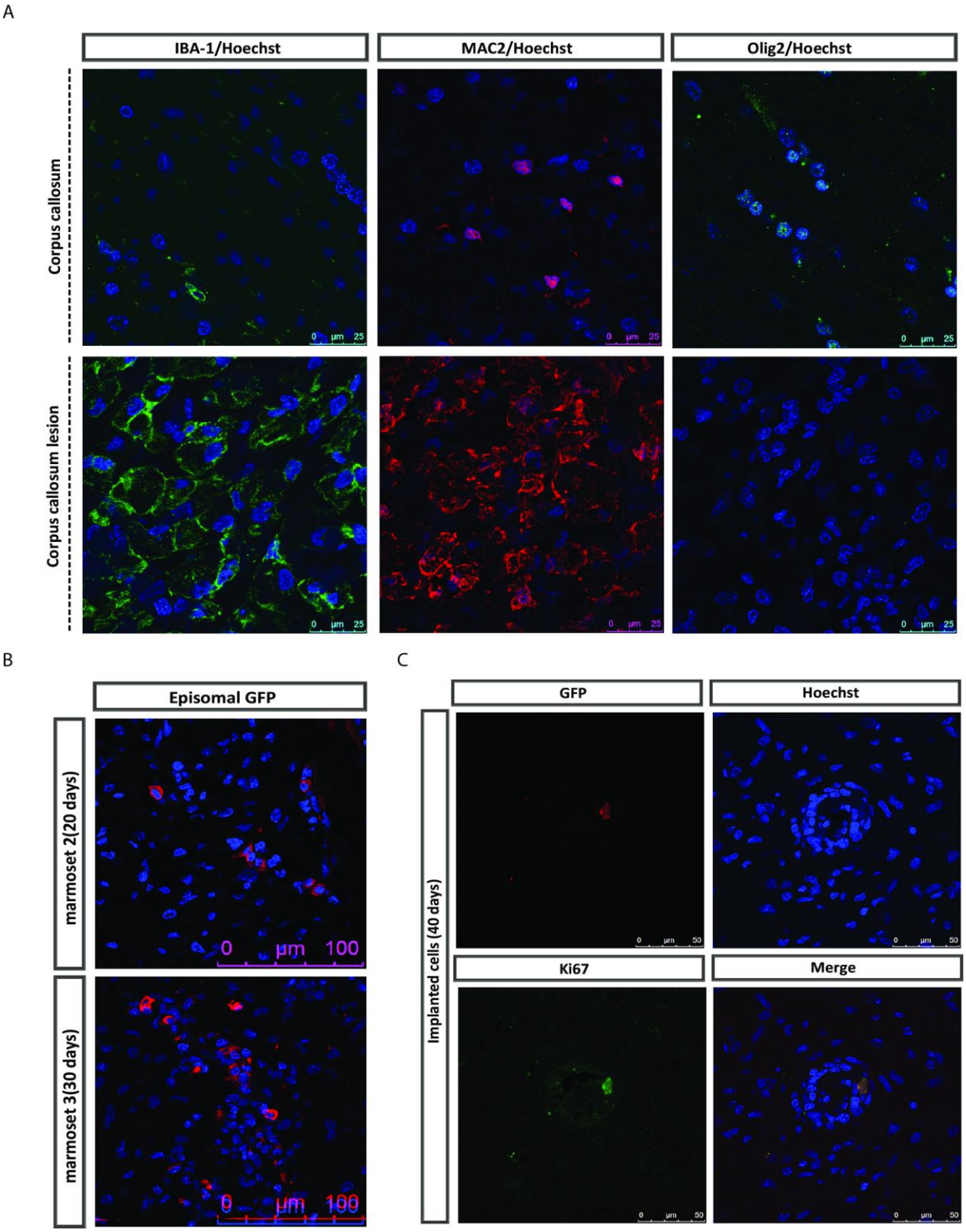

A
IBA-1/Hoechst
MAC2/Hoechst
Olig2/Hoechst
Corpus callosum
Corpus callosum lesion
B
Episomal GFP
marmoset 2(20 days)
marmoset 3(30 days)
0 µm 100
C
GFP
Hoechst
Ki67
Merge
Implanted cells (40 days)

Figure S6. EAE marmosets and implanted hiPSC-derived OPCs. (A) Immunostaining for IBA, MAC2 in the marmoset EAE brain shows activated microglial cells in the lesion and no OLIG-2 positive cells. Scale bars: 25µm. **(B)** GFP-positive implanted hiPSC-derived OPCs within the marmoset brain (animals 20 and 30 days post implantation). Scale bars: 100µm. **(C)** Ki67/GFP double immunostaining reveals only a few proliferating hiPSC-derived OPCs within the marmoset EAE brains at 40 days post implantation. Scale bars: 50µm.

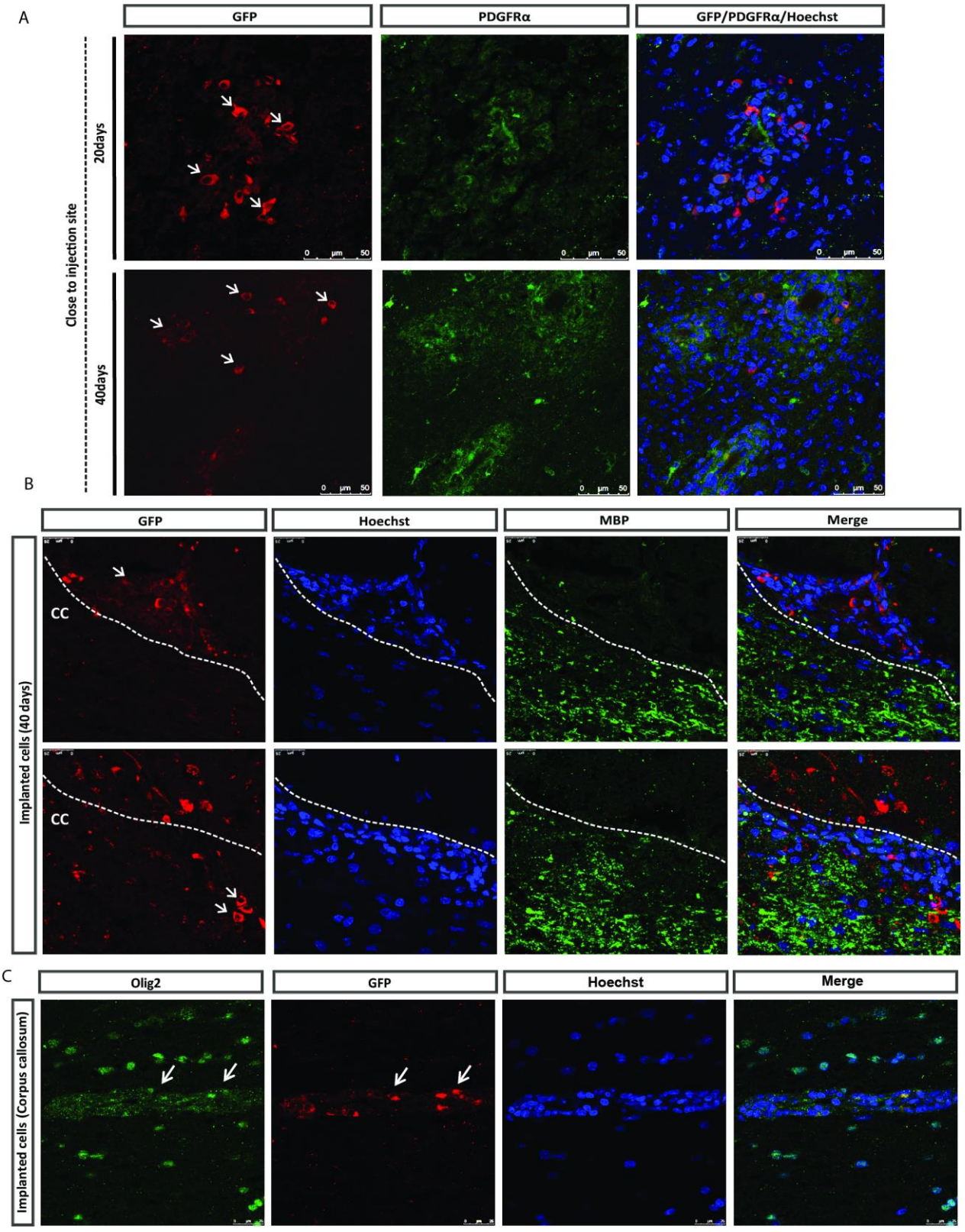

A
GFP
PDGFRα
GFP/PDGFRα/Hoechst
Close to injection site
20days
40days
B
GFP
Hoechst
MBP
Merge
Implanted cells (40 days)
CC
CC
C
Olig2
GFP
Hoechst
Merge
Implanted cells (Corpus callosum)

Figure S7. Migration of implanted hiPSC-derived OPCs in EAE marmosets. (A) GFP/PDGFRα double immunostaining reveals immature OPCs (arrows) among the implanted cells near the injection site (20 and 40 days post implantation) and negative for MBP (data not shown). Scale bars: 50µm **(B)** MBP/Hoechst counterstained GFP-labeled hiPSC-derived OPCs migrate in the corpus callosum from the site of injection and express MBP. Scale bars: 25 µm. **(C)** GFP/OLIG2 immunostaining shows the typical location and morphology of the implanted OPCs progenitors within the CC (arrows). Scale bars: 25 µm.

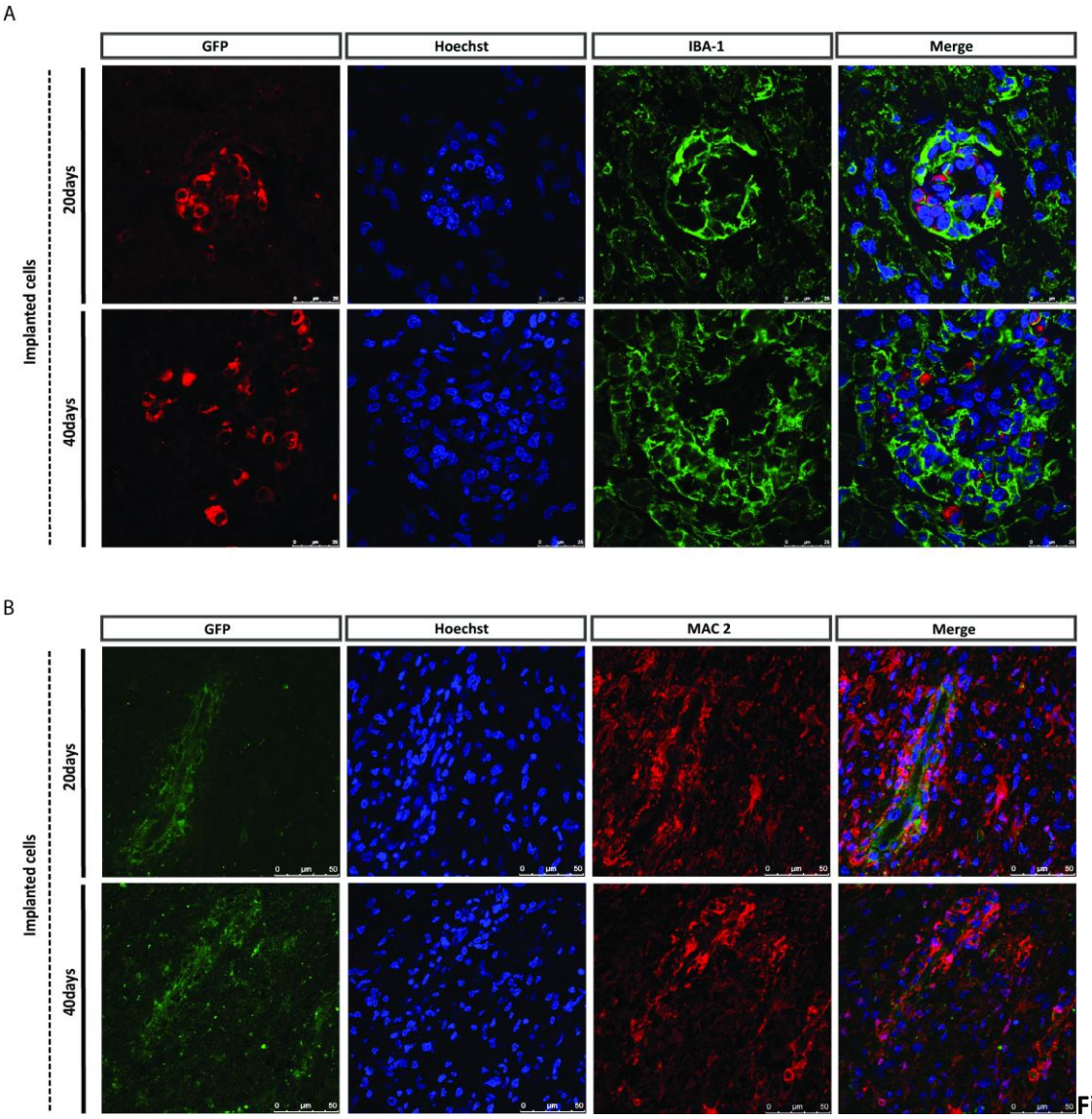


Figure S8. Response of microglia/macrophages to implanted hiPSC-derived OPCs in EAE marmosets. (A & B). At 40 days post implantation, viable implanted GFP-labeled hiPSC-derived OPCs are surrounded by IBA and MAC2 positive microglia/macrophages. Scale bars: 25µm (IBA-1) and 50µm (MAC2).