Nature Neuroscience 16, 1211–1218 (2013) doi:10.1038/nn.3469

Supplemental Information

Title:

M2 microglia/ macrophages drive oligodendrocyte differentiation during CNS remyelination

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Supplementary Figure 1

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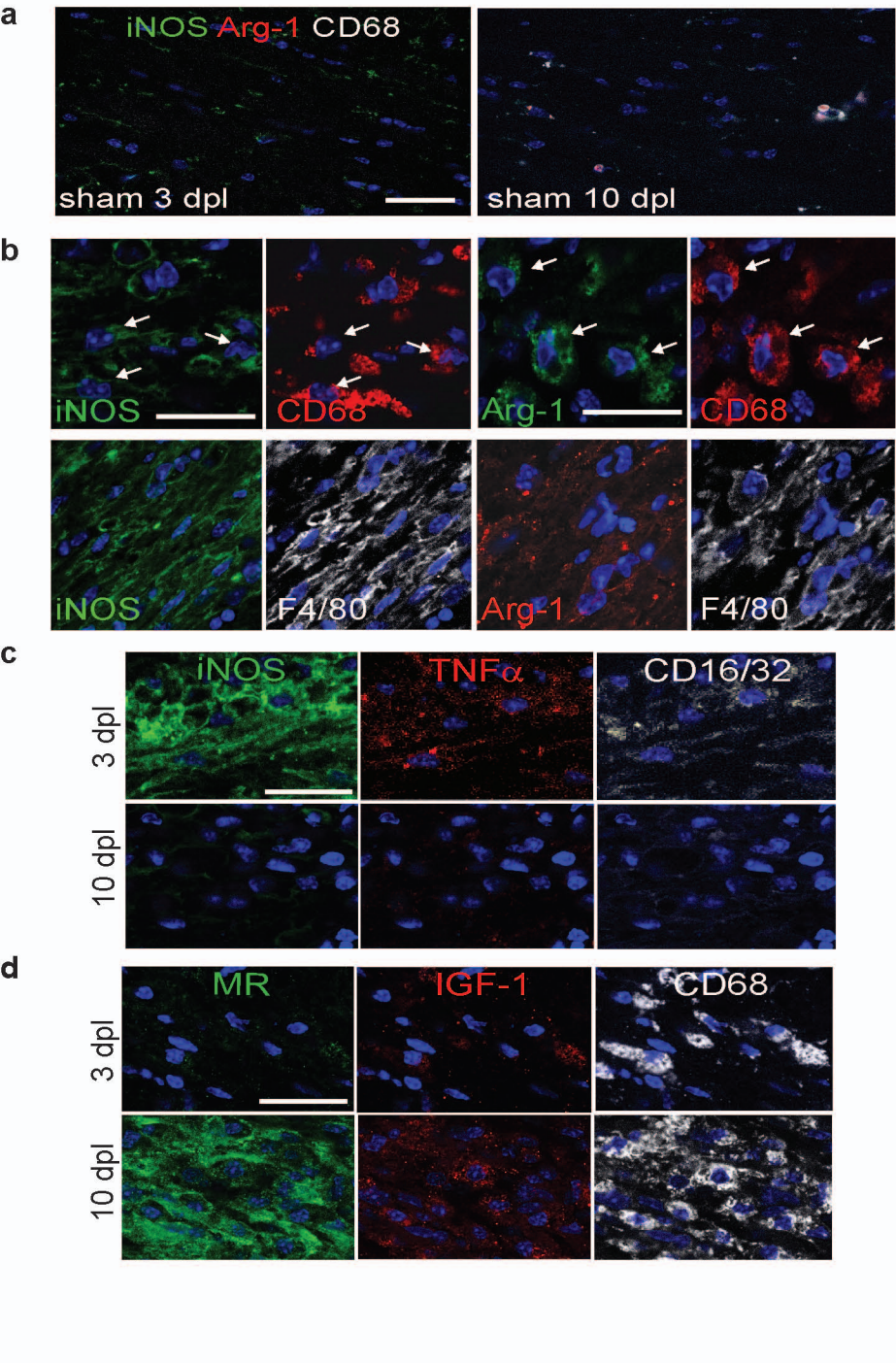

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Supplementary Figure 1 Additional characterization of polarization in corpus callosum lesions.

(a) Representative images of sham PBS-injected lesions at 3 and 10 dpl immunostained for iNOS (green), Arg-1 (red), and CD68 (white). (b) Co-localization of iNOS and Arg-1 in microglia/ macrophage cell bodies (CD68; arrows) and processes (F4/80). 3 and 10 dpl lesions immunostained for additional M1 markers (c; TNFα, CD16/32) and M2 markers (d; MR, IGF-1). All scale bars, 25 μm.

Supplementary Figure 2

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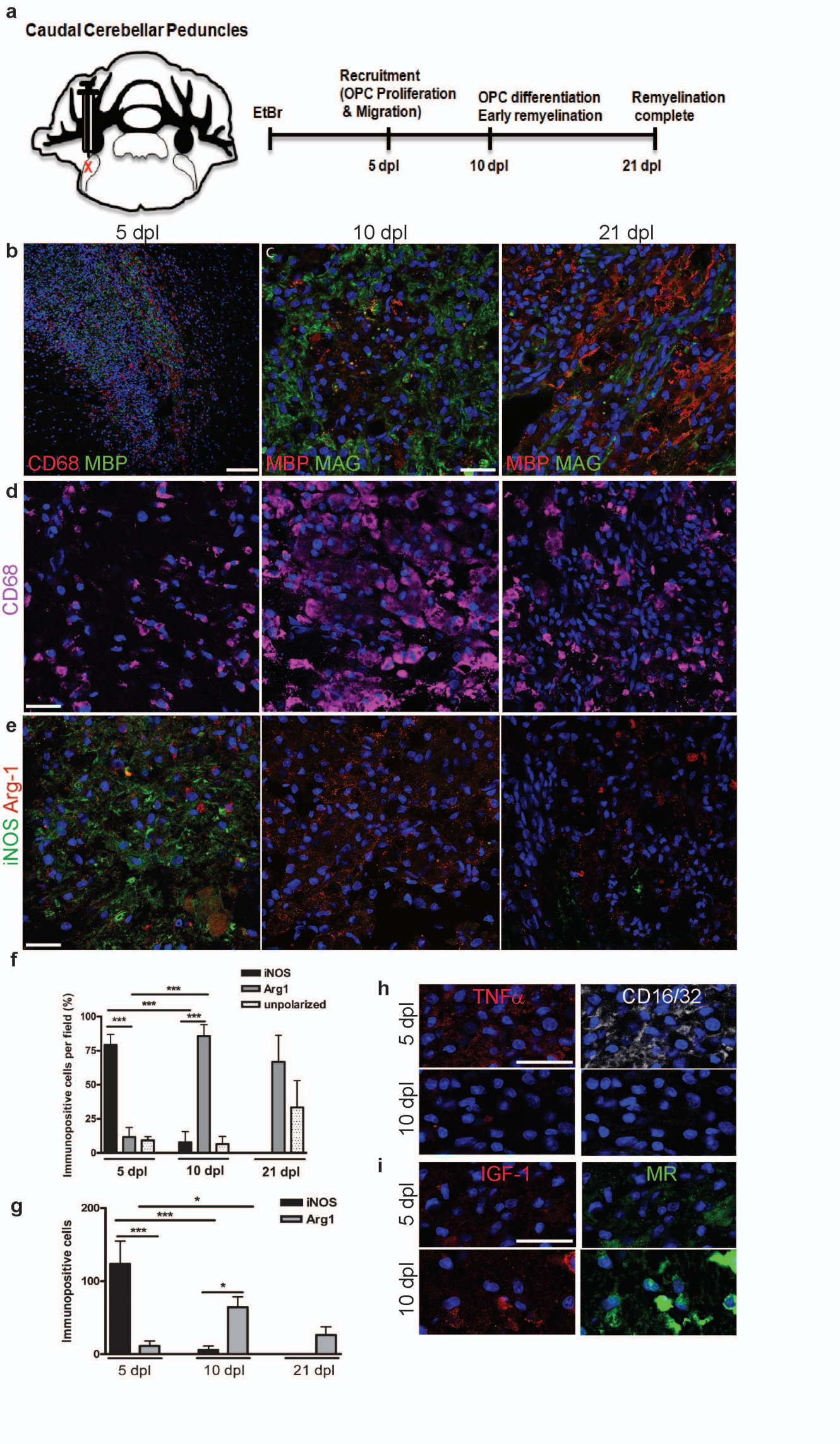

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Supplementary Figure 2 Switch from M1- to M2-dominant response in remyelinating lesions of the rat caudal cerebellar peduncle.

(a) Oligodendroglial lineage cell responses following induction of demyelination of the caudal cerebellar peduncle by stereotaxic injection of ethidium bromide (EtBr). Scale bar, 100 μm. (b) Injection of demyelinating toxin induces accumulation of microglia (CD68+; red) at 5 dpl. Recovery of myelin protein expression is observed with re-expression of MAG at 10 dpl (c, centre) and MBP at 21 dpl (c, right). (d) Microglia/ macrophages (CD68+; purple) are present at 5 dpl, increase in abundance at 10 dpl, and decrease in number by 21 dpl. (e) Representative images of lesions immunostained for iNOS (green) and Arg-1 (red). Percentage (f) and mean number (g) of iNOS+ M1, Arg-1+ M2, and unpolarized (iNOS– Arg1–) cells per field ± s.e.m. at 5, 10, and 21 dpl. One-way ANOVA and Newman-Keuls post test, *$p<0.05$, **$p<0.01$. (n=3, df=26, 17, respectively). (h) 5 and 10 dpl sections immunostained for additional M1 (TNFα, CD16/32) and M2 (IGF-1, MR) markers. All other scale bars, 25 μm.

Supplementary Figure 3

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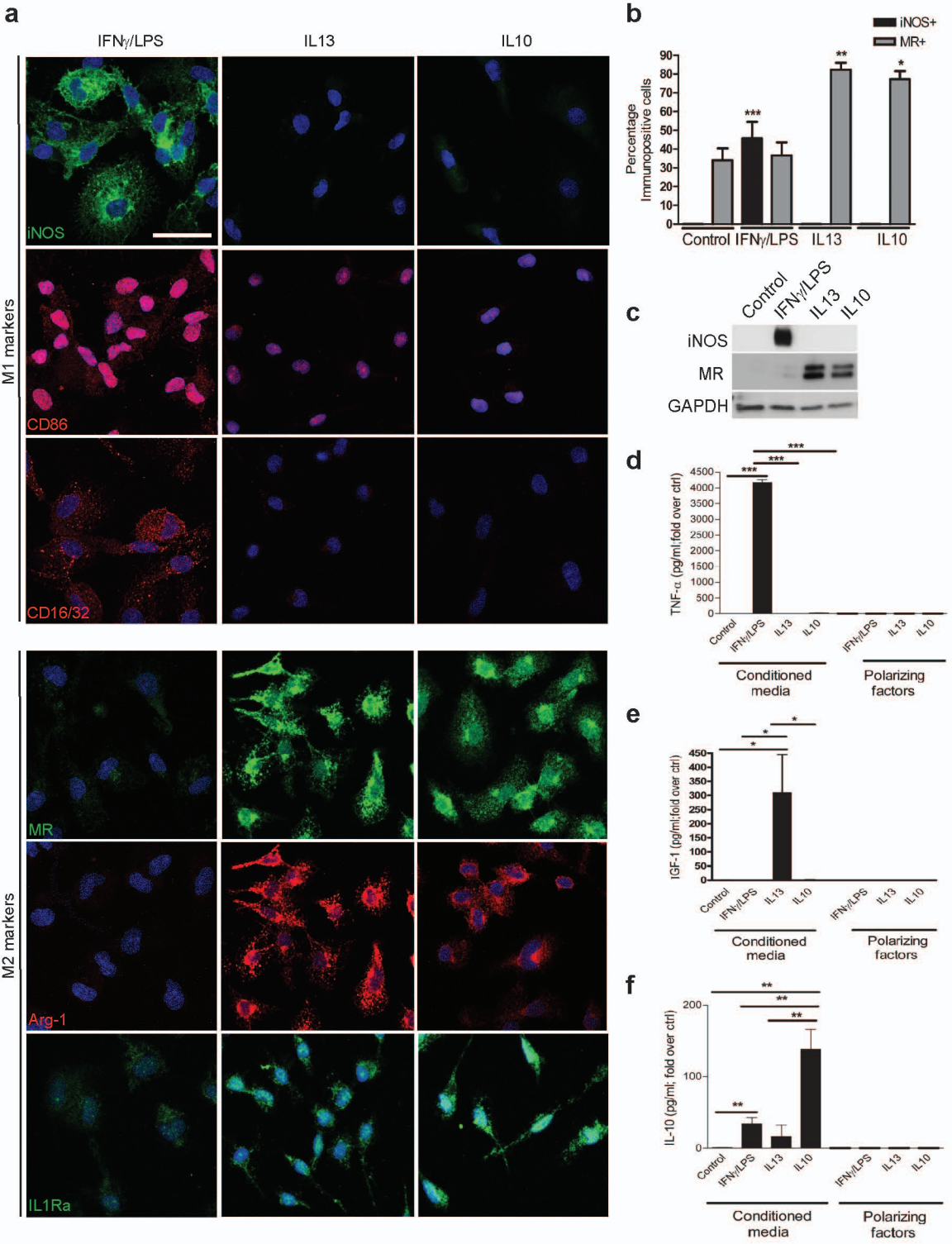

Supplementary Figure 3 Polarization of cultured microglia to M1 and M2 phenotypes.

(a) Representative images of microglia immunostained against M1 markers iNOS, CD86, CD16/32 (top) and M2 markers MR, Arg-1, and interleukin-1 receptor antagonist (IL1Ra) (bottom). Scale bar, 25 μm. (b) Mean percentage of iNOS+ or MR+ cells of total CD68+ cells ± s.e.m.. Kruskal-Wallis test and Dunn's multiple comparison post-hoc test, *p<0.05, **p<0.01, ***p<0.001. (*n*=4) (c) Cropped Western blots showing expression of iNOS with IFNγ/LPS treatment and increase in MR expression with IL-13 or IL-10 treatment, with loading control GAPDH. ELISAs used to assay conditioned media for levels of TNFα (d; *P*=0.0004, *n*=5, df=8), IGF-1 (e; *P*=0.0330, *n*=3, df=4), and IL-10 (f; *P*=0.0051, *n*=5, df=8), presented as mean fold over M0 control ± s.e.m. demonstrating polarization to M1, M2a, and M2c, respectively. A small yet significant increase in IL-10 secretion was observed with IFNγ/ LPS treatment (*P* =0.0081). Polarizing factors alone were included in the assay as a control and did not show detectable levels relative to those measured in conditioned media (d-f). 2-tailed Student's *t*-test.

Supplementary Figure 4

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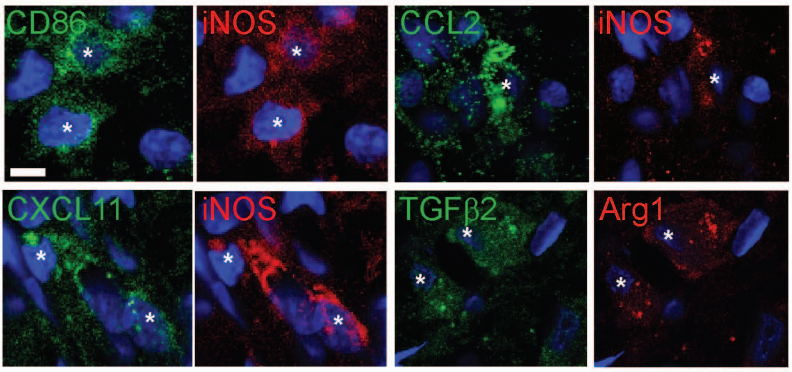
a

Upregulated M1 genes:	Upregulated M2 genes:
Cd86	*Mrc1 (MR)*
Cxcl11	*Tgfb2*
Ccl2	*Arg1*
Fcgr2a (Cd32)	

b



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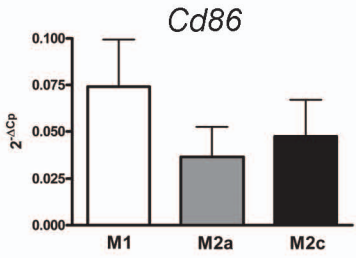

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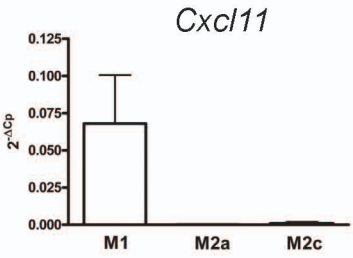

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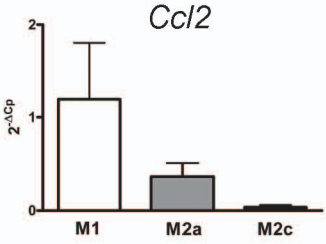

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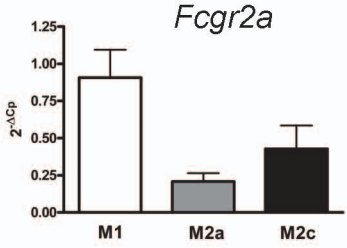

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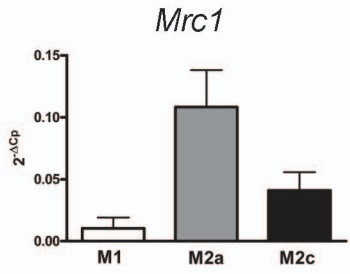

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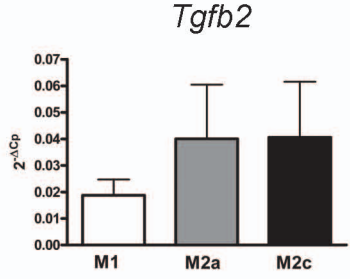
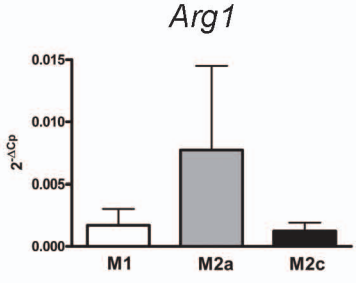


i



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Supplementary Figure 4 M1 and M2 associated gene expression in cultured microglia.

(a) M1- and M2-associated genes were identified from a previously performed microarray[18] as being significantly upregulated during remyelination of the rat caudal cerebellar peduncles (CCP). These genes were selected for a custom qPCR array to assess gene expression profiles of polarized microglia *in vitro*. (b) Confirmation of expression of additional polarization markers CD86, CCL2, CXCL11, and TGFβ2 in iNOS+ or Arg-1+ cells in the rat CCP. Scale bar, 5 μm. Microglia treated with IFNγ/LPS (M1), IL-13 (M2a), and IL-10 (M2c) were analyzed for gene expression levels of (c) *Cd86*, (d) *Cxcl11*, (e) *Ccl2*, (f) *Fcgr2a* (*Cd32*), (g) *Mrc1*(mannose receptor), (h) *Tgfb2*, and (i) *Arg1*, values are represented as $2^{-\Delta Cp}$ ± s.e.m.

Supplementary Figure 5

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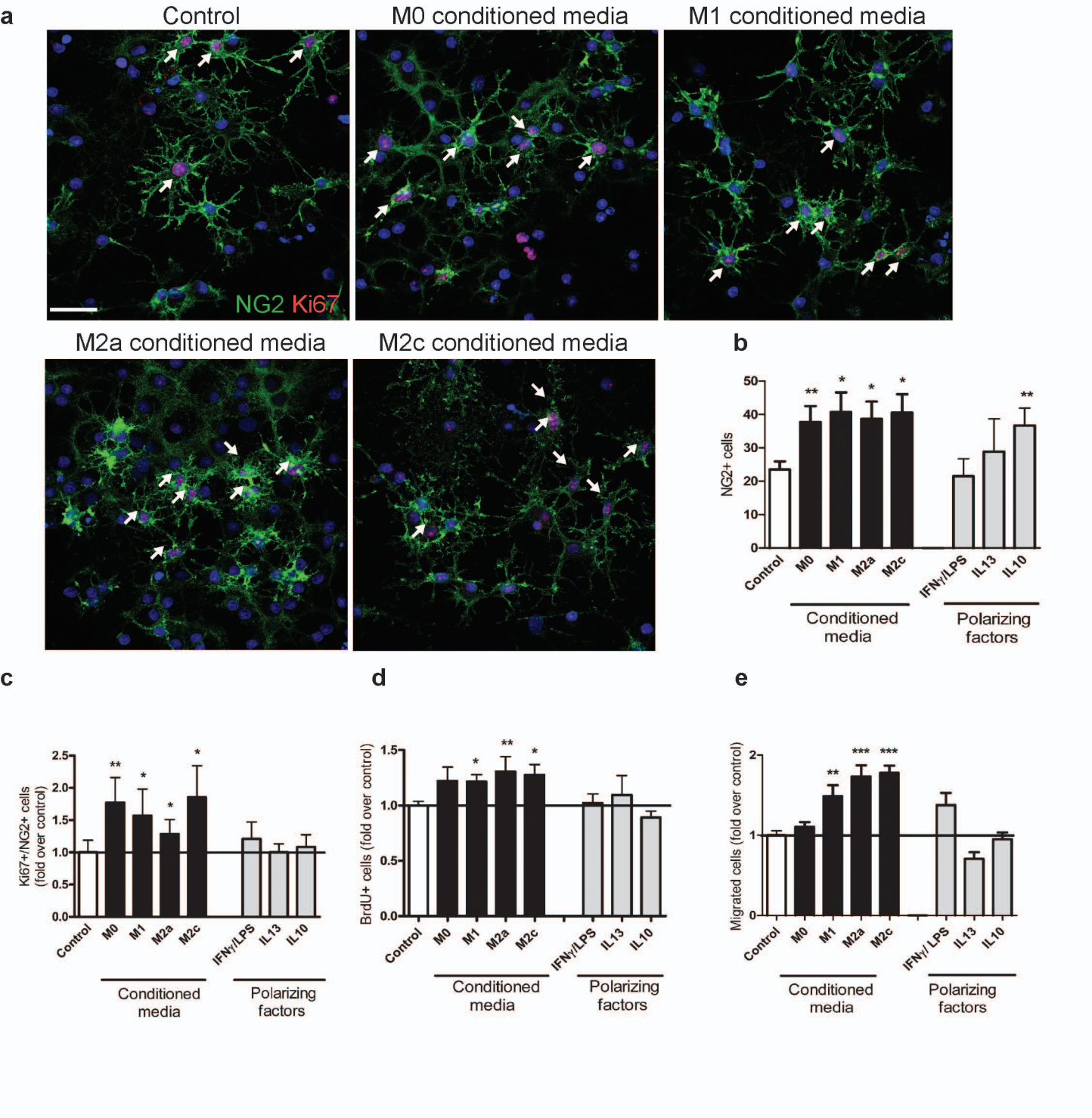

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Supplementary Figure 5 M1 and M2 microglia conditioned media increase OPC proliferation and migration.

OPCs were treated with microglia conditioned media for 3d. Polarizing factors present in the conditioned media (IFNγ/LPS, IL-13, and IL-10) were directly applied to OPCs as a control. (a) Representative images of OPCs immunostained against NG2 (green) and the proliferative marker Ki67 (red). Double-positive cells are highlighted by arrows. Scale bar, 25 μm. (b) Number of NG2+ cells per field was increased with M0 (P=0.005), M1 (P=0.00188), M2a (P=0.00382), M2c (P=0.0119) conditioned media (CM), and IL-10 (P=0.0029) compared to control (n=5, df=8). (c) Ki67+/NG2+ cells were increased with M0 (P=0.0084), M1 (P=0.0362), M2a (P=0.0194), M2c (P=0.0192) conditioned media (n=4, df=6) (d) BrdU+ cells were increased with M1 (P=0.042), M2a (P=0.0083) and M2c conditioned media (P=0.0423) (n=4, df=6). Values from conditioned media-treated conditions (black bars) and polarizing factors alone (grey bars) were normalized to control (unconditioned media, white bars) in (c) and (d), and are represented as mean ± s.e.m., 2-tailed Student's t-test. (e) Number of OPCs ± s.e.m. migrated towards microglia conditioned media (black bars), or polarizing factors alone (grey bars), normalized to values in control (unconditioned media, white bar) showing chemotactic properties of both M1 and M2 conditioned media. Kruskal-Wallis test and Dunn's multiple comparison post-test, **p<0.01, ***p<0.001. (n=3).

Supplementary Figure 6

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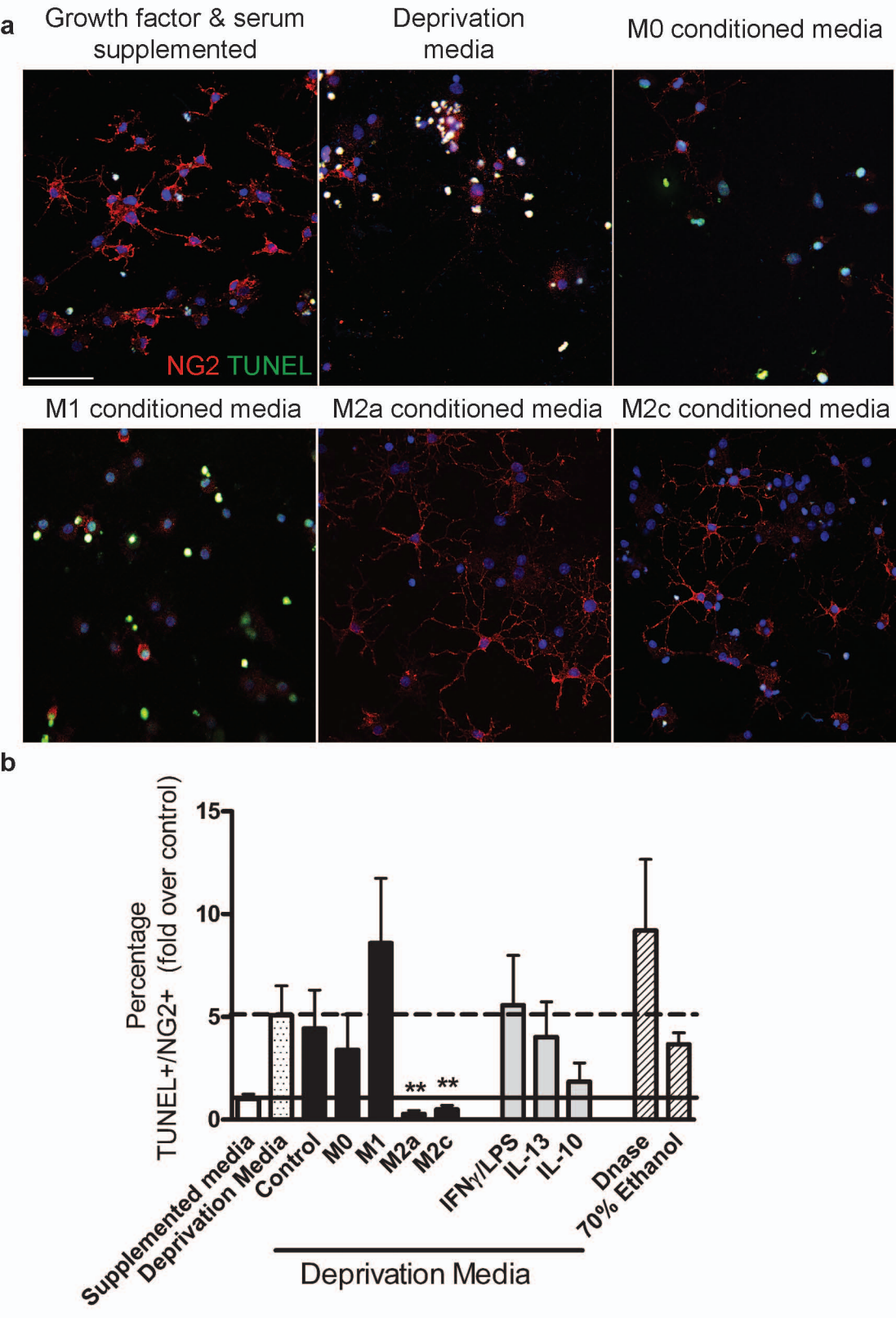

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Supplementary Figure 6 M2 polarized microglia conditioned media promotes the survival of OPCs in a death-inducing environment.

OPCs were treated with microglia conditioned media for 3d in media devoid of serum and growth factors. Polarizing factors present in the conditioned media (IFNγ/LPS, IL-13, and IL-10) were directly applied to OPCs as a control. (a) Representative images of OPCs under basal growth supplemented conditions, grown in deprivation media alone, or supplemented with microglia conditioned media. OPCs were immunostained against NG2 (red) and apoptotic OPCs were visualized by TUNEL assay (green). Scale bar, 50 μm. (b) Mean percentage of TUNEL+ NG2+ OPCs normalized to control supplemented with growth factors and media ± s.e.m.. DNase and ethanol treatment were positive controls for TUNEL positivity. Application of microglia conditioned media to OPCs under normal growth conditions did not induce apoptosis (data not shown). Kruskal-Wallis test and Dunn's multiple comparison post-test, *$p<0.05$, **$p<0.01$. ($n=6$).

Supplementary Figure 7

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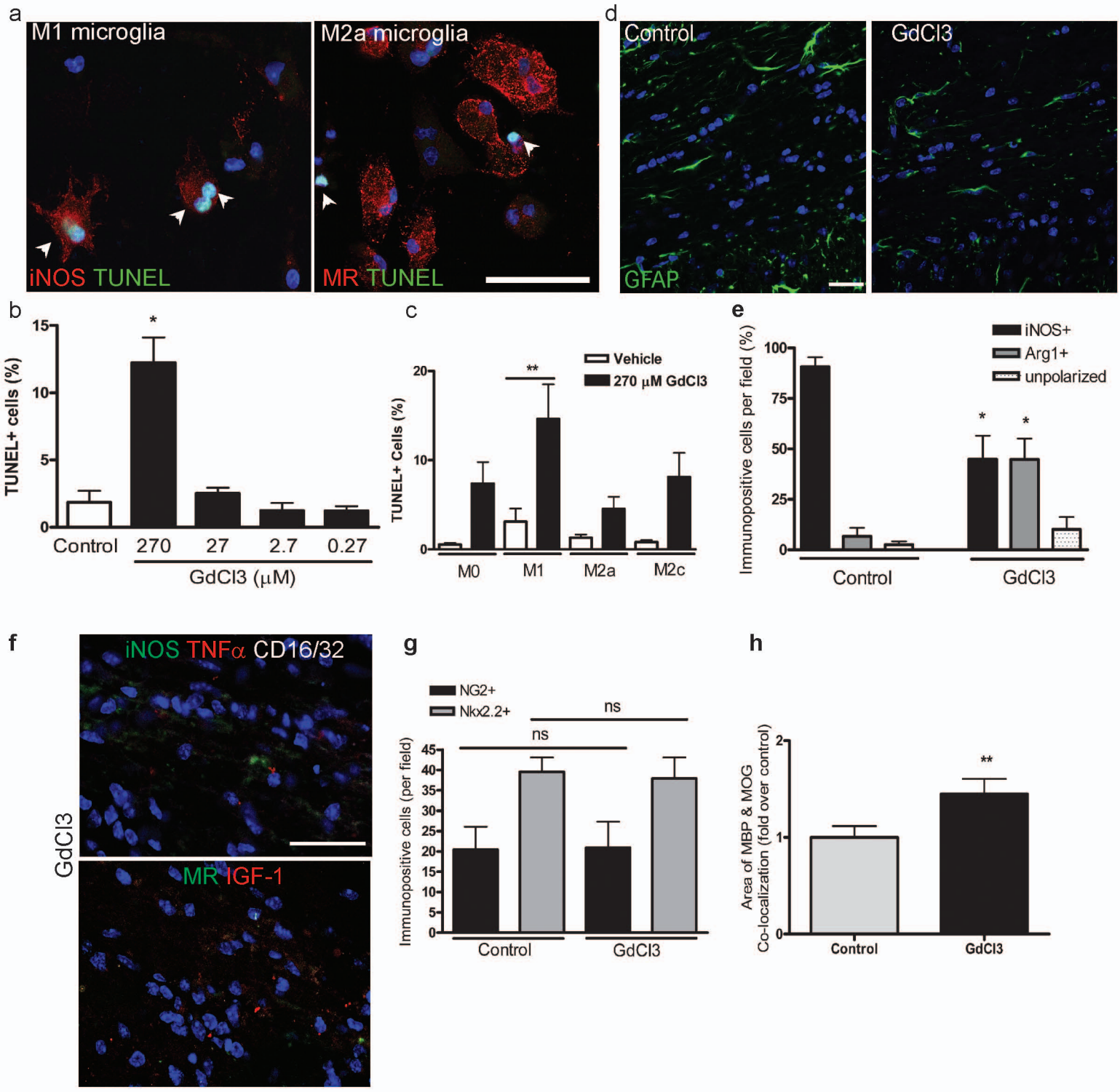

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Supplementary Figure 7 Selective depletion of M1 polarized microglia/ macrophages using gadolinium chloride.

(a) Representative images of M1 (iNOS+; left) and M2a (MR+; right) microglia treated with 270 µm GdCl3 and apoptosis assessed by TUNEL assay (green nuclei). Apoptotic cells are indicated by arrowheads, and were iNOS+ and MR–. Scale bar, 25 µm. (b) Percentage of TUNEL+ M1 microglia treated with vehicle (ctrl), or GdCl3 (0.27-270 µm). Mann-Whitney test, P=0.0286. (n=4). (c) Mean percentage of TUNEL+ cells ± s.e.m. in M0, M1, M2a, and M2c polarized microglia treated with vehicle or 270 µm GdCl3. One-way ANOVA and Newman-Keuls post-hoc test, **p<0.01 (n=6, df=54). (d) GFAP immunostaining in representative control and GdCl3-injected lesions shows no difference in astrocyte reactivity. Scale bar, 25 µm. (e) Percentage of iNOS+ or Arg1+ cells in control or GdCl3-injected lesions, P=0.0135 (iNOS), 0.0319 (Arg1), 2-tailed Student's t-test (n=5, df=8). (f) GdCl3-injected lesions immunostained for M1 markers (iNOS, TNFα, CD16/32) and M2 markers (MR, IGF-1) at 3 dpl. Scale bar, 25 µm. (g) NG2+ (n=4, df=6) and Nkx2.2+ (n=5, df=8) cells per field in control and GdCl3-treated lesions at 3 dpl. 2-tailed Student's t-test, p>0.05. (h) Mean area of MBP and MOG co-localization, fold over control ± s.e.m. in lesions at 21 dpl. P=0.081, 2-tailed Student's t-test (n=6, df=10).

Supplementary Figure 8

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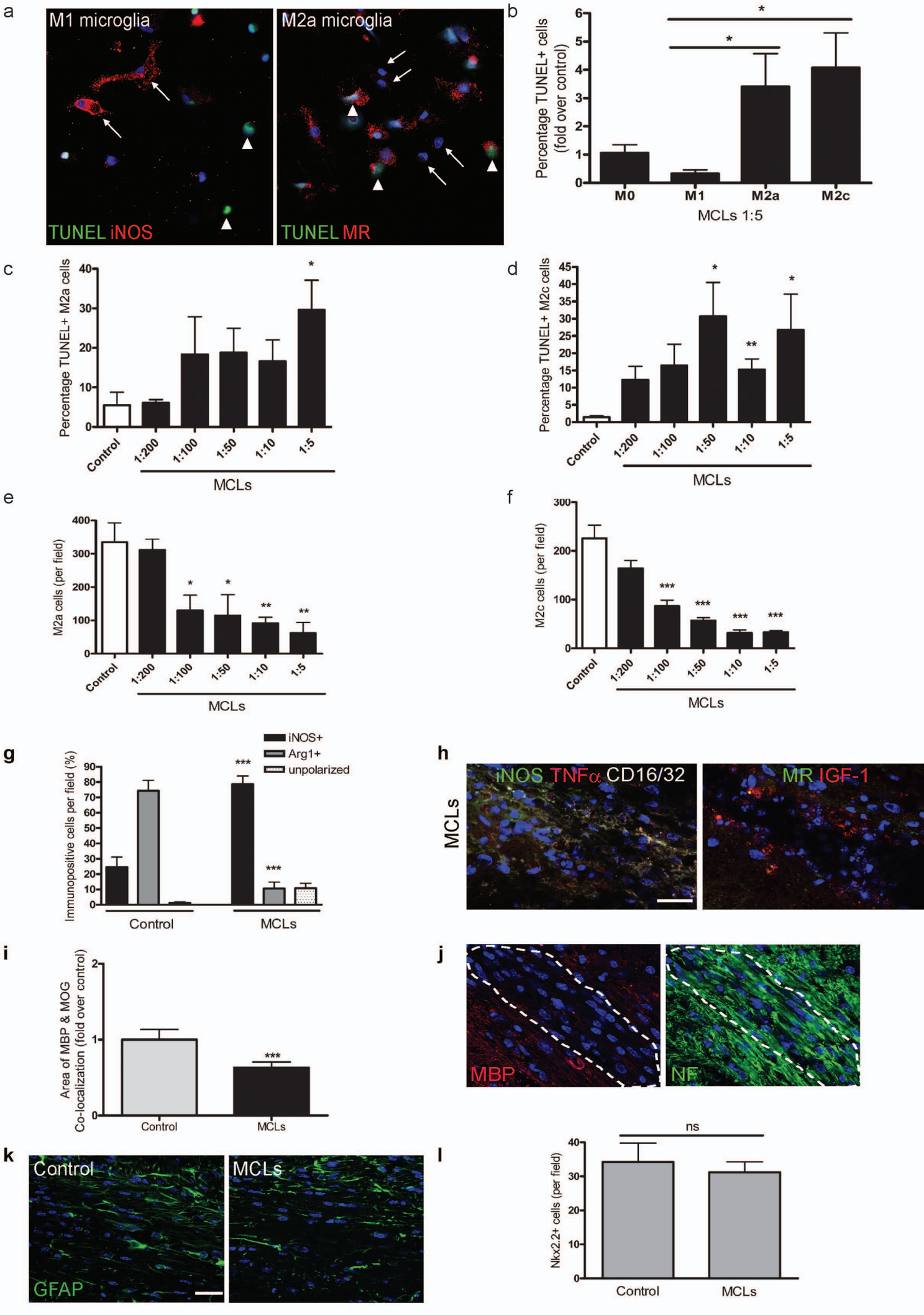

Supplementary Figure 8 Selective depletion of M2 polarized microglia/ macrophages using mannosylated clodronate liposomes.

(a) Representative images of iNOS+ M1 and MR+ M2a microglia treated with MCLs (1:5) and assessed for apoptosis by TUNEL assay. In M1 polarizing conditions, iNOS+ cells were TUNEL– (arrows); TUNEL+ cells were iNOS– (arrowheads). In M2a polarizing conditions, TUNEL+ cells were MR+ (arrowheads), whereas MR– cells were TUNEL– (arrows). (b) Mean percentage of TUNEL+ cells ± s.e.m. in M0, M1, M2a, and M2c polarized microglia treated MCLs (1:5 dilution), normalized to values from M0 conditions. P=0.027 (M1 vs. M2a), P=0.013 (M1 vs. M2c), 2-tailed Student's t-test (n=6, df=10). Mean percentage of TUNEL+ cells ± s.e.m. in M2a (c, P=0.015, df=7) and M2c (d, P=0.0251, 0.0016, 0.03 for 1:50, 1:10, 1:5, respectively, df=6) microglia treated with MCLs (1:200-1:5; black bars) compared to control (white bars) (n=4). Mean numbers of microglia per field ± s.e.m. for M2a (e, P=0.02, 0.03, 0.009, 0.007 for 1:100, 1:50, 1:10, 1:5, respectively, df=6) and M2c (f; P=0.0003, <0.0001, 0.0005, 0.0005 for 1:100, 1:50, 1:10, 1:5, respectively, df=6) polarized cells treated with MCLs (1:200-1:5; black bars) compared to control (white bars), 2-tailed Student's t-test (n=4). (g) Percentage of iNOS+ M1, Arg-1+ M2, or unpolarized (iNOS–, Arg-1–) cells per field ± s.e.m. in control and MCL-injected lesions at 10 dpl. P<0.0001, 2-tailed Student's t-test (n=5, df=8). (h) MCL-injected lesions immunostained for additional M1 (iNOS, TNFα, CD16/32) and M2 markers (MR, IGF-1). Scale bar, 25 μm. (i) Mean area of MBP and MOG colocalization fold over control ± s.e.m. in lesions at 21 dpl. 2-tailed Student's t-test, P=0.0002 (n=6, df=10). (j) Axons (NF+) were detectable running through the lesion (MBP–) at 10 dpl following MCL injection. (k) MCL injection did not influence astrocytes (GFAP+)

within the lesion. Scale bar, 25 μm. (l) Total numbers of Nkx2.2+ OPCs ± s.e.m. in control and MCL injected lesions. 2-tailed Student's *t*-test, p>0.05 (*n*=4, df=6).

Supplementary Figure 9

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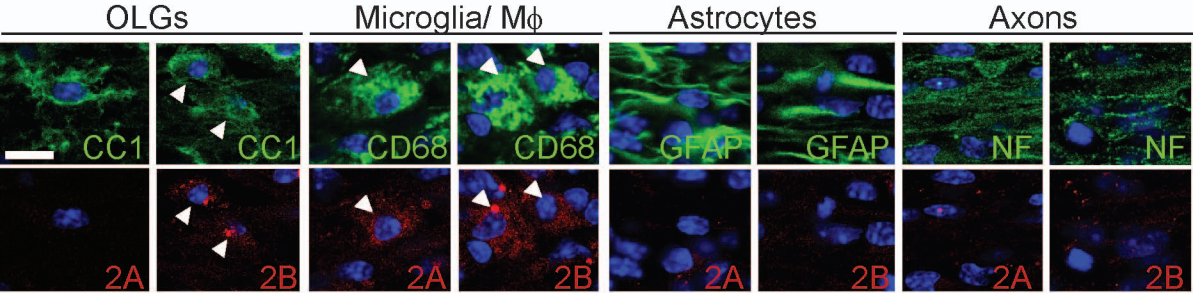

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Supplementary Figure 9 Expression of activin-A receptors on cells in remyelinating lesions.

Representative images of oligodendrocytes (CC1+), microglia/ macrophages (CD68+), astrocytes (GFAP+), and neurons (axons; NF+) with expression (arrows), or lack thereof, of activin-A receptors Acvr2A and Acvr2B. Scale bar, 10 μm.

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Supplemental Table 1. Human brain tissue samples

	Classification	Sex	Age	Disease duration (yrs)	Block	Lesions Analyzed			
						Active	Chronic Active	Chronic Inactive	Remyelinated
Multiple Sclerosis Patients	SPMS	M	46	8	P2E3	0	0	2	2
					P3E3	1	0	1	1
	SPMS	F	49	14	A3D3	3	0	0	0
	SPMS	M	40	9	P3B4	1	0	1	3
	SPMS	F	60	21	P3C3	1	1	1	0
	SPMS	F	34	21	A2D7	1	0	0	0
					A2E2	1	0	1	1
	PPMS	M	37	27	P4D1	0	0	1	3
	SPMS	F	57	27	P3D1	0	0	0	2
	SPMS	F	46	25	A2B3	0	1	1	0
					P2C4	0	2	0	1
	SPMS	F	42	19	A2C2	2	0	0	1
	SPMS	F	57	19	P3C4	0	2	3	0
					TOTAL	**10**	**6**	**11**	**14**
Non-Neurological Controls	Carcinoma of the lung metastasized	M	77	-	P3B4	-	-	-	-
	Cardiac failure	M	64	-	P2D2	-	-	-	-
	Carcinoma of the tongue	M	35	-	P3B3	-	-	-	-
	Ovarian cancer	F	60	-	P3D2	-	-	-	-
	Myelodysplast-ic syndrome, Rheumatoid Arthritis	M	82	-	A2C7	-	-	-	-