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Selective depletion of Mac-1-expressing microglia in rat subventricular zone does not alter neurogenic response early after stroke

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[1]Abbreviations: insulin-like growth factor-1(IGF-1), lipopolysaccharide (LPS), macrophage antigen complex-1 (Mac-1), middle cerebral artery occlusion (MCAO), stromal cell-derived factor-1α (SDF-1α), subventricular zone (SVZ), triggering receptor expressed on myeloid cells-2 (TREM-2), tumor necrosis factor (TNF)

Abstract

Ischemic stroke induces migration of newly formed neuroblasts, generated by neural stem cells in the adult rat subventricular zone (SVZ), towards the injured striatum where they differentiate into mature neurons. Stroke also leads to accumulation of microglia in the SVZ but their role for neurogenesis is unclear. Here we developed a method for selective depletion of the macrophage antigen complex-1 (Mac-1)-expressing microglia population in the SVZ by intraventricular injection of the immunotoxin Mac-1-saporin in rats. We found that the vast majority of Mac-1+ cells were Iba-1+ microglia. The Mac-1+ population was heterogeneous and included both a small proliferative pool of cells, which was not affected by middle cerebral artery occlusion (MCAO), and a larger subpopulation that changed morphologically into a semi-activated state in response to the insult. This subpopulation did not increase its expression of the phagocytic marker ED1 but exhibited high levels of triggering receptor expressed on myeloid cells-2 (TREM-2), associated with alternative microglia activation. A minor portion of the SVZ Mac-1+ cells originated from the blood early after stroke, but this macrophage population became much more substantial at later stages. Almost 80% reduction of Mac-1-expressing microglia, caused by Mac-1 saporin delivered just before and at 1 week after MCAO, did not alter the numbers of newly formed neuroblasts in the striatum or their migratory distance. These findings indicate that the Mac-1-expressing microglia in the SVZ do not play a major role either for the number of neuroblasts which exit the SVZ or their migration in the striatum early following stroke.

Key words

macrophage antigen complex-1, Iba1, microglia, triggering receptor expressed on myeloid cells-2, subventricular zone, neuroblast, neural stem cell, stroke

Introduction

In the intact, adult brain the formation of new neurons is confined to two regions: the subgranular zone in the dentate gyrus, giving rise to granule cells, and the subventricular zone (SVZ), lining the lateral ventricle, generating olfactory bulb neurons (Zhao, et al., 2008). Pathological conditions can trigger neurogenesis also in other brain areas. Ischemic stroke caused by middle cerebral artery occlusion (MCAO) induces increased progenitor proliferation in the SVZ and long-term formation of neuroblasts, which migrate **radially into striatum towards the ischemic damage** and develop into mature projection neurons (Arvidsson, et al., 2002, Li, et al., 2010, Parent, et al., 2002, Thored, et al., 2006, Yamashita, et al., 2006, Yang, et al., 2007, Zhang, et al., 2004). New neurons may also be generated in the cerebral cortex under ischemic conditions (Leker, et al., 2007, Ohira, et al., 2010). Neurogenesis following brain insults leading to neuronal death has been proposed to be part of a self-repair mechanism, which potentially could contribute to functional recovery.

Pathological conditions in the brain are often associated with inflammation, and microglia cells, which are the main innate immune cells, influence several steps of adult neurogenesis (Carpentier and Palmer, 2009, Ekdahl, et al., 2009). Activated microglia release pro-inflammatory cytokines like tumor necrosis factor (TNF)-α, which suppresses SGZ and SVZ progenitor proliferation through TNF-receptor 1(R1) signaling after status epilepticus (SE) and stroke (Iosif, et al., 2008, Iosif, et al., 2006). Conversely, resident microglia interact with CNS-specific T-cells and promote progenitor proliferation in the SGZ and SVZ (Ziv, et al., 2006). Microglia activated early after stroke or SE, or by administration of the bacterial endotoxin lipopolysaccharide (LPS) are detrimental for the survival and differentiation of newly formed hippocampal or striatal neurons (Ekdahl, et al., 2003, Hoehn, et al., 2005, Liu, et al., 2007, Monje, et al., 2003). However, microglia may also be beneficial for the survival of the new neurons. Seizure-generated hippocampal neurons can survive for at least 6 months despite a chronic inflammatory environment comprised of increased numbers of activated phagocytic microglia (Bonde, et al., 2006). In agreement, chronically activated microglia are permissive to neuronal differentiation and survival in adult mouse SVZ cultures (Cacci, et al., 2008). Microglia and microglia-conditioned medium rescue the *in vitro* formation of neuroblasts from SVZ neural

stem cells (NSCs), which otherwise is lost with continued culture (Aarum, et al., 2003, Walton, et al., 2006). Release of factors such as stromal cell-derived factor (SDF)-1α (Thored, et al., 2006) and osteopontin (Yan, et al., 2009) from activated microglia is involved in the migration of neuroblasts towards the ischemic regions. Interestingly, microglia activation induced by LPS influences the development of functional synaptic connectivity of the surviving new hippocampal neurons (Jakubs, et al., 2008).

We recently found increased numbers of microglia in the ipsilateral SVZ concomitant with the continuous production of new neuroblasts migrating into the striatum up to 4 months after stroke (Thored, et al., 2009). A substantial proportion of the microglia expressed insulin-like growth factor-1 (IGF-1), which is proneurogenic, suggesting a supportive action of these cells for neurogenesis. In order to explore in more detail the involvement of SVZ microglia in stroke-induced neurogenesis, we injected intraventricularly the ribosome-inactivating protein saporin coupled to an antibody against macrophage antigen complex-1 (Mac-1), a β2-integrin glycoprotein found on the cell surface and expressed mainly on cells of the myeloid lineage, including microglia. This immunotoxin, Mac-1-saporin, has previously been injected intraperitoneally to deplete circulating monocytes or resident microglia in mouse pups subjected to excitotoxic lesions (Dommergues, et al., 2003), or applied to hippocampal slice cultures exposed to oxygen-glucose deprivation (Montero, et al., 2009) or SVZ dissociates *in vitro* (Walton, et al., 2006). Mac-1 (complement type 3 receptor / CD11b) functions both as an adhesion molecule mediating the diapedesis of leukocytes across the endothelium, thereby promoting neutrophil inflammatory responses, and as a receptor for the iC3b fragment of complement responsible for phagocytic/degranulation responses to microorganisms. Mac-1/CR3 can also recognize microbial surface polysaccharides like LPS and form membrane complexes with glycosylphosphatidylinositol (GPI)-anchored receptors, providing a transmembrane signaling mechanism for these outer membrane-bound receptors that allows them to mediate cytoskeleton-dependent adhesion or phagocytosis and degranulation (Ross, 2000).

The objectives of the present study were three-fold: First, to identify a useful regime for microglia depletion in SVZ, with respect to magnitude and duration, by intraventricular injection of Mac-1-saporin; Second, to analyze the specificity in the

depletion of Mac-1-expressing microglia and define some characteristic properties of this microglia population including origin, proliferation, recruitment and activation; Third, to investigate the role of Mac-1-expressing microglia in the early phase of stroke-induced neurogenesis by selective ablation using the immunotoxin.

Material and methods

Mac1-saporin infusion and group assignment

Adult male Wistar rats (Charles-River, Sulzfeld, Germany, weighing 290-310g) were housed under 12hrs light/12hrs dark cycle with unlimited access to food and water. All experimental procedures followed the guidelines set by the Malmö-Lund Ethical Committee for the use of laboratory animals and were conducted in accordance with European Union directive on the subject of animal rights.

Animals were anesthetized with isofluorane and a stainless-steel guiding cannula (Bilaney Consultants GmbH, Germany) was stereotactically implanted into the left lateral ventricle (coordinates: 0.48mm rostral and 1.0mm lateral to bregma and 3.9mm ventral from dura, toothbar at -3.3mm (Paxinos and Watson, 1997)). The first group of rats received an intracerebroventricular (i.c.v) injection immediately after surgery containing either 5μg (n=5) or 10μg (n=4) of Mac-1-saporin (Advanced Targeting Systems, San Diego, CA) dissolved in 7μl vehicle (phosphate-buffered saline, PBS), or 7μl vehicle (n=4), or served as intact non-cannula-implanted controls (n=4). Animals were perfused 1 week after injection. The second group received 5μg of Mac-1-saporin or vehicle i.c.v. (n= 7 in each group) and rats were perfused 1, 2 and 4 weeks thereafter. The third group (n=6 in each group) received 5μg of Mac-1-saporin or vehicle i.c.v. twice, 1 week apart, and was perfused 2 weeks after the first injection. The fourth group of rats received 5μg of Mac-1-saporin or vehicle i.c.v. immediately before undergoing middle cerebral artery occlusion (MCAO) (Mac-1-saporin + MCAO: n=8; Vehicle + MCAO: n=7), was given another injection 1 week later, and was perfused 2 weeks after the insult together with sham-operated, non-cannula-implanted control rats (n=4). Five-bromo-2-deoxyuridine (BrdU 50mg/kg, Sigma Aldrich, St. Louis, MO) was injected intraperitoneally twice daily for 14 days from the day after the first injection of Mac-1-saporin. Finally, the fifth group of rats

underwent MCAO or sham surgery and was perfused 2 weeks later (n= 13 and n=13, respectively).

Middle cerebral artery occlusion

Under isofluorane anesthesia, the middle cerebral artery (MCA) was occluded on artificially ventilated rats. A nylon filament was inserted through the common carotid artery, into the internal carotid artery, past the origin of the MCA (Zhao, et al., 1994), and left in place for 2 h. In sham-operated rats, the filament was advanced only a few mm inside the internal carotid artery and did not occlude the MCA. The cannula for injection of Mac-1-saporin or vehicle was implanted on the side contralateral to the MCAO.

Adult, male CD45BL/6 (CD45.1) mice (Møllegaard, Copenhagen, Denmark) were lethally irradiated (Gy 9.75) and 4 h thereafter transplanted with 1000–1500 LSK stem cells from congenic female transgenic C57BL/6 (CD45.2) mice expressing green fluorescent protein (GFP) (Nygren, et al., 2004). All mice were given sterile food and autoclaved acidified water and housed under pathogen-free conditions. Ten weeks after bone marrow reconstitution, animals were tested for donor-derived multi-lineaged chimerism in the blood as previously described (Nygren et al., 2004). Chimeric mice were subjected to 30 minutes of MCAO (n=6) or sham surgery (n=4) under isofluorane anesthesia (Thored, et al., 2009) and transcardially perfused 5 weeks thereafter. In another experiment, adult male SV129 mice (Charles-River, Sulzfeld, Germany) received 30 minutes of MCAO (n=2) or sham surgery (n=2), and were perfused 2 weeks later.

Immunohistochemistry

After transcardial perfusion with 4% ice-cold phosphate-buffered paraformaldehyde (PFA), brains were postfixed in PFA overnight, cryoprotected in 20% sucrose and then cut coronally in 30 μm thick sections on powdered dry ice. Prior to staining for **1,** BrdU, sections were incubated in 1M HCl at 65°C for 10 minutes and at room temperature for 20 minutes and 2, CD45, sections were treated with sodium citrate buffer at 90°C for 20 min. Following preincubation with appropriate normal sera, sections were incubated with the following primary antibodies overnight or during 48h at 4°C: rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba1) (a Ca-

binding protein detecting both active and quiescent forms of microglia (Imai and Kohsaka, 2002); 1:1000; Wako Chemicals, Osaka, Japan), mouse anti-ED1 (1:200; Serotec, Oxford, UK), rat anti-mouse CD11b/Mac-1 (1:100; Serotec), mouse anti-rat CD11b (1:500; BD Biosciences, San José, CA), rabbit anti-GFP (1:10000; Abcam, Cambridge, UK), rat anti-BrdU (1:100; Oxford Biotechnology, Oxford, UK), goat anti-doublecortin (Dcx, 1:400, Santa-Cruz Biotechnology), mouse anti-Ki67 (1:50; Novocastra Laboratories, Newcastle, UK), rabbit anti-CD45 (1:100, sc-25590), Santa-Cruz Biotechnology), sheep anti-mouse recombinant mouse triggering receptor expressed on myeloid cells 2b (TREM-2) (1:100, R&D System, Abingdon, UK), and mouse anti-NeuN (1:100; Chemicon, Temecula, CA). Staining was visualized by incubation for 2 h with Cy3-conjugated donkey anti-rabbit / goat / rat / mouse antibody (1:200; Jackson Immunoresearch, West Grove, PA), and biotinylated secondary antibodies (1:200; Vector, Burlingame, CA) followed by Alexa 488-conjugated streptavidin (1:200; Molecular Probes, Eugene, OR), for 2 h for double staining. Finally, sections were rinsed, counterstained with Hoechst (0.25 μl/ml; Molecular Probes), mounted and cover-slipped.

Microscopical analysis

An observer blind to treatment conditions performed all assessments. Numbers of immunoreactive cells were counted in SVZ and striatum at 40x magnification using an epifluorescence microscope in 4 coronal sections at +1.60, +1.00, +0.48 and -0.26 mm from bregma. All cells were counted in the SVZ, both ipsi- and contralaterally. In the striatum, cells were counted in 12 (4 X 3) continuous fields using a 0.0625 mm^2 square grid (Thored, et al., 2006). Double-labeling of cells in epifluorescence was validated using confocal microscopy. Percentage of striatal damage was calculated by measuring the area of remaining NeuN-labeled cells on the ipsilateral side and comparing it to the total striatal area on the contralateral side to the MCAO in 4 coronal section at +1.60, +1.00, +0.48 and -0.26 mm from bregma, using light microscopy and stereological equipment, driven by CAST 2 Software (Olympus).

Statistical analysis

Comparisons were performed using one-way and two-way analysis of variance (ANOVA) with Bonferroni-Dunn *post-hoc* test and paired and unpaired Student's t-

test. Data are presented as means ±SEM, and differences considered significant at $P < 0.05$.

Results

Selective depletion of Mac-1+ microglia in subventricular zone

In order to develop a method for depleting microglia in the SVZ, we first injected either 5 and 10µg of Mac-1-saporin unilaterally in the lateral ventricle and perfused the rats one week thereafter. The immunotoxin gave rise to a marked reduction of both the total number of microglia (Iba1+ cells) compared to control and the number of activated, phagocytic (Iba1+/ED1+) and less phagocytic microglia (Iba1+/ED1-) in the SVZ (**Fig. 1A-C**). We observed no significant differences in the effect on the numbers of SVZ microglia either between the sides ipsi- and contralateral to the injection, or between the animals given 5 and 10µg Mac-1-saporin (**Fig. 1A-C**). In the following experiments we therefore used the 5µg dose.

We next wanted to determine the duration of the microglia-depleting effect following a single dose of Mac-1-saporin. Rats were given 5µg of Mac-1-saporin i.c.v. and perfused 1, 2, and 4 weeks later. The bilateral reduction of the numbers of microglia (Iba1+, Iba1+/ED1+, Iba1+/ED1- cells) at 1 week was no longer observed at 2 and 4 weeks following administration of the toxin **(Fig. 1D-F).** Thus, Mac-1-saporin induced only a transient depletion of microglia in the SVZ.

We then explored whether ablation of SVZ microglia for longer time periods, allowing for assessment of the consequences for neurogenesis, could be achieved by repeated Mac-1-saporin injections. We gave 2 i.c.v. injections of 5µg Mac-1-saporin with 1-week interval and killed the animals 2 weeks after the first injection. Compared to control, there was a substantial, bilateral reduction of both the total number of Iba1+ microglia and the number of Iba1+/ED1- microglia in the Mac1-saporin-treated rats **(Fig. 2A,C),** while the number of Iba1+/ED1+ microglia did not differ between groups **(Fig. 2B, D-F)**. Our results indicate that using this injection paradigm, there is a marked depletion of microglia both at 1 and 2 weeks, and especially of the Iba1+/ED1- microglia.

We finally assessed the specificity in the effect of the immunotoxin for Mac-1+ microglia in the SVZ. Following the two injections of 5µg Mac-1-saporin, we

found a dramatic, about 90%, bilateral depletion of the number of Iba1+/Mac1+ microglia in the SVZ, whereas the number of Iba1+/Mac-1- cells was unaffected **(Fig. 3).** Thus, the immunotoxin very efficiently and selectively ablates the Mac-1+ microglia population in the SVZ of the intact brain.

Selective depletion of Mac-1+ microglia in subventricular zone following stroke

We next induced stroke by 2h MCAO in animals which had been injected with Mac-1-saporin or vehicle in the contralateral lateral ventricle just prior the insult. The same injections were then given at 1 week, and the rats were killed 1 week thereafter. At 2 weeks after MCAO, the damage, as assessed by measuring the area of remaining NeuN-labeled cells, was about 80% of the total striatal volume and did not differ between the groups **(Fig. 4A).** The Mac-1-saporin injections gave rise to 43% reduction of the number of Iba1+ microglia and 40% decrease of the Iba1+ED1- microglia in the SVZ ipsilateral to the MCAO, whereas the number of Iba1+/ED1+ cells was unchanged **(Fig. 4B-F).** Importantly, also after stroke the immunotoxin was selective for Iba1+/Mac-1+ cells, which were depleted by 73% in SVZ. In contrast, the number of Iba1+/Mac-1- cells was not affected **(Fig. 4G-J)**. Iba1-/Mac-1+ cells in the SVZ were fewer than 1% of the total number of Mac-1+cells (data not shown). The Mac-1-saporin injections did not cause any loss of Iba1+/Mac-1+ cells in the striatum ipsilateral to the MCAO (2 136±582 in vehicle vs 2 050±601 cells in Mac-1-saporin-treated stroke rats), indicating that the effect of the intraventricularly administered immunotoxin was confined to the SVZ also after stroke.

Properties and origin of Mac-1+ microglia in subventricular zone following stroke

We wanted to explore in more detail the characteristics of the Mac-1 population after stroke. Consistent with our previous data (Thored, et al., 2009), we detected a significant increase of the total number of Iba1+ cells in the ipsilateral SVZ after stroke as compared to control (363±40.4 in sham-operated vs 538±21.4 cells in MCAO rats). However, the number of Iba1+/Mac-1+ cells was unchanged after the insult (98.0±5.7 in sham-operated vs 72.6±11.4 cells in MCAO rats). In line with this observation, stroke did not alter the number of proliferating, Ki67+/Mac-1+ cells **(Fig. 5A)** in the SVZ (1.8±1.1 in sham-operated vs 0.17±0.17 cells in MCAO rats). We wanted to explore whether stroke induced a change in the activation state of the microglia, as reflected by the morphological and/or molecular phenotype of the

Iba1+/Mac-1+ cell population. First, we classified Iba1+/Mac-1+ cells in the SVZ of stroke-damaged and sham-operated rats into ramified, intermediate, amoeboid, or round phenotypes using the morphological criteria described previously (**Fig. 5B**; (Lehrmann, et al., 1997, Thored, et al., 2009)). These morphological phenotypes represent at least partly different levels of microglia activation, round phenotype signifying the most activated ones**.** We found all four phenotypes in the SVZ Iba1+/Mac-1+ population in both sham-operated and stroke-damaged rats **(Fig. 5C)**. Stroke gave rise to a significant change of the incidence of different microglia phenotypes (two-way ANOVA). In the sham-operated SVZ, almost 80% of microglia were ramified, which after stroke decreased to about 40%. Conversely, microglia exhibiting the intermediate phenotype increased from less than 20% to almost 40%. Amoeboid and round cells remained at low levels after stroke. These findings provide evidence for increased activation (semi-activation) state of Iba1+/Mac-1+ microglia after stroke. Interestingly, the phenotypic change at 2 weeks after stroke may be specific to Iba1+ microglia expressing Mac-1 in the SVZ, since we have previously observed that the total Iba1+ microglia population has a predominantly ramified morphology at this time point and changes in phenotype occur later (6 weeks after stroke) (Thored, et al., 2009).

Secondly, we determined to what extent the Mac-1+ microglia expressed the macrophage lysosomal antigen ED1, which is associated with increased activation and phagocytic capacity. Very few of the Mac-1+ cells were double-labeled with ED1 in the SVZ of both intact and stroke-damaged rats, while double-labeling was more frequent in the damaged striatum.

Thirdly, we explored the possibility of an alternative activation of the Mac-1+ microglia population and evaluated TREM-2 immunoreactivity in Mac-1+ cells. TREM-2 expression in microglia impairs TNF-α and nitric oxide synthase-2 transcript expression even if it is stimulated by and takes part in the phagocytosis of apoptotic neurons by adhering to the TREM-2-ligand expressed on neurons (Hsieh, et al., 2009). We found that about 70% of the SVZ Mac-1+ cells expressed TREM-2 regardless of morphological phenotype **(Fig. 5A).** Taken together, our data indicate that the Iba1+/Mac-1+ microglia population in the SVZ is heterogeneous with low proliferation rate independent of stroke. A substantial part of the Iba1+/Mac-1+

microglia population change into a more activated phenotype after stroke, and a large proportion of these microglia may exhibit alternative activation.

We wanted to determine whether the Mac-1+ cells in SVZ after stroke were resident microglia or bone marrow-derived macrophages. We have previously demonstrated (Thored, et al., 2009) that recruitment of macrophages from the bone marrow contributes to the long-term increase of microglia in SVZ after stroke. Here we used lethally irradiated mice that had been reconstituted with bone marrow from GFP-expressing transgenic mice. When these mice were analyzed 5 weeks after stroke, Thored and coworkers (Thored, et al., 2009) found substantial numbers of GFP+ cells in ipsilateral SVZ whereas only scattered GFP+ cells were detected in SVZ of sham-operated mice. We co-stained for Mac-1 and observed that 38±1.9% of the Mac1+ cells were also GFP+ **(Fig. 5A)**. We also evaluated the expression of CD45, a putative marker for myeloid cells (Donnou, et al., 2005) in the Mac1+ cells at 2 weeks after stroke. A small percentage of Mac1+cells expressed CD45 in vehicle-treated stroke rats and a significantly lower percentage in the Mac-1-saporin-treated group (3.36±0.68 vs 0.77±0.53%, respectively). Taken together, our findings indicate that the Mac-1+ cells in SVZ after stroke are both resident microglia and bone marrow-derived macrophages and that both populations are depleted by the Mac1-saporin immunotoxin. In line with our previous findings (Thored, et al., 2009) the present data suggest that a substantial recruitment of blood born Mac1+ cells occurs beyond 2 weeks post stroke. Mac-1 can also be expressed on neutrophils but their number was very low in the SVZ 2 weeks after stroke, less than 1% of the Mac-1+cells lacking the microglia-specific marker Iba1.

Lack of effect of intraventricular Mac-1-saporin on neuroblast recruitment to striatum early following stroke

We wanted to explore whether the immunotoxin-induced depletion of the Mac-1+ microglia population influenced striatal neurogenesis after stroke. Since animals were killed at 2 weeks after the insult, we could only determine the effect on the initial part of the neurogenic response, i.e., the recruitment of the neuroblasts into striatum and the distance the new neurons had migrated from SVZ towards the injury. Animals had been given daily BrdU injections during the entire post-insult period. Therefore, we could estimate both the total number of immature neurons in the striatum (Dcx+),

and the number of neuroblasts formed after the stroke (Dcx+/BrdU+), and, at least partly, before the insult (Dcx+/BrdU-).

Very few Dcx+ cells were detected in the striatum of intact rats or on the side contralateral to MCAO (data not shown). Stroke gave rise to a marked increase of the number of Dcx+ cells in the ipsilateral striatum, but the variability was high both in Mac-1-saporin- and vehicle-treated animals. No difference in number of newly formed neuroblasts (Dcx+/BrdU+) was found. Although the mean number of Dcx+/BrdU- cells (and consequently also of Dcx+ cells) was higher following Mac-1-saporin as compared to vehicle, this difference was not statistically significant **(Fig 6A-C).** We explored whether the high variation in the level of neurogenesis may be related to the extent of the ischemic damage or to the cell number in different microglia populations (Iba1+, Iba1+/ED1+, Iba1+/ED1-, Iba1+/Mac1+, Mac1+/Ki67+, Mac1+/TREM2+, and Mac1+/CD45+ cells). However, no significant correlation was found between either of these parameters and the numbers of Dcx+, Dcx+/BrdU+, or Dcx+/BrdU- neuroblasts (data not shown)**.**

We finally divided the stroke-damaged striatum into columns, located at different distances from the border of SVZ, to assess whether the loss of Mac-1+ microglia in the SVZ had affected the migratory capacity of the Dcx+ neuroblasts. No differences in the relative distribution in different columns of Dcx+, Dcx+/BrdU+, or Dcx+/BrdU- cells were found between the Mac-1-saporin- and vehicle-treated groups **(Fig. 6D-F).**

Discussion

Here we show that microglia expressing Mac-1 can be selectively depleted in the intact rat SVZ and following stroke using i.c.v. injection of the immunotoxin Mac-1-saporin. We find that Mac-1-expressing microglia are a heterogeneous cell population both under normal conditions and after stroke. These microglia comprise a minor proliferating pool being unaffected by stroke, and a large subpopulation, which changes from a mostly surveying, ramified phenotype to a more semi-activated intermediate phenotype 2 weeks after the insult. Concomitantly, a majority of Mac-1+ cells exhibit alternative activation. A minor portion of the SVZ Mac-1-expressing cells originates from the blood at this time point, but this macrophage population becomes much more substantial at later stages. Ablating the Mac-1-expressing microglia in SVZ during the first 2 weeks after stroke does not change the initial part

of the neurogenic response, i.e., neither the number of neuroblasts recruited into striatum, nor their migration towards the injury. Since the Mac-1-saporin lesion was confined to microglia in the SVZ, it was possible to distinguish the effects of this microglia population from the role of striatal microglia, which are known to influence the migration of new neuroblasts to the injured, ischemic region through release of, e.g., SDF-1α (Thored, et al., 2006) and monocyte chemoattractant protein-1 (Yan, et al., 2007).

Mac-1 expression is coupled to several intra- and intercellular functions. In SVZ microglia and macrophages, Mac-1-associated cell-cell adhesion capacity could be of importance for interaction with surrounding non-immune cells, recruitment of other immune cells, and complement activation, since Mac-1 interacts with several endogenous ligands, including extracellular matrix protein, the counterreceptor intracellular adhesion molecule-1, and complement iC3b (Ross, 2000, Ross and Vetvicka, 1993). Mac-1 may also be involved in microglia-enhanced neurotoxicity. Mac-1 deficiency greatly attenuated dopaminergic degeneration induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a model for Parkinson´s disease, and Mac-1-/- mice lacked MPTP-induced microglial activation (Hu, et al., 2008). In contrast, selective ablation of the proliferating pool of Mac-1/CD11b-expressing cells in transgenic mice exacerbated infarct size and increased apoptosis after stroke, indicating a neuroprotective action of these microglia (Gowing, et al., 2008, Lalancette-Hebert, et al., 2007).

We found only a small percentage of Mac-1+ cells with amoeboid and round morphologies at 2 weeks after stroke consistent with what we have previously observed for the total Iba1+ cell population (Thored, et al., 2009). Thus, the morphologically most activated stage is not typical of SVZ microglia after stroke. Instead, stroke increased the percentage of microglia in the intermediate, semi-activated stage. The observed morphological change was not associated with increased ED1 expression, a marker of high phagocytic capacity. This finding illustrates that determining the activation state of microglia/macrophages by assessing morphological phenotype and expression of phagocytic markers is complex. Activated macrophages may not be more phagocytic than “resting” cells (Mosser, 2003). In fact, microglia can switch to a phenotype contributing to neuronal damage without morphological change (Perry, et al., 2007).

More than 70% of SVZ Mac-1+ cells expressed TREM-2, supporting the occurrence of alternative activation in these microglia. The alternative activation pathway is associated with down-regulation of pro-inflammatory cytokines and promotion of tissue repair through removal of apoptotic cells (Hsieh, et al., 2009, Takahashi, et al., 2005). In mice with experimental autoimmune encephalitis (EAE), blockade of TREM-2 exacerbated the disease, while treatment with TREM-2-expressing myeloid cells reduced inflammation and improved the disease (Piccio, et al., 2007, Takahashi, et al., 2007). Absence of TREM-2 expression on microglia impairs their capacity to phagocytose cell membrane debris and increases their gene transcription of pro-inflammatory cytokines. Despite TREM-2 expression in SVZ Mac-1+ cells, we found no differences in numbers of new striatal neuroblasts after stroke following Mac-1-saporin injections. This could be due to the fact that about 20% of the Mac-1+ microglia were not ablated by the immunotoxin, and remaining microglia may be able to maintain a presumed supportive effect on the formation of new neurons. Another explanation could be that other signaling pathways compensate for the reduction in TREM-2+/Mac-1+ cells. Alternatively activated anti-inflammatory cells have previously been shown to be pro-neurogenic with induction of neurogenesis from adult stem cells by IL-4-activated microglia and promotion of a regenerative growth response in adult sensory axons by arginase 1 and CD206+ macrophages (Butovsky, et al., 2006, Kigerl, et al., 2009).

In the present study, Mac-1+ microglia in the SVZ were ablated only during the first 2 weeks after stroke. However, the total SVZ microglia population has a more delayed activation peak around 6 weeks, and microglia accumulation in SVZ continues at 16 weeks post-stroke (Thored, et al., 2009). During this time period, there is a persistent production of neuroblasts in SVZ and recruitment into damaged striatum (Thored, et al., 2006). We have previously found elevated levels of IGF-1 mRNA expression in SVZ at 1 and 6 weeks after stroke, with progressive increase of percentage of microglia expressing IGF-1 protein between 2 and 16 weeks (Thored, et al., 2009). IGF-1 mitigates apoptosis and promotes proliferation and differentiation of neural stem cells (Camarero, et al., 2003, Kalluri, et al., 2007, Otaegi, et al., 2006). These findings raise the possibility that Mac-1+ microglia in SVZ support neurogenesis at a later stage after the insult, which would explain why such an effect was not detected by the early Mac-1-saporin lesion used here.

In summary, we report a new method for selective depletion of the Mac-1-expressing microglia population in the adult rat SVZ. The heterogeneity of SVZ Mac-1+ microglia under normal conditions and after stroke in terms of proliferation, activation and origin is most likely reflected by simultaneous expression of both pro-inflammatory and pro-regenerative components and alternative activation. This complex pattern could explain why no common role for the SVZ Mac-1+ microglia in neuroblast recruitment and migration was revealed here. Improving our understanding how inflammatory mechanisms in the neurogenic areas regulate the different steps of neurogenesis in the adult brain is of fundamental importance for the development of regenerative strategies after stroke.

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Legends to figures

Fig. 1. Effect of intraventricular (i.c.v.) injection of Mac-1-saporin toxin on numbers of SVZ microglia in intact rats. **A-C**: Total number of microglia (Iba1+ cells, **A**), number of activated microglia (Iba1+/ED1+ cells, **B**), and number of less-phagocytic microglia (Iba1+/ED1- cells, **C**) in SVZ 1 week after 5μg or 10μg Mac-1-saporin or vehicle injection. **D-F**: Total number of microglia (Iba1+ cells, **D**), number of activated (Iba1+/ED1+ cells, **E**), and less-phagocytic microglia (Iba1+/ED1- cells, **F**) in the SVZ at 1, 2 and 4 weeks after 5μg Mac-1-saporin injection. Means ± SEM (ipsi to cannula) in **A-C** 5μg (n=5), 10μg (n=4), vehicle (n=4) and in **D-F** n = 7 for each time-point. *, $P < 0.05$, one-way ANOVA with Bonferroni-Dunn post-hoc test.

Fig. 2. Effect of two i.c.v. injections of Mac-1-saporin toxin, 1 week apart, on

numbers of SVZ microglia in intact brain. **A-C**: Total number of microglia (Iba1+ cells, **A**), number of activated (Iba1+/ED1+ cells, **B**) and less-phagocytic microglia (Iba1+/ED1- cells, **C**) in SVZ 2 weeks after the first Mac-1-saporin or vehicle injection. Means ± SEM, n = 6 for each group. * and †, P < 0.05 unpaired t-test compared to vehicle group. **D–F**: Confocal images of Iba1+/ED1+ microglia in SVZ showing Iba1 (**D**) and ED1 (**E**) immunoreactivity separately or as merged image (**F**). Orthogonal reconstructions from confocal z-series are presented as viewed in *x-z* (bottom) and *y-z* (right) planes. Scale bar is 10 µm (D-F).

Fig. 3. Specificity of Mac-1-saporin immunotoxin for Mac-1-expressing microglia in intact brain. **A-C:** Confocal images of Iba1+/Mac-1+ microglia in SVZ showing Iba1 (**A**) and Mac-1 (**B**) immunoreactivity separately or as merged image (**C**). Orthogonal reconstructions from confocal z-series are presented as viewed in *x-z* (bottom) and *y-z* (right) planes. **D-E:** Number of Mac-1-expressing microglia (Iba1+/Mac-1+ cells, **D**) and number of non-Mac-1-expressing microglia (Iba1+/Mac-1- cells, **E**) in SVZ 2 weeks after the first Mac-1-saporin or vehicle injection. Means ± SEM, n = 6 for each group. * and †, P < 0.05 unpaired t-test compared to vehicle group. Scale bar is 10 µm (A-C).

Fig. 4. Effect of Mac-1-saporin toxin on striatal damage and numbers of SVZ microglia 2 weeks after MCAO. **A**, Percentage of damaged striatal area ipsilateral to the MCAO. **B-D**: Total number of microglia (Iba1+ cells, **B**), number of activated (Iba1+/ED1+ cells, **C**) and less-phagocytic microglia (Iba1+/ED1- cells, **D**) in SVZ 2 weeks after the first Mac-1-saporin or vehicle injection delivered immediately before MCAO. **E-F:** Photomicrographs showing distribution of Iba1+ (green) and ED1+ (red) and Iba1+/ED1+ cells (yellow) in the SVZ and striatum on the MCAO side after vehicle (**E**) or Mac-1-saporin injections (**F**). **G, J:** Number of Mac-1-expressing (Iba1+/Mac-1+ cells, **G)** and non-Mac-1-expressing microglia (Iba1+/Mac-1- cells, **J**) in SVZ 2 weeks after MCAO. **H-I**: Photomicrographs showing distribution of Iba1+ (green) and Iba1+/Mac-1+ cells (yellow) in SVZ on the MCAO side after vehicle (**H**) or Mac-1-saporin injections (**I**). Means ± SEM, Mac-1-sap n = 8 and vehicle n=7. * and †, P < 0.05 unpaired t-test compared to vehicle group. Scale bar is 75 µm (E, F) and 30 µm (H, I).

Fig. 5. Properties of Mac-1-expressing microglia in the SVZ after MCAO. **A**: Confocal images of Ki67+/Mac-1+, TREM2+/Mac-1+, and GFP/Mac-1+ cells in SVZ showing Ki67, TREM2, or GFP and Mac-1 immunoreactivity separately or as merged images. Orthogonal reconstructions from confocal z-series are presented as viewed in *x*-*z* (bottom) and *y*-*z* (right) planes. **B**: Photomicrographs showing 4 different phenotypes of Iba1+ (green) /Mac-1+ (red) cells in the SVZ after MCAO. **C:** Percentage of Iba1+/Mac-1+ microglia with different morphological phenotypes in the ipsilateral SVZ after MCAO compared to sham-treated rats. Means ± SEM, MCAO n=7 sham-operated n=4. Significant difference in incidence of different microglia phenotypes between stroke-damaged and sham-treated animals, $P < 0.05$ with two-way ANOVA. Scale bar is 10 µm (A, B).

Fig. 6. Effects of selective ablation of SVZ Mac-1-expressing microglia on numbers of neuroblasts leaving the SVZ and their migratory distance towards the ischemic core after MCAO. Total number (**A-C**) and relative distribution in continuous striatal columns (**D-F**) of striatal neuroblasts (DCX+ cells, **A, D**), number of striatal neuroblasts formed after (DCX+/BrdU+ cells, **B, E**) and partly before MCAO (DCX+/BrdU- cells, **C, F**) evaluated 2 weeks after the first Mac-1-saporin or vehicle injection delivered immediately before MCAO. Means ± SEM, Mac-1-sap n=8, vehicle n=7. *, $P < 0.05$ unpaired t-test compared to vehicle group and one-way ANOVA with Bonferroni-Dunn post-hoc test comparing continuous columns.

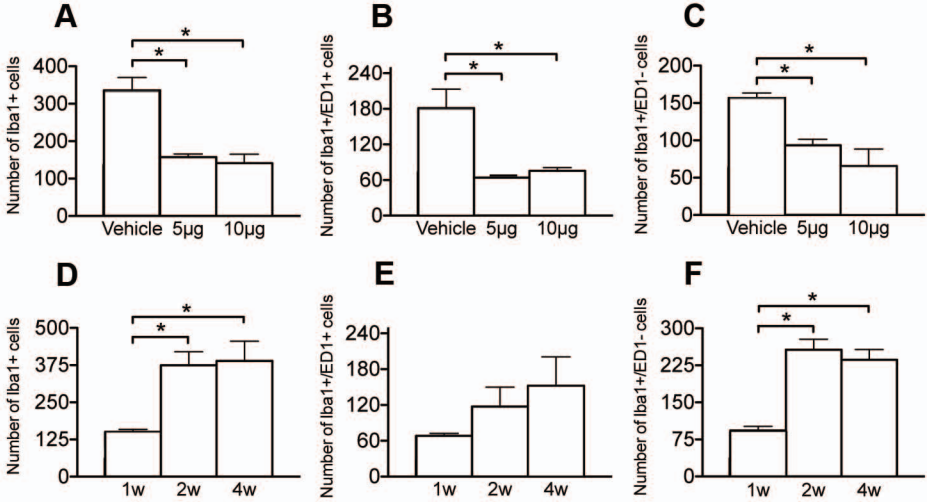

A
Number of Iba1+ cells
400
300
200
100
0
Vehicle 5μg 10μg
B
Number of Iba1+/ED1+ cells
240
180
120
60
0
Vehicle 5μg 10μg
C
Number of Iba1+/ED1- cells
200
150
100
50
0
Vehicle 5μg 10μg
D
Number of Iba1+ cells
500
375
250
125
0
1w 2w 4w
E
Number of Iba1+/ED1+ cells
240
180
120
60
0
1w 2w 4w
F
Number of Iba1+/ED1- cells
300
225
150
75
0
1w 2w 4w

Fig. 1.

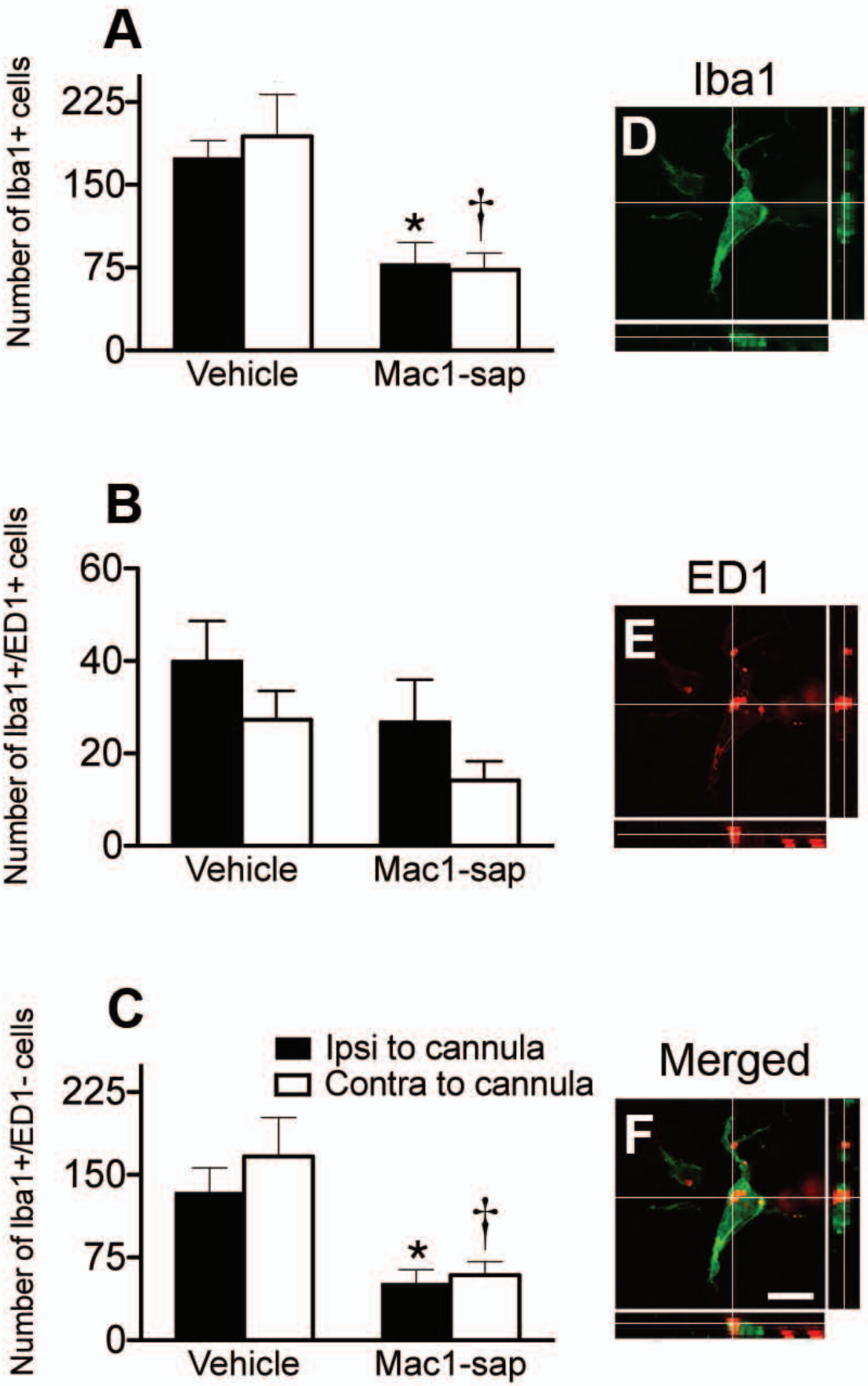

A
Number of Iba1+ cells
225
150
75
0
*
†
Vehicle
Mac1-sap
Iba1
D
B
Number of Iba1+/ED1+ cells
60
40
20
0
Vehicle
Mac1-sap
ED1
E
C
Number of Iba1+/ED1- cells
Ipsi to cannula
Contra to cannula
225
150
75
0
*
†
Vehicle
Mac1-sap
Merged
F

Fig. 2.

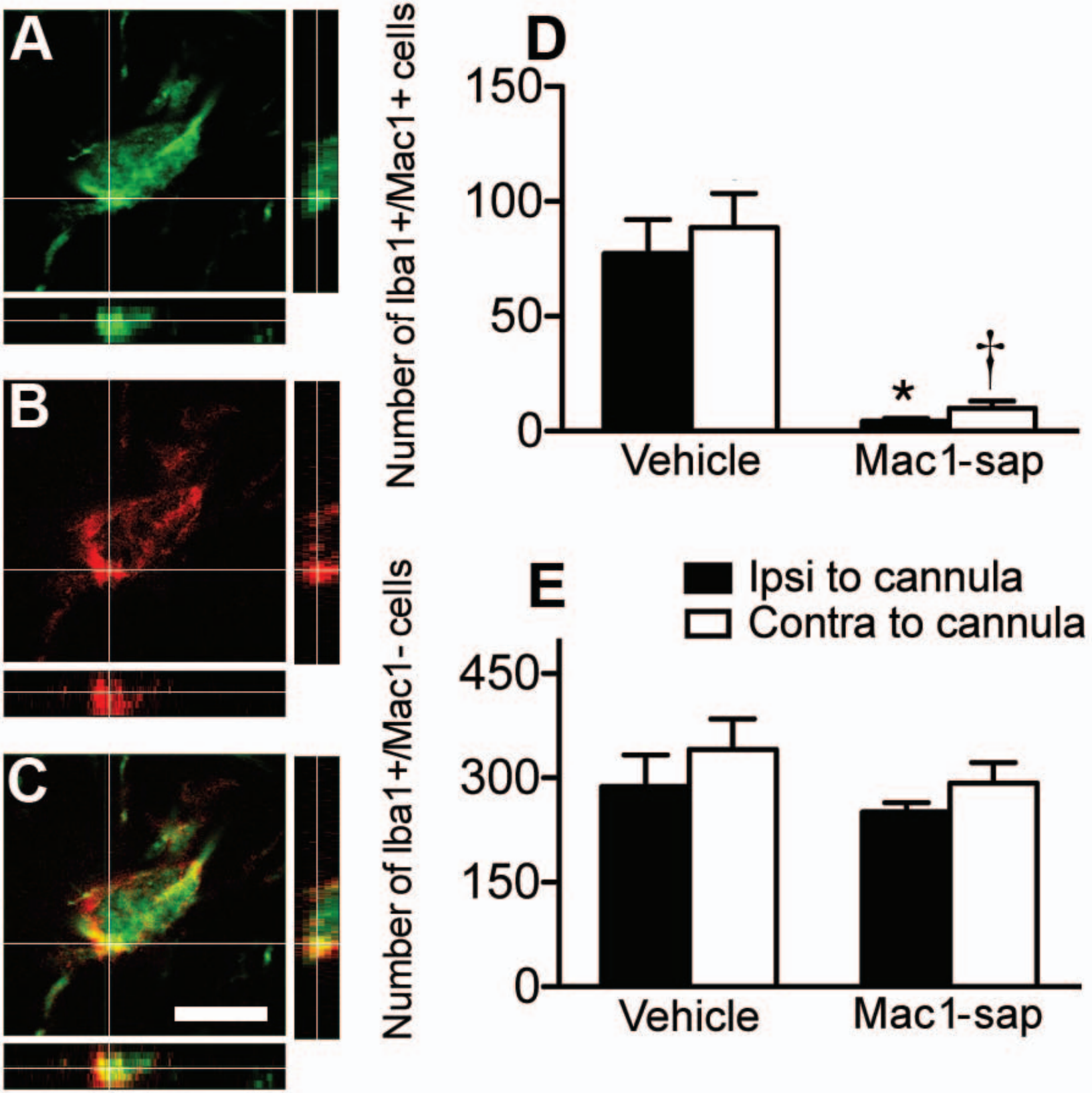

A
B
C
D
Number of Iba1+/Mac1+ cells
150
100
50
0
*
†
Vehicle
Mac1-sap
E
Ipsi to cannula
Contra to cannula
Number of Iba1+/Mac1- cells
450
300
150
0
Vehicle
Mac1-sap

Fig. 3.

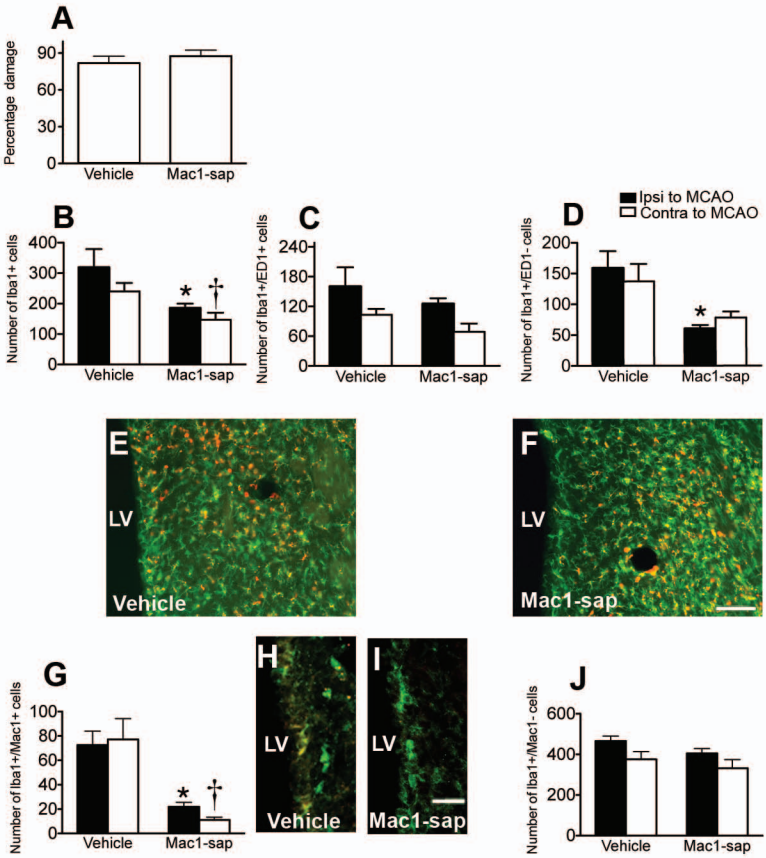
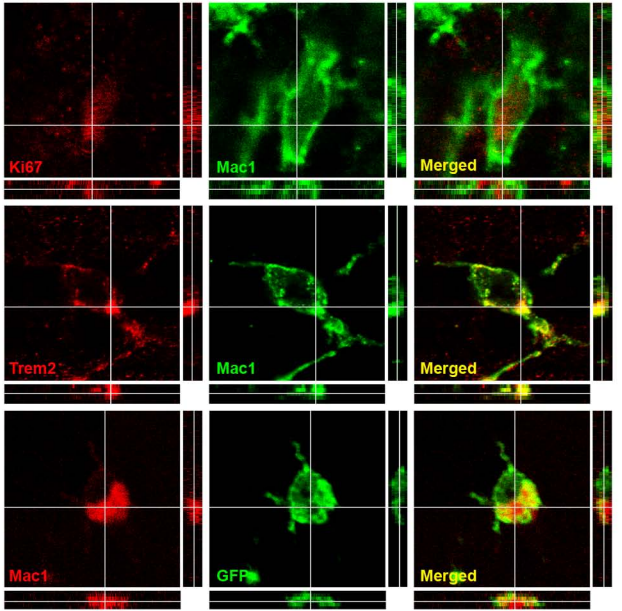
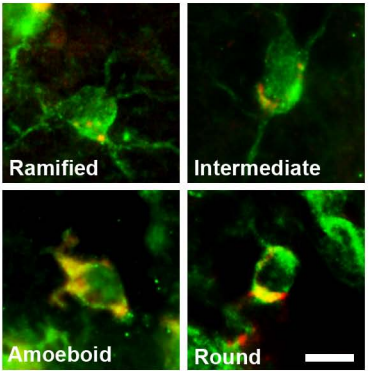

A
Percentage damage
90
60
30
0
Vehicle
Mac1-sap
B
Number of Iba1+ cells
400
300
200
100
0
*
†
Vehicle
Mac1-sap
C
Number of Iba1+/ED1+ cells
240
180
120
60
0
Vehicle
Mac1-sap
D
Ipsi to MCAO
Contra to MCAO
Number of Iba1+/ED1- cells
200
150
100
50
0
*
Vehicle
Mac1-sap
E
LV
Vehicle
F
LV
Mac1-sap
G
Number of Iba1+/Mac1+ cells
100
80
60
40
20
0
*
†
Vehicle
Mac1-sap
H
LV
Vehicle
I
LV
Mac1-sap
J
Number of Iba1+/Mac1- cells
600
400
200
0
Vehicle
Mac1-sap

Fig. 4.


A
Ki67
Mac1
Merged
Trem2
Mac1
Merged
Mac1
GFP
Merged


B
Ramified
Intermediate
Amoeboid
Round

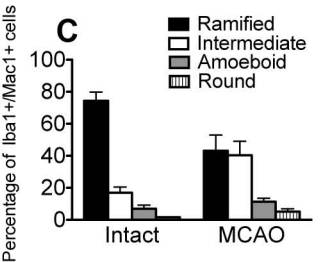

C
Ramified
Intermediate
Amoeboid
Round
Percentage of Iba1+/Mac1+ cells
100
80
60
40
20
0
Intact
MCAO

Fig. 5.

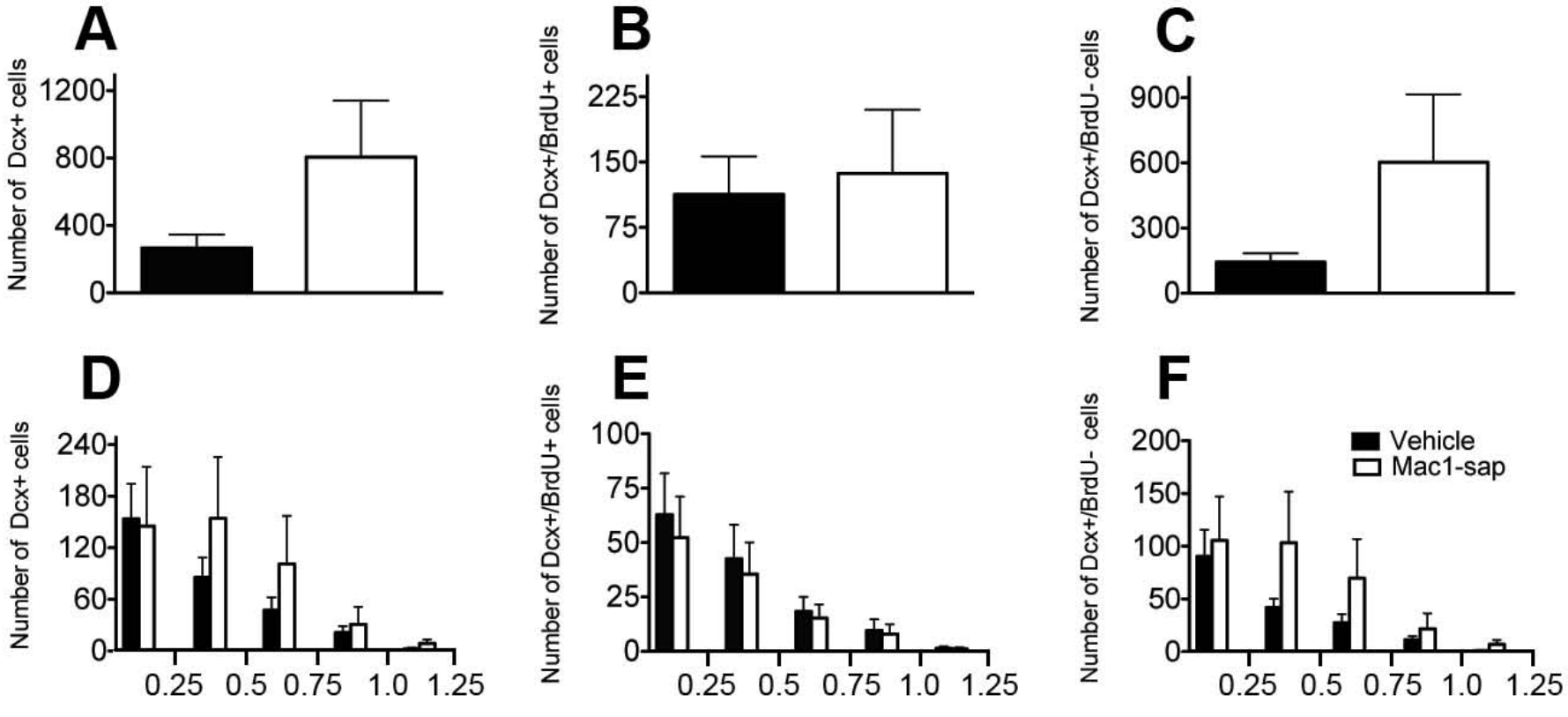

A
Number of Dcx+ cells
1200
800
400
0
B
Number of Dcx+/BrdU+ cells
225
150
75
0
C
Number of Dcx+/BrdU- cells
900
600
300
0
D
Number of Dcx+ cells
240
180
120
60
0
0.25 0.5 0.75 1.0 1.25
E
Number of Dcx+/BrdU+ cells
100
75
50
25
0
0.25 0.5 0.75 1.0 1.25
F
Number of Dcx+/BrdU- cells
200
150
100
50
0
0.25 0.5 0.75 1.0 1.25
Vehicle
Mac1-sap
Distance from SVZ (mm)

Fig. 6.

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