



CD200 restrains macrophage attack on oligodendrocyte precursors via toll-like receptor 4 downregulation

Citation

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Resubmission of JCBFM-0361-15-ORIG by Hayakawa et al.

Dear Drs. Dirnagl and Lauritzen,

My colleagues and I are grateful for all the very helpful comments from all reviewers. Basically, we agree and have tried to incorporate all these important suggestions into our revised paper. Point-by-point responses are listed below.

Reviewer 1

1. There are some mistakes. In the Abstract and Materials and Methods, the authors stated that; “Treatment with CD200-Fc upregulated alternatively activated macrophage markers such as Arg1, TGM2, and TGF- β , in part via enhanced C/EBP-beta transcriptional pathways”. “immunoprecipitation assay was performed between CREB and C/EBP-beta”, “FACS analysis: CD206 (1:100, Abcam, ab195191)”; “Western blot analysis: anti-pSTAT1 (1:500, Santacruz, sc-8394), anti-pSTAT6 (1:500, Santacruz, sc-11762R), anti-Arg1 (1:500, Santacruz, sc-20150), anti-TGM2 (1:500, Santacruz, sc-20621), anti-TLR4 (1:500, Santacruz, sc-12511), anti-C/EBP-beta (1:500, Santacruz, sc-7962), anti-pCREB (1:1000, #9198), anti-CREB (1:500, #9104)”. However, in the result and discussion, no measurements or data was presented for Arg1, TGM2, and TGF- β , CREB, C/EBP-beta, pSTAT1, pSTAT6. Please define what is the maker of CD206? Authors should carefully check the Abstract and Methods.

Response: We apologize for our mistakes. Some text was copied from earlier drafts and a different pilot study. We did not examine these endpoints in the present study. We have removed these incorrect sentences in our revision.

2. Introduction: Page 3, “Myelin sheaths are generated by oligodendrocyte precursor cells (OPCs) that are capable of migrating into demyelinated areas to promote remyelination^{5, 6}.” The authors are suggested to precisely state the concept, for example, myelin sheaths are generated by mature oligodendrocytes but not OPCs. OPCs are generated and are capable of migrating into demyelinated areas and differentiated into oligodendrocytes, promoting remyelination in the adult brain. Please also indicate where the OPCs come from.

Response: We apologize for this misleading sentence. We have rewritten the sentence to indicate that OPCs mature to oligodendrocytes that then synthesize myelin. And we have added sentences to describe the origin and migration of OPCs.

3. Results: Fig.2E shows that macrophages attack OPCs (% LDH increased) but not OL. Please explain why OLs are not attacked in the Discussion?

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5 **Response:** We are sorry for not being clear previously. Basically, our hypothesis states that macrophages
6 attack OPCs because of TLR4 interactions with the primary OPC antigen NG2. Mature oligodendrocytes do
7 not express NG2. Furthermore, recent reports indicate that mature oligodendrocytes express CD47 as a "don't
8 eat me" signal, which also prevents attack by microglia or macrophages (Gitik et al., J Neuroinflammation
9 2011). Therefore, endogenous NIREgs expression in mature oligodendrocytes might be an important
10 mechanism to escape from macrophage attack and phagocytosis. We have expanded on these points in our
11 revised Discussion.
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18 4. Minor concerns: Please use abbreviations consistently, for example, PDGFRa, PDGF-R-a; Figure legend 1;
19 the labels of images in Figure 1C may be switched "PDGF-R- α -positive OPCs (green?) were surrounded by
20 F4/80-positive macrophages (red?)".
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23 **Response:** We are sorry for the inconsistent abbreviations and the typo. We now corrected both.
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27 5. Minor concerns: Figure legends and Figures do not match with Methods, Results and Abstract. For example,
28 APC, SSC, Olig2, CSPG4 are presented in Figure legends or in Figures, however, there is no introduction in
29 Methods and Results, what are the markers for? Please define.
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32 **Response:** We now include information about APC, PE, FITC, SSC, Olig2 in Methods and Legends. CSPG4
33 is an alternative name for NG2 and we now use "NG2" instead of "CSPG4" in whole sections in our
34 manuscript.
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40 **Reviewer 2**

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43 1. Major comments: CD200 is a 45kDa protein. It is not clear whether it can cross the blood brain barrier to
44 bind to CD200R. Moreover, CD200R is also expressed by many peripheral immune cells, which may deplete
45 the CNS availability of CD200-Fc.
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49 **Response:** We apologize for not being clear. We agree that CD200 receptor is highly expressed in peripherally
50 circulating myeloid lineage cells including monocyte/macrophage and dendritic cell (Barclay et al., Trends in
51 Immunology 2002). In fact, our use of CD200-Fc treatment is meant to target peripherally circulating
52 monocyte/macrophages. Our hypothesis is that intraperitoneal CD200-Fc treatment downregulates TLR4
53 expression in peripheral blood monocytes/macrophages and reduce TLR4 function, thus decreasing the ability
54 of these macrophages to subsequently go onto attack OPCs in damaged brain compartments. To assess this
55 initial effect in the non-CNS compartment, we used FACS analysis of blood samples in ET-1-induced white
56 matter injury models (Figure 5). We demonstrated that CD200-Fc treatment significantly reduced TLR4
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4 expression in circulating blood F4/80 positive cells, suggesting that the initial effect may occur in the systemic
5 non-CNS compartment, i.e. CD200 ameliorates the overall macrophage response that then prevents these
6 phagocytic cells from attacking OPCs in white matter. Of course, the caveat remains that besides targeting
7 circulating macrophages/monocytes, off-target effects on other CD200R-bearing (non-monocyte) cells in the
8 circulation should be taken into consideration. We have tried to highlight these findings and potential
9 interpretations more clearly in the revised Results and Discussion sections.
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14 2. More discussion on the CD200-Fc is needed. Why use CD200-Fc and why not CD200 itself? How it works
15 and how this strategy is better compared to using anti-CD200 or anti-CD200R antibodies? Does the presence
16 of 'Fc' in CD200 would affect its binding to CD200R?
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21 **Response:** We apologize for not being clear. Our intention is to use CD200-Fc to bind the CD200 receptor
22 and downregulate TLR4 expression. First, the use of CD200-Fc as a selective way to activate the CD200
23 receptor has been reported and well established in vitro and in vivo (Snelgrove et al., Nat Immunology 2008;
24 Simelyte et al., Arthritis Rheum 2008; Guarda et al., Nature 2009; Lyons et al., J Neuroinflammation 2012).
25 Therefore, we hope that this approach is reasonable in our experiments. Second, we use CD200-Fc that
26 produces 50% of the optimal binding response to CD200R-Fc is approximately 5-30 ng/ml. In this study, we
27 treated CD200-Fc in a concentration of 10 µg/ml in vitro and 10 µg/ml (100 µl/10 g) in vivo which are
28 therefore reasonable concentrations to stimulate CD200 receptor. These references and background
29 calculations are now included. In contrast, anti-CD200 or CD200 receptor blocking antibodies may be used to
30 interfere with this pathway instead of activating it. We only performed gain-of-function experiments here.
31 Loss-of-function experiments are being planned for future studies.
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39 3. Description of E. Coli (K-12) phagocytic assay (Fig. 2A) is missing in the methods
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42 **Response:** We are sorry for this omission. We have included the information in Methods and Materials.
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45 4. Description of rat brain primary microglia (fetal/adult etc.?) is also missing in the methods.
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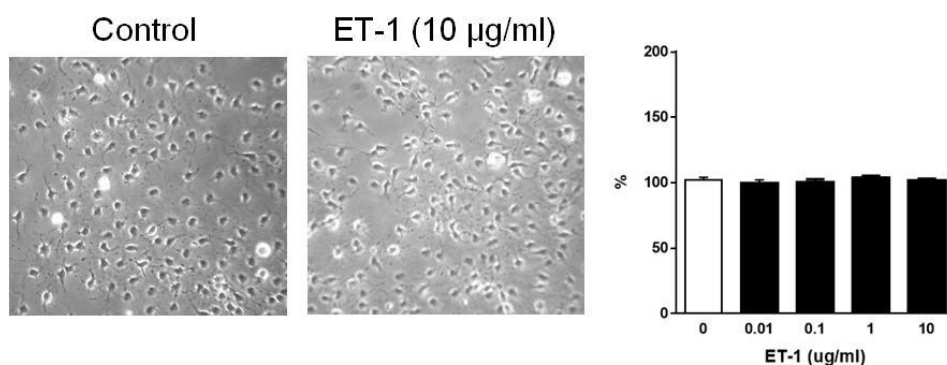
48 **Response:** We are sorry for not including the method for rat brain primary microglial isolation. We now
49 include the information in Methods and Materials.
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53 5. Abstract/methods: CD200-Fc mediated upregulation of alternative activation markers (Arg1, TGM2 and
54 TGF-beta) has mentioned, but there is no data in support of this in the manuscript.
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57 **Response:** We are really sorry for this confusion. This text was cut and pasted from an earlier draft and
58 another pilot study. We did not formally measure these markers in the present experiment. We carefully
59 checked the Abstract and Methods, and these extra information or sentences are now deleted.
60

6. Is there any possibility that OPCs are undergoing ET1 mediated direct dying (apoptosis) or is it exclusively active phagocytosis by TLR4+ macrophages?? Because an earlier study demonstrated the direct effect of ET1 on OPC proliferation and differentiation (Gadea A et al., J Neurosci 2009).

Response: Thank you for raising this potential caveat. We are aware of several important papers (e.g. Hammond et al, Neuron 2014; Gadea et al, J Neurosci 2009) suggesting that ET-1 can influence OPC migration and differentiation. However, in our in vivo ET-1 injection model, there was no apparent effect on OPC numbers even after 3 days. OPC numbers only seemed to go down when macrophages begin to attack on day 7 onwards. We also investigated if ET-1 directly affects OPC proliferation in cell culture, and we found that the levels of ET-1 used here (10 $\mu\text{g/ml}$: same concentration in vivo) did not appear to significantly affect OPC survival or proliferation in WST assay (please see figure below; also included as Supplementary Data). Taken together, it seems that ET-1 may not directly affect OPCs in our model systems. Nevertheless, we include this important caveat in our revised Discussion.



7. In Fig. 2E, how much of LDH release is contributed by death of macrophages themselves? Looks like macrophages are also dying following phagocytosis in Fig. 2C.

Response: Thank you for raising this important point. In Figure 2E, extracellular LDH was significantly increased at 3, 6 and 24 hours after macrophage-OPC co-culture. Thus, we clarified which cells, OPCs or macrophages, were dying by pre-fixation propidium iodide (PI) staining. As expected, many PDGFR α -positive OPCs were positive for PI but macrophages were negative for PI at 6 hours after the co-culture, suggesting that macrophages killed OPCs, thus accounting for the elevation in extracellular LDH. We now include our new PI data in Figure 2F. In addition, we showed that healthy OPC was eventually killed and eaten by macrophages during the co-culture in supplemental movie 1 (please see newly attached movie file). Images in figure 1C consist of images from time points at 15, 75, 135, and 195 min after the co-culture in the supplemental movie 1.

8. Fig. 3D/3E, not clear how the reduced phagocytosis (with anti-TLR4 and anti-NG2 antibodies) can be

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3 inferred with these phase contrast images? Also, quantification would be helpful.
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7 **Response:** Thank you for this suggestion. We now include slightly clearer images to show the anti-TLR4 and
8 anti-NG2-mediated reduction of macrophage attack on OPCs in Figure 3D. Additionally, we quantified
9 protective effect of the neutralizing antibody using LDH assay. We include these new data in Figure 3E.
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13 1. Minor comments: More information in the background or discussion on various other (other than
14 CD200-CD200R) 'macrophage-downregulation signaling' may be useful.
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18 **Response:** Thank you for the suggestion. We now included discussion of other inhibitory signaling pathway
19 to downregulate macrophages. In addition, we included some recent reports which have shown mechanisms of
20 monocyte/macrophage polarization after CNS injury.
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24 2. Methods: Line 4: "C56BL6" should be "C57BL6"
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27
28 **Response:** Sorry for the typo. We corrected it.
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31 3. Few sentences on the ET1 model (effect of ET1, reversible/irreversible and half-life etc.) in the introduction
32 would be helpful.
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35 **Response:** Thank you for the suggestion. ET-1 is a potent vasoconstrictor agent and exhibits long-lasting
36 actions for at least 48 hours when injected into cerebral cortex and produces ischemic injury. Additionally,
37 when injected into white matter area, ET-1 produces hallmarks of white matter injury observed in humans,
38 such as axonal damage, demyelination, inflammation, and glial scar formation. We have included more
39 background information on this standard ET-1 model in the Methods section.
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44 4. Methods: Page 5: Line 16: is it "CD206" or "CD200"? If it is mannose receptor (CD206), then the use of
45 this antibody has not been described.
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49 **Response:** We are sorry for typo. Should be CD200.
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52 5. Some of the abbreviations can be expanded at their first appearance (eg. TGM2, EBP, CREB etc.).
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55 **Response:** We apologize for the mistake. The extra sentences about TGM2, EBP, CREB have been deleted.
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58 6. Fig. 1A: What is shown in green? The label says "myelin", is it an antibody based labeling? If so, which
59 one? Or is it GFP conjugated ET1?
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4 **Response:** Sorry for being unclear. We performed fluoromyelin staining using FluoroMyelin Green
5 Fluorescent staining kit (Life technologies, F34651) and therefore green fluorescence shows myelin in corpus
6 callosum. We now included this information in the Methods section.
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10 7. Some of the western blots don't have Molecular Weights. It would be helpful to show molecular weights.
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12 **Response:** Thanks for this suggestion. Molecular weights (kDa) are included in all western blot figures.
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16 8. Fig. 5C middle panel: Why the IgG administration significantly increased F4/80+ monocytes/macrophages
17 in the blood (while the authors did not mention this neither in the results nor in figure legends)
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20 **Response:** We apologize for not being clear. The increase in F4/80 positive monocyte/macrophages is due to
21 ET-1-induced white matter ischemic injury. The use of IgG is only meant as a "control" for comparison vs
22 CD200 treatments. In Figure 5C, IgG or CD200-Fc was treated at day1 after ET-1 injection. We demonstrated
23 that blood F4/80 positive monocyte/macrophages were increased in peripheral blood along with upregulation
24 of TLR4 expression at day 3 after white matter stroke onset. Importantly, treatment with CD200-Fc
25 suppressed TLR4 expression at day 3 after ET-1 injection compared to IgG treatment. We have tried to clarify
26 this effect of CD200-Fc treatment in peripheral monocyte/macrophages compared to control IgG treatment in
27 the Results and Figure Legend. The increase in F4/80 positive monocyte/macrophages is not because of IgG
28 (injected as control antibody); it is due to ET-1-induced white matter ischemic injury.
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36 9. This reviewer couldn't find the supplementary movie along with the manuscript file.
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39 **Response:** We are sorry that we may have made a mistake during file uploading. Supplemental movie 1 is a
40 MP4 video file that can be opened by any video player programs such as media player.
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43 We hope that all these revisions have improved our paper. We still feel that this may be an important study that
44 links CD200 mechanisms in macrophages to white matter remodeling via inhibition of killing and
45 phagocytosis of OPCs. We hope you can give us the chance to report our findings in Journal of Cerebral
46 Blood Flow & Metabolism.
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50
51 Respectfully,

52 Kazuhide Hayakawa and Eng H. Lo,

53 On behalf of all co-authors
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Original Article**Title:** CD200 restrains macrophage attack on oligodendrocyte precursors via TLR4 downregulation

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Running headline: (limit 50 characters): CD200 promotes white matter repair after injury

Abstract (161/200 words)

There are numerous barriers to white matter repair after CNS injury and the underlying mechanisms remain to be fully understood. In this study, we propose the hypothesis that inflammatory macrophages in damaged white matter attack oligodendrocyte precursor cells (OPCs) via TLR4 signaling thus interfering with this endogenous progenitor recovery mechanism. Primary cell culture experiments demonstrate that peritoneal macrophages can attack and digest OPCs via TLR4 signaling, and this phagocytosis of OPCs can be inhibited by using CD200-Fc to downregulate TLR4. In an *in vivo* model of white matter ischemia induced by endothelin-1, treatment with CD200-Fc suppressed TLR4 expression in peripherally circulating macrophages, thus restraining macrophage phagocytosis of OPCs and leading to improved myelination. Taken together, these findings suggest that deleterious macrophage effects may occur after white matter ischemia, whereby macrophages attack OPCs and interfere with endogenous recovery responses. Targeting this pathway with CD200 may offer a novel therapeutic approach to amplify endogenous OPC-mediated repair of white matter damage in mammalian brain.

Key words: CD200, macrophage, phagocytosis, oligodendrocyte precursors, white matter injury

Introduction

One of major findings in neuroscience in the past decade is that adult mammalian brain can be surprisingly plastic after stroke and brain injury.¹ In the context of functional recovery, white matter connectivity may be especially crucial, and many cells and processes contribute to endogenous mechanisms that promote axonal recovery.² But white matter recovery is often incomplete because repair processes are also impaired by the development of inhibitory responses in glial cells. Current knowledge is mostly focused on the potential role of inhibitory matrix substrates such as chondroitin sulfate proteoglycans and NOGO.^{3, 4}

Beyond axonal growth and reconnections, remyelination should also be an essential component of white matter recovery. Oligodendrocytes can be generated from V-SVZ cells in the adult brain, and newly generated oligodendrocyte precursor cells (OPCs) migrate towards the corpus callosum and the white matter tracts of striatum and fimbria fornix.⁵ After white matter injury, recovery occurs when myelin sheaths are generated by oligodendrocytes that mature from OPCs that are capable of migrating into demyelinated areas to promote remyelination.^{6, 7} But unlike the developing brain, OPCs are now moving into damaged tissue with a complex inflammatory milieu. How OPCs survive and respond in the inflammatory environment remains to be fully understood.

In this study, we used a combination of cell culture and the *in vivo* model to examine how OPCs respond to inflammatory macrophages in damaged white matter. Traditionally, “M2-like” macrophages are thought to perform tissue clean-up and debris removal duties that promote repair and recovery. In this study, our findings suggest that in addition to these beneficial actions, potentially deleterious “M1-like” macrophages can attack OPCs via TLR4-dependent pathways. By attacking OPCs, these macrophages prevent endogenous oligodendrocyte replacement and interfere with recovery. However, treatment with CD200-Fc may downregulate TLR4, prevent this macrophage attack on OPCs, and enhance white matter recovery.

Methods and Materials

In vivo white matter injury models: All experiments were performed following an institutionally approved protocol in accordance with National Institutes of Health guidelines and with the United States Public Health Service's Policy on Human Care and Use of Laboratory Animals and following Animals in Research: Reporting *In vivo* Experiments (ARRIVE) guidelines.⁸ Male C57BL6 mice (11-12 weeks, Charles River Laboratories) were deeply anaesthetized with isoflurane (5% to 1.5%) in 30%/70% oxygen/nitrous oxide. Endothelin 1 (ET-1, Sigma, E7764) at a concentration of 10 µg/ml was injected through a 30-gauge needle over 5 minutes into the left corpus callosum as performed before (anterior: 0.5 mm from bregma, lateral: 1.0 mm from bregma, depth: 2.3 mm from the skull surface).⁹ ET-1 is a potent vasoconstrictor and exhibits long-lasting actions for at least 48 hours when

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4 injected into brain as a standard model for producing focally restricted ischemic injury.¹⁰ Additionally, when
5 injected into white matter area, ET-1 produces hallmarks of white matter injury observed in humans, such as
6 axonal damage, demyelination, inflammation, and glial scar formation.¹¹ Myelin damage was detected by
7 fluoromyelin staining (1:300, Life technologies, F34651). Since CD200-Fc treatment to target CD200R has been
8 reported in an *in vivo* study,^{12,13} we treated mouse CD200-Fc in our *in vivo* model. Mouse CD200 fusion protein
9 (100 µg/kg, R&D systems, 3355-CD-050) or mouse control IgG (100 µg/kg, Santacruz, sc-2025) was injected
10 intraperitoneally on days 1, 5, and 10 after ET1 injection.
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16 **Macrophages (Mφ) isolation:** Mφs were isolated from rat peritoneal cavity. After collecting cells from the cavity,
17 cells were seeded on non-coated six-well plates and grown in RPMI medium 1640 containing 10% FBS, 1%
18 penicillin/streptomycin. Twenty four hours later, attached cells were used for further experiment.
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22 **Oligodendrocytes precursor cells (OPCs)/oligodendrocyte/microglia culture:**

23 OPCs/oligodendrocyte/microglia cultures were prepared as previously described.^{14,15} Briefly, cerebral cortices
24 from 1-2 day old Sprague-Dawley rats were dissected, minced, and digested. Dissociated cells were plated in
25 poly-D-lysine-coated 75-cm² flasks, and maintained in Dulbecco's Modified Eagle's medium containing 20%
26 heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. After the cells were confluent (~10
27 days), the flasks were shaken for 1 hour on an orbital shaker (220 rpm) at 37°C to remove microglia.
28 Non-adherent cells were collected as microglia following shaking the flasks and cultured in DMEM/F12
29 containing 10% FBS. Twenty four hours later, microglia culture was used for further stimulation or experiment.
30 After shaking the flasks for 1h, the medium was changed with a new medium and shaken overnight (~20 hours).
31 The medium was then collected and plated on non-coated tissue culture dishes for 1 hour at 37°C to eliminate
32 possible contamination by astrocytes and microglia. The non-adherent cells were collected and re-plated in
33 Neurobasal Media containing glutamine, 1% penicillin/streptomycin, 10 ng/mL PDGF, 10 ng/mL FGF, and 2%
34 B27 supplement onto poly-DL-ornithine-coated plates. Oligodendrocyte precursor cells were grown in 10 ng/ml
35 T3, and 10 ng/ml CNTF for 7 days to get matured oligodendrocytes.
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45 **GST-pull down assay:** GST-pull down assay experiment was performed between His-tagged NG2 recombinant
46 protein (1 µg, R&D systems, 2585-PG-050) and GST-tagged TLR4 (0.1 µg, Abnova, H00007099-Q01) or GST
47 control (0.1 µg, Santacruz, sc-4033). Total amount of 400 µl of each combination was incubated with 10 µl of a
48 50% slurry of glutathione-agarose beads for 4 hours at 4°C with end-over-end mixing. Agarose beads were
49 collected after centrifugation at 1,000g for 10 seconds, and washed three times with 50% PRO-PREP lysis buffer
50 (iNtRON Biotechnology, 17081) diluted by PBS. NG2 recombinant protein associated with the GST fusion
51 protein was detected by SDS-PAGE-western blot method.
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57 **Macrophage-mediated bacteria phagocytosis assay:** Rat peritoneal macrophages were stimulated by
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3 interleukin-1 β (IL-1 β , 100 ng/ml) for 24 hours and the ability of non-stimulated or IL-1 β -stimulated macrophages
4 to incorporate bacterial particles was assessed by Vybrant Phagocytosis Assay Kit (Life technologies, V-6694). *E.*
5 *coli* particles (K-12 strain) labeled by fluorescein was added to macrophages for 2 hours and washed twice with
6 PBS to remove free floating debris. Finally, the incorporation of bacterial particles in macrophage was analyzed
7 by fluorescence microplate reader at Ex (488 nm)/Em (515 nm).
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12 **Macrophage-mediated OPC or myelin debris phagocytosis assay:** OPCs were labeled by diI-ac LDL
13 (Invitrogen, L3484) for 2 hours at 37°C incubator and wash three times with PBS. Isolated macrophages were
14 directly co-cultured with OPCs in Neurobasal Media containing glutamine, 1% penicillin/streptomycin, 10 ng/mL
15 PDGF, 10 ng/mL FGF, and 2% B27 supplement onto poly-DL-ornithine-coated plates and the percentage of
16 ac-LDL incorporated CD68 (anti-CD68, 1:100, Abcam, ab955) -positive macrophages were identified by flow
17 cytometry at 24 hours after starting the co-culture. To block macrophage phagocytosis, antibodies (normal mouse
18 IgG ; 5 or 10 μ g/ml, Santacruz, sc-2025, anti-TLR4 ; 10 μ g/ml, R&D systems, AF1478, or anti-NG2 ; 5 μ g/ml,
19 Invitrogen, 37-2700) or CD200 Fc (10 μ g/ml, R&D systems, 3355-CD-050) were pre-incubated for 1 hour and
20 co-incubated according to each experimental schedule. Time-lapse image during the coculture was performed
21 using Nikon Eclipse Ti microscope and NIS-Elements software in 5% CO₂ incubator at 37°C. To prepare
22 fluorescent labeled myelin debris, oligodendrocytes were stained with fluoromyelin (Invitrogen, F34651) then
23 gently broke down with ultrasound sonicator. Myelin pellet was collected following filtration with 1.2 μ m syringe
24 filter and centrifugation at 2,000 rpm for 10 minutes. Macrophages were incubated with fluorescein-labeled
25 myelin debris for 2 hours and washed twice with PBS to remove free floating debris. Finally, the incorporation of
26 myelin debris in macrophage was analyzed by fluorescence microplate reader at Ex (488 nm)/Em (515 nm).
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38 **LDH cytotoxicity assay:** OPCs or mature oligodendrocytes were co-incubated with IL-1 β -stimulated rat
39 peritoneal macrophages or IL-1 β -stimulated rat brain microglia for 24 hours. Since LDH release is commonly
40 observed during phagocytosis,^{16,17} extracellular LDH was detected by LDH Cytotoxicity Detection kit (Roche
41 Applied Science, 11644793001). Then the absorbance of the culture medium was measured with a microplate
42 reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. Macrophage or microglia was
43 identified by CD68 antibody (1:100, Abcam, ab955) and OPCs were labeled by diI-ac LDL, PDGFR α antibody
44 (1:200, R&D Systems, AF1062) or Olig2 antibody (1:200, Santacruz, sc-19969). To determine which cells, OPCs
45 or macrophages, were killed and releasing LDH, propidium iodide (PI 1:300, Invitrogen, P3566) staining for 10
46 min prior to fixation (pre-fixation PI staining)^{18, 19} was performed.
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55 **WST assay:** Cell proliferation/viability was assessed by WST reduction assay (Dojindo), which detects
56 dehydrogenase activity of viable cells. The cells were incubated with 10% WST solution for 1 h at 37°C. Then the
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4 absorbance of the culture medium was measured with a microplate reader at a test wavelength of 450 nm and a
5 reference wavelength of 630 nm.
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8 **FACS analysis:** For analyses of *in vivo* brain samples, tissues are minced and then digested at 37°C for 30 min
9 with an enzyme cocktail (Collagenase type IV; Sigma-Aldrich, C5138, DNase I; Sigma-Aldrich, D4263) as
10 previously described.²⁰ Single cell suspensions were prepared by filtering through a 40- μ m strainer. Cell
11 suspensions are pre-blocked with 3% BSA and then incubated with the following primary antibodies against
12 TLR4 (1:100, Abcam, ab45104), NG2 (1:100, Abcam, ab81104), PDGFR α (1:200, BD biosciences, 558774).
13 F4/80 (1:100) was purchased from Biolegend (123102). Fab specific secondary antibodies tagged by
14 phycoerythrin (PE), fluorescein isothiocyanate (FITC), and allophycocyanin (APC) from Jackson laboratories are
15 incubated for 30 min at room temperature. Labeled cell populations are measured by FACSCalibur (BD
16 biosciences). FACS data are analyzed by Cellquest pro software (BD biosciences). FACS analysis are performed
17 using a variety of controls including unstained samples, isotype antibodies and single stained samples for
18 determining appropriate gates, voltages, and compensations required in multivariate flow cytometry.
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26 **Western blot analysis:** Western blot procedure was performed as described before.²⁰ After blocking, membranes
27 were then incubated overnight at 4°C with following primary antibodies; anti-NG2 (1:500, Santacruz, sc-166251),
28 monoclonal anti-MBP (1:500, Abcam, ab62631), monoclonal anti-beta actin (1:5000, Sigma, A2228) were
29 obtained from Cell Signaling Technology. after incubation with peroxidase-conjugated secondary antibodies (GE
30 Healthcare, NA931- anti-mouse, or NA934- anti-rabbit, or NA935- anti-rat) and visualization by enhanced
31 chemiluminescence (Thermo Scientific, 34078).
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37 **Immunocytochemistry or immunohistochemistry:** Immunocytochemistry or immunohistochemistry was
38 performed as described before.²⁰ After staining with primary antibody, fluorescent-tagged secondary antibody,
39 nuclei were counterstained with or without 4,6-diamidino-2-phenylindole (DAPI), and coverslips were placed.
40 Immunostaining was analyzed with a fluorescence microscope (Nikon ECLIPSE Ti-S) interfaced with a digital
41 charge-coupled device camera and an image analysis system or using Carl Zeiss Laser Scanning Confocal
42 Microscope Pascal 5 LSM and Pascal 5 LSM software Version 3.2.
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48 **Electron Microscopy:** Tissues were fixed in 2.0% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4
49 (Electron Microscopy Sciences, Hatfield, PA) overnight at 4°C. They were rinsed in buffer, post-fixed in 1.0%
50 osmium tetroxide in cacodylate buffer for one hour at room temperature, rinsed in buffer again and dehydrated
51 through a graded series of ethanol to 100%. They were then infiltrated overnight with Eponate resin (Ted Pella,
52 Redding, CA) in a 1:1 solution of Eponate : propylene oxide. The following day they were placed in fresh
53 Eponate for several hours and then embedded in Epon overnight at 60°C. Thin sections were cut on a Leica EM
54 UC7 ultramicrotome, collected onto formvar-coated grids, stained with uranyl acetate and lead citrate and
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4 examined in a JEOL JEM 1011 transmission electron microscope at 80 kV. Images were collected using an AMT
5 digital imaging system (Advanced Microscopy Techniques, Danvers, MA). Using the calibrated measurement tool
6 within the AMT Image Capture Engine V601 software package, myelin measurements were drawn (inner axonal
7 diameter from myelin edge to myelin edge, and total axonal diameter) and values (mm) embedded on images at
8 magnification x60,000 at the time of data collection.
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13 **Statistical analysis:** Results were expressed as mean±SEM. When only two groups were compared, Student's
14 t-test was used. Multiple comparisons were evaluated by Tukey-Kramer's test after one-way or two-way ANOVA.
15 $P < 0.05$ was considered to be statistically significant. All experiments and analyses were conducted in a
16 randomized and blinded manner in accordance with NIH guidelines on rigor and reproducibility.
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19 20 21 Results

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24 Acute injury in white matter upregulates endogenous OPCs populations as part of the compensatory
25 response for repair and recovery.^{21, 22} But how well do these newly generated cells survive the
26 inflammatory environment that is known to exist in damaged brain tissue?²³ Endothelin-1 (ET-1) was
27 stereotaxically injected into the corpus callosum of adult male C57Bl6 mice in order to induce focal
28 ischemic damage and demyelination. As the lesion developed over the first few days after ET-1 injection
29 (Fig. 1A), macrophages were observed to accumulate within the damaged white matter zone (Fig. 1B).
30 Confocal microscopy suggested that macrophages often surrounded PDGFR α -positive OPCs (Fig. 1C),
31 hence raising the possibility that this inflammatory process may somehow interfere with the endogenous
32 progenitor cell response. Cell counts demonstrated that OPC density began to decrease from day 3 to
33 day 7 (Fig. 1D), along with an increase in accumulated toll-like receptor 4 (TLR4)-expressing
34 macrophages (Fig. 1E). Correspondingly, GSTpi-positive mature oligodendrocytes showed a decreasing
35 trend over 14 days after white matter injury (Fig. 1F).
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44 The temporal profile of decreasing OPCs along with accumulating macrophages as well as the spatial
45 co-localization of these cells over time suggest that macrophages can attack OPCs. To assess the mechanisms that
46 may underlie this phenomenon, we turned to cell culture models. First, we showed that within the range of
47 concentrations used here, ET-1 did not appear to directly kill OPCs (Suppl. Fig. 1), suggesting that the effects
48 documented in our *in vivo* model may be due to white matter ischemia and not cytotoxicity of ET-1 per se. Next,
49 we conducted *in vitro* phagocytosis assays where rat peritoneal macrophages were stimulated with
50 interleukin-1beta (IL-1 β) to increase phagocytic activity and TLR4 expression (Fig. 2A and 2B). When
51 macrophages were then added onto primary rat OPCs, they attached to, ingested, and digested the live OPCs (Fig.
52 2C-2F). Conditioned media collected from macrophages did not kill OPCs (Fig. 2G), suggesting that direct
53 cell-on-cell attacks may be required. In contrast, macrophages did not attack mature oligodendrocytes in our
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4 system (Fig. 2E). Additionally, we investigated whether brain microglia attacked OPCs. In our cell culture system,
5 IL-1 β -stimulated brain microglia could attack OPCs, but the degree of phagocytotic attack was smaller compared
6 to macrophages (Fig. 2H).
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10 Because of this differential vulnerability between OPCs and oligodendrocytes, we next asked whether the
11 ability of macrophages to target susceptible cells may be mediated by the expression of immature signals in OPCs
12 such as NG2 (Fig. 3A). Toll-like-receptors (TLRs) comprise a family of proteins known to be essential for
13 macrophage function,²⁴ so we used a GST-pull-down assay to screen this TLR family for NG2-binding ability.
14 Our initial data suggest that TLR4 can bind to NG2 proteins (Fig. 3B). Moreover, TLR4 from macrophages
15 appeared to overlap with NG2 in OPCs when these cells were co-cultured (Fig. 3C), and application of anti-TLR4
16 or anti-NG2 neutralizing antibodies reduced OPC phagocytosis (Fig. 3D and 3E).
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22 If macrophages can attack and phagocytose OPCs via TLR4 signaling, is it possible to prevent this
23 deleterious process and promote white matter recovery by suppressing the macrophage response? To answer this
24 question, we focused on the immunoglobulin superfamily CD200, which is known to interact with its receptor
25 CD200R on immune cells to deactivate inflammation after stroke²³. Cultured macrophages expressed CD200R
26 (Fig. 4A), and treatment with the fusion protein CD200-Fc reduced their surface levels of TLR4 (Fig. 4B). Next,
27 conditioned media from IL-1 β -stimulated macrophages following IgG or CD200-Fc treatment were collected and
28 transferred into OPCs. We confirmed that CD200-Fc-stimulated macrophage conditioned media did not affect
29 OPC proliferation (Fig. 4C). Interestingly, treatment with CD200-Fc significantly decreased the ability of
30 macrophages to attack and ingest OPCs (Fig. 4D) without affecting the ability of macrophages to incorporate
31 myelin debris (Fig. 4E), suggesting that CD200-Fc may protect OPC from macrophage attack through
32 downregulating TLR4 expression and function.
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40 Based on these *in vitro* findings, we then asked whether CD200-Fc administration could similarly suppress
41 macrophages and promote white matter recovery *in vivo*. Mice were stereotaxically injected with ET-1 to induce
42 focal ischemic injury in the corpus callosum, and then were treated with CD200-Fc fusion protein (100 μ g/kg
43 intraperitoneal) or control IgG at 1, 5 and 10 days later. To assess whether CD200-Fc suppressed TLR4 expression
44 in blood monocyte/macrophages, FACS analysis was performed on blood samples collected at 3 days after injury
45 (Fig. 5A). This analysis showed that treatment with CD200-Fc successfully increased plasma CD200 protein level
46 (Fig. 5B). After 3 days following ET-1 injection, the number of circulating F4/80 positive monocyte/macrophages
47 found in blood were significantly increased in accompanied with upregulation of TLR4 expression compared to
48 sham-operated control group in FACS analysis (Fig. 5C). Treatment with CD200-Fc suppressed TLR4 expression
49 at day 3 after ET-1 injection (Fig. 5C), suggesting that intraperitoneally injected CD200-Fc may be able to
50 ameliorate circulating monocytes/macrophages before they target towards the injured corpus callosum. Confocal
51 microscopy images showed that macrophages surrounding OPCs were decreased in corpus callosum of
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4 CD200-treated mice (Fig. 6A). Mice that were treated with CD200-Fc showed a significant suppression in TLR4
5 positive macrophage accumulation within the lesioned white matter compared to controls (Fig. 6B) at days 3 and
6 7 after ET-1 injection. Concomitantly, the number of OPCs and mature oligodendrocytes were also preserved in
7 the CD200-Fc-treated brains (Fig. 6C and 6D). Finally, using electron microscopy, we confirmed that CD200-Fc
8 treatment significantly improved myelination at day 14 after ET-1-induced white matter stroke (Fig. 6E),
9 suggesting that protecting OPCs from macrophage attack may facilitate newly generated oligodendrocytes for
10 white matter remodeling.
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14 15 16 Discussion

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19 After stroke and brain injury, endogenous attempts at self-repair are complex and poorly understood. Many
20 cell types and intercellular signals are involved, and often, these pathways comprise a shifting balance between
21 beneficial and deleterious responses. Furthermore, beyond cell-cell signaling within injured brain per se, dynamic
22 crosstalk between brain and systemic responses such as circulating blood cells may also be important.²⁵ In
23 particular, monocyte/macrophage cells may contribute to both brain damage and remodeling,^{26, 27} depending on
24 the inflammatory circumstance.²³ The present study may be consistent with this overall conceptual framework.
25 Our primary cell culture and *in vivo* data suggest that macrophages can attack OPCs via TLR4-NG2 signaling,
26 and treatment with CD200-Fc administration boosts white matter recovery by suppressing the macrophage attack
27 and phagocytosis of susceptible OPCs. Macrophages are traditionally thought to be somewhat beneficial by
28 mediating tissue clean-up and debris clearance, thus facilitating repair and recovery. Our findings here suggest
29 that in addition to positive “M2-like” phenomenon, activated macrophages may also possess detrimental
30 “M1-like” actions in damaged white matter.
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39 TLRs are traditionally considered as sensors for danger associated molecules produced by bacteria and
40 viruses to discriminate between "self" and "non-self" in innate immune system. More recently, emerging data
41 demonstrate that TLRs signaling may have critical roles in both detrimental inflammatory responses and
42 beneficial repair processes after CNS injury. For example, TLR4-deficient mice show smaller infarction along
43 with less inflammatory substances such as iNOS and cyclooxygenase compared to wild type.²⁸ LPS did not affect
44 cell survival of oligodendrocyte precursors, but microglial TLR4 activation by LPS caused oligodendrocyte
45 precursor injury.²⁹ On the other hand, TLR4 and TLR2 mutations may cause impairments of myelination and
46 long-term recovery of locomotor function after spinal cord injury,³⁰ suggesting that TLRs may give various
47 impact upon the oligodendrocyte function and homeostasis after CNS injury. Our current findings may provide a
48 novel mechanism that macrophage expressing TLR4 can directly interact with OPC-derived NG2 and promote
49 OPC phagocytosis in acute stage thus interfering endogenous oligodendrogenesis in late stage after white matter
50 injury. This TLR4-NG2-mediated attack on OPCs may indeed comprise an underlying mechanism that potentially
51 explains the damaging effects of macrophages in damaged white matter in our model systems.
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5 The key role of neuroimmune regulatory proteins (NIRegs) have well been evidenced in the control of
6 macrophage or microglia activation. For example, death signaling pathways based on CD95L and CD95
7 interaction transmits an apoptotic signal to target T cells.³¹ CD47-SIRP α signaling behaves as “don't eat me”
8 signals to prevent unwarranted phagocytosis in macrophage or microglia.³² Siglecs are known as a class of
9 receptors that can bind to sialylated glycoproteins and glycolipids contain immunoreceptor tyrosine - based
10 inhibitory motifs (ITIMs) in their cytoplasmic tail which may negatively regulate the function of phagocytes,
11 including microglia.³³ More recently, the concept of macrophage polarization toward different phenotypes after
12 CNS injury has also been explored. In general, macrophage phenotype can be characterized as classically
13 activated pro-inflammatory forms (M1-like) or alternatively activated forms (M2-like). Inflammatory
14 microglia/macrophages promote secondary injury,³⁴ whereas M2-like macrophages may participate to debris
15 clean-up and remodeling.³⁵ Recent report showed the important role of CCR2 in the phenotype polarization in
16 monocyte. Blockade of CCR2 increased M1 phenotype and resulted in increased infarct volume along with
17 exacerbated functional deficits.³⁶ Additionally, it has been reported that HDAC inhibition shifted
18 microglia/macrophage to M2 phenotype and prevented white matter injury.³⁷ As a compartment of NIRegs,
19 CD200 can bind to CD200 receptor which predominantly expresses on myeloid lineage cells and suppress the
20 inflammatory responses.³⁸ In this study, we demonstrate another example which CD200-Fc administration boosts
21 white matter recovery by suppressing macrophage attack and phagocytosis of susceptible OPCs through TLR4
22 downregulation. Additionally, CD200-Fc may not alter the ability of debris clearance in macrophages. Thus, these
23 novel pathways may lead to new therapeutic opportunities for promoting white matter recovery after injury and
24 disease.
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37 Nevertheless, there are several issues that warrant further consideration. First, we only focus on macrophages
38 as a major representative cell of the inflammatory response. But other cells may also participate in white matter
39 injury. For example, many studies have implicated microglia in a wide spectrum of CNS disorders.^{39,40} In our
40 OPC cultures, microglia can attack OPCs, but compared to macrophages, the degree of phagocytotic attack was
41 smaller. The difference of cellular responses should be further investigated, especially in blood where besides
42 monocytes, potentially beneficial cells such as endothelial progenitor cells (EPCs) may also be recruited.^{20,41} To
43 study the effects of CD200 on all cell types is outside our scope for now. Whether and how CD200 affects other
44 inflammatory and circulating cell types bearing CD200 receptors should be carefully examined in future studies.
45 Second, our data suggest that TLR4 in macrophages would recognize NG2 in OPCs for the initiation of their
46 phagocytosis. However, other brain cells such as pericytes may also express NG2.⁴² Activated NG2 positive
47 pericytes triggers the chemotactic migration of leukocytes and upregulation of TLRs, integrins, MMPs on
48 neutrophils and monocytes.⁴³ The importance of NG2-positive pericytes in CNS disorders is well recognized now
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42. Whether and how our hypothesized CD200 mechanism affects pericytes should be further investigated. Third,
rat peritoneal macrophages attacked OPCs but not mature oligodendrocytes in our *in vitro* model system,

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4 potentially due to the differential expression of NG2. But exactly how mature oligodendrocytes able to avoid
5 macrophage attack remains to be fully elucidated. After CNS injury, degenerated myelin is phagocytosed by
6 activated microglia or macrophages. However, healthy oligodendrocytes express CD47 that may serve as a “don’t
7 eat me” signal to prevent phagocytosis by microglia or macrophages.⁴⁴ Besides our hypothesis which is that
8 OPC-derived NG2 enhances the ability of macrophages to target OPCs, endogenous NIREgs expression in mature
9 oligodendrocytes might be an important complementary mechanism that allow oligodendrocytes to avoid
10 macrophage attack. Fourth, we should also consider the roles of CD200 in vascular remodeling after white matter
11 damage. Neurovascular and gliovascular remodeling are important facets in the chronic phase of almost all CNS
12 diseases.^{45, 46} In the so-called “oligovascular niche” in white matter, trophic coupling between OPCs and vascular
13 compartments regulates both angiogenesis and oligodendrogenesis.¹⁴ Our current study demonstrates that CD200
14 may promote oligodendrogenesis recovery mechanisms. Future studies may need to examine whether this
15 mechanism may also affect angiogenesis via oligovascular signaling. Finally, it must be acknowledged that there
16 are no perfect models for white matter injury and disease. In this study, data from both ET-1 injections *in vivo* and
17 primary OPC/macrophage cultures *in vitro* were consistent with hypothesis. But further studies in other model
18 systems are warranted in order to assess the generalizability of our hypothesized phenomenon.
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28 The proliferation and maturation of progenitor cells may provide an important endogenous protective and
29 repair mechanism in CNS disease. However, this internally programmed protective system may be self-limited
30 because of the continuing development of a potentially hostile inflammatory environment in damaged or diseased
31 tissue. In gray matter, compensatory neurogenesis may be dampened by activated microglia.^{47, 48} Here, we show
32 that an analogous phenomenon may operate in damaged white matter. Accumulated macrophages appear to
33 recognize and attack susceptible OPCs via TLR4-NG2 signaling. Treatment with CD200-Fc may downregulate
34 TLR4, dampen the macrophage attack on OPCs, and promote white matter recovery. Further investigation into
35 these mechanisms may lead to novel therapeutic opportunities for modulating the balance between positive versus
36 negative effects of macrophage inflammation, and promote white matter repair in CNS diseases.
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51 **Conflict of Interest:** The authors declare they have no competing financial interest.
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54 **Authors' contribution:** K.H. contributed to manuscript preparation, experimental design/analysis and conducted
55 experiments. L.D.P., J.H.S., N.M., T.M., and Y.T., contributed to conducted experiments. S.S., and C.W.,
56 contributed to data analysis. D.B., K.W.K., K.A., and E.H.L contributed to manuscript preparation and
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4 experimental design.
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9 Supplementary information is available at the JCBM web site www.nature.com/jcbfm.
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Figure legend:**Figure 1. Reduction of OPCs and macrophage accumulation after focal white matter ischemia: (A)**

Stereotaxic injection of endothelin-1 (ET-1) into the corpus callosum induced focal demyelination in the white matter tracts. DAPI (blue) staining showed cell accumulation in the lesion area. Scale bar: 200 μ m. **(B)** Immunohistochemistry showed an accumulation of F4/80-positive macrophages in demyelinated white matter on day 3 after ET1 injection. Scale bar: 100 μ m. **(C)** PDGFR α -positive OPCs (green) were surrounded by F4/80-positive macrophages (red) on day 7 post-injury. Scale bar: 50 μ m. **(D)** Flow cytometry analysis (FACS) showed that the number of OPCs (NG2/PDGFR α -double positive cells) were decreased on day 7. Quantitation of the FACS data confirmed the significant decrease of OPCs. * P <0.05 vs sham (Sham; n=3, Day 3; n=4, Day 7; n=5, Day 14; n=4). **(E)** FACS analysis demonstrating an accumulation of TLR4-positive macrophages (F4/80-positive cells) on day 7 in the injured white matter. Quantitation of the FACS data confirmed the significant increase of total macrophages and TLR4-positive macrophages. * P <0.05, ** P <0.01 vs sham (Sham; n=3, Day 3; n=4, Day 7; n=4, Day 14; n=4). **(F)** FACS analysis showed a gradual reduction in GSTpi-positive mature oligodendrocytes over the course of 14 days after ET-1 injection (Sham; n=3, Day 3; n=4, Day 7; n=4, Day 14; n=4). SSC; side scatter. All data are presented as mean \pm SEM.

Figure 2. OPC phagocytosis by macrophage *in vitro*: **(A)** Rat peritoneal macrophages were stimulated with interleukin-1beta (IL-1 β 100 ng/ml) for 24 hours. IL-1 β significantly enhanced *E-coli* (K-12) phagocytic function in macrophages (Control; n=4, IL-1 β ; n=3). **(B)** FACS analysis was performed using TLR4 antibody conjugated with PE. Membrane expressing TLR4 in macrophages was upregulated by IL-1 β stimulation (100 ng/ml) for 24 hours. **(C-E)** Rat peritoneal macrophage stimulated with IL-1 β (100 ng/ml) killed and ingested rat OPCs *in vitro*. **(C)** time-lapse imaging was performed for 280 minutes during the co-culture (Suppl. Movie 1), scale bar: 50 μ m, **(D)** 24 hours after the co-culture, scale bar: 100 μ m. **(E)** OPCs but not mature oligodendrocytes were damaged when they were co-cultured with macrophages. * P <0.05, ** P <0.01 vs single OPC (3 hours - OPC; n=4, OPC+M ϕ ; n=4, OL; n=3, OL+M ϕ ; n=6. 6 hours - OPC; n=8, OPC+M ϕ ; n=4, OL; n=3, OL+M ϕ ; n=6. 24 hours - OPC; n=8, OPC+M ϕ ; n=4, OL; n=3, OL+M ϕ ; n=6). **(F)** PDGFR α -positive OPCs (green) were positive for PI (red) at 6 hours after the co-culture. However, macrophages were negative for PI after pre-fixation PI staining. **(G)** Macrophage conditioned media did not affect OPCs, as measured using a standard WST cell viability assay (n=3). **(H)** Rat brain microglia stimulated with IL-1 β (100 ng/ml) for 24 hours killed rat OPCs (n=3). All data are presented as mean \pm SEM.

Figure 3. Macrophage TLR4 interacts with NG2 to promote OPC phagocytosis *in vitro*: **(A)** Western blot analysis confirmed that OPCs expressed NG2, and mature oligodendrocytes expressed MBP in our culture system. **(B)** In the cell-free system, His-tagged NG2 bound to GST-tagged TLR4 assessed by western blot after GST pull-down. **(C)** Immunocytochemistry showed that NG2 positive fraction was incorporated in macrophages 24 hours after macrophage/OPC co-culture. Scale bar: 50 μ m. **(D)** Blockade of TLR4 (10 μ g/ml) or NG2 (5 μ g/ml)

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4 with a neutralizing antibody attenuated macrophage attack on OPCs. (E) Treatment with neutralizing antibody
5 significantly decreased LDH release at 90 minutes after the co-culture (n=4). ** $P < 0.01$. All data are presented as
6 mean \pm SEM.
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10 **Figure 4. CD200-Fc suppresses OPC phagocytosis by macrophage without affecting clearance of myelin**
11 **debris *in vitro*:** (A) Immunocytochemistry showed that rat peritoneal macrophages (CD68 positive cells)
12 expressed CD200 receptor. (B) IL-1 β (100 ng/ml)-stimulated macrophages were co-incubated with CD200-Fc (10
13 μ g/ml) for 24 hours. FACS analysis showed that CD200-Fc treatment significantly suppressed TLR4 expression
14 on macrophage cell surface. (C) Macrophage conditioned media were collected from IL-1 β (100
15 ng/ml)-stimulated macrophages treated by IgG or CD200-Fc (10 μ g/ml). Conditioned media did not increase OPC
16 proliferation. (D) FACS confirmed that macrophages ingested OPCs at 24 hours after starting the co-culture.
17 Co-incubation of CD200-Fc (10 μ g/ml) significantly reduced the OPC phagocytosis by macrophages *in vitro*.
18 (IgG; n=7, CD200-Fc; n=6). (E) Fluorescent myelin debris were prepared from mature oligodendrocytes stained
19 with fluoromyelin. CD200-Fc (10 μ g/ml) did not affect the incorporation of myelin fragment in IL-1 β (100
20 ng/ml)-stimulated macrophages (IgG; n=4, CD200-Fc; n=4). All data are presented as mean \pm SEM.
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28 **Figure 5. Intraperitoneal administration of CD200-Fc reduced TLR4 expression in blood**
29 **monocyte/macrophages after white matter ischemia *in vivo*:** (A) Western blot or FACS analysis was performed
30 using blood samples from ET-1-induced white matter stroke in mice. CD200-Fc (100 μ g/kg) or mouse IgG (100
31 μ g/kg) was administered on day 1 after ET-1 injection, and then TLR4 expression in blood
32 monocyte/macrophages was assessed on day 3. (B) Western blot analysis confirmed that intraperitoneal treatment
33 with CD200-Fc increased plasma CD200 expression (IgG; n=3, CD200-Fc; n=3). (C) FACS analysis revealed
34 that CD200-Fc did not strongly affect the count of total monocyte/macrophage in the blood, but significantly
35 reduced TLR4 expression compared to IgG-treated group at day 3 after ET-1 injection. * $P < 0.05$, ** $P < 0.01$ vs sham.
36 (Total F4/80 or F4/80/TLR4; Sham n=3, IgG n=4, CD200-Fc n=4). All data are presented as mean \pm SEM.
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44 **Figure 6. CD200-Fc treatment enhances remyelination after ET-1 induced white matter ischemia *in vivo*:**
45 (A) Confocal microscope images indicated that treatment with CD200-Fc (100 μ g/kg) at days 1 and 5 after
46 ET-1-injection into mouse corpus callosum preserved PDGFR α -positive OPCs (green) at day 7. Scale bar: 20 μ m,
47 macrophage: F4/80 (red). (B) CD200-Fc treatment significantly suppressed the accumulation of TLR4-positive
48 macrophages in the ET-1-damaged white matter area (Day 3 - IgG; n=4, CD200 Fc; n=4, Day 7 - IgG; n=4,
49 CD200 Fc; n=3, Day 14 - IgG; n=4, CD200 Fc; n=4). (C) FACS analysis showed that the number of OPCs was
50 significantly preserved after multiple treatments with CD200-Fc on day 7 after white matter injury (IgG; n=3,
51 CD200 Fc; n=3). (D) The number of GSTpi-positive mature oligodendrocytes was significantly increased after
52 multiple treatments with CD200-Fc on day 14 after ET-1 injection (IgG; n=5, CD200 Fc; n=3). (E) Electron
53 micrograph of axons in the corpus callosum from day 14 after ET-1 treatment. Quantification of g ratio showed
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the significant improvement of myelination in CD200 Fc-treated mice (Control; n=100 axons, IgG; n=108 axons, CD200-Fc; n=101 axons). Scale: 1 μ m. All data are presented as mean \pm SEM.

Confidential: For Review Only

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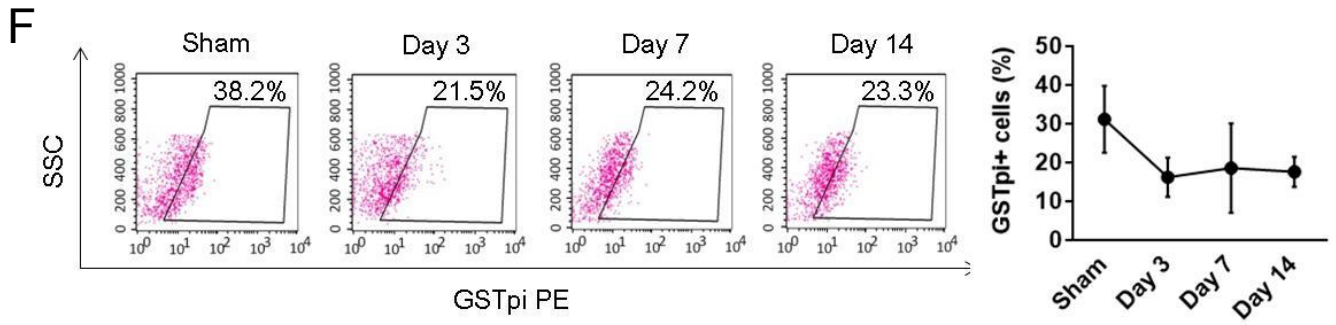
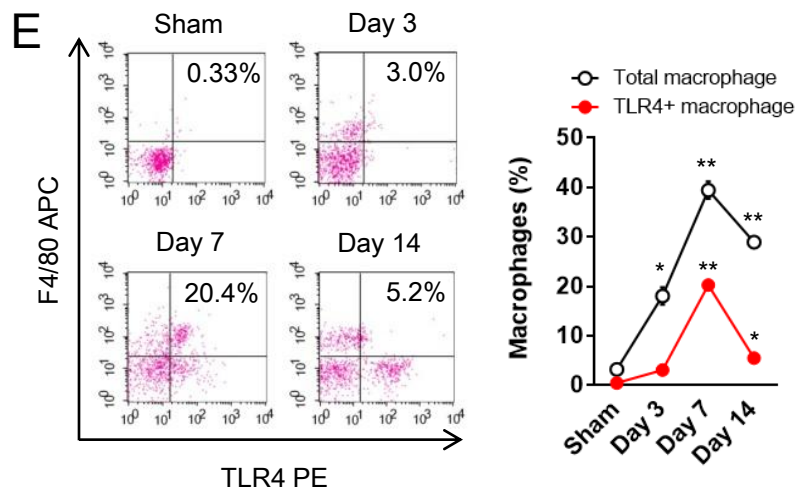
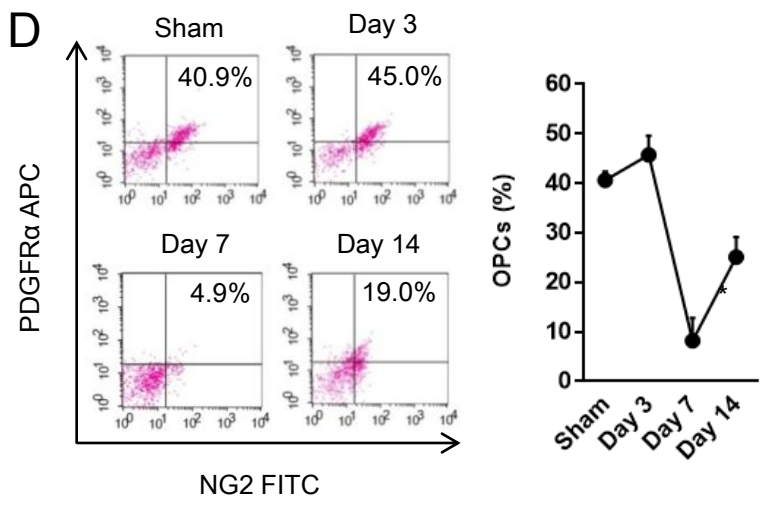
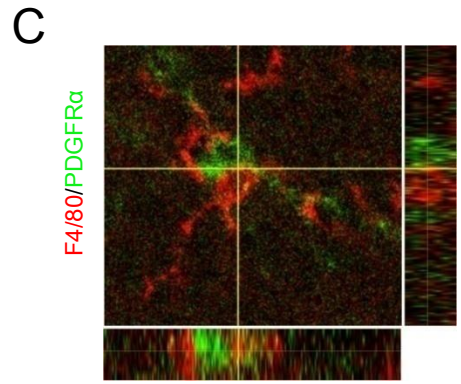
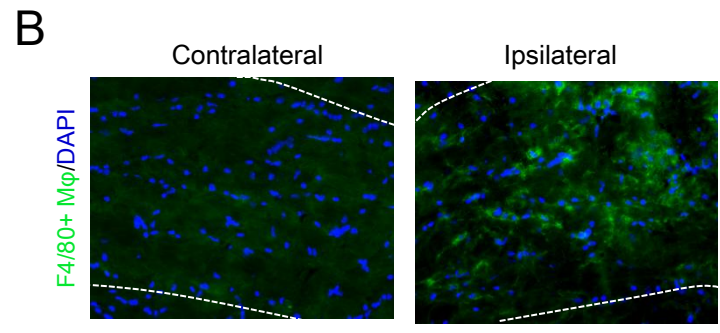
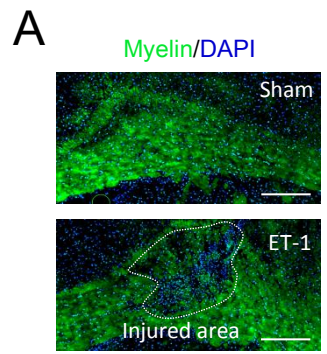
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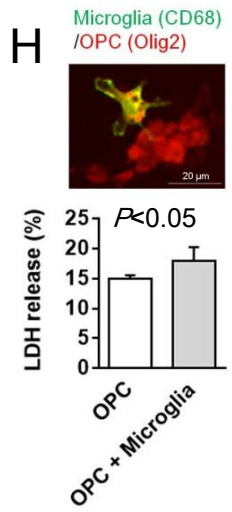
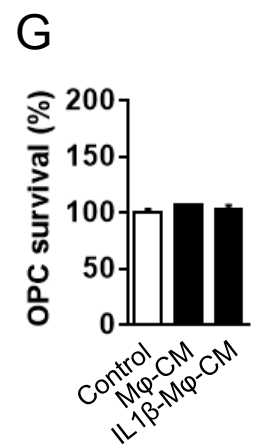
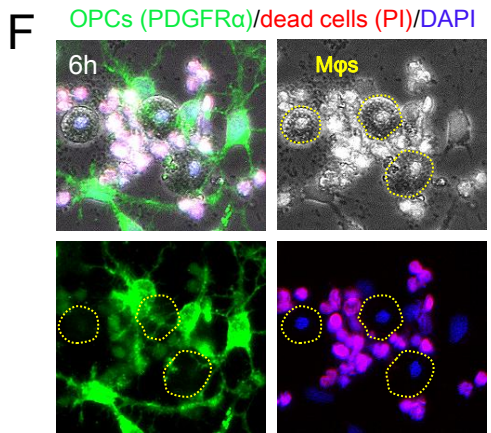
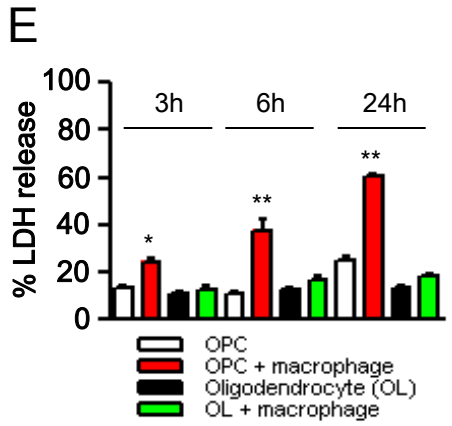
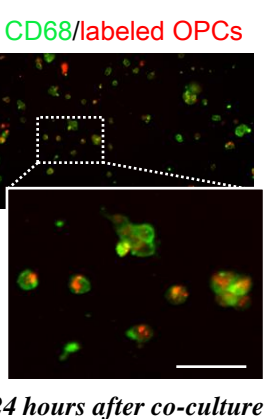
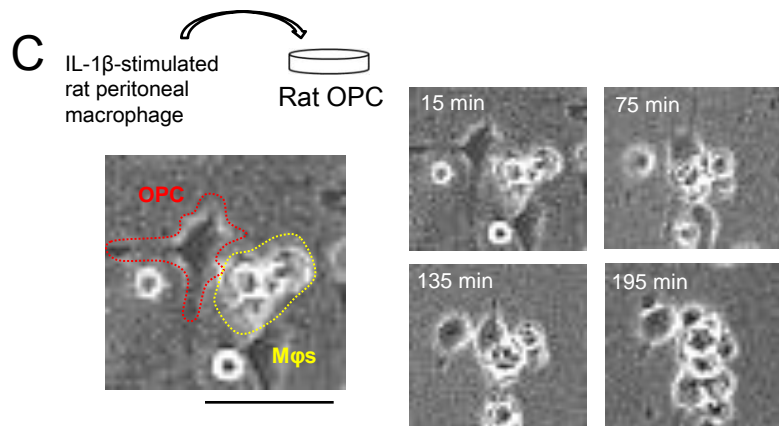
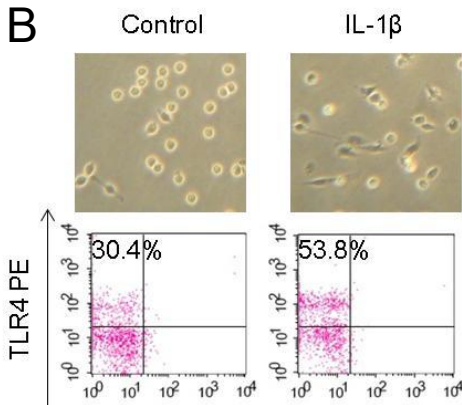
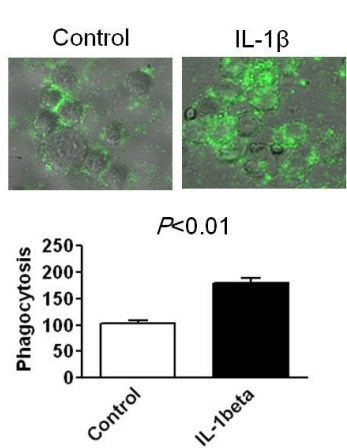
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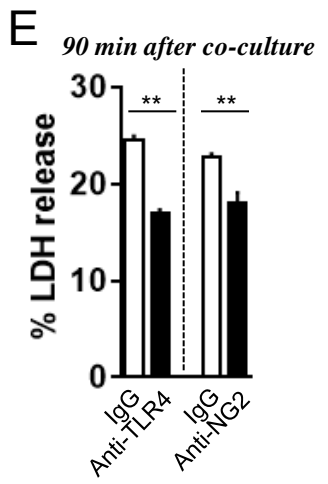
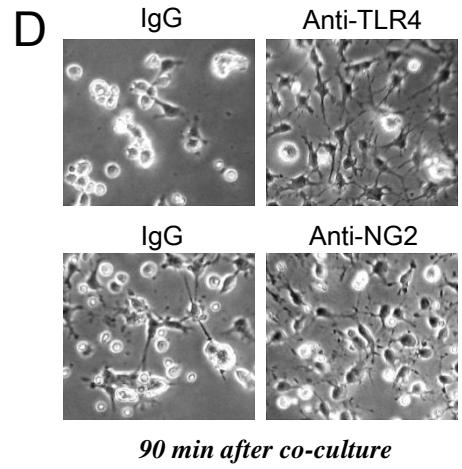
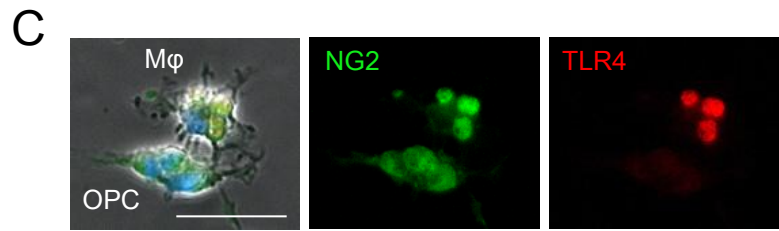
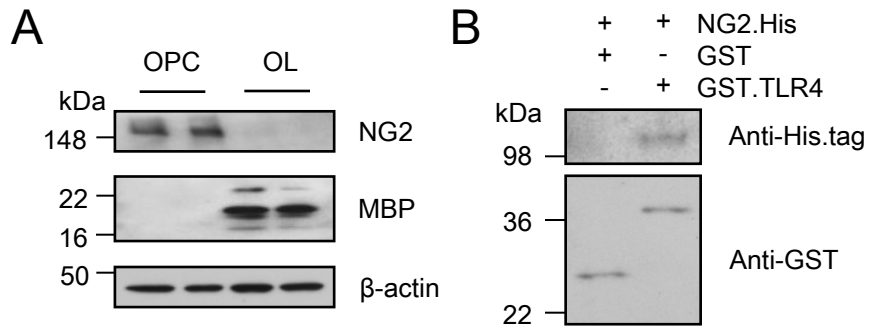
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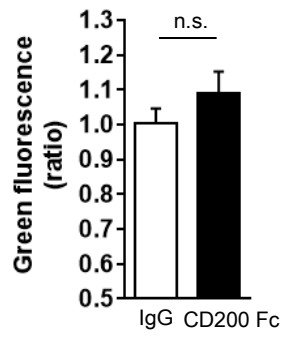
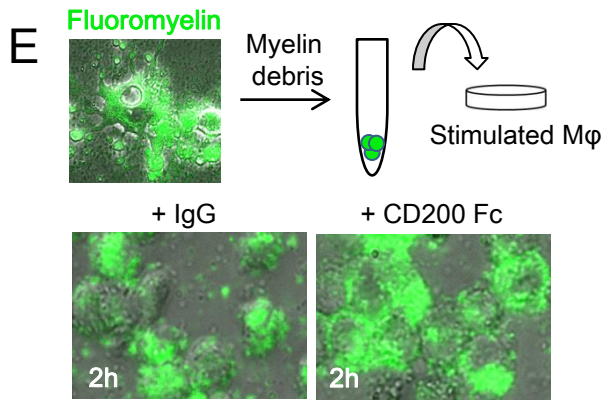
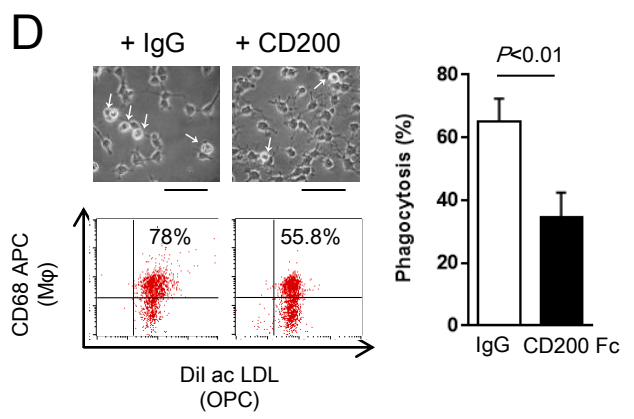
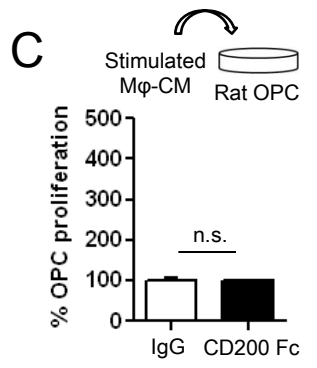
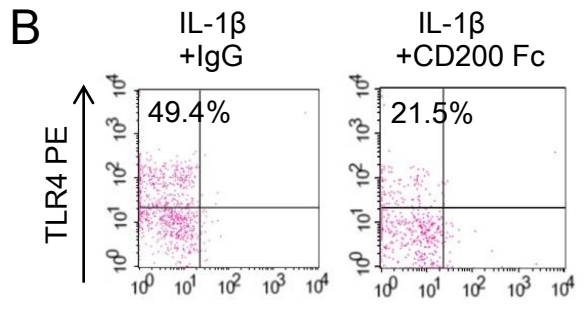
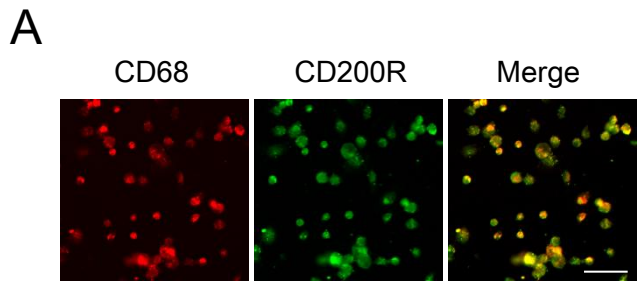
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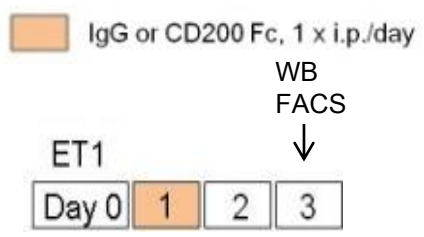


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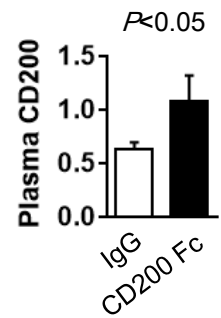
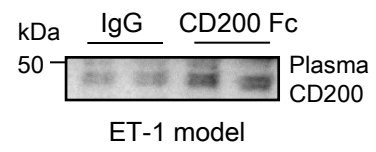


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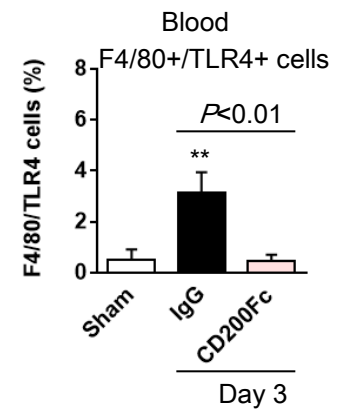
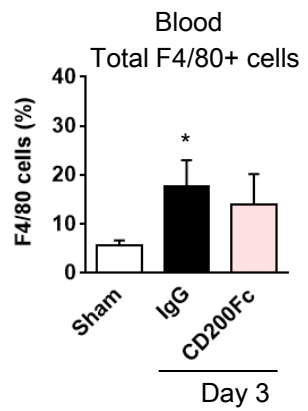
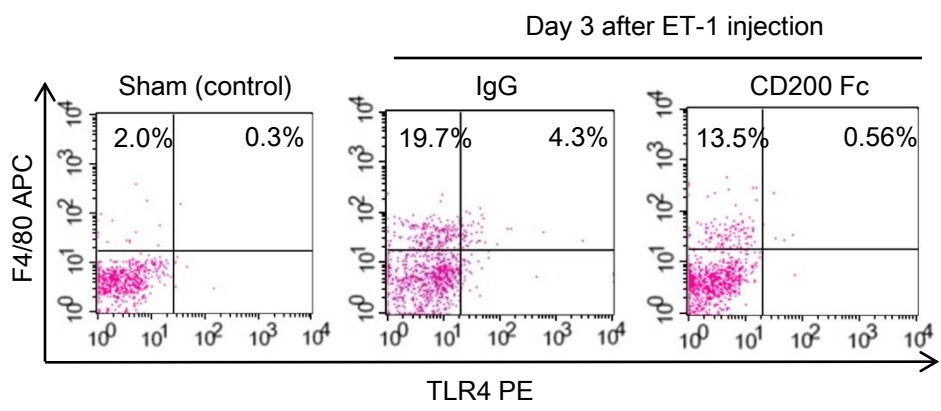
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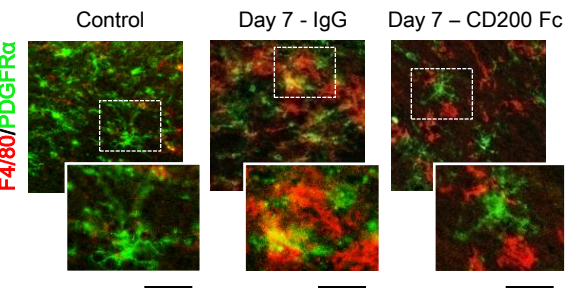


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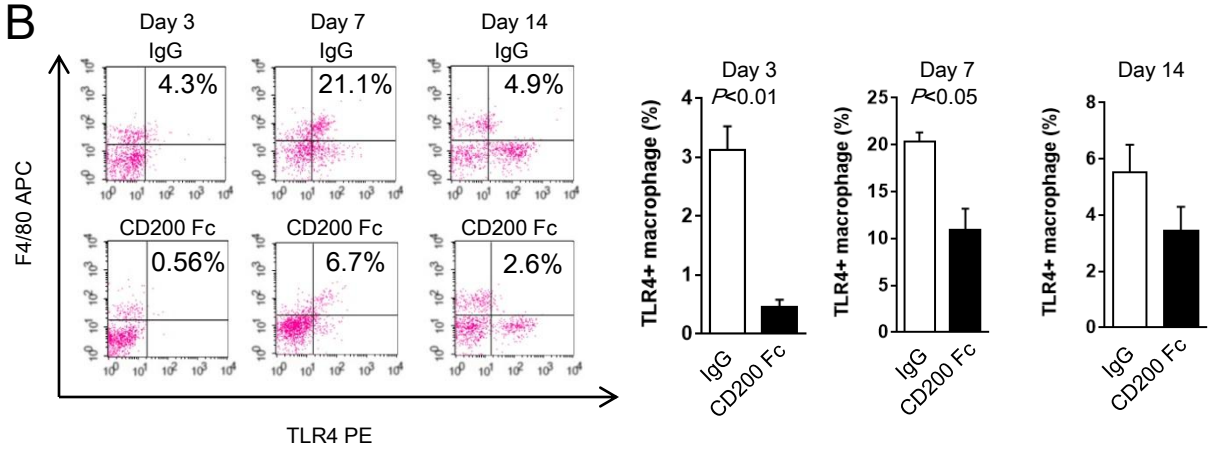


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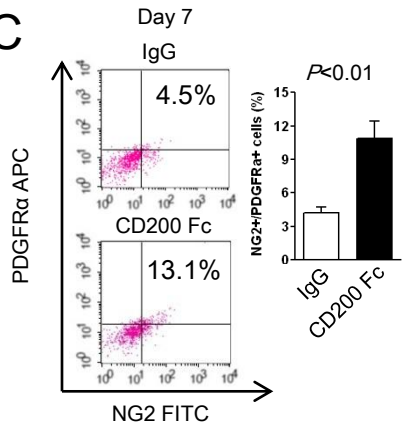
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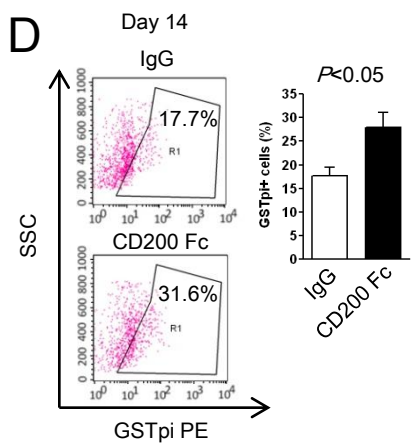
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