CSF1R inhibition by a small molecule inhibitor affects hematopoiesis and the function of macrophages.

Fengyang Lei MD, PhD,[1,2,3] Naiwen Cui, PhD,[4] Chengxin Zhou PhD,[1,2,3] James Chodosh, MD, MPH,[1,2,3] Demetrios G. Vavvas MD, PhD,[1,5] Eleftherios I. Paschalis MSc, PhD,[1,2,3]

[1]Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, MA
[2]Boston Keratoprosthesis Laboratory, Harvard Medical School, Boston, MA
[3]Disruptive Technology Laboratory, Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, MA
[4]John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA
[5]Angiogenesis Laboratory, Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, MA

Corresponding author and reprints: Eleftherios I. Paschalis, Department of Ophthalmology, Boston Keratoprosthesis Laboratory, Massachusetts Eye and Ear and Schepens Eye Research Institute, Harvard Medical School, Boston, MA 02114, USA;
e-mail: eleftherios_paschalis@meei.harvard.edu

Conflict of Interest: The authors have declared that no conflict of interest exists.
Key Words: Eye; neuroglia, microglia; macrophage; remodeling, CSF1R.
Running title: Long-term effect of CSF1R inhibition on hematopoiesis.

Abstract
Colony-stimulating factor 1 receptor (CSF1R) inhibition has been proposed to specifically deplete microglia without affecting peripheral immune cells. Here, we show that CSF1R inhibition by PLX5622 causes changes in the myeloid and lymphoid compartments and long-term functional impairment of bone marrow-derived macrophages by suppressing their IL-1β expression, phagocytosis, and M1, but not M2 phenotype. Thus, CSF1R inhibition with small molecule inhibitors is not restricted to microglia only, but rather causes strong effects on peripheral macrophages that perdure long after cessation of the treatment. These changes may have significant implications on the interpretation of relevant experimental data.

\body

Colony stimulating factor 1 receptor (CSF1R) inhibition has been proposed as a specific microglia depletion method(1-3) that does not affect peripheral immune cells(2, 4-6). However, this assumption has been based solely on cell count measurements of blood monocytes and evaluation of blood brain barrier rather than direct assessment of cellular subtypes and their function. Given that peripheral monocytes have been shown to participate in CNS disease via both infiltration and repopulation of neuroglia following microglia depletion(6-12), it is important to determine if CSF1R inhibition depletes solely microglia or it also affects the function of peripheral immune cells(3, 5, 6, 12-15).

Here we show that, contrary to the accepted notion(2, 3), PLX5622, a commonly used small molecule CSF1R inhibitor for microglia depletion(3, 10, 12), does not affect only microglia but also leads to long-term changes in the myeloid and lymphoid compartments of the bone marrow by suppressing $CCR2^+$ monocyte progenitor cells, $CX3CR1^+$ bone marrow-derived macrophages (BMDM), $CD117^+$ (C-$KIT^{+)}$ hematopoietic progenitor cells, and $CD34^+$ hematopoietic stem cells **(fig. 1 A-G)**. Most importantly, these cell populations either do not recover or rebound after cessation of CSF1R inhibition, with the exception of $CD45^+$ $CD11b^+$ cells which remain unaffected **(fig. 1 A-G)**. Besides the effects on the myeloid compartment, CSF1R inhibition also alters the lymphoid compartment of the bone marrow by suppressing T-cells ($CD3^+$, $CD4^+$, and $CD8^+$), **(fig. 1 G)** and by upregulating $CD19^+$ B cells **(fig. 1 G)**. Cessation of CSF1R inhibition causes rebound of some, but not all, lymphoid cells (**fig. 1 G)**.

In addition to the effects on bone marrow cells, CSF1R inhibition also suppresses splenic $CX3CR1^+$ cells and this inhibition persist for at least 3 weeks after cessation of treatment **(fig. 1 H)**. Moreover, splenic $CD3^+$ T cells (primarily $CD8^+$) become suppressed, whereas splenic $CD19^+$ B cells are not affected by the inhibitor **(fig. 1 H)**.

CSF1R inhibition also suppresses the proliferation of bone marrow and spleen macrophages **(fig. 2 A-D)**, and impairs the function of bone marrow-derived macrophages (BMDM) for the long-term. In fact, 3 weeks after cessation of CSF1R inhibition, BMDMs

display reduced IL-1β expression in response to endotoxin **(fig. 2 E, F)**, diminished phagocytic capacity **(fig. 2 G)** and suppressed M1-, but not M2- like phenotype **(fig. 2 H-K)**. This latter finding is consistent with a previous study showing that recombinant CSF1-Fc protein induces M1, but not M2-like phenotype on macrophages(16, 17).

Previous studies have suggested that microglia depletion by CSF1R inhibition can either promote or exacerbate neurodegeneration(6, 12, 18). This puzzling and contradictory results may not only be due to the differing role of microglia in various disease models but also due to the varying relative contribution of peripheral macrophages on disease phenotype. Our study demonstrates that peripheral macrophages are also affected by CSF1R inhibition which may explain in part the reason for the discrepancy in recent investigations.

In conclusion, this study provides compelling evidence that CSF1R inhibition by PLX5622 does not simply deplete microglial cells, but also affects the myeloid and lymphoid compartments and causes functional impairment in BMDMs. Considering that BMDMs permanently engraft into diseased CNS tissue(10, 12), this work suggests that microglia depletion studies with small molecule inhibitors are not specific to microglia but additionally affect the number and function of peripheral macrophages. These effects perdure long after cessation of the treatment and has implications in the interpretation of relevant experimental data.

Materials and Methods

Mouse model: All animal experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the National Institutes of Health (NIH) Guidance for the Care and Use of Laboratory Animals. This study was approved by the Mass. Eye and Ear Animal Care Committee. Mice at 6-12 months old were used: C57BL/6J (Stock#: 000664), B6.129(Cg)-Ccr2tm2.1lfc/J (Stock#: 017586) and B6.129P- Cx3cr1tm1Litt/J (Stock#: 005582) Jackson Laboratory. $CCR2^{RFP/+}$::$CX3CR1^{EGFP/+}$ generated by crossing B6.129(Cg)-Ccr2tm2.1lfc/J with B6.129P- Cx3cr1tm1Litt/J. $CX3CR1^{EGFP/+}$ generated by crossing male B6.129P- Cx3cr1tm1Litt/J with female C57BL/6J. Mice were bred in house. Microglia depletion was performed by chow administration for 3 weeks of PLX5622 (Plexxikon Inc., Berkeley, CA). Flow cytometry, and *ex vivo* BMDM evaluation was performed as previously described(10, 12).

Flow cytometry markers: Bone marrow and spleen cells from $CX3CR1^{+/GFP}$ and $CX3CR1^{+/EGFP}$::$CCR2^{+/RFP}$ reporter mice were blocked with CD16/32 (Clone: 2.4G2), analyzed with IL-1β (Clone: NJTEN3) eBiosciences (San Diego, CA); CD45 (Clone: 104), CD11b (Clone: M1/70), CD11c (Clone: N418), CD3 (Clone: 17A2), CD4 (Clone: GK1.5), CD8 (Clone: 53-5.8), CD19 (Clone: 6D5), CD117 (Clone:2B8), CD34 (Clone: HM34), CD115 (Clone: AFS98), CD68 (Clone: FA-11), CD206 (Clone: C068C2), CCR2(Clone: SA203G11), BrdU(Clone: Bu20a)

BioLegend (San Diego, CA). Intracellular staining was performed by fixing cells in Paraformaldehyde-based Fixation buffer (BioLegend) followed by permeabilization with Perm/ Wash buffer (BioLegend). Cells were analyzed on a BD LSR II cytometer (BD Biosciences, San Jose, CA, USA) using FlowJo software (Tree Star, Ashland, OR, USA).

LPS stimulation assay: BMDM were primed with 150U/mL interferon-γ (IFNγ) for 6 hours followed by LPS at final concentration of 10ng/mL (Sigma-Aldrich, St. Louis, MO) added into culture medium for 20 hours. Brefeldin A 5μg/mL (BD Pharmingen, Bedford, MA) was added 4 hours before cell harvest and flow cytometry.

Phagocytosis Assay: The pHrodo™ Red BioParticles® Conjugates for Phargocytosis (P35364) (Molecular Probes, Eugene, OR) kit was used. Six days after cell plating with mCSF, and one day prior to the assay, BMDMs were recovered from culture and seeded. Cells were stimulated with IFNγ for 4 hours and then culture medium was replaced with reconstituted red Zymosan A BioParticles. Cells were incubated at 37°C for 2 hours, trypsinized, and evaluated with flow cytometry.

Statistical analysis: Data were analyzed with GraphPad (Prism 2.8.1, San Diego, CA) using two-tailed unpaired t-test and ordinary one-way ANOVA with Dunnet's correction for multiple comparisons. Statistical significance was determined at $P < 0.05$.

Author contributions: FL designed experiments, acquired data and analyzed data; NC, CZ analyzed data; DGV wrote and reviewed the manuscript; JC reviewed the manuscript; EIP designed experiments, analyzed data, and wrote the manuscript.

Acknowledgments: This work was supported by the Boston Keratoprosthesis Research Fund, Massachusetts Eye and Ear, the Eleanor and Miles Shore Fund, the Massachusetts Lions Eye Research Fund, an unrestricted grant to the Department of Ophthalmology, Harvard Medical School, from Research to Prevent Blindness, NY, NY, NIH National Eye Institute core grant P30EY003790; Yeatts Family Foundation; Monte J Wallace Chair; Macula Society Research Grant award; an RPB Physician Scientist Award; NEI R21EY023079; NEI grant EY014104 (MEEI Core Grant); Loeffler Family fund; R01EY025362-01; ARI Young investigator Award. PLX5622 was kindly provided by Plexxikon Inc.

Figure Legends

Fig. 1. CSF1R inhibition by PLX5622 affects the myeloid and lymphoid compartments of the bone marrow and spleen.

Flow cytometric analysis of bone marrow cells isolated from $CCR2^{+/RFP}$::$CX3CR1^{+/GFP}$ mice

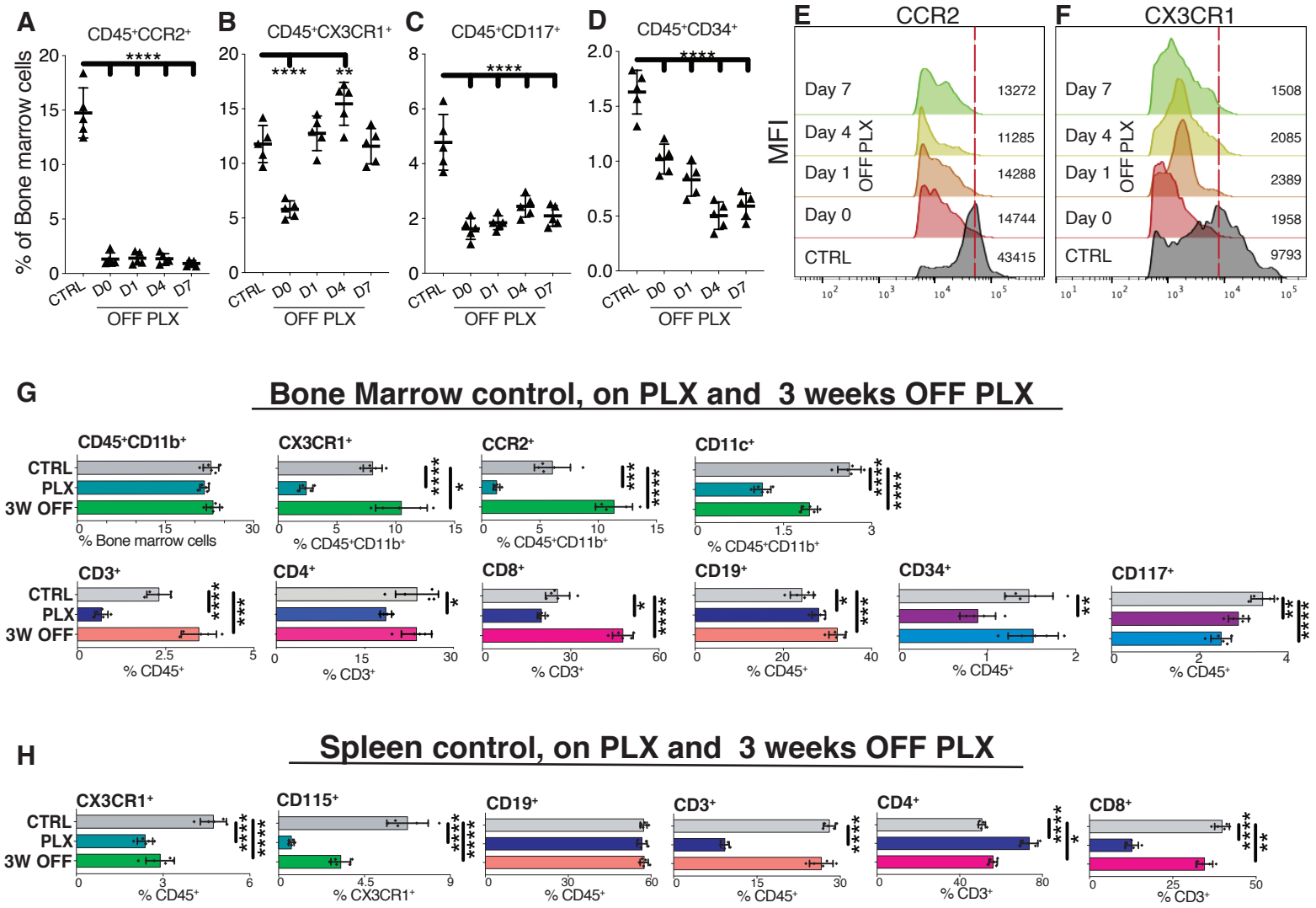
immediately, after 3-week treatment with CSF1R inhibitor (PLX5622), and at different timepoints after cessation of the CSF1R inhibitor. **(A - F)** CSF1R inhibition suppresses CCR2$^+$, CX3CR1$^+$, CD117$^+$, and CD34$^+$ cells. One week after cessation of CSF1R inhibitor, only macrophages recover in number, although with a lower expression of CX3CR1. **(G)** CSF1R inhibition does not affect CD45$^+$, CD11b$^+$ and Ly6C$^+$ bone marrow myeloid cell populations, but does suppress CD11c$^+$ dendritic cells, CD4$^+$ and CD8$^+$ T lymphocytes, as well as, CD115$^+$, CD117$^+$ and CD34$^+$ hematopoietic subsets, and upregulates CD19$^+$ B cells. Three weeks after cessation of CSF1R inhibition, CX3CR1$^+$, CCR2$^+$, Ly6C$^+$ CD3$^+$ and CD8$^+$ sub-populations rebound; Ly6G$^+$ granulocytes, CD115$^+$, and CD117$^+$ cells remain suppressed; CD4$^+$ T cells and CD34$^+$ cells recover; and CD19$^+$ B cells remain upregulated. **(H)** Effects of CSF1R inhibition on the spleen's myeloid and lymphoid populations. Only CD19$^+$ B cells remain unaffected. n=5 per group, mean±SD, One-way analysis of variance with Dunnett's correction for multiple comparisons, * *$P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.*

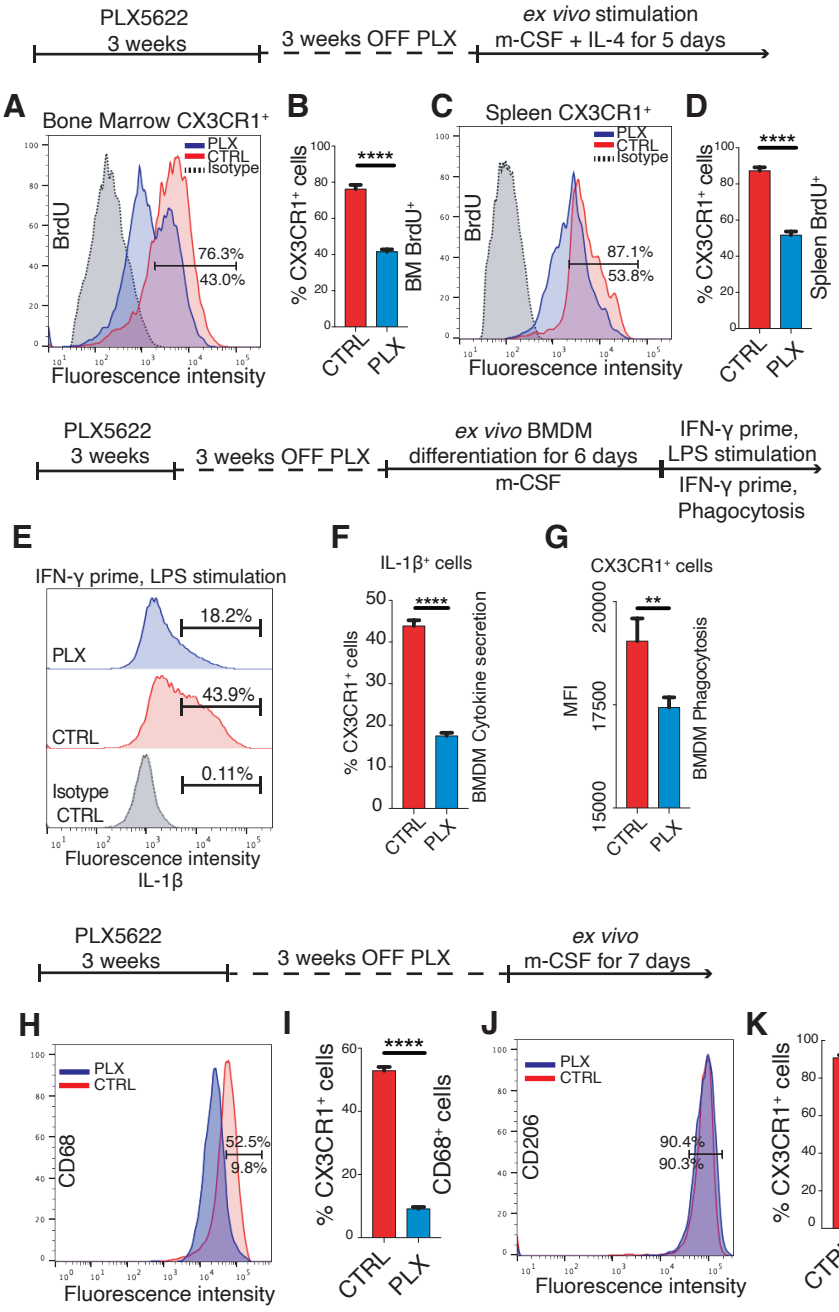
Fig. 2. CSF1R inhibition by PLX5622 affects the function of peripheral macrophages. **(A-D)** *ex vivo* evaluation of the function of bone marrow-derived macrophage (BMDM) from CX3CR1$^{+/GFP}$ mice 3 weeks after cessation of CSF1R inhibitor. Macrophages from the bone marrow or spleen exhibit reduced proliferation 3 weeks after cessation of CSF1R inhibition. **(E-G)** CSF1R inhibition suppresses IL-1β expression and phagocytosis of bone marrow-derived macrophage following exposure to lipopolysaccharide. **(H-K)** These changes are associated with suppression of CD68$^+$ M1-like but not CD206$^+$ M2-like phenotype. n=5 per group, mean±SD, Independent t-test, ** *$P<0.01$, **** $P<0.0001$.*

Bibliography

1. B. Erblich, L. Zhu, A. M. Etgen, K. Dobrenis, J. W. Pollard, Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits. *PLoS ONE* **6**, e26317 (2011).
2. M. R. P. Elmore *et al.*, Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron* **82**, 380-397 (2014).
3. N. N. Dagher *et al.*, Colony-stimulating factor 1 receptor inhibition prevents microglial plaque association and improves cognition in 3xTg-AD mice. *Journal of neuroinflammation* **12**, 139 (2015).
4. S. Mok *et al.*, Inhibition of CSF-1 Receptor Improves the Antitumor Efficacy of Adoptive Cell Transfer Immunotherapy. *Cancer research* **74**, 153-161 (2014).
5. A. M. Hilla, H. Diekmann, D. Fischer, Microglia Are Irrelevant for Neuronal Degeneration and Axon Regeneration after Acute Injury. *Journal of Neuroscience* **37**, 6113-6124 (2017).
6. V. Bellver-Landete *et al.*, Microglia are an essential component of the neuroprotective scar that forms after spinal cord injury. *Nature communications* **10**, 518 (2019).
7. T. A. Evans *et al.*, High-resolution intravital imaging reveals that blood-derived macrophages but not resident microglia facilitate secondary axonal dieback in traumatic spinal cord injury. *Experimental neurology* **254**, 109-120 (2014).
8. T. A. Wynn, K. M. Vannella, Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity* **44**, 450-462 (2016).
9. S. J. Karlen *et al.*, Monocyte infiltration rather than microglia proliferation dominates the early immune response to rapid photoreceptor degeneration. *Journal of neuroinflammation* **15**, a020545-020516 (2018).
10. E. I. Paschalis *et al.*, Permanent neuroglial remodeling of the retina following infiltration of CSF1R inhibition-resistant peripheral monocytes. *Proceedings of the National Academy of Sciences of the United States of America* **115**, E11359-E11368 (2018).
11. E. I. Paschalis *et al.*, The Role of Microglia and Peripheral Monocytes in Retinal Damage after Corneal Chemical Injury. *The American journal of pathology* **188**, 1580-1596 (2018).
12. E. I. Paschalis *et al.*, Microglia Regulate Neuroglia Remodeling in Various Ocular and Retinal Injuries. *Journal of immunology (Baltimore, Md. : 1950)* **202**, 539-549 (2019).
13. D. Kokona, A. Ebneter, P. Escher, M. S. Zinkernagel, Colony-stimulating factor 1 receptor inhibition prevents disruption of the blood-retina barrier during chronic inflammation. *J Neuroinflammation* **15**, 340 (2018).
14. S. K. Halder, R. Milner, A critical role for microglia in maintaining vascular integrity in the hypoxic spinal cord. *Proc Natl Acad Sci U S A* **116**, 26029-26037 (2019).
15. J. M. Hutchinson, L. G. Isaacson, Elimination of microglia in mouse spinal cord alters the retrograde CNS plasticity observed following peripheral axon injury. *Brain Res* **1721**, 146328 (2019).
16. D. J. Gow *et al.*, Characterisation of a novel Fc conjugate of macrophage colony-stimulating factor. *Mol Ther* **22**, 1580-1592 (2014).
17. J. G. van den Boorn, G. Hartmann, Therapeutic Tissue Regeneration by a Macrophage Colony-Stimulating Factor Fc Conjugate. *Molecular Therapy* **22**, 1577-1579 (2014).

18. Y. Okunuki *et al.*, Retinal microglia initiate neuroinflammation in ocular autoimmunity. *Proceedings of the National Academy of Sciences* **156**, 201820387-201820310 (2019).


A
CD45+CCR2+
% of Bone marrow cells
CTRL D0 D1 D4 D7
OFF PLX
B
CD45+CX3CR1+
C
CD45+CD117+
D
CD45+CD34+
E
CCR2
F
CX3CR1
MFI
Day 7
Day 4
Day 1
Day 0
CTRL
OFF PLX
13272
11285
14288
14744
43415
1508
2085
2389
1958
9793
G
Bone Marrow control, on PLX and 3 weeks OFF PLX
CD45+CD11b+
CX3CR1+
CCR2+
CD11c+
CTRL
PLX
3W OFF
% Bone marrow cells
% CD45+CD11b+
CD3+
CD4+
CD8+
CD19+
CD34+
CD117+
% CD45+
% CD3+
H
Spleen control, on PLX and 3 weeks OFF PLX
CX3CR1+
CD115+
CD19+
CD3+
CD4+
CD8+
% CD45+
% CX3CR1+
% CD3+


PLX5622
3 weeks
3 weeks OFF PLX
ex vivo stimulation
m-CSF + IL-4 for 5 days
A
Bone Marrow CX3CR1+
PLX
CTRL
Isotype
BrdU
76.3%
43.0%
Fluorescence intensity
B
% CX3CR1+ cells
****
BM BrdU+
CTRL
PLX
C
Spleen CX3CR1+
PLX
CTRL
Isotype
BrdU
87.1%
53.8%
Fluorescence intensity
D
% CX3CR1+ cells
****
Spleen BrdU+
CTRL
PLX
PLX5622
3 weeks
3 weeks OFF PLX
ex vivo BMDM
differentiation for 6 days
m-CSF
IFN-γ prime,
LPS stimulation
IFN-γ prime,
Phagocytosis
E
IFN-γ prime, LPS stimulation
PLX
18.2%
CTRL
43.9%
Isotype
CTRL
0.11%
Fluorescence intensity
IL-1β
F
IL-1β+ cells
****
% CX3CR1+ cells
BMDM Cytokine secretion
CTRL
PLX
G
CX3CR1+ cells
**
MFI
BMDM Phagocytosis
CTRL
PLX
PLX5622
3 weeks
3 weeks OFF PLX
ex vivo
m-CSF for 7 days
H
PLX
CTRL
CD68
52.5%
9.8%
Fluorescence intensity
I
% CX3CR1+ cells
****
CD68+ cells
CTRL
PLX
J
PLX
CTRL
CD206
90.4%
90.3%
Fluorescence intensity
K
% CX3CR1+ cells
CD206+ cells
CTRL
PLX