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

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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.

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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 argument has been solely based 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 are known to participate in CNS disease(6-12) and in the repopulation of microglia following their depletion,(10, 12) it is important to definitively determine if CSF1R inhibition 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) leads to long-term changes in the myeloid and lymphoid compartments of the bone marrow and spleen by suppressing CCR2^{+} monocyte progenitor cells, CX3CR1^{+} bone marrow-derived macrophages (BMDM), CD117^{+} (C-KIT$^{+)}$ hematopoietic progenitor cells, and CD34^{+} hematopoietic stem cells, and Ly6G neutrophils **(fig. 1 A-G)**. Most importantly, these cell populations do not recover or rebound after cessation of CSF1R inhibition, with the exception of CD45^{+} CD11b^{+} cells **(fig. 1 A-G)**.
CSF1R inhibition also suppresses bone marrow-derived lymphoid CD3^{+}, CD4^{+}, and CD8^{+} cells **(fig. 1 G)**, and upregulates CD19^{+} cells **(fig. 1 G)**. Cessation of CSF1R inhibition causes rebound of some but not all lymphoid cells (**fig. 1 G)**.

CSF1R inhibition also affects splenic CX3CR1^{+} cells and these changes persist for at least 3 weeks after cessation of the inhibitor **(fig. 1 H)**. In addition, splenic CD3^{+} T cells (primarily CD8^{+}) are suppressed with CSF1R inhibitor whereas CD19^{+} B cells are not affected **(fig. 1 H)**.

CSF1R inhibition also suppresses the proliferation of bone marrow and spleen macrophages **(fig. 2 A-D)**, and impairs the inflammatory response of BMDMs to endotoxin by suppressing IL-1β expression **(fig. 2 E, F)**, reducing phagocytic capacity **(fig. 2 G)** and suppressing M1-like but not M2- like phenotype **(fig. 2 H-K)**. The latter finding is consistent

with a previous study that showed recombinant CSF1-Fc protein to induce M1-like macrophage phenotype, but not M2(16, 17).

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 the role of peripheral monocytes in CNS disease,(9, 10) 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, and these effects perdure long after cessation of the treatment and may have significant implications on 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), Ly6C (Clone: HK1.4), Ly6G (Clone:1A8), 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*.

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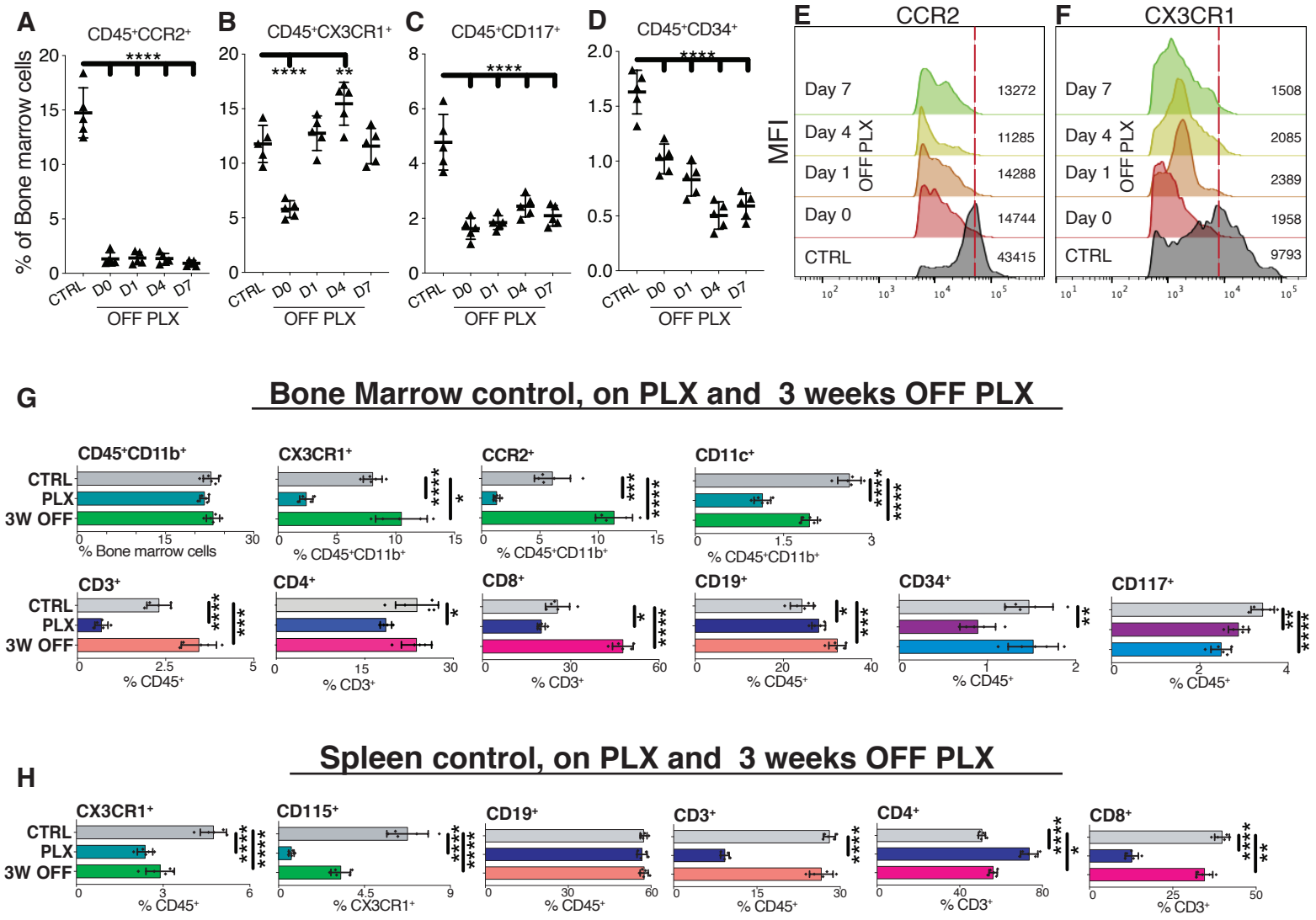
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9793
G
Bone Marrow control, on PLX and 3 weeks OFF PLX
CD45+CD11b+
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CD19+
CD34+
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Spleen control, on PLX and 3 weeks OFF PLX
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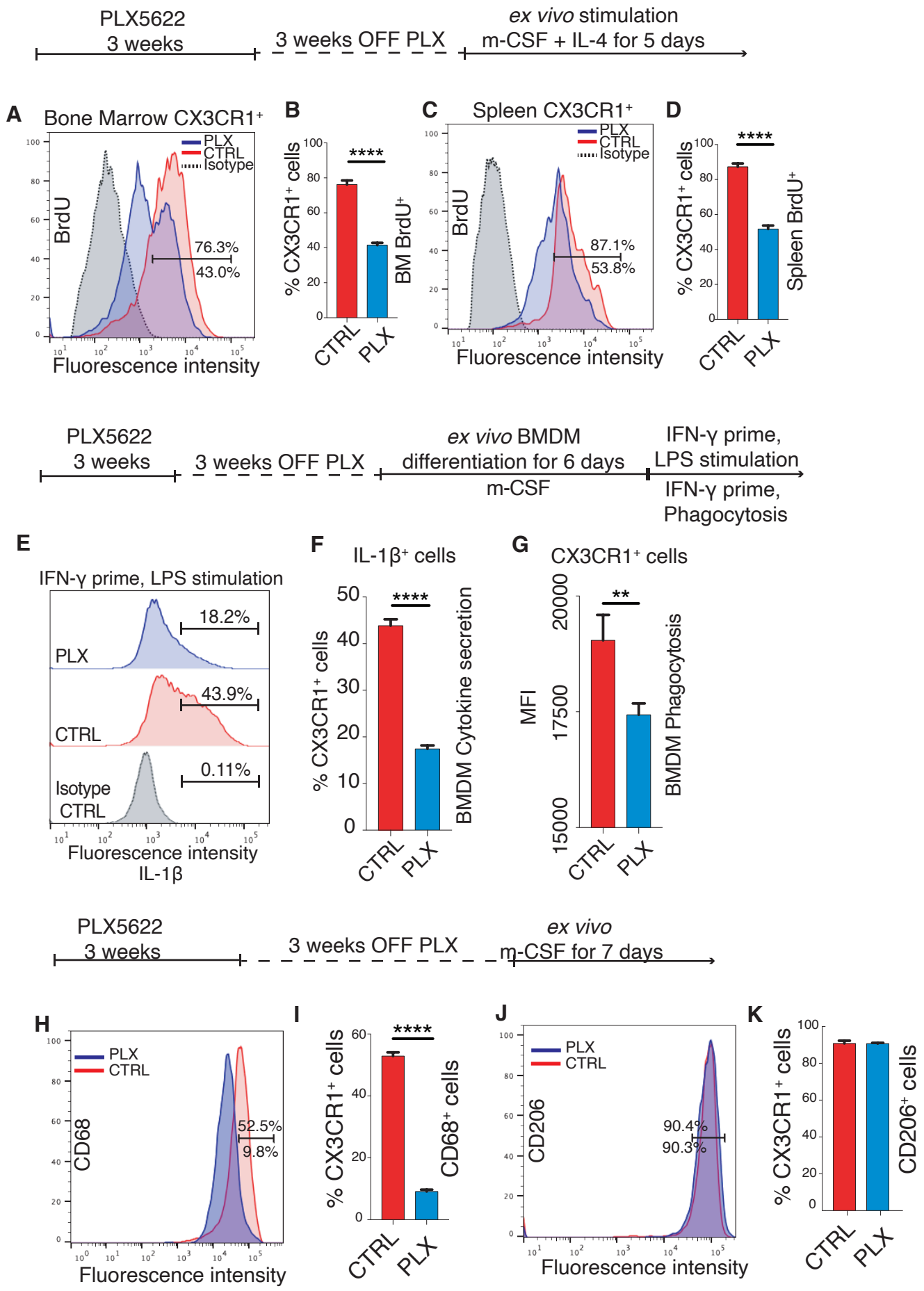
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m-CSF
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CTRL
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CX3CR1+ cells
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BMDM Phagocytosis
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