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

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Abstract
Previous studies have suggested that microglia depletion using small molecule colony-stimulating factor 1 receptor (CSF1R) inhibitor, does not affect peripheral immune cells, and, therefore, disease phenotype changes are attributed to microglia depletion only. Here, we show that CSF1R inhibition by PLX5622 causes long-term changes in the myeloid and lymphoid compartments, impairs the inflammatory and phagocytic response of bone marrow-derived macrophages (BMDM), and suppresses their M1 but not M2 phenotype. These effects are associated with long-term changes in *Il-10, CD14, Ncoa6* gene expression, activation of epigenetic reprogramming marker histone deacetylase 9 (*Hdac9*), and changes in transcription factors *Pax5, Mek, Erk1/2*, and *Notch1*. Future microglia depletion studies with small molecule inhibitor need to consider these changes in the interpretation of the results.

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Colony stimulating factor 1 (CSF1R) inhibition has been proposed as a specific microglia depletion method (1-3) that does not affect peripheral immune cells or CNS disease phenotype.(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 the function of bone marrow-derived macrophages (BMDM). Given that peripheral monocytes were recently shown to participate in CNS disease by permanently engrafting into the tissue,(6-12) and that bone marrow-derived macrophages participate in microglia repopulation in the retina after microglia depletion,(10, 12) possible changes in their function by CSF1R manipulations could greatly affect experimental outcomes, and might explain the growing discrepancy in recent published literature, showing that microglia depletion can be detrimental, protective, or irrelevant to neuronal survival in various CNS pathologies.(3, 5, 6, 12-15)
Here we show that, contrary to the accepted notion,(2, 3) PLX5622, a common small molecule CSF1R inhibitor for microglia depletion,(3, 10, 12) leads to long-term changes in hematopoiesis, affecting the myeloid and lymphoid compartments of the bone marrow and spleen by suppressing the $CD34^+$ hematopoietic stem cells, $CD117^+$ (C-$KIT^{+)}$ hematopoietic progenitor cells, $CCR2^+$ monocyte progenitor cells, and $CX3CR1^+$ BMDMs **(fig. 1 A-F)**. Most importantly, these cell populations do not recover even after 7 days of cessation of CSF1R inhibition, with the exception of $CD45^+$ $CX3CR1^+$ macrophages, which remain altered in that they exhibit low expression of endogenous CX3CR1**(fig. 1 A-F)**. In the bone marrow, CSF1R inhibition does not affect the overall $CD45^+$ $CD11b^+$ myeloid cell counts but leads to suppression of $CX3CR1^+$ macrophages, $CCR2^+$ progenitor monocytes, and $CD11c^+$ dendritic cells, without affecting the $Ly6c^+$ and $Ly6G^+$ populations **(fig. 1G)**. Three weeks after cessation of CSF1R inhibition, myeloid cells rebound, with subsequent increase in $CX3CR1^+$, $CCR2^+$, and $Ly6c^+$, and a reduction in $Ly6G^+$ cell populations as compared to baseline levels **(fig. 1 G)**. However, $CD11c^+$ cell numbers remain suppressed **(fig. 1 G)**. In addition, CSF1R inhibition suppresses $CD4^+$ and $CD8^+$ expression by bone marrow cells **(fig. 1 G)**, and upregulates $CD19^+$ expression **(fig. 1 G)**.

Three weeks after cessation of CSF1R inhibition, lymphoid $CD3^+$ cells rebound above normal expression levels (**fig. 1 G)**.

CSF1R inhibition also suppresses $CX3CR1^+$ myeloid cells in the spleen and their CSF1R expression. These changes persist even 3 weeks after cessation of the inhibitor **(fig. 1 H)**. In contrast, CSF1R inhibition does not affect the frequency of splenic $CD19^+$ B cells **(fig. 1 H)** but suppresses both $CD4^+$ and $CD8^+$ T cells. Three weeks after cessation of CSF1R inhibition, the $CD8^+$ T cells in the spleen remain suppressed, however, the total number of $CD3^+$ lymphocytes returns to normal **(fig. 1 H)**.

CSF1R inhibition not only alters the number of myeloid and lymphoid cells, but also the function of macrophages. CSF1R inhibition causes bone marrow and spleen macrophages to exhibit reduced proliferation **(fig. 1 L-O)**, while bone marrow derived macrophages (BMDMs) display impaired inflammatory response to endotoxin, with low IL-1β expression and reduced phagocytic capacity **(fig. 1 I-K)**. Impairments in the inflammatory response were associated with selective suppression M1-like phenotype as indicated by CD68 suppression without affecting the M2-like phenotype CD206 expression **(fig. 1 P-S)**.

CSF1R inhibition leads to immediate and long-term changes in the expression of common hematopoietic genes **(fig. 2 A, B)**, with *Il-10* being upregulated up to one month after cessation of CSF1R inhibition. *Il-10* is a repressor of cytokine secretion, antigen presentation, and $CD4^+$ T cell activation(16) and promotes inhibition of lipopolyscacharide (LPS)-mediated TNF-α, IL-1β, IL-12 and IFNγ expression by macrophages.(17, 18) Two months after cessation of CSF1R inhibitor, hematopoietic genes *Ncoa6* and *Cd14* remain down-regulated **(fig. 2 A, B)**. *Ncoa6* is a non-redundant transcriptional regulator and co-activation of *c-Fos*, *c-Jun*, *Creb*, *Nf-kb*, *Atf-2*, heat shock factors, *E2f-1*, *Srf*, *Rb*, *p53* and *Stat2*,(19) and its suppression leads to defective wound healing(20) and lethality by apoptosis.(20) *Cd14*, on the other hand, facilitates endocytosis of the TLR4 complex and mediates LPS-induced macrophage activation.(21) These genetic changes corroborate the functional changes in BMDMs that occur after CSF1R inhibition.

Using principal component analysis (PCA) we confirmed that *Il-10* and *Ncoa6*, among other genes **(fig. 2 E)**, participate in transcriptional heterogeneity of bone marrow cells immediately, 1, and 2 months after cessation of CSF1R inhibition **(fig. 2 C)**. Using ingenuity pathway analysis (IPA) we confirmed that CSF1R inhibition is implicated in the control of epigenetic marker *Hdac9* **(fig. 2 D)**, and transcription factors *Pax5*, *Mek*, *Erk1/2*, and *Notch1* **(fig. 2 F-H)**, all of which involved in the regulation of immune response, lymphoid and dendritic cell maturation, neuroinflammation, and activation of the liver/retinoid X receptor (LXR/RXR), **(fig. 2 I)**. Upregulation of *Hdac9* prevents chromatin expansion and genetic transcription of inflammatory genes following exposure to LPS,(22-24) which can explain the observed suppression in the inflammatory response of BMDMS in this study. Comparative IPA showed that these changes are likely to promote disease phenotypes associated with organ death, disturbances in the hematopoiesis of mononuclear leukocytes, lymphopoiesis, T cell development, and recruitment of phagocytes **(fig. 2 G)**. Upstream analysis identified LPS, poly rI:C-RNA, CSF2, napabucasin,

HIF1A, TNF as major regulators affecting hematopoiesis, lymphopoiesis, and T cell development, consistent with the flow-cytometric analyses performed in this study.
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. These changes are not transient. Rather, they persist long after cassation of CSF1R inhibitor. Considering the role of peripheral monocytes in CNS disease,(9, 10) this work suggests that future microglia depletion studies need to consider the effect of CSF1R inhibition on peripheral immune cells.

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, 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: BMDMs 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, JC reviewed the manuscript; EIP designed experiments, analyzed data, and wrote the manuscript.

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Figure Legends

Fig. 1. CSF1R inhibition affects hematopoiesis and the function of peripheral macrophages. 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 cessations 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 in the spleen's myeloid and lymphoid populations. Only $CD19^+$ B cells remain unaffected. **(I-K)** *ex vivo* evaluation of the function of bone marrow-derived macrophage (BMDM) from $CX3CR1^{+/GFP}$ mice 3 weeks after cessation of CSF1R inhibitor. CSF1R inhibition suppresses IL-1β expression and phagocytosis. **(L-O)** Macrophages from bone marrow or spleen exhibit reduced proliferation 3 weeks after cessation of CSF1R inhibition. **(P-S)** These changes are associated with suppression of $CD68^+$ M1-like phenotype but not $CD206^+$ M2. n=5 per group, mean±SD, Independent t-test or one-way analysis of variance with Dunnett's correction for

multiple comparisons - Group comparison with controls (CTRL), * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Fig. 2. CSF1R inhibition leads to immediate and long term changes in the expression of common hematopoietic genes and affects major epigenetic and developmental markers. **(A)** Heat map of common hematopoietic transcripts in bone marrow cells of C57BL/6J mice immediately, 1 and 2 months after cessation of CSF1R inhibition with PLX5622. **(B)** Scatter-plot shows genes *Il-10, Ncoa6,* and *Cd14* being significantly differentially expressed. **(C)** Principal component analysis (PCA) shows heterogeneous transcriptional profile between the groups. **(E)** Table of genes contributing to PCA separation. All genes involved in the 3-dimensional PCA separation are included. **(A-C)** Normalized gene expression against *Il11* (stable gene) using (2^ (- Delta CT)) in logarithmic scale. **(D)** Ingenuity pathway analysis (IPA) shows activation of epigenetic marker *Hdac9*. **(F- H)** Cessation of CSF1R inhibitor upregulates *Csf1* and *Csf1r and* changes *Cd8a*, *Spp1*, *Notch2/4*, *Pax5* and *B2m* gene expression. **(I)** Comparative IPA analysis of the effects of CSF1R inhibition on canonical pathways of hematopoiesis (**G)** and disease/function shows association with organism death, hematopoiesis of mononuclear leukocytes, lymphopoiesis, and other developmental abnormalities of the immune system.

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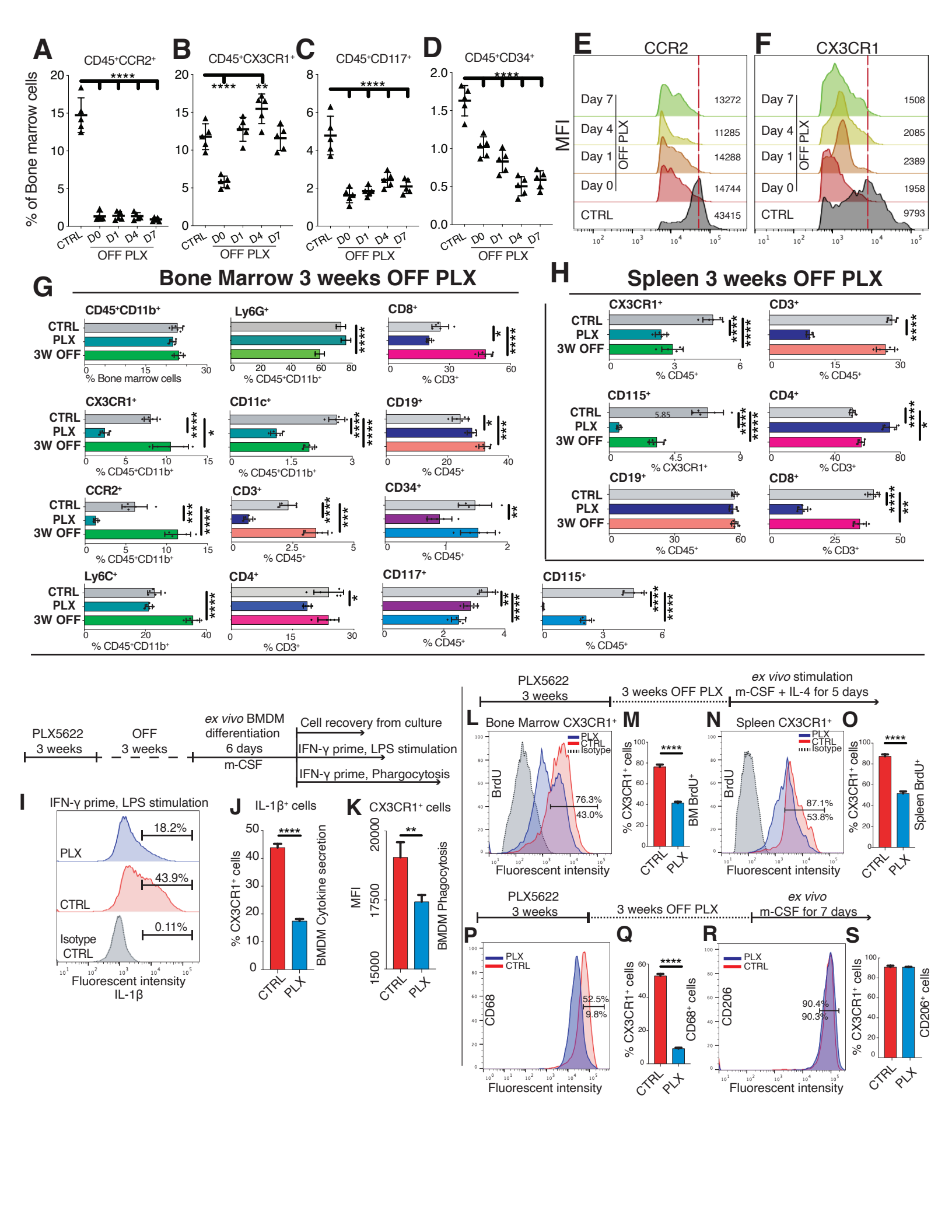
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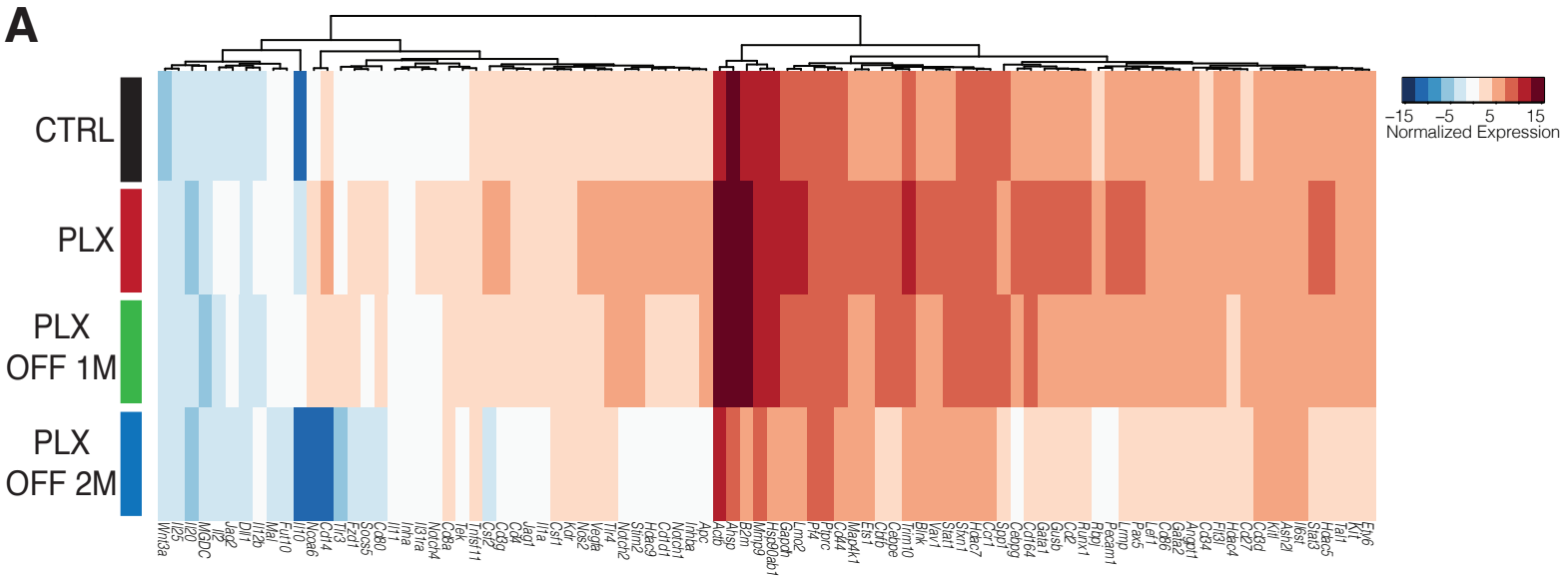

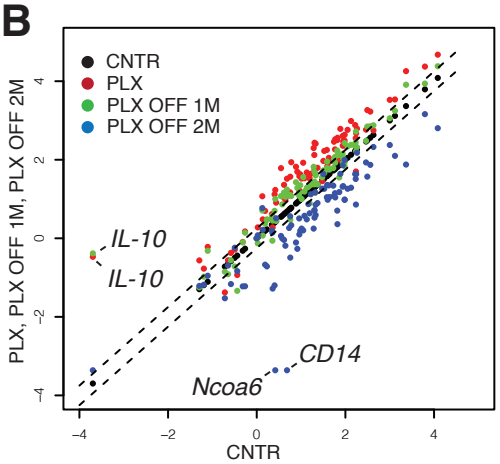

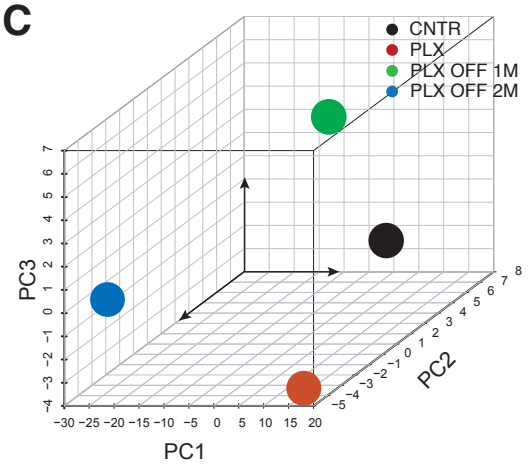

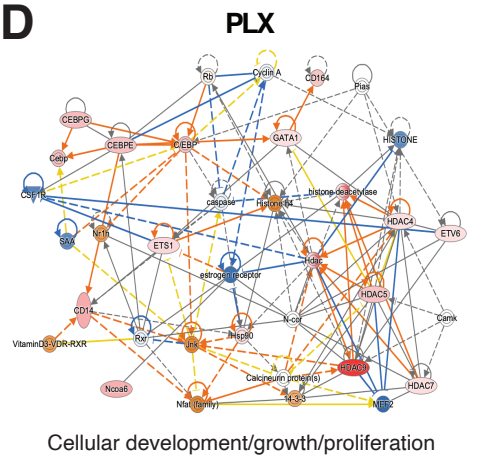

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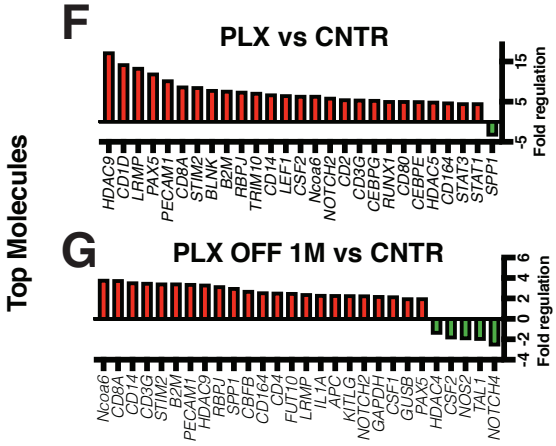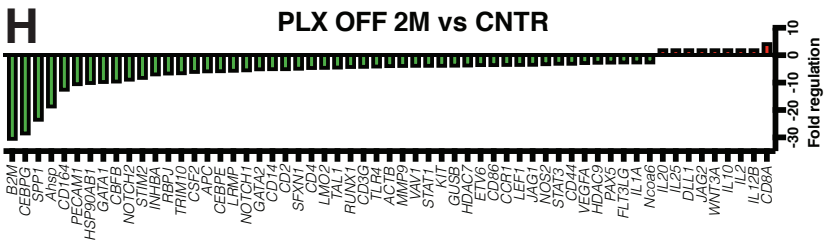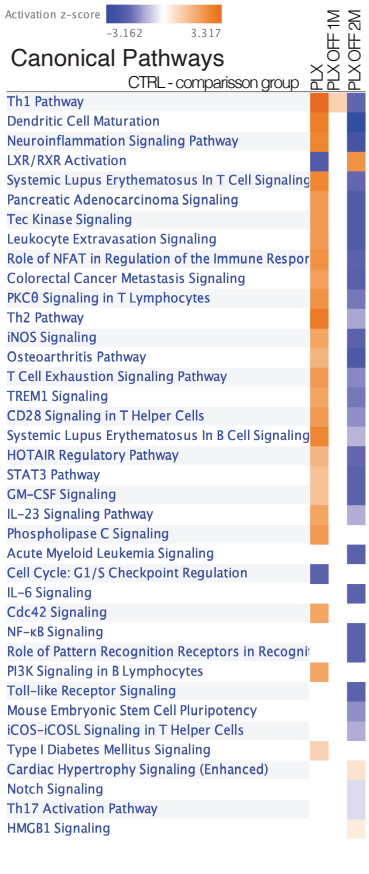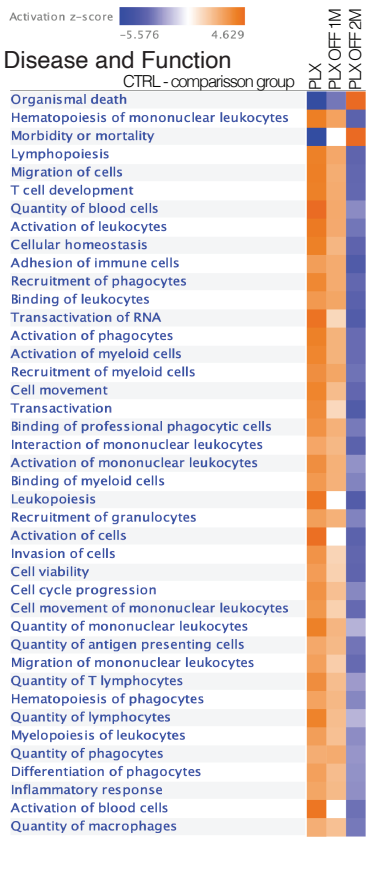
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PC3	**IL10**	Spp1	Jag2	IL20	CD4	Kitl	**Ncoa6**	Pf4	Fut10	IL1a
Downregulated										
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PC2	**IL10**	CD8a	Jag2	Hdac9	CD1d1	Wnt3a	Pax5	Blnk	Lrmp	CD3d
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