An overlooked subset of $\text{Cx3cr1}^{\text{wt}/\text{wt}}$ microglia in the $\text{Cx3cr1}^{\text{CreER-Eyfp}/\text{wt}}$ mouse has a repopulation advantage over $\text{Cx3cr1}^{\text{CreER-Eyfp}/\text{wt}}$ microglia following microglial depletion

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Abstract

Background: Fluorescent reporter labeling and promoter-driven Cre-recombinant technologies have facilitated cellular investigations of physiological and pathological processes, including the widespread use of the $\text{Cx3cr1}^{\text{CreER-Eyfp}/\text{wt}}$ mouse strain for studies of microglia.

Methods: Immunohistochemistry, Flow Cytometry, RNA sequencing and whole-genome sequencing were used to identify the subpopulation of microglia in $\text{Cx3cr1}^{\text{CreER-Eyfp}/\text{wt}}$ mouse brains. Genetically mediated microglia depletion using $\text{Cx3cr1}^{\text{CreER-Eyfp}/\text{wt}}, \text{Rosa26}^{\text{DTA}/\text{wt}}$ mice and CSF1 receptor inhibitor PLX3397 were used to deplete microglia. Primary microglia proliferation and migration assay were used for in vitro studies.

Results: We unexpectedly identified a subpopulation of microglia devoid of genetic modification, exhibiting higher $\text{Cx3cr1}$ and $\text{CX3CR1}$ expression than $\text{Cx3cr1}^{\text{CreER-Eyfp}/\text{wt}}, \text{Cre}^{+}\text{Eyfp}^{+}$ microglia in $\text{Cx3cr1}^{\text{CreER-Eyfp}/\text{wt}}$ mouse brains, thus termed $\text{Cx3cr1}^{\text{high}}, \text{Cre}^{–}\text{Eyfp}^{–}$ microglia. This subpopulation constituted less than 1% of all microglia under homeostatic conditions, but after Cre-driven DTA-mediated microglial depletion, $\text{Cx3cr1}^{\text{high}}, \text{Cre}^{–}\text{Eyfp}^{–}$ microglia escaped depletion and proliferated extensively, eventually occupying one-third of the total microglial pool. We further demonstrated that the $\text{Cx3cr1}^{\text{high}}, \text{Cre}^{–}\text{Eyfp}^{–}$ microglia had lost their genetic heterozygosity and become homozygous for wild-type $\text{Cx3cr1}$. Therefore, $\text{Cx3cr1}^{\text{high}}, \text{Cre}^{–}\text{Eyfp}^{–}$ microglia are $\text{Cx3cr1}^{\text{wt}/\text{wt}}, \text{Cre}^{–}\text{Eyfp}^{–}$. Finally, we demonstrated that CX3CL1–CX3CR1 signaling regulates microglial repopulation both in vivo and in vitro.

Conclusions: Our results raise a cautionary note regarding the use of $\text{Cx3cr1}^{\text{CreER-Eyfp}/\text{wt}}$ mouse strains, particularly when interpreting the results of fate mapping, and microglial depletion and repopulation studies.

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Background

Microglia are derived from the yolk sac during early embryonic development and represent approximately 10% of the healthy adult brain’s total cell population [1, 2]. They play critical roles in maintaining brain development and function [3, 4]. In their homeostatic state, they display a highly ramified morphology, efficiently surveying the central nervous system (CNS) microenvironment, recognizing and clearing cell debris [5]. Microglia play pivotal roles in diverse CNS diseases including neurodevelopmental disorders, neurodegenerative disorders, and high-grade glioma [6–13]. Targeting microglia has thus emerged as an attractive strategy to modulate neuroinflammation in the context of various CNS diseases [14, 15].

The Cx3cr1 gene is mainly expressed in microglia in the CNS parenchyma, and the Cx3cl1/CX3CR1 axis plays a crucial role in microglia–neuron communication [16, 17]. Cx3cr1 is widely used to genetically label microglia by inserting Gfp [18, 19] since this enables microglia tracing, visualization and sorting [1, 20–23]. Cx3cr1Cre/wt and Cx3cr1CreER-Eyfp/wt mouse strains are potent, commonly used tools for studies of microglial fate mapping [1, 24], microglial depletion [25–28] and modification of the microglial genome by leveraging floxed target genes [29–31]. These methods have greatly increased our understanding of microglia in CNS homeostasis and disease conditions.

In this study, we report the existence of an unexpected, small population of Cx3cr1CreER-Eyfp/Cx3cr1CreER-Eyfp− microglia in Cx3cr1CreER-Eyfp/wt mice. After genetically mediated microglial depletion using Cx3cr1CreER-Eyfp/wt Rosa26DTA/wt mice, Cx3cr1CreER-Eyfp− microglia escape depletion, display a repopulation advantage and eventually constitute one-third of the total repopulated microglial pool. We further determined that the Cx3cr1CreER-Eyfp− microglia are Cx3cr1CreER-Eyfp+. Finally, we demonstrate the vital role of Cx3cl1/CX3CR1 signaling in regulating microglial repopulation post-depletion. Not being aware of this population may result in misinterpretation of the results generated since these cells escape detection (not carrying the Eyfp or Gfp) and cannot be modified (lacking Cre expression) as expected.

Methods

Mice
Breeding pairs of Rosa26DTA/DTA and Cx3cr1CreER-Eyfp/Cx3cr1CreER-Eyfp mice were bought from the Jackson Laboratory (Bar Harbor, ME, USA) with stock numbers 009669 and 021160, respectively. Second generation pups with genotypes Cx3cr1CreER-Eyfp/wt Rosa26wt/wt, Cx3cr1CreER-Eyfp/wt Rosa26DTA/wt, Cx3cr1CreER-Eyfp/Cx3cr1CreER-Eyfp Rosa26wt/wt and Cx3cr1CreER-Eyfp/Cx3cr1CreER-Eyfp Rosa26DTA/wt were used for all experiments. Cx3cr1Gfp/Gfp mice breeding pairs were bought from the Jackson Laboratory (005582, Bar Harbor, ME, USA), C57BL/6 mice were bought from Charles River (Sulzfeld, Germany), and the pups of Cx3cr1Gfp/Gfp and C57BL/6 mice were used for the experiments. All mice were housed with a 12:12 h light–dark cycle and had free access to food and water.

Tamoxifen
Tamoxifen (T5648–1G, Sigma-Aldrich, Merck, Germany) was dissolved in corn oil (C8267, Sigma-Aldrich, Merck, Germany), and 125 mg/kg or 62.5 mg/kg was injected i.p. every 24 h for 3 (postnatal days 18, 19 and 20) or 10 consecutive days (postnatal days 18–27).

PLX3397 chow treatment
PLX3397 (HY-16749, MedChemExpress) was formulated with standard chow at 290 mg/kg by SAFE Nutrition Service, France. Four-week-old mice were treated with PLX3397 chow for 3 weeks and sacrificed at different timepoints afterwards.

Genotyping
DNA was extracted from ear punches and microglia. The following primer sets were used for the genotyping, Cx3cr1CreER-Eyfp−: common 5′-AAG ACT CAC GTG GAC CTG CT-3′, Wild-type reverse 5′-AGG ATG TTG ACT TCC GAG TTG-3′; C57BL/6 mice, and C57BL/6 mice breeding pairs were used for the breeding pairs.

Immunohistochemistry, cell counting, and image analysis
After perfusion with cold PBS, the brain was dissected, immersed in 4% paraformaldehyde (PFA) for 2 days, followed by immersion in 30% sucrose in 0.1 M phosphate buffer until fully saturated. Brain tissues were sagittally sliced into 25 μm sections and stored in tissue cryoprotectant solution (25% ethylene glycol and 25% glyc erin in 0.1 M phosphate buffer). The sections were rinsed in Tris-buffered saline (TBS, 50 mM Tris–HCl in 150 mM...
NaCl, pH 7.5) and then blocked with 3% donkey serum in TBS with 0.1% Triton X-100. After blocking, the sections were incubated overnight in primary antibody solution (Goat anti-Iba-1, 1:500, ab5076, Abcam; Chicken anti-GFP, 1:800, ab13970 Abcam; Rabbit anti-Tmem119, 1:500, ab209064, Abcam; Rabbit anti-P2ry12, 1:500, generated at Harvard Medical School; Rat anti-Ki67, 1:500, 14-5698-82, eBioscience). After washing, the sections were incubated for 2 h at room temperature with a secondary antibody (Donkey anti-goat, 1:1,000, Alexa Fluor 633, 20127, BIOTIUM; Donkey anti-chicken, 1:1000, Alexa Fluor 488, 703-545-155, Jackson Immuno Research; Donkey anti-rabbit, 1:1000, Alexa Fluor 555, 1945911, Invitrogen; Donkey anti-Rat, 1:1,000, Alexa Fluor 555, ab150154, Abcam) together with Hoechst (1:1000, 33342 Life Technologies). The sections were mounted onto glass slides with ProLong® Gold antifade reagent (1925239, Invitrogen). For Iba-1+ counting, the sections were blocked with 3% donkey serum in TBS with 0.1% Triton X-100, and then incubated in Rabbit anti-Iba-1 1:1,000 (Wako) overnight. After that, sections were incubated with the secondary antibody (donkey anti-rabbit 1:500, 20215, BIOTIUM) for 2 h, and then endogenous peroxidase activity was blocked with 3% H2O2 for 10 min. Visualization was performed using Vectastain ABC Elite with 3,3-diaminobenzidine.

Iba-1+ microglia were counted using an Axio Imager M2 microscope with Apotome attachment (Carl Zeiss). Cells were counted exhaustively on day 1, day 3, day 7 and day 10 after the final Tam injection. While a fractionator was used to estimate the positive cells on day 42 after the final Tam injection, the counting frame width and height are 150 μm, sample grid X and Y are 300 μm. Thus, all the data has a 2nd estimated coefficient of errors (Schmitz-Hof) less than 0.1 [32]. Then, relative microglia number ratios were calculated with reference to the control group.

All the fluorescence images were captured using an LSM700 laser scanning confocal microscope (Axioobserver Z1; CarlZeiss microscopy, Germany) and analyzed using ZEN software (the black edition; Zeiss).

Morphological analysis
Confocal microscopy was used to acquire images at 3 μm intervals, with a 40× objective lens used for Cx3cr1 depletion analysis (Fig. 2g and Additional file 1: Fig. S4G) and a 20× lens used for Cx3cr1CreER: EYFP/wt physiological analysis (Additional file 1: Fig. S5A) (Plan-Apochromat lens, 20×/0.8 and 40×/1.3 Oil objective, Carl Zeiss). Microglial morphology was analyzed with the skeleton analysis method using FIJI open-source image analysis software (Image J 1.51s, NIH) [33, 34]. Briefly, the maximum intensity projections of z-series stacks were created. The Iba-1+ channel and the CX3CR1-EYFP channel were enhanced in brightness to visualize all processes, followed by de-speckling to eliminate single-pixel backgrounds. Then, each channel image was processed into a binary image, and a topological skeleton image was created (Additional file 1: Fig. S2A). The Analyze Skeleton plugin feature was applied to the whole image frame, and the process length and the number of endpoints were normalized by the cell number per frame. Three morphological parameters were used: (1) total length of the processes, (2) number of endpoints, and (3) microglial process area. The process length and the number of endpoints of CX3CR1+/EYFP Iba-1+ cells were calculated by subtracting the CX3CR1-EYFP channel data from the Iba-1 channel data. Each cell’s process area was represented as the convex hull area by connecting the process ends using the polygon tool (Additional file 1: Fig. S2B, C) [35]. The selection criteria for the process area analysis were relatively isolated from the processes of the surrounding cells, and whole processes were not truncated and are within the image.

Microglial isolation
Single-cell suspensions were prepared using a Neural Tissue Dissociation Kit (T; Miltenyi Biotec), and myelin was removed using 38% Percoll. After passing through a 40 μm cell strainer, cells were incubated with the CD11b magnetic bead for 20 min. CD11b+ cells were isolated and collected using a magnetic field and MS column (Miltenyi Biotec, Germany).

Flow cytometry
After microglia isolation, cells were transferred into 96-well V-bottom plates and incubated at 4 °C for 20 min with the following antibodies: yellow dead cell stain kit (L34959, Thermo Fisher Scientific), Near-IR dead cell stain kit (L34975, Thermo Fisher Scientific), CD11b (PerCP-Cyanine5.5, Clone M1/70, BioLegend), CD45 (PE-Cyanine7, Clone 30F11, Biolegend), Ly-6G (V450, Clone 1A8, BD Biosciences), F4/80 (APC, Clone BM8, BioLegend), MHCII (Alexa Fluor 700, Clone M5/114.15.2, BioLegend), Cx3cr1 (Alexa Fluor 700, Clone SA011F11, Biolegend), Cre Recombinase (PE, Clone D7L7L, Cell Signaling Technology) and Ki67 (V450, Clone B56, BD). Cells were run on a Gallios flow cytometer (Beckman Coulter), and the data were analyzed with Kaluza software (Beckman Coulter).

Cell sorting and bulk RNA sequencing
Cells were sorted into CD11b+CD45+Ly6C−CX3CR1+EYFP+ and CD11b+CD45+Ly6C−CX3CR1+EYFP− populations.
using a BD Influx Cell Sorter. Briefly, 100 cells were collected directly into lysis buffer, and the library was built by the smart-seq method. Gene expression data were analyzed using Quercus Omics Explorer 3.4. For two-group and multi-group comparisons of candidate genes, expression was considered as significantly different if \( p < 0.05 \) using a heteroscedastic two-tailed Student’s \( t \) test or an \( F \) test, respectively. For transcriptome-wide analyses, the Benjamini–Hochberg method was used to correct multiple tests.

Whole-genome sequencing

Whole-genome sequencing was performed essentially as in DNTR-seq [36] with minimal modifications. Cell nuclei (1,500–5,000 nuclei per sample) were treated with 15 \( \mu l \) proteinase K (0.4 ml/ml) for 2 h at 50 °C followed by heat inactivation at 80 °C for 30 min. 2 \( \mu l \) of the solution was taken for tagmentation with 1 \( \mu l \) Tn5 stock solution and 1.6 \( \mu l \) reaction buffer (50 mM TAPS, 25 mM MgCl2, 40% PEG 8k; final concentration 8% PEG, 5 mM MgCl2, 10 mM TAPS) and 3.4 ml H2O (final volume 8 \( \mu l \)), incubated at 55 °C for 10 min. For barcoding PCR, each sample was split into 4 individual reactions, each barcoded with a unique molecular barcode (fastq files for the replicate reactions were merged after sequencing). 1 \( \mu l \) of the tagmented molecular barcode (fastq files for the replicate reactions were merged after sequencing). 1 \( \mu l \) of the tagmented DNA solution was used as the template for each barcoding PCR reaction. The PCR program was 72 °C/3 min, 95 °C/30 s, [95 °C/15 s, 67 °C/30 s, 72 °C/1 min] \( \times \) 15 cycles, 72 °C/5 min, and then 10 °C hold. After PCR amplification, the reactions were cleaned-up by SPRI-beads (at 0.9 \( \times \) volume) for size selection and were pooled together according in equal parts according to their DNA concentration (qubit dsDNA quantification assays, ThermoFisher). The pooled library was then cleaned-up more time by SPRI-beads (at 0.9 \( \times \) volume) and sequenced on an Illumina NextSeq 550 using the high output 75 bp kit.

Genomic reads were trimmed for adaptor sequences using TrimGalore and mapped to human genome reference build hg38 with the non-genomic parts of the Cx3cr1-CreERT2 targeting vector added as a separate template. Duplicates were removed using Picard Mark-Duplicate, along with read pairs with MAPQ<20. Reads mapping to the part of the vector which is inserted into the genome (position 1173–6936), and to the region surrounding the insertion site (chr9:120,040,000–120,080,000) were extracted from the bam files. Physical coverage (eg. the number of times a base is spanned by paired read mapping positions) was visualized using a custom R script.

Primary microglia proliferation and migration assay

Primary microglia were cultured as follows: cerebrum tissues were collected from postnatal days 3 mice and used for generating mixed glia cultures in T75 flasks. After 2 weeks of primary culture (DMEM F-12 with 20 ng/ml M-CSF), the microglia were isolated with CD11b magnetic bead sorting (MACS, Miltenyi Biotec) according to the manufacturer’s protocol. For proliferation assay, isolated CD11b positive cells were seeded at a density of \( 0.2 \times 10^5 \) on the coverslips (83.1840.002, Sartstedt) in 24-well plate for 2 days with DMEM F-12 medium containing 10% FCS and 20 ng/ml M-CSF, followed by replacement with serum-free medium (DMEM F-12) and serum-free medium (DMEM F-12) with 100 ng/ml CX3CL1 (472-FF-025/CF, R&D systems). 24 h later the cells were fixed by 4% paraformaldehyde, then incubated overnight in primary antibody solution (Goat anti-Iba-1, 1:500, ab5076, Abcam; Rat anti-Ki67, 1:500, 14-5698-82, eBioscience). After washing, the coverslips were incubated for 2 h at room temperature with a secondary antibody (Donkey anti-goat, 1:1000, Alexa Fluor 488, 1942238, invitrogen; Donkey anti-Rat, 1:1000, Alexa Fluor 555, ab150154, Abcam). The rate of ki67 positive cells in Iba-1 positive cells were calculated as the proliferating rate of microglia. For migration assay, isolated CD11b positive cells were seeded at a density of \( 0.5 \times 10^5 \) in an Incucyte ImageLock 96-well plate (4379, Sartorius) for overnight with the addition of 10% FCS and 20 ng/ml M-CSF. On the following day, a standardized scratched wound was induced in each well simultaneously using an Incucyte WoundMaker kit (Cat. No. 4493, Sartorius), followed by replacement with serum-free, M-CSF free DMEM/F-12 medium containing indicated concentration of CX3CL1 (472-FF-025/CF, R&D systems). The plate was then placed in an Incucyte Zoom System, and live images were taken every 2 h. The images were analyzed according to the manual of Incucyte Zoom.

Results

Identification of a subset of Cx3cr1\textsuperscript{Cre\textsuperscript{EYFP}}+ parenchymal microglia in Cx3cr1\textsuperscript{Cre\textsuperscript{ER-T2}} mice

In Cx3cr1\textsuperscript{Cre\textsuperscript{ER-Eyfp/wt}} mice, the protein-coding exon of one of the Cx3cr1 alleles is replaced by the Cre-ERT2 fusion gene and the enhanced yellow fluorescent protein gene (EYFP), implying that all CX3CR1+ cells (mainly microglia in the CNS parenchyma) should express both Cre-ERT2 and EYFP. Unexpectedly, we observed a subpopulation of Iba-1\textsuperscript{+}Tmem119\textsuperscript{+}EYFP\textsuperscript{+} cells with microglia-like morphology in the CNS parenchyma of Cx3cr1\textsuperscript{Cre\textsuperscript{ER-Eyfp/wt}} mice under homeostatic conditions (Fig. 1a and Additional file 1: Fig. S1a). These Iba-1\textsuperscript{+}Tmem119\textsuperscript{+}EYFP\textsuperscript{+} cells and Iba-1\textsuperscript{+}Tmem119\textsuperscript{+}EYFP\textsuperscript{+} microglia exhibited similar morphologies as measured by process complexity...
and EYFP CD45 + CD11b Ly6C–Ly6G– cells at 3–15 weeks, with no significant differences between ages. One outlier sample in 9-week age mice was excluded from the analysis, having 6% of EYFP– cells of the total CD11b+CD45+ Ly6C–Ly6G– pool (Fig. 1c and Additional file 1: Fig. S3b). Furthermore, we observed that both EYFP+ microglia and EYFP– microglia-like cells were CX3CR1+ (Fig. 1d). These data indicate that CX3CR1+EYFP– cells are resident microglia.

To further confirm this finding, we performed fluorescence-activated cell (FAC) sorting of CX3CR1+EYFP+ and CX3CR1+EYFP– cells (gated as CD11b+CD45+ Ly6C–Ly6G– CX3CR1+) followed by RNA sequencing (Fig. 1d). To investigate the Cre and Eyfp expression, the RNA sequencing reads were mapped on the mouse genome integrated with the CreERT2-Eyfp knockin-allele. We determined that both CX3CR1+EYFP+ and CX3CR1+EYFP– cells expressed similar levels of specific markers for myeloid cells expressing Hoechst, Iba‑1, Tmem119, but not GFP, and microglia (P2ry12, Tmem119, and Fceir1g) and microglia (P2ry12, Tmem119, and Sall1) (data not included), further confirming that both CX3CR1+EYFP– and CX3CR1+EYFP+ cells were CNS parenchymal microglia. Next, the transcriptomes of CX3CR1+EYFP– and CX3CR1+EYFP+ microglia were compared, indicating 32 differently expressed genes (DEGs) with a false discovery rate (FDR)< 0.05 (Additional file 2: Table S1). The top 5 downregulated genes in CX3CR1+EYFP+ microglia were WdfEc21, Camp, Chil3, Lif, and Cd177, while all the upregulated genes in CX3CR1+EYFP– microglia were Eyfp, Cre, H2-Aa, Ipf47, Zfp940, Msi4a4a, and Itsn1 (Fig. 1e). Furthermore, we determined that CX3CR1+EYFP– microglia expressed twice as much Cx3cr1 mRNA compared with CX3CR1+EYFP+ microglia (Fig. 1f). Moreover, CX3CR1+EYFP– microglia did not express Eyfp or Cre mRNA (Fig. 1g, h). We thus defined that CX3CR1+EYFP– microglia are CX3cr1hiCre-Eyfp–.

To assess the variation of Cx3cr1 expression in C57BL/6 mice, single-cell RNA sequencing data from our previous publication [37] were reanalyzed in CX3CR1+ microglia from the hippocampus. No clusters with high or low Cx3cr1 expression were detected (Additional file 1: Fig. S3c). The Cx3cr1 expression was normally distributed in CX3CR1+ microglia (Additional file 1: Fig. S3d), supporting that the difference in Cx3cr1 expression levels between CX3cr1hiCre-Eyfp– and CX3cr1CreER-Eyfp/wt Cre+Eyfp+ microglia in CX3cr1CreER-Eyfp/wt mice was attributed to the genetic modification.

To confirm that CX3cr1hiCre-Eyfp– microglia were not an anomaly of one specific mouse strain, we investigated the microglial populations in CX3cr1CreER-Eyfp/wt mice (JAX, 005582) by flow cytometry (gating strategy depicted in Additional file 1: Fig. S3a) and immunohistochemistry. The results revealed that Iba-1+Tmem119+GFP+ cells with microglial morphology could also be detected in the CX3cr1CreER-Eyfp/wt adult mouse brain, constituting 0.59 ± 0.27% of total microglia (Fig. 1i, j).

Cx3cr1hiCre-Eyfp– microglia repopulated and became a dominant subgroup of the repopulated microglial pool following microglial depletion

To investigate whether CX3cr1hiCre-Eyfp– microglia are capable of filling the vacant microglial niche following microglial depletion, we first crossed Cx3cr1CreER-Eyfp/Cx3cr1GFP/wt mice to breed Cx3cr1CreER-Eyfp/wt Rosa26DTA/DTA mouse strains (Fig. 2a, IAX, 009669) to breed Cx3cr1CreER-Eyfp/wtRosa26DTA/DTA mice. Following tamoxifen (Tam) injections, Cx3cr1CreER-Eyfp/wt cells in the brain parenchyma (mainly CX3cr1CreER-Eyfp/wt Cre+Eyfp+ microglia) can be depleted through the intracellular release of diphtheria toxin [38]. To track dynamic microglial alterations in the brain, Cx3cr1CreER-Eyfp/wtRosa26DTA/DTA and Cx3cr1CreER-Eyfp/wt mice were sacrificed at different timepoints (days 1, 3, 7, 8, 10, 21, and (see figure on next page.)

Fig. 1 Cx3cr1hiCre-Eyfp– microglia in CX3cr1CreER-Eyfp/wt mice. a Representative images of Hoechst, EYFP, Iba-1 and Tmem119 immunohistochemical staining in the brains of CX3cr1CreER-Eyfp/wt mice. White arrows point to the EYFP–Iba-1+Tmem119+ cells. Scale bar, 50 μm. b Comparison of morphometry between EYFP–Iba-1+ microglia and EYFP–Iba-1+ microglia in CX3cr1CreER-Eyfp/wt mice. The graph shows process endpoints (left, p = 0.00773), process length (middle, p = 0.0647), and process occupied area (right, p = 0.1253) by Student’s two-tailed unpaired t test. n = 59 for EYFP–Iba-1+ cells and n = 31 for EYFP–Iba-1+ cells from four CX3cr1CreER-Eyfp/wt mice, mean ± s.d. C Representative dot plots of EYFP+ microglia in the brains of CX3cr1CreER-Eyfp/wt mice (gated as CD11b+CD45+Ly6C–Ly6G–). d Representative dot plots of expression of EYFP and Cx3cr1, n = 8. e Volcano plot showing the DEGs with FDR < 0.05, the indicated genes are Cx3cr1, all upregulated genes and the top 5 down-regulated genes. f The reads per kilobase of transcript, per million mapped reads (RPKM) of Cx3cr1 between EYFP+ and EYFP– microglia from the RNA-seq data, p < 0.001 by paired Student’s two-tailed t test. g, h The RPKM of EYFP and Cre between EYFP+ and EYFP– microglia from the RNA-seq data. **p < 0.01, ***p < 0.001 by paired Student’s two-tailed t test. I Dot plots of flow cytometry analysis of Cx3cr1CreER-Eyfp+ mice, values in plots are the ratio of GFP+ microglia to total microglia, n = 6 mice, mean ± s.d. j Representative images of GFP, Iba-1 and Tmem119 triple staining in CX3cr1GFP/wt mice, the arrows indicating cells expressing Hoechst, Iba-1, Tmem119, but not GFP, n = 6 mice. Scale bar, 50 μm.
Fig. 1 (See legend on previous page.)
42) after three consecutive Tam injections on postnatal days 18, 19, and 20, respectively (Fig. 2b). Iba-1+ microglia were quantified by immunohistochemistry, revealing that approximately 89.7±2.4%, 90.9±0.3%, and 94.5±1.4% of the microglia were depleted by the first day after the final Tam injection in the hippocampus, cortex, and cerebellum, respectively, (Fig. 2c, d, Additional file 1: Fig. S4a, b). Moreover, newly repopulated microglia were noted 1 week later, amounting to 21.9±8.5% in the cortex and 34.9±5.9% in the cerebellum compared to control Cx3cr1<sup>CreER-Eyfp</sup>/wt mice (Additional file 1: Fig. S4a, b). We also recorded an overabundance of microglia 10 days after the final Tam injection, 123.3±2.7% in the hippocampus and 139.8±18.6% in the cerebellum compared to the control group (Fig. 2d and Additional file 1: Fig. S4a). No significant differences were observed in the numbers of newly repopulated microglia 42 days after the final Tam injection, compared with the baseline level (Fig. 2c, d, Additional file 1: Fig. S4a, b).

The numbers of Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>+</sup></sup> (EYFP<sup>+</sup>Iba1<sup>+</sup>) microglia increased from day 1 to day 3 after the final Tam injection (Additional file 1: Fig. S4c, d). Distinct clusters of Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>+</sup></sup> microglia were formed by day 7, further expanded on days 8 and 10, and then maintained 42 days after the final Tam injection (Fig. 2e, f). Interestingly, newly repopulated Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>+</sup></sup> microglial clusters were distributed in a stochastic manner with a considerable regional variation between different mice, not co-localizing with Cx3cr1<sup>CreER-Eyfp/wt Cre<sup>+</sup>Eyfp<sup>+</sup></sup> microglia clusters (Fig. 2e, f). We have previously demonstrated that high and low expression of F4/80 can be used to distinguish resident repopulated microglia and peripheral infiltrating microglia-like cells following experimental microglial depletion [38]. Flow cytometric analysis revealed 3 main subsets of microglia-like cells after repopulation in Cx3cr1<sup>CreER-Eyfp/wt Rosa26<sup>DTA/wt</sup></sup> mice (Fig. 2g). These included EYPF<sup>+</sup>F4/80<sup>low</sup>, EYPF<sup>+</sup>F4/80<sup>low</sup>, and EYPF<sup>+</sup>F4/80<sup>hi</sup>, likely representing resident Cx3cr1<sup>CreER-Eyfp/wt Cre<sup>+</sup>Eyfp<sup>+</sup></sup> microglia, resident Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>−</sup></sup> microglia, and peripherally derived microglia-like cells, respectively (Fig. 2g). The finding was further confirmed using Immunohistochemistry (IHC), again demonstrating mainly 3 repopulated microglial subgroups expressing EYPF<sup>+</sup>Tmem119<sup>−</sup>, EYPF<sup>+</sup>Tmem119<sup>−</sup>, and EYPF<sup>+</sup>Tmem119<sup>+</sup>, respectively (Fig. 2h).

We next addressed if these newly repopulated Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>−</sup></sup> microglia were derived from the periphery. We and others have previously demonstrated that the empty microglial niche can be repopulated within weeks through resident microglia proliferation and concomitant infiltration of monocytes [38, 39]. Two novel specific surface markers (Tmem119 and P2ry12) were used to identify CNS-resident microglia [40, 41]. Our results demonstrated that all newly repopulated Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>−</sup></sup> microglia expressed both Tmem119 (Fig. 2h) and P2ry12 (Fig. 3a). Furthermore, the vast majority of Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>−</sup></sup> microglia had low expression of F4/80 (Fig. 2g).

To confirm the CNS origin of Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>−</sup></sup> microglia, we reanalyzed data from our previous publication [38]. Specifically, Cx3cr1<sup>CreER-Eyfp/wt Rosa26<sup>DTA/wt</sup></sup> and Cx3cr1<sup>CreER-Eyfp/wt</sup> mice (CD45.2) were exposed to head-protected irradiation and transplanted with congenic CD45.1 bone marrow, Tam was injected 8 weeks after reconstitution, and the chimeras were analyzed another 8 weeks later (Fig. 3b). We determined that the vast majority of the F4/80<sup>high</sup> cells were derived from CD45.1<sup>+</sup> donor cells, while all Cx3cr1<sup>CreER-Eyfp/wt Cre<sup>+</sup>Eyfp<sup>+</sup></sup> (EYPF<sup>+</sup>F4/80<sup>low</sup>) microglia originated from CD45.2<sup>+</sup> resident microglia in both Cx3cr1<sup>CreER-Eyfp/wt</sup> and Cx3cr1<sup>CreER-Eyfp/wt Rosa26<sup>DTA/wt</sup></sup> chimeras, and that the vast majority of the Cx3cr1<sup>high Cre<sup>−</sup>Eyfp<sup>−</sup></sup> (EYPF<sup>+</sup>F4/80<sup>low</sup>) microglia were derived from CD45.2<sup>+</sup> resident microglia, indicating their CNS origin.

This notion was further confirmed by RNA sequencing of the 4 groups of repopulated microglia expressing EYPF<sup>+</sup>F4/80<sup>low</sup>, EYPF<sup>+</sup>F4/80<sup>low</sup>, EYPF<sup>+</sup>F4/80<sup>hi</sup>, and EYPF<sup>+</sup>F4/80<sup>hi</sup>. We first addressed if these newly repopulated
Fig. 2 (See legend on previous page.)
EYFP<sup>−</sup>F4/80<sup>high</sup>, respectively (Fig. 2g). Specifically, our results demonstrated that the F4/80<sup>low</sup> groups had lower expression of signature genes of engrafted macrophages, including Ms4a7 and Lyz2, compared to the F4/80<sup>high</sup> groups (Fig. 3c, d). Principle component analysis (PCA) indicated that the greatest difference was evident between F4/80<sup>low</sup> and F4/80<sup>high</sup> groups (Fig. 3e). We further investigated the expression levels of 14 macrophage- and microglia-related genes [42], and EYFP<sup>+</sup>F4/80<sup>low</sup> and EYFP<sup>−</sup>F4/80<sup>low</sup> microglia expressed similar patterns, while EYFP<sup>+</sup>F4/80<sup>high</sup> and EYFP<sup>−</sup>F4/80<sup>high</sup> microglia expressed similar patterns (Fig. 3f). For example, the F4/80<sup>high</sup> groups expressed higher levels of Adgre1 (F4/80) and Ptprc (CD45) than did the F4/80<sup>low</sup> groups (Fig. 3f), supporting the efficiency of our sorting strategy and the peripheral source of the F4/80<sup>high</sup> groups. F4/80<sup>high</sup> and F4/80<sup>low</sup> groups expressed similar levels of Fcgr1, Aif1 (Iba-1), and Cd68. Moreover, F4/80<sup>high</sup> groups expressed lower levels of Fcgr1, C1qa, Cx3cr1, Sall1, Tmem119, sparcl, P2ry12, Hexb and Itgam.

![Fig. 3 Repopulated EYFP<sup>−</sup> microglia originate from the resident microglia pool.](image-url)

a Representative images of EYFP, Iba-1, P2ry12 triple staining on newly repopulated microglia of Cx3cr1<sup>CreER<sup>EYFP<sup>+</sup> Rosa26<sup>DTA<sup>/</sup> mice. Scale bar, 20 μm. b Experimental chimerism setup to determine the origin of repopulated Cx3cr1<sup>Cre<sup>EYFP<sup>−</sup> microglia after microglia depletion. Middle-left panel gated on CD11b<sup>+</sup>CD45<sup>+</sup>Ly6C<sup>−</sup>Ly6G<sup>−</sup>EYFP<sup>−</sup> microglia. Middle-right panel with red color rectangle gated on F4/80<sup>low</sup>EYFP<sup>+</sup> microglia. Bottom-left panel with blue color gated on F4/80<sup>low</sup> EYFP<sup>−</sup> microglia. Bottom-right with purple color gated on F4/80<sup>high</sup> cells. n = 5–12, mean ± s.d. c, d Bar graphs show RPKM of Ms4a7 and Lyz2, n = 5, mean ± s.d. e Principal component analysis of genes p < 0.05. f Heat map visualization of specific genes on macrophage and microglia. Data is presented as mean-centered sigma-normalized
CD11b) (Fig. 3f). Taken together, newly repopulated Cx3cr1<sup>high</sup>Cre<sup>−/−</sup>Eyfp<sup>−</sup> microglia originate from the resident Cx3cr1<sup>high</sup>Cre<sup>−<sup>−</sup></sup>Eyfp<sup>−</sup> microglia pool.

Collectively, although only less than 1% Cx3cr1<sup>high</sup>Cre<sup>−/−</sup>Eyfp<sup>−</sup> microglia exist in the Cx3cr1<sup>CreER-Eyfp/wt</sup> mouse brain, they can account for one-third of the newly repopulated microglia pool following conditional genetic depletion in Cx3cr1<sup>CreER-Eyfp/wt</sup>Rosa26<sup>DTA/wt</sup> mice.

**Cx3cr1<sup>high</sup>Cre<sup>−/−</sup>Eyfp<sup>−</sup> microglia have lost the CreERT2-Eyfp fusion gene and reveal a wild-type Cx3cr1 genotype**

We next investigated the newly repopulated CNS-derived Cx3cr1<sup>high</sup>Cre<sup>−/−</sup>Eyfp<sup>−</sup> microglia. The experimental Cx3cr1<sup>high</sup>Cre<sup>−/−</sup>Eyfp<sup>−</sup> microglia contain the inserted CreERT2-Eyfp (EYFP<sup>−</sup>) microglia to target longer sequences, including the whole Cx3cr1<sup>high</sup>Cre<sup>−/−</sup>Eyfp<sup>−</sup> microglia. Given the lack of Cre and Eyfp expression on the mRNA and protein levels, we reasoned that the CreERT2-Eyfp knockin-allele was either epigenetically/ genetically silenced or deleted. We thus investigated Cx3cr1<sup>high</sup>Cre<sup>−/−</sup>Eyfp<sup>−</sup> (EYFP<sup>−</sup>) microglia compared to Cx3cr1<sup>high</sup>Cre<sup>−/−</sup>Eyfp<sup>−</sup> (EYFP<sup>−</sup>) microglia (Additional file 1: Fig. S5a–c). Moreover, flow cytometric analysis indicated a higher expression of CX3CR1 protein (Additional file 1: Fig. S5d, e).

To further confirm the role of CX3CR1 in regulating microglial repopulation, we depleted microglia using PLX3397 chow in Cx3cr1<sup>wt/wt</sup>, Cx3cr1<sup>CreER-Eyfp/wt</sup>, and Cx3cr1<sup>CreER-Eyfp/CreER-Eyfp</sup> mice. Previous studies have
**Fig. 4** Cx3cr1<sup>high</sup>Crem<sup>Cre</sup> EYFP<sup>-</sup> microglia are Cx3cr1<sup>wt/wt</sup>Crem<sup>Cre</sup> EYFP<sup>-</sup> microglia. **a, b** DNA electrophoresis of PCR product from EYFP<sup>-</sup> and EYFP<sup>+</sup> microglia. **c** Normalized coverage of the first 290 bps in exon 2 of Cx3cr1 in both Cx3cr1<sup>high</sup>Crem<sup>Cre</sup> EYFP<sup>-</sup> and Cx3cr1<sup>wt/wt</sup>Crem<sup>Cre</sup> EYFP<sup>+</sup> microglia, indicating the integrity of the protein-coding exon 2 of Cx3cr1, mouse 1 and mouse 2 are two biological replicates. **d, e** Whole-genome sequencing of Cx3cr1<sup>high</sup>Crem<sup>Cre</sup> EYFP<sup>-</sup> microglia (d) and Cx3cr1<sup>wt/wt</sup>Crem<sup>Cre</sup> EYFP<sup>+</sup> microglia (e). The top panel represents the sequencing mapping on the CreERT2-EYFP vector; the middle panel represents the sequencing mapping on chromosome 9; the bottom panel is a schematic graph showing the homozygosity of the exon 2 of Cx3cr1.

**Fig. 5** Competitive advantage of higher CX3CR1 expressed microglia during microglial repopulation. **a** Different percentages of EYFP<sup>-</sup>F4/80<sup>low</sup>, EYFP<sup>-</sup>F4/80<sup>边界</sup>, EYFP<sup>+</sup>F4/80<sup>low</sup>, and EYFP<sup>+</sup>F4/80<sup>边界</sup> microglia in total microglia at Ctrl, day 1, 21 and 42 after Tam injections. **b** Representative images of Hoechst, Iba-1 and Ki67 staining in the primary cultured microglial. **c** Bar graph showing the rate of Ki67<sup>+</sup> cells in Iba-1<sup>+</sup> cells in Cx3cr1<sup>wt/wt</sup>, Cx3cr1<sup>CreER-EYFP/wt</sup> and Cx3cr1<sup>CreER-EYFP/CreER-EYFP</sup> primary microglia, and the rate of Ki67<sup>+</sup> cells in Iba-1<sup>+</sup> cells in Cx3cr1<sup>CreER-EYFP/wt</sup>, Cx3cr1<sup>CreER-EYFP/CreER-EYFP</sup> and Cx3cr1<sup>CreER-EYFP/CreER-EYFP</sup> primary microglia after adding 100 ng/ml CX3CL1 in the medium. **d** Migration assay: the left panel showing would density in Cx3cr1<sup>wt/wt</sup>, Cx3cr1<sup>CreER-EYFP/wt</sup>, Cx3cr1<sup>CreER-EYFP/CreER-EYFP</sup> primary microglia culture. **e** Graph showing cell density in the whole brain of control group, day 0, day 3 day 7 and day 14 after ceasing PLX3397 chow treatment in Cx3cr1<sup>wt/wt</sup>, Cx3cr1<sup>CreER-EYFP/wt</sup>, Cx3cr1<sup>CreER-EYFP/CreER-EYFP</sup> mice, n = 3–8, mean ± s.d. *p < 0.05 by One-way ANOVA.
Fig. 5 (See legend on previous page.)
demonstrated that blood-borne infiltrating cells do not contribute to microglia repopulation following microglial depletion using PLX3397 administered in chow [43]. Approximately 70% of the microglia were depleted after 21 days PLX3397 treatment and there was no difference in depletion efficiency between the 3 genotypes. Microglia repopulated quickly 3 days after ceasing treatment, without any differences between the 3 genotypes. However, 7 days after repopulation the Cx3cr1wt/wt group exhibited more microglia than did the Cx3cr1CreER::Ai14 group, reaching 20% higher numbers than the control group, while the Cx3cr1CreER-Eyfp/CrER-Eyfp group stopped at the control, pre-depletion level and the Cx3cr1CreER-Eyfp/wtRosa26DTA/wt (Fig. 5e), further indicating that CX3CR1 plays a role in microglial depletion using the CSF1 receptor inhibitor PLX5622 in mouse brain, which can be repopulated after microglial depletion following Tam injections in Cx3cr1CreER::Ai14 mice. One previous study reported that all repopulated microglia were derived from CX3CR1+ cells, as measured by fate mapping in Cx3cr1CreER::Ai14 mice. All CX3CR1+ microglia were tdTomato+, and all Iba-1+ cells were tdTomato+, leading to the interpretation that all the repopulated Iba-1+ cells arose from the surviving CX3CR1+ cells following microglial depletion [43]. However, in both our Cx3cr1CreER-Eyfp/wt and Cx3cr1GFP/wt transgenic mice, the presence of Cre−EYFP− and GFP− microglia was recorded. Proliferating GFP− microglia in Cx3cr1GFP/+ mice have also been reported following microglia depletion using the CSF1 receptor inhibitor PLX3397 [18]. In that study, the authors showed that proliferating GFP− cells could become Iba-1− microglia; however, whether these Iba-1−GFP− microglia were also CX3CR1− was not addressed [18]. Our results indicated that these cells could be de facto microglia expressing all thus far established canonical markers and that they existed before microglial depletion and repopulation. Another study reported that most newly repopulated microglia were tdTomato+ using the CSF1 receptor inhibitor PLX5622 in Cx3cr1CreER+/+tdTomato mice, and the Iba1−tdTomato− cells were interpreted as being infiltrating peripheral monocytes [26]. However, based on our study, we consider that the Iba1−tdTomato− cells could be derived from the local microglial cell pool, which does not carry the transgene Cx3cr1CreER. Furthermore, another study demonstrated that EYFP− microglia escaped Cre-mediated recombination and could repopulate the CNS following microglial depletion in Rosa26STOP−EYFP/Cx3cr1CreER::iDTR mice [39]. Phagocytosis and activation of microglia is, at least partly, dependent on the CX3CR1/CX3CRL axis [44–49]. Hence, the EYFP–microglia, expressing twice the amount of CX3CR1, might have a different phagocytosis capacity. In light of these findings, interpretation of results from Cx3cr1CreER/wt and Cx3cr1GFP/wt transgenic mice should be made with caution.

We consider two possible explanations for the presence of Cx3cr1wt/wtCre−EYFP− microglia in the Cx3cr1CreER-Eyfp/wt mouse brain: (i) maternal-derived macrophages or microglia, or (ii) LOH of microglia during mitosis. The possibility of maternal-derived microglia or macrophages was excluded by our results, as the Cx3cr1wt/wtCre−EYFP− microglia inherited the paternal DTA gene. Alternatively, LOH due to homologous recombination could explain the existence of Cx3cr1wt/wtCre−EYFP− microglia in the Cx3cr1CreER::Ai14 mouse brain. LOH is a phenomenon whereby the cells only possess the genetic information from one of the parental chromosomes, as previously described in the cancer cells [50] and mammalian cells in vivo and in vitro [51–53]. Our results showing a similar percentage of Cx3cr1wt/wtCre−EYFP− microglia in each of the heterozygous Cx3cr1CreER-Eyfp/wt mouse brains and the absence of Cx3cr1wt/wtCre−EYFP− microglia in the homozygous Cx3cr1CreER-Eyfp/CrER-Eyfp mouse brain support the theory of LOH through homologous recombination during mitosis. The mechanisms of microglial LOH in Cx3cr1CreER-Eyfp/wt and Cx3cr1GFP/wt mouse strains and whether the phenomena of LOH also occurs in other analogous genetic modified heterozygous strains need to be further investigated.

Microglial depletion and repopulation studies have expanded our knowledge of microglia in physiological and pathological states, exerting favorable effects in different preclinical disease models [54, 55]. Moreover, new microglia rapidly repopulate the brain parenchyma following microglial depletion, although the origin of these newly repopulated microglia has been debated. Elmore and colleagues reported that CX3CR1+GFP− cells were potential microglial precursor cells during the microglial repopulation period. However, this viewpoint has been challenged, and newly repopulated microglia are proposed to only arise from the surviving microglia [56]. This is further supported using fate mapping approaches showing that The new forming microglia only temporary
expression nestin, and no microglia were derived from None microglia nestin+ cells [43]. Our RNA sequencing data revealed no increased expression of precursor of stem cell genes in Cx3cr1wt/wtCre-Eyfp− microglia. Moreover, microglial single-cell data from wild type mice revealed no high Cx3cr1-expressing microglia that clustered separately from other microglia. Rather, the cells expressing higher levels of Cx3cr1 were evenly distributed (Additional file 1: Fig. S3C, D). Taken together, these data support that Cx3cr1wt/wtCre-Eyfp− microglia are not precursors or stem cells. How these surviving microglia could survive depletion and whether they were unaffected by the depletion period remain unclear. Microglia cannot actually be fully depleted using currently available depletion models [54] and the existence of a microglial subset that may be resistant to depletion has been previously proposed [57]. Cx3cr1wt/wtCre-Eyfp− microglia could be such subset, at least in the Cx3cr1-Cre derived depletion setting. Our results indicate that both unaffected Cx3cr1wt/wtCre+Eyfp+ microglia and surviving Cx3cr1CreER-Eyfp/wtCre+Eyfp+ microglia repopulate the brain competitively, with contribution from peripheral derived macrophages. Moreover, it is widely known that individual microglia occupy non-overlapping spatial territories [58, 59]. Herein, we report a novel finding that non-overlapping niches exist between repopulated Cx3cr1wt/wtCre+Eyfp− and Cx3cr1CreER-Eyfp/wtCre+Eyfp+ microglia; however, related signal pathways need to be further investigated.

Tissue macrophages with a self-renewing capacity are seeded during embryonic development, and some macrophages can be replaced or renewed postnatally by peripheral monocytes [60–65]. In the brain, the resident microglial pool is self-renewing without contribution from peripheral monocytes during the whole life span [1]. However, in disease states, such as CNS injury and neurodegenerative diseases, monocytes can infiltrate the brain and become microglia-like cells, albeit with different functionalities [66]. Here we used a mouse model in which peripheral monocytes enter the brain after microglial depletion [38]. Our results demonstrate that the main transcriptomic difference is noted between the F4/80high and F4/80low groups, but not between the EYFP− and EYFP+ groups after competitive microglial repopulation, which indicates that the infiltrating monocytes, imprinted by the CNS microenvironment, are different from the resident repopulated microglia, and confirming our previous publication [38].

Microglia replacement therapy has been proposed for CNS diseases linked to microglial dysfunctions or gene mutations. Replacing microglia by genetically modified or engineered cells may hold promise for distinct CNS diseases, yet the factors regulating competitive engraftment of different populations including microglia, monocytes and engineered cells are poorly understood [67, 68]. CX3CR1 regulates microglia colonization and distribution in the brain [69], and Cx3cr1 gene-deleted mice exhibited lower microglial density in the developing brain [70]. The CX3CL1–CX3CR1 axis also regulates microglial repopulation following microglial depletion in the mouse retina [26], but whether this axis regulates competitive microglial repopulation in the brain has not previously been addressed. Our results indicate that repopulating microglia originate from three different predominant sources, resident microglia, including both Cx3cr1wt/wtCre+Eyfp− and Cx3cr1CreER-Eyfp/wt microglia, competing with infiltrating peripheral-derived microglia-like cells. Our data demonstrate that Cx3cr1wt/wtCre+Eyfp− microglia have a competitive advantage over Cx3cr1CreER-Eyfp/wt microglia. Furthermore, resident microglia lacking Cx3cr1 (from Cx3cr1CreER-Eyfp/Cx3cr1CreER-Eyfp mice) were unable to compete with the peripheral-derived microglia-like cells following microglial depletion. This indicates that the resident microglia repopulation, but not peripheral derived microglia-like cell repopulation relies on CX3CR1. The proliferation rate of microglia in vitro was decreased in Cx3cr1 gene-depleted primary microglia, and this was not affected when the cells were challenged with CX3CL1 protein. This likely explains the repopulation advantage of Cx3cr1wt/wtCre+Eyfp− over Cx3cr1CreER-Eyfp/wt microglia post-depletion, at least partly. Cx3cr1 deficiency decreased the microglial migration rate, consistent with previous studies reporting that Cx3cr1 deficiency impaired microglia migration in vivo [71, 72] and in vitro [73]. However, microglial migration was not impaired in Cx3cr1CreER-Eyfp/wt microglia. CX3CL1 increased migration rates of Cx3cr1wt/wtCre+Eyfp− microglia but not of Cx3cr1CreER-Eyfp/wt and Cx3cr1CreER-Eyfp/Cx3cr1CreER-Eyfp microglia, which indicates that CX3CL1–CX3CR1 regulates microglial migration. The migration rate is higher in Cx3cr1wt/wt Cre+Eyfp− than Cx3cr1wt/wt Cre+Eyfp+ microglia after adding CX3CL1, which further explains the repopulation advantage of Cx3cr1wt/wt Cre+Eyfp− over Cx3cr1 wt/wt Cre+Eyfp− microglia post-depletion. Taken together, we conclude that the CX3CL1–CX3CR1 axis is important for the resident microglial repopulation and for competition with peripheral monocyte-derived microglia-like cells. Thus, limiting residential microglia repopulation by inhibiting CX3CL1–CX3CR1 signaling improves the microglial replacement efficiency by peripheral derived monocytes.
Conclusions
A small portion (less than 1%) of Cx3cr1<sup>wt/wt</sup>Cre<i> Eyfp</i>-microglia do not carry genetic labels in the widely used Cx3cr1<sup>EGFP/wt</sup> or Cx3cr1<sup>CreER-Eyfp/wt</sup> mouse models. Not being aware of this population may lead to significant data misinterpretation since these cells may escape detection (not carrying the <i>Eyfp</i> or <i>Gfp</i>) and cannot be modified (lacking Cre expression) as expected. We further demonstrate the important role of the CX3CL1–CX3CR1 axis in regulation of microglial repopulation post-depletion. These findings raise an important cautionary note, not only when using the strains mentioned above but also for other strains that might display similar phenomena.

Abbreviations
DTA: Diphtheria toxin subunit A; LOH: Loss of heterozygosity; CNS: Central nervous system; Eyfp: Enhanced yellow fluorescent protein gene; FAC: Fluorescence-activated cell; Tam: Tamoxifen.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12974-022-02381-6.

Additional file 1: Figure S1. Additional data related to Fig. 1. A Representative 8 sections of Hoechst staining of Cx3cr1<sup>CreERT2-Eyfp</sup> mouse brain sequential sagittal slices, the numbers representing the location and number of EYFP<sup>−</sup> microglia in that location, each color represents one mouse, n=6. Figure S2. Morphology analysis. A The process to prepare topological skeleton from the original photomicrographs. The full-size maximum intensity projection images (top) were processed into binary images (middle) and then skeletonized (bottom) following the ImageJ polygon tool. Cropped images (right) from the original full-size maximum intensity projection images (top) were processed into binary images (middle) and then skeletonized (bottom) following the ImageJ polygon tool. B The Analyze Skeleton plugin was applied, and skeletonized endpoints were tagged purple, the slab is orange, and the junction is pink. The tagged data are summarized as total length (sum of endpoints, slab, and junction) and the total number of endpoints. C The process area is represented as the convex hull area by connecting the process ends using the ImageJ polygon tool. Figure S3. Additional data related to Fig. 1. A Gating strategies of flow cytometry analyses. B EYFP<sup>−</sup> microglia ratio of total microglia at 3 weeks, 4 weeks, 6 weeks, 9 weeks and 15 weeks old mice, n=4, 6, 4, 6, 4, respectively, mean ± s.d. No significant difference by one-way ANOVA. C UMAP plots from single-cell sequencing, each dot represents one cell, the color represents the expression levels of Cx3cr1. D Histogram plot of Cx3cr1. The x-axis represents Cx3cr1 expression read counts; the y-axis represents cell numbers. Figure S4. Additional data related to Fig. 2. A Graph showing relative iba1<sup>−</sup> microglia at 1 day, 3 days and 7 days after the final Tam injection, respectively, n=3–4, mean ± s.d. *p < 0.05, **p < 0.01, ***p < 0.001 by Student’s two-tailed unpaired t test. B Representative images of iba1<sup>−</sup> microglia staining of Cx3cr1<sup>CreERT2-Eyfp</sup> Rosa26DTA<sup>wt/wt</sup> mice at 1 day after the final Tam injection in cortex and cerebellum. Scale bar, 200 μm. C Representative 3 sequential sagittal sections (25 μm/section, with 36 sections interval) after Hoechst staining of Cx3cr1<sup>CreERT2-Eyfp</sup> Rosa26DTA<sup>wt/wt</sup> mice brains. The numbers represent the location and number of Cx3cr1<sup>CreERT2-Eyfp</sup> microglia at 1 day and 3 days after the final Tam injection, each color represents one mouse, n=3. D Quantitative data showing the total number of microglia in the 3 sections in Ctrl group, 1 day and 3 days after the final Tam injection, n=4, 3, 3, mean ± s.d. Figure S5. Additional data related to Fig. 4. A-C Bar graphs showing RPMK of Cre, EYFP, and Cx3cr1, respectively, n=5, mean ± s.d. ***p < 0.001 by Student’s two-tailed unpaired t test. D The expression of Cx3cr1 in both Cx3cr1<sup>CreERT2-Eyfp/wt</sup> and Cx3cr1<sup>wt/wt</sup>Cre<i>Eyfp</i>-microglia in Cx3cr1<sup>CreERT2-Eyfp/Rosa26DTA<sup>wt/wt</sup></sup> mice at day 42 after the final Tam injection. E Bar graph showing the mean fluorescence intensity (MFI) of Cx3cr1<sup>−/−</sup> n=4, mean ± s.d. ***p < 0.001 by Student’s two-tailed unpaired t test. Figure S6. Additional data related to Fig. 4. A Schematic diagram showed how the primers were designed. The red color indicates the common primers used in all the PCR reactions; the green and blue color indicate the WT primer and Mutant primer used in PCR reaction for Fig. 4f; the orange color indicates the long PCR product targeting primer used in PCR reaction for Fig. 4g. B Whole-genome sequencing of Cx3cr1<sup>wt/wt</sup>Eyfp<sup>−</sup>-microglia in mouse 2. The top panel is the sequencing mapping on the CreERT2-Eyfp vector; the middle panel is the sequencing mapping on chromosome 9; the bottom panel is the schematic graph showing the homozygosity of the exon 2 of Cx3cr1. C Whole-genome sequencing of Cx3cr1<sup>CreERT2-Eyfp</sup> microglia. The top panel is the sequencing mapping CreERT2-Eyfp vector; the middle panel is the sequencing mapping the chromosome 9; the bottom panel is the schematic graph showing the heterogeneity of the exon 2 of Cx3cr1. D DNA electrophoresis of PCR product from EYFP<sup>−</sup> and EYFP<sup>+</sup> microglia. E Representative dot plots showing the percentage of EYFP<sup>F<sub>4</sub>/80<sup>4</sup></sup>, EYFP<sup>F<sub>4</sub>/80<sup>−</sup></sup>, EYFP<sup>F<sub>4</sub>/80<sup>−</sup></sup>, and EYFP<sup>F<sub>4</sub>/80<sup>−</sup></sup> repopulated microglia at day 42 after Tam in Cx3cr1<sup>CreERT2-Eyfp/Rosa26DTA<sup>wt/wt</sup></sup> mice, mean ± s.d.

Additional file 2: Table S1. Gene list including Gene symbols, Log2 fold changes, P-values and FDR values. The table shows FDR values and P-values in ascending order.

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Authors’ contributions
KZ and KB conceived the study and designed experiments. The RAH lab developed the microglia depletion mouse strains. KZ performed all the animal experiments and histological analysis. KZ, JH, YW and CX performed the flow cytometry analysis. HL, CZ, BJ, XZ, and RAH performed the morphological analysis. NRB prepared the RNA sequencing experiments and histological analysis. KZ performed all the animal experiments. KZ, JH, YW and CX performed the flow cytometry analysis. HL, CZ, BJ, XZ, and RAH edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data supporting the findings are available upon request from the corresponding author. The DNA and RNA sequencing data generated during the current study are available in the public repository GEO with accession numbers GSE186700 and PRJNA778933, respectively.
Declarations

Ethics approval
All experiments in this study were approved and performed following the Swedish National Board for Laboratory Animals’ guidelines and the European Community Directive (86/609/EEC) under the ethical permits N163/15, N127/16, and 13676-2020.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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