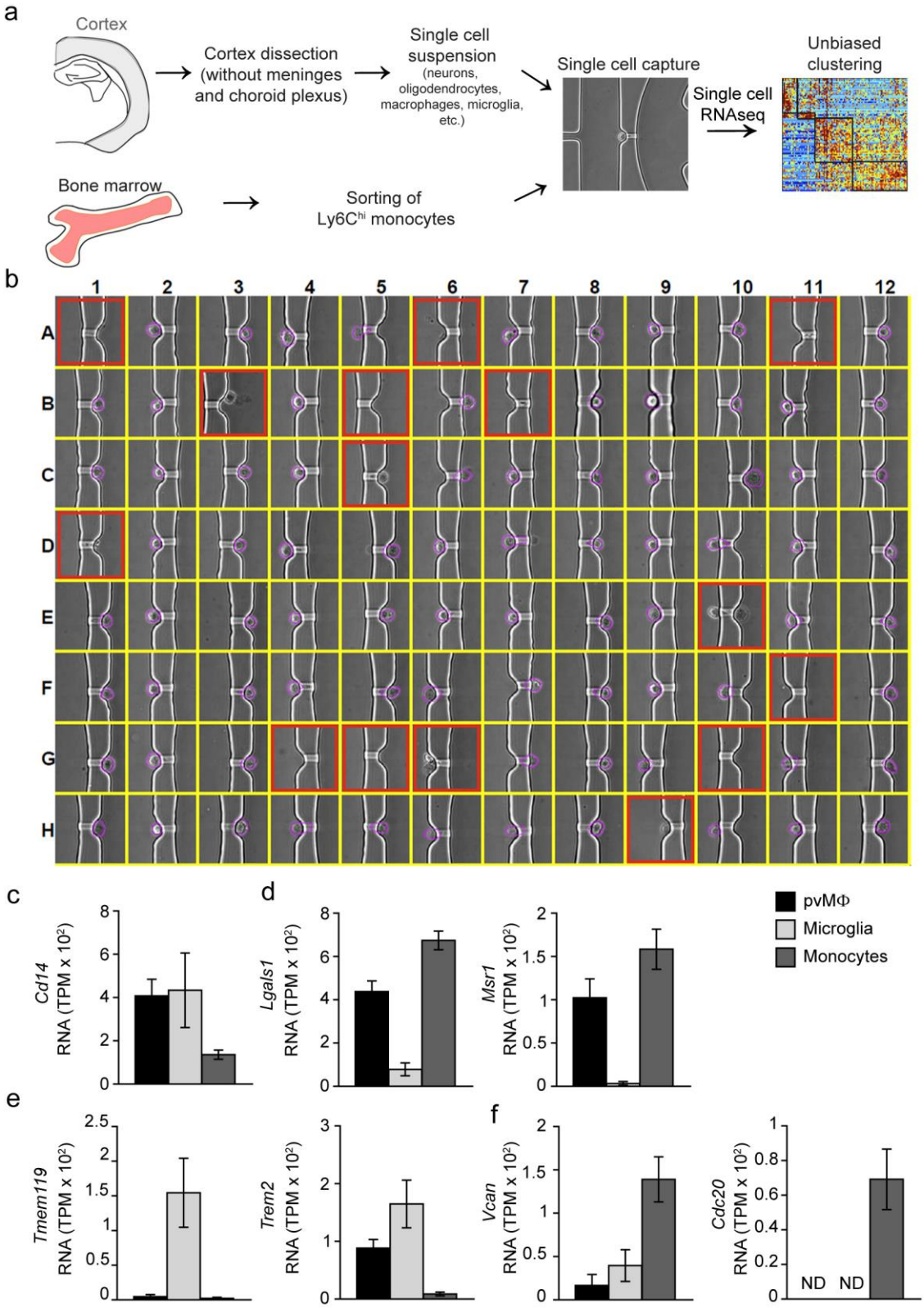


Supplementary Figure 1

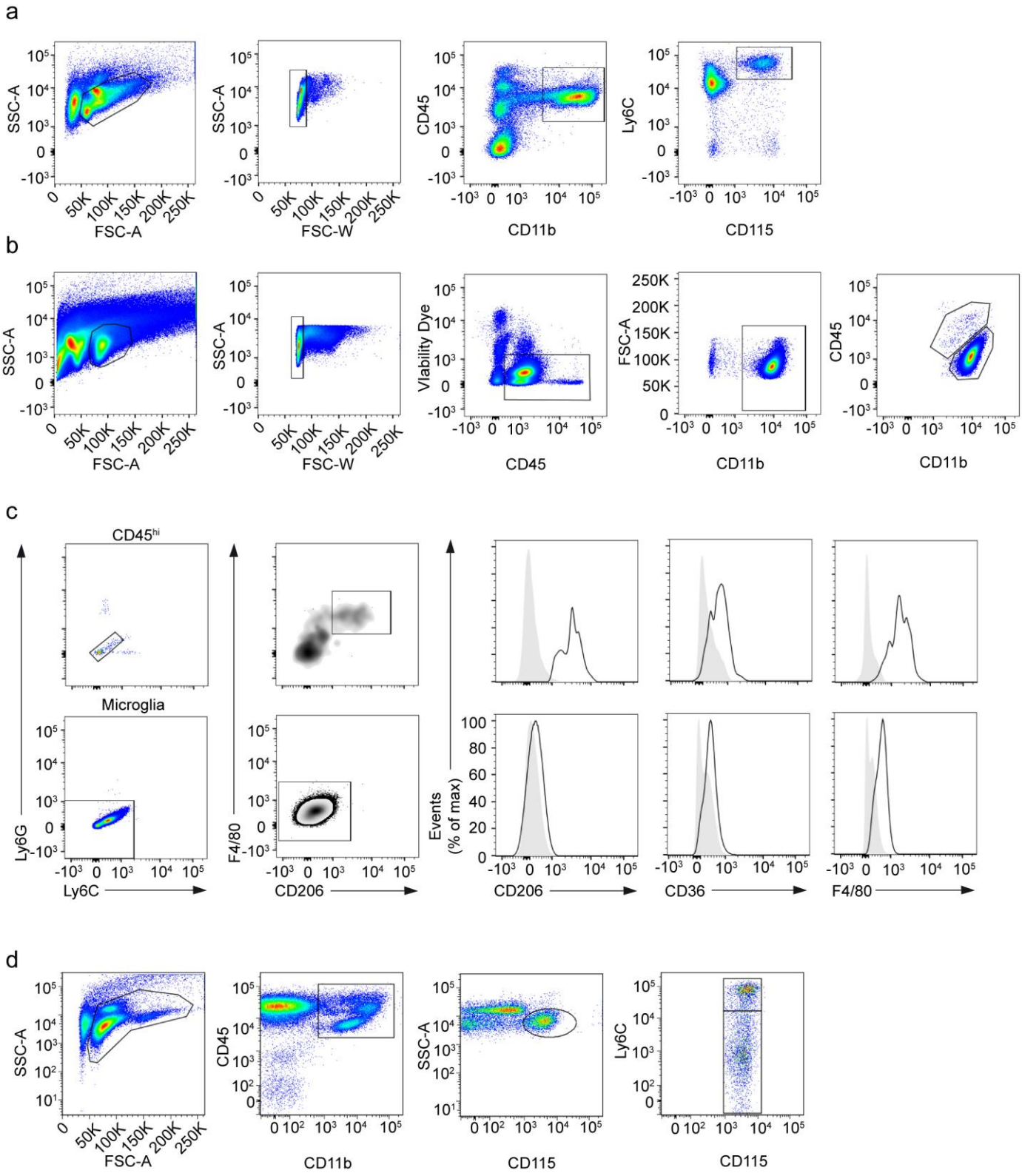
Single-cell RNA sequencing of pvMΦ, microglia and monocytes.

(a) Work flow for obtaining and analyzing single-cell RNA-seq from mouse cortical cells and monocytes from dissection to single-cell RNA-seq and unbiased biclustering.

(b) Representative image of single cells captured in the chip. All cells were checked individually by light microscopy and chambers with no or damaged cells (red squares) were omitted from subsequent analysis.

(c-f) Bar graphs for commonly expressed genes (c), markers selectively expressed by pvMΦ (**d**), selectively expressed by cortical microglia (e) or just by monocytes (f) evaluated by single cell RNA-seq.

Picture from cell capture for RNAseq is representative of one independent experiment (**b**). Single cell RNAseq data (**c-e**) is representative of two independent experiments with 167 ptMΦ, 246 monocytes, 33 microglia and 65 pvMΦ. Data is represented as mean ± s.e.m.



Supplementary Figure 2

Flow cytometry of cortical pvMΦ and microglia.

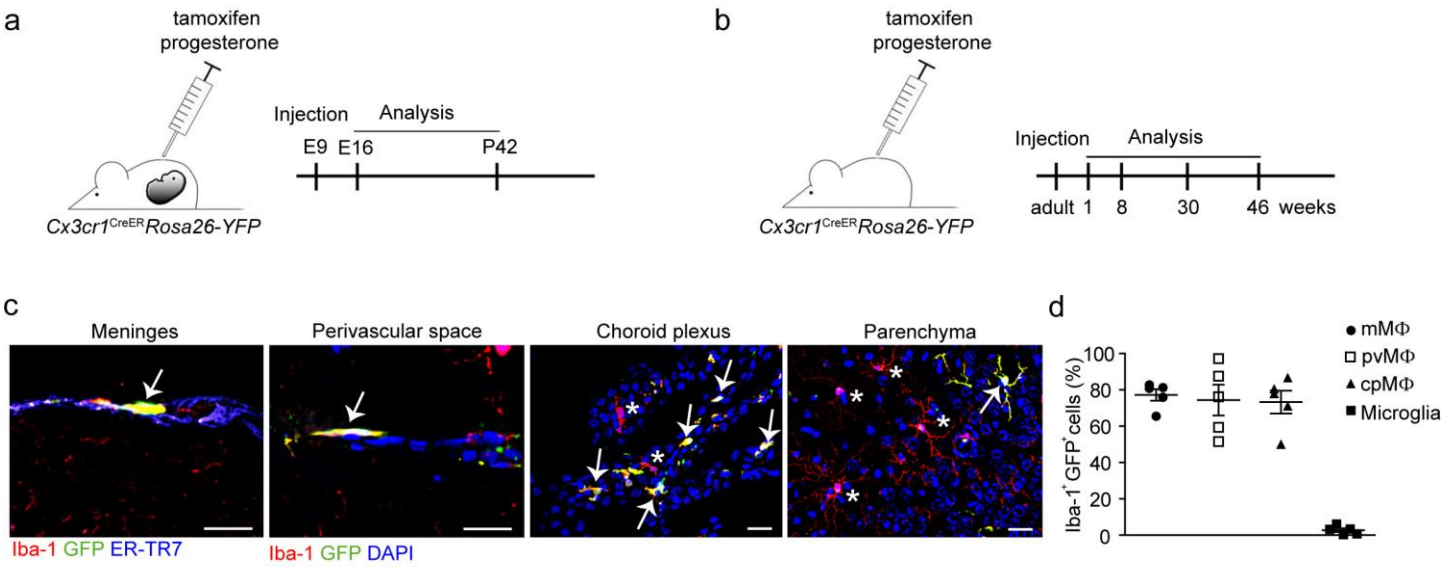
(a) Representative gating strategy for the flow cytometry-based isolation of bone marrow monocytes for subsequent single-cell RNA-sequencing as shown in Fig. 1d-g.

(b) Representative gating strategy for the isolation of cortical $CD11b^{+}$ $CD45^{lo}$ microglia and $CD11b^{+}$ $CD45^{hi}$ cells from the cortex (meninges and choroid plexus were removed) as shown in Fig. 1j-k.

(c) $CD11b^{+}$ $CD45^{hi}$ pvMΦ can be further separated from $CD11b^{+}CD45^{lo}$ microglia by gating them as $CD45^{hi}$ $CD11b^{+}$ $Ly6C^{-}$ $Ly6G^{-}$ $CD206^{+}$ $F4/80^{+}$ cells. Representative histograms for CD206, CD36 and F4/80 staining on pvMΦ and microglia (black line) are shown over isotype controls (filled gray).

d) Representative gating strategy for the flow cytometry-based characterization of Ly6C cells as shown in Fig. 6a-b. Peripheral blood leukocytes were gated according to physical parameters and further subdivided in myeloid cells by the expression of CD45 and CD11b. $CD45^{+}$ $CD11b^{+}$ SSC^{lo} $CD115^{+}$ monocytes are then further discriminated into $Ly6C^{hi}$ inflammatory monocytes and $Ly6C^{lo}$ patrolling monocytes.

Gating strategies are representative of six mice from two independent experiments (**a**, **d**) or from three biological replicates from two independent experiments (**b**, **c**).



Supplementary Figure 3

Irradiation induces engraftment of bone-marrow-derived CNS macrophages and microglia.

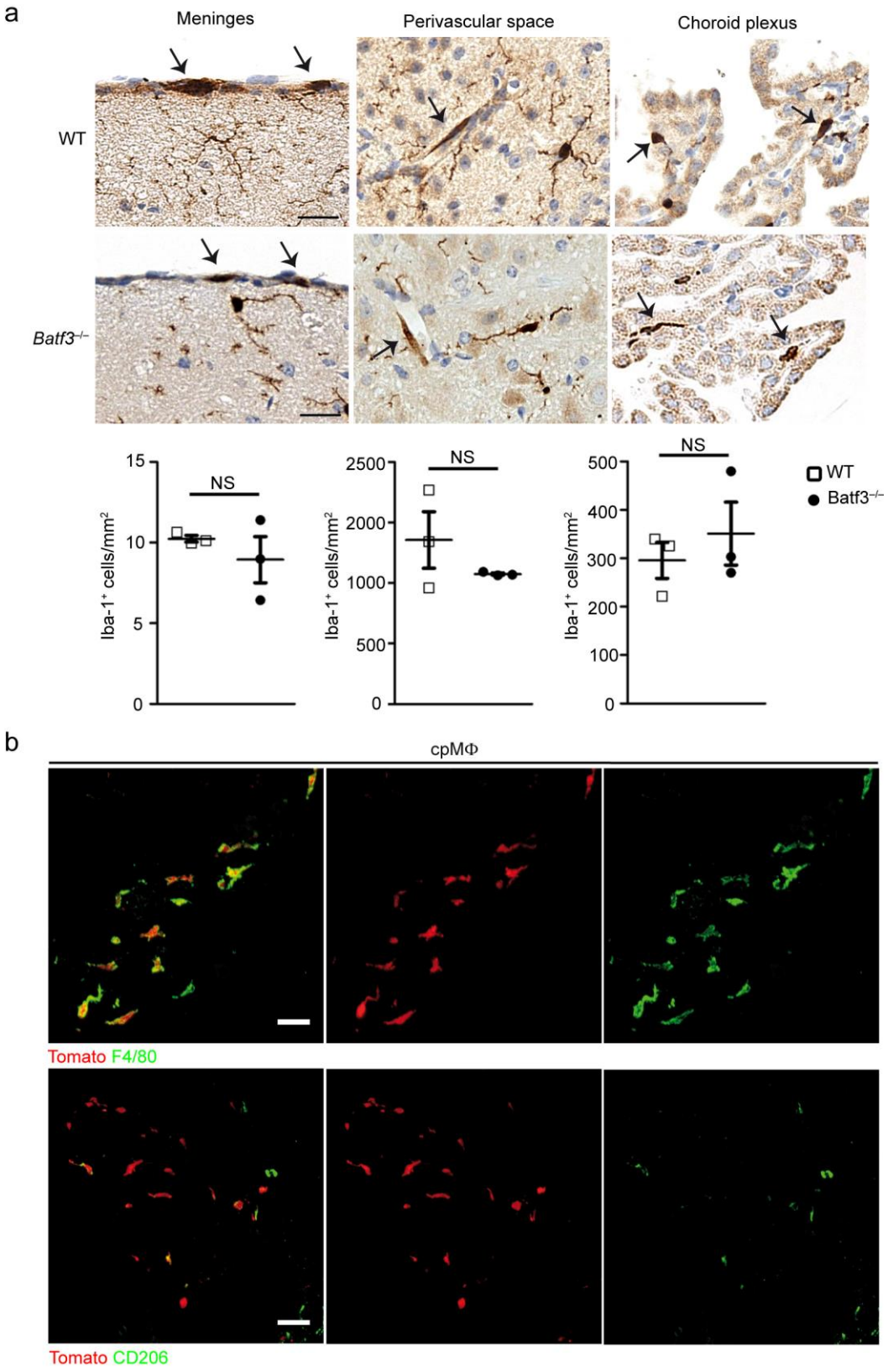
a) Scheme for the induction of recombination (injection of tamoxifen [TAM]) and subsequent analysis in *Cx3cr1*^{CreER}*Rosa26-YFP* animals.

b) Scheme and timeline for labelling and analyses of pvMΦ, mMΦ and cpMΦ in adulthood using TAM injection in adult *Cx3cr1*^{CreER}*Rosa26-YFP* animals.

c) Direct fluorescence microscopic visualization revealed numerous GFP+ donor-derived Iba-1^{+} pvMΦ, mMΦ, cpMΦ and few microglia 20 weeks after transfer of bone marrow from *Acta1*-GFP mice into lethally irradiated wild-type mice. Arrows indicate double positive, asterisks single Iba-1 (red) positive cells. Scale bar = 25 µm.

d) Quantification of donor-derived GFP+ Iba-1+ cells.

Immunofluorescence pictures (**c**) are representative of four mice from one independent experiment. Data were obtained from five mice per group from one independent experiment (**d**). Each symbol represents one mouse and three tissue sections per mouse were quantified. (means ± s.e.m.)



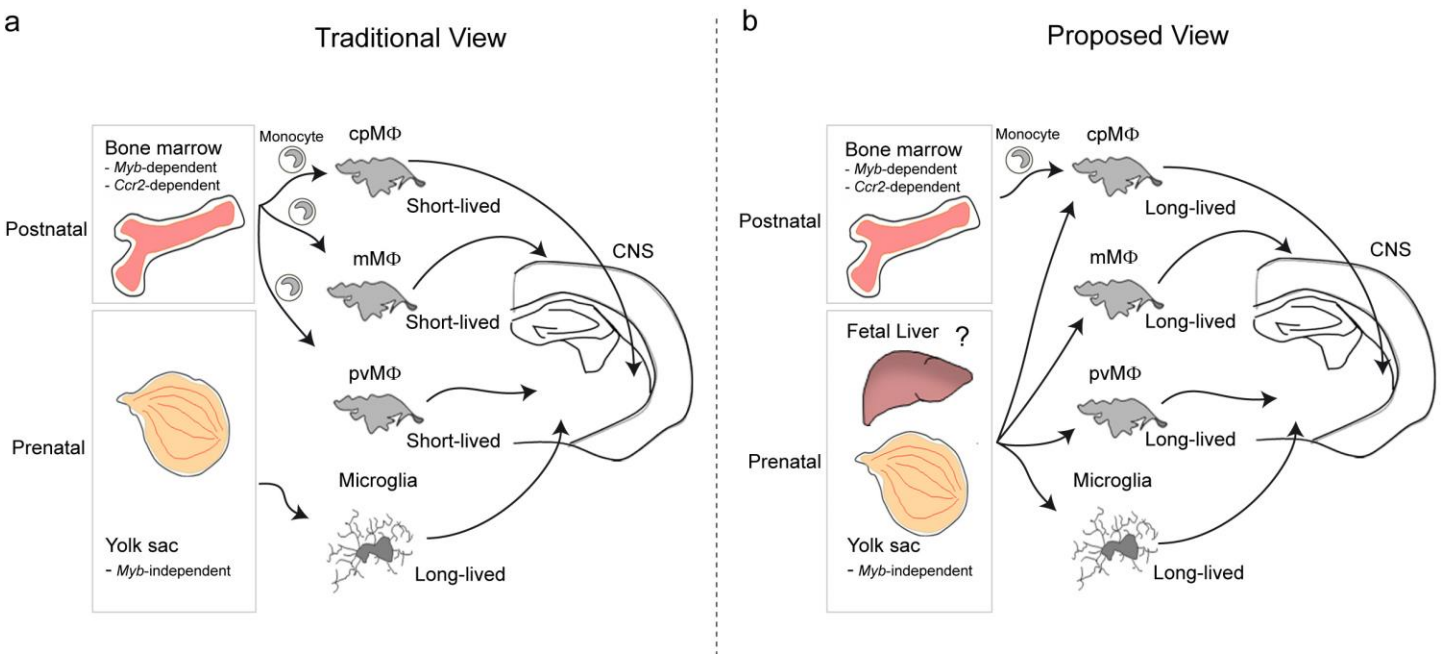
Supplementary Figure 4

CNS macrophages do not require *Batf3*.

a) Localization and presence of pvMΦ, mMΦ and cpMΦ in adult wild-type (WT) and *Batf3*$^{-/-}$ mice evaluated using Iba-1 immunohistochemistry. Representative figure are presented (upper images) and quantification thereof.

b) Confocal pictures showing Tomato^{+} cells in the choroid plexus of *Cx3cr1*^{CreER}*Rosa26-Tomato* mice (tomato, red) at 8 weeks after TAM expressing the macrophage marker F4/80 and to a much lesser extent CD206. Scale bar: 25 µm.

Immunofluorescence pictures are representative of of five mice from two independent experiments (**b**). Immunohistochemistry pictures (**a**) are representative of three mice per genotype from one independent experiment. Macrophage density (a) is representative of three mice per genotype from one independent experiment (Meninges: P= 0.7; perivascular space: P=0.7; choroid plexus P= 1). Mann Whitney test was applied. Each symbol represents one mouse with quantification of a minimum of three tissue sections. (error bars, s.e.m.). N.S. = not significant.



Supplementary Figure 5

Model for experimental findings on the origin, fate and dynamics of microglia and macrophages at CNS boundaries.

Traditional and proposed view on the origin, fate and turnover of non-parenchymal CNS macrophages and microglia.