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Microglia in the adult brain arise from Ly-6C^{hi}CCR2^{+} monocytes only under defined host conditions

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Microglia are crucially important myeloid cells in the CNS and constitute the first immunological barrier against pathogens and environmental insults. The factors controlling microglia recruitment from the blood remain elusive and the direct circulating microglia precursor has not yet been identified *in vivo*. Using a panel of bone marrow chimeric and adoptive transfer experiments, we found that circulating Ly-6C^{hi}CCR2^{+} monocytes were preferentially recruited to the lesioned brain and differentiated into microglia. Notably, microglia engraftment in CNS pathologies, which are not associated with overt blood-brain barrier disruption, required previous conditioning of brain (for example, by direct tissue irradiation). Our results identify Ly-6C^{hi}CCR2^{+} monocytes as direct precursors of microglia in the adult brain and establish the importance of local factors in the adult CNS for microglia engraftment.

Microglia are an integral part of the resident mononuclear phagocyte population in the CNS. These cells share many phenotypical and functional characteristics with other tissue macrophages and with peripheral blood monocytes, suggesting that microglia participate in many immune reactions of the brain[1]. Microglia continuously monitor their local microenvironment with highly motile processes and constitute the first line of defense against invading pathogens[2,3].

Microgliosis is a rather poorly understood process, despite it having a probable role in the pathogenesis of various CNS diseases[4]. One major issue is to determine the extent to which circulating monocytes contribute to the microglial response in the brain. Despite tremendous efforts in the past years, monocyte-derived and resident CNS parenchymal microglia remain virtually indistinguishable on the basis of known immunophenotypic markers, although they might be functionally heterogeneous[5]. Using chimeric mice, in which bone-marrow cells (BMCs) were marked with the green fluorescent protein (GFP), MHC class II, Y chromosome, congenic CD45.1/CD45.2 molecules and others, bone marrow–derived cells were shown to enter the brain during postnatal development and to differentiate into microglia[6–8], but not into other glial cell types[9]. Subsequent studies in mice have even demonstrated enhanced microglia engraftment in several disease models, such as spongiform encephalopathies[10,11], bacterial meningitis[12], experimental autoimmune encephalomyelitis[13], Parkinson's disease[14] and Alzheimer's disease[5,15,16]. Notably, bone marrow–derived microglia appeared to be functionally relevant in the brain; for example, they phagocytosed β-amyloid in Alzheimer's disease transgenic mice, whereas resident microglia seemed to be rather ineffective in removing β-amyloid[5]. All of these chimeric mice studies used total body irradiation as the conditioning regimen for the bone-marrow transplantation, thereby exposing the brain to potential irradiation-induced changes in the local microenvironment. Indeed, the few studies using nonirradiated parabiotic mice detected substantially less engrafted microglia in the brains[17]. Therefore, we designed a set of experiments using bone-marrow chimeric approaches to define the local conditions in the brain that allow the entry of blood-derived monocytes into the CNS and their differentiation into parenchymal microglia.

Furthermore, the direct circulating precursor of microglia in the peripheral blood has not yet been identified *in vivo*. Prevailing concepts view monocytes as intermediary cells that continuously develop in the bone marrow, circulate in the blood and migrate into tissues, where they become local macrophages, dendritic cells or other tissue descendants[18]. The identification of monocyte heterogeneity has led to the hypothesis that monocytes commit for specific functions while still in the circulation[19]. Indeed, both human and mouse monocytes fall into at least two phenotypically distinct subsets: Ly-6C^{hi} (which are Gr-1^{+}CCR2^{+}CX$_3$CR1^{lo}) and Ly-6C^{lo} (which are Gr-1^{-}CCR2^{-}CX$_3$CR1^{hi}) mouse monocytes, which correspond to human CD14^{hi}CD16^{-} and CD14^{+}CD16^{+} monocytes, respectively[20,21]. It is generally thought that Ly-6C^{hi} cells preferentially populate sites of experimentally induced inflammation, whereas their Ly-6C^{lo} counterparts can enter lymphoid and nonlymphoid tissues under homeostatic conditions[22].

Here we demonstrate that circulating Ly-6C^{hi}CCR2^{+} monocytes are direct circulating precursors of microglia in the blood. Genetic or antibody-mediated depletion of this specific monocyte subtype vastly prevented the recruitment of monocytes into the lesioned brain.

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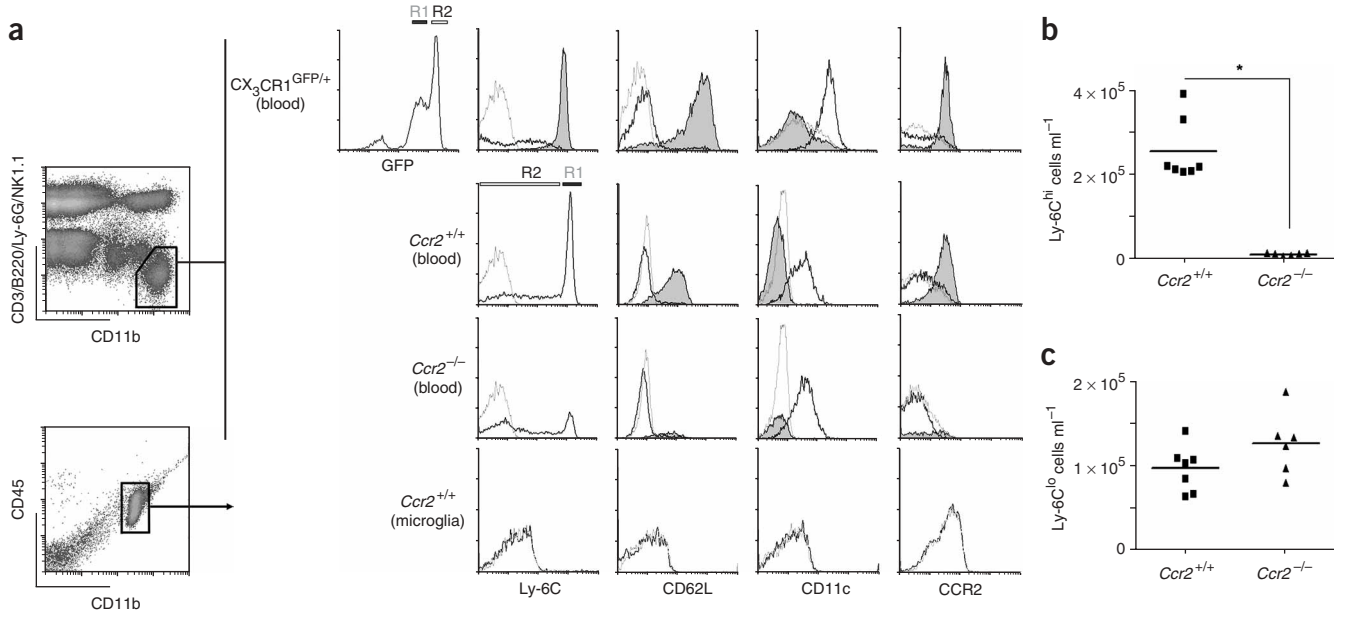

Figure 1 Brain-specific macrophages (microglia) have an immunomarker profile similar to that of resident blood monocytes. (**a**) Blood monocytes (upper left density plot) of *Ccr2*$^{+/+}$, *Ccr2*$^{-/-}$ and CX$_3$CR1$^{GFP/+}$ mice, and *ex vivo*–isolated microglia (lower left density plot) of *Ccr2*$^{+/+}$ mice were characterized by surface expression of the markers Ly-6C, CCR2, CD62L, CD11b and CD11c. Living blood cells were gated to determine the presence of CD11b^{hi}CD3^{lo}B220^{lo}Ly-6G^{lo}NK1.1^{lo} monocytes (upper left). CX$_3$CR1$^{GFP/+}$ mice were used to distinguish between CX$_3$CR1^{hi} (R2, Ly-6C^{lo}) and CX$_3$CR1^{lo} (R1, Ly-6C^{hi}) blood monocytes. Percoll gradient–isolated microglia were gated as a CD45^{lo}CD11b^{+} cell population in the brain (lower left). R2 gates (thick lines) and R1 gates (filled curves) in the blood indicate Ly-6C^{lo} and Ly-6C^{hi} monocytes, respectively. Isotype controls are shown as thin lines. (**b**) Adult CCR2-deficient mice showed a distinct lack of Ly-6C^{hi} monocytes in the peripheral blood. Each symbol represents one individual mouse. The mean is indicated. Asterisk indicates statistical significance ($P < 0.05$). (**c**) No significant difference in the amount of Ly-6C^{lo} monocytes was found in the peripheral blood of WT versus *Ccr2*$^{-/-}$ mice. Each symbol represents one individual mouse. The mean is indicated.

Demyelinating and neurodegenerative CNS disease models without blood-brain barrier disruption were not sufficient to induce substantial microglia engraftment during adulthood, but additional host endogenous factors, such as irradiation-induced gene expression, were required to condition the adult brain for microglia engraftment.

RESULTS

Microglia display surface markers of specific monocytes

To test the hypothesis that a distinct subset of circulating monocytes represents the direct precursor of microglia in the adult brain, surface immunomarker expression of microglia was compared with circulating monocyte populations (**Fig. 1**). Monocytes in the peripheral blood were defined as CD11b^{hi}CD3^{lo}B220^{lo}Ly-6G^{lo}NK1.1^{lo} mononuclear cells by flow cytometry as described previously (**Fig. 1a**)[23]. We took advantage of the CX$_3$CR1$^{GFP/+}$ mice (GFP is inserted into one allele of the CX$_3$CR1 locus), which allow division of the Ly-6C^{hi} and Ly-6C^{lo} fractions on the basis of GFP expression levels. Microglia were subsequently defined as being CD45^{lo} and CD11b^{+} according to the literature[24]. Notably, isolated microglia were CD62L^{-}CCR2^{-}Ly-6C^{lo}, which was similar to the immunoprofile found on resident Ly-6C^{lo} monocytes. This monocyte population, however, was reported to be CD11c^{+} (ref. 23), which was not the case in isolated adult microglia.

A previous report claimed that the chemokine receptor CCR2 might also be required for the emigration of Ly-6C^{hi}CCR2^{+} monocytes from the bone marrow into the circulation[25]. Theoretically, this could cause a deficiency of this specific monocyte subpopulation in the peripheral blood stream. To test this hypothesis, we counted the number of Ly-6C^{hi} monocytes in the blood of 8-week-old adult *Ccr2*$^{-/-}$ mice and in age- and sex-matched *Ccr2*$^{+/+}$ mice (**Fig. 1b**). The amount of Ly-6C^{hi} monocytes was substantially reduced in CCR2-deficient mice compared with the wild-type (WT) situation (mean ± s.e.m., 1.2 ± 0.1 × 10^4 cells ml^{-1} in *Ccr2*$^{-/-}$ and 25.6 ± 2.8 × 10^4 cells ml^{-1} in WT mice). To ensure that only this specific monocyte subtype was affected, we examined the amount of Ly-6C^{lo} monocytes (**Fig. 1c**). Notably, we found an unchanged amount of Ly-6C^{lo} monocytes in CCR2-deficient mice compared to WT (mean ± s.e.m., 12.6 ± 1.7 × 10^4 cells ml^{-1} in *Ccr2*$^{-/-}$ and 9.7 ± 1.0 × 10^4 cells ml^{-1} in WT mice).

CCR2-independent development and function of microglia

Because the chemokine receptor CCR2 is crucial for monocyte and macrophage function (for example, for the chemotactic attraction during inflammation[26]), we investigated a potential role for CCR2 in the development and immunological properties of microglia. Morphology of brain microglia in the absence of CCR2 was assessed by using immunofluorescence staining with the macrophage and microglia marker Iba-1 (red, **Fig. 2a**). In different regions of the cerebellum (molecular layer, granular cell layer and white matter), Iba-1^{+} parenchymal cells with typical round to spindle-shaped microglial morphology and distinct arborization pattern were undistinguishable in *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ brains (**Fig. 2a**, left). Quantitative morphometric analysis revealed comparable numbers of parenchymal Iba-1^{+} microglia in different regions of the cerebellum. Cell dimension, as judged by the microglia occupying area (908 ± 40 μm^2 in *Ccr2*$^{-/-}$ and 957 ± 14 μm^2 in *Ccr2*$^{+/+}$), was similar irrespective of the genotype (**Fig. 2a**, right).

We next examined the microglial ability to respond to the Toll-like receptors 2 and 4 ligands, lipopolysaccharide (LPS) and the lipopeptide Pam$_3$CSK$_4$, in a dose-dependent manner (**Fig. 2b**). Both CCR2-deficient and competent microglia produced similar amounts of the proinflammatory cytokines interleukin 6 (IL-6) and tumor

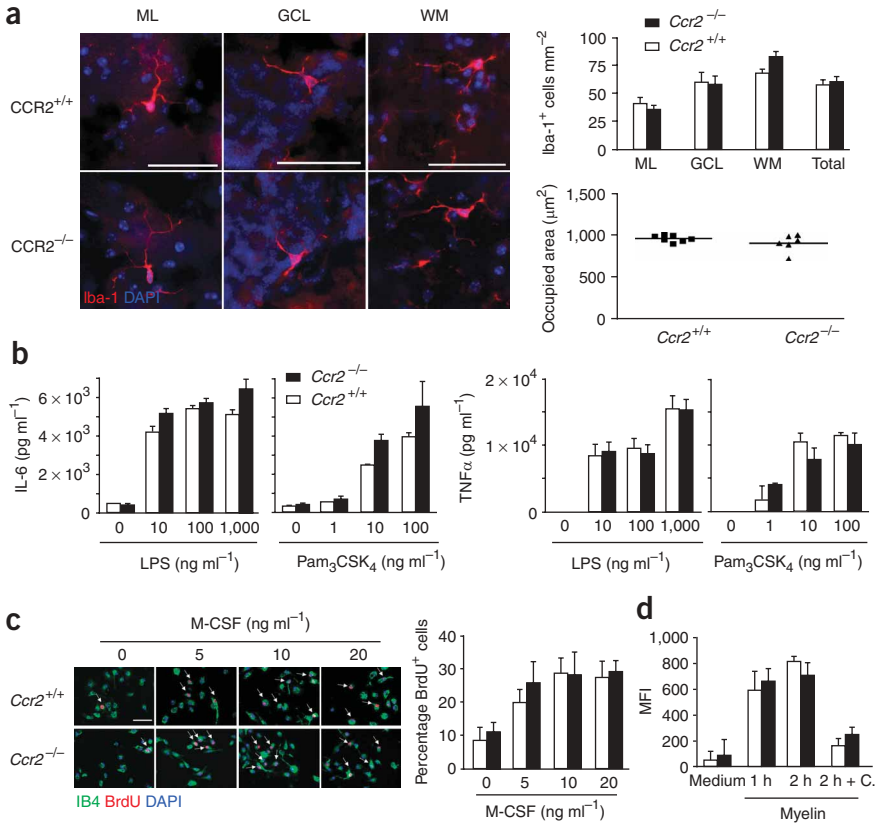

Figure 2 Normal development, morphology and function of microglia in the absence of CCR2. (**a**) Iba-1 immunohistochemistry (red) and DAPI staining of nuclei (blue) of cerebellar sections from 8-week-old $Ccr2^{+/+}$ and $Ccr2^{-/-}$ mice revealed the same morphology and arborization pattern of parenchymal microglia in the molecular layer (ML), granule cell layer (GCL) and in the white matter (WM, upper left). Scale bars represent 40 μm. The same number of Iba-1$^+$ ramified parenchymal cells was found in different localizations of the cerebellum (upper right). Data are expressed as means ± s.e.m. At least eight mice were examined for each group. Iba-1–immunoreactive areas in the cerebellar WM were quantified by determining the areas covered by the outer-most cell processes (lower right). For each mouse, at least 25 cells were assessed quantitatively. Each symbol represents the mean measurements in one mouse. The mean is indicated for each group. (**b**) Unaltered cytokine response in $Ccr2^{-/-}$ microglia. $Ccr2^{+/+}$ (white bars) or $Ccr2^{-/-}$ microglia (black bars) were exposed to increasing dosages of either LPS or Pam_3CSK_4, and production of TNFα and IL-6 was measured by ELISA 18 h after exposition. Data represent means ± s.e.m. of three independent experiments. No statistically significant differences were detected ($P > 0.05$). (**c**) Microglia cell proliferation does not require CCR2. Primary microglia were treated with different concentrations of M-CSF for 48 h. We added 10 μM BrdU for 1 h and the cells were stained for isolectin B4 (green), BrdU (red) and DAPI (blue). Representative images are shown in the left panel. Scale bar represents 50 μm. Quantification of the percentage of BrdU$^+$ microglia revealed no differences in the proliferative responses of $Ccr2^{+/+}$ (white bars) or $Ccr2^{-/-}$ (black bars) microglia to M-CSF. (**d**) Mean fluorescence intensities (MFI) of $Ccr2^{+/+}$ (white bars) and $Ccr2^{-/-}$ (black bars) microglia ingesting fluorescein isothiocyanate–labeled myelin after 1 h, 2 h or after exposure of myelin with cytochalasin D for 2 h (+ C). Data represent means ± s.e.m. of three independent experiments.

necrosis factor α (TNFα) after ligand challenge. In addition, surface expression of MHC class II molecules was upregulated to a similar degree after interferon γ (IFNγ) stimulation (**Supplementary Fig. 1** online). Recombinant IFNβ, in turn, counteracted the IFNγ-induced MHC class II expression levels to a similar extent in $Ccr2^{+/+}$ and $Ccr2^{-/-}$ primary microglia. Because CCR2 may not only affect the activation response, we also investigated the effect of CCR2 on microglia proliferation (**Fig. 2c**). No difference was found in the ability of macrophage colony-stimulating factor (M-CSF) to stimulate microglia proliferation in the presence or absence of CCR2. We next exposed cultured microglia to fluorescently labeled myelin and measured the kinetics and quantity of myelin phagocytosis (**Fig. 2d**). Myelin uptake by microglia was independent of CCR2 expression, and could be specifically inhibited by incubation with cytochalasin D. Taken together, our data indicate that microglial development, morphology and innate immune functions are preserved in the absence of CCR2.

Postnatal microglia derive from Ly-6C^{hi}CCR2$^+$ monocytes

As a result of the strong and specific reduction of the Ly-6C^{hi} subpopulation in the absence of CCR2, $Ccr2^{-/-}$ mice are a suitable tool for determining the monocyte fraction that might be the direct precursor of adult microglia in the peripheral blood. To do so, we generated double-transgenic mice by intercrossing ACTβ (β-actin)-EGFP mice with $Ccr2^{-/-}$ mice. These mice express GFP on the $Ccr2^{+/+}$ ($Ccr2^{+/+}$GFP) or the $Ccr2^{-/-}$ ($Ccr2^{-/-}$GFP) background, respectively. To distinguish invading bone marrow–derived monocytes from brain endogenous microglia during health and disease, we created bone-marrow chimeras. Respective GFP-marked BMCs were mixed with unlabeled WT BMCs (at a 1:3 ratio) and transplanted into lethally whole-body irradiated (including the brain) adult recipient mice. In the resulting $Ccr2^{+/+}$GFP → $Ccr2^{+/+}$ chimeras ($Ccr2^{+/+Ch}$), both monocyte subsets express GFP, whereas chimeric $Ccr2^{-/-}$ GFP → $Ccr2^{+/+}$ mice ($Ccr2^{-/-Ch}$) express the fluorophore predominantly on Ly-6C^{lo} monocytes. As expected, peripheral blood Gr-1^{hi} (Ly-6C^{hi}) monocytes were strongly diminished in chimeric $Ccr2^{-/-Ch}$ mice compared with $Ccr2^{+/+Ch}$ mice 4 weeks after bone-marrow transfer (exemplified in **Fig. 3a**). The general reconstitution efficacy, however, was similar in $Ccr2^{-/-Ch}$ and $Ccr2^{+/+Ch}$ mice, as indicated by comparable numbers of GFP$^+$CD3$^+$ and GFP$^+$B220$^+$ lymphocytes (**Fig. 3b**).

To assess the ability of GFP-marked monocyte subsets derived from transplanted bone marrow to differentiate into microglia in the nondiseased brain, we analyzed recipient mice histologically 12 weeks after transplantation (**Fig. 3c–e**). Microscopical investigation of several CNS regions, such as the cerebellum, brain stem (**Fig. 3c**) and spinal cord (**Fig. 3e**), revealed some parenchymal cells with typical microglial morphology. These donor-derived GFP$^+$ cells were Iba-1$^+$ (**Fig. 3c,e**), as well as for the pan-macrophage marker F4/80 (**Fig. 3d**). Moreover, the engrafted cells were MHC class II$^-$ and were CD45^{lo}, as determined by fluorescent-activated cells sorting (FACS) analysis (data not shown), providing further evidence for their microglial identity. Notably, however, engrafted GFP$^+$Iba-1$^+$ cells were predominantly found in the CNS of $Ccr2^{+/+Ch}$ mice, whereas the number of engrafted microglia