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The Fractalkine Receptor but Not CCR2 Is Present on Microglia from Embryonic Development through Adulthood

Makiko Mizutani,* Paula A. Pino,†‡ Noah Saederup,§ Israel F. Charo,§ Richard M. Ransohoff,* and Astrid E. Cardona†‡

Microglial cells are difficult to track during development because of the lack of specific reagents for myeloid subpopulations. To further understand how myeloid lineages differentiate during development to create microglial cells, we investigated CX3CR1 and CCR2 transcription unit activation in Cx3cr1<sup>−/−</sup>GFPCCR2<sup>−/−</sup>RFP knockin fluorescent protein reporter mice. The principal findings include: 1) CX3CR1<sup>+</sup> cells localized to the aorta–gonad–mesonephros region, and visualized at embryonic day (E)9.0 in the yolk sac and neuroectoderm; 2) at E10.5, CX3CR1 single-positive microglial cells were visualized penetrating the neuroepithelium; and 3) CX3CR1 and CCR2 distinguished infiltrating macrophages from resident surveillant or activated microglia within tissue sections and by flow cytometric analyses. Our results support the contribution of the yolk sac as a source of microglial precursors. We provide a novel model to monitor chemokine receptor expression changes in microglia and myeloid cells early (E8.0–E10.5) in development and during inflammatory conditions, which have been challenging to visualize in mammalian tissues.


Microglia, the resident tissue macrophages and intrinsic “immune effector” cells of the CNS, constitute ~20% of the total glial population and are distributed throughout the brain, spinal cord, and optical tissues (1). In CNS leukocyte preparations, CD45 is used to separate the CD45<sup>hi</sup> hematogenous population from CD45<sup>lo</sup> resident microglia by flow cytometry (2, 3). Characterization of MHC class II (MHC-II) expression and upregulation of activation markers such as CD40, CD44, and CD86 have provided insights into the effector functions of activated microglia during CNS inflammation (4). However, there are still debates regarding the alterations of CD45 expression by monocytes and CNS-born microglial cells. Currently, no simple combination of markers facilitates the identification and differentiation of resident surveillant or activated microglia from hematogenous monocytes.

The discovery of the chemokine receptor CX3CR1 in 1994 and its unique ligand fractalkine in 1997 represented a major advancement in the understanding of microglial and monocyte function (5–8). Fractalkine is a distinct chemokine, expressed as a transmembrane glycoprotein on neurons and peripheral, but not CNS, endothelial cells (5, 9). The fractalkine receptor (CX3CR1) is present on microglia and circulating monocytes, dendritic cells, NK cells, and T cells (10–12). Membrane-bound fractalkine on endothelial cells mediates adhesion of CX3CR1<sup>+</sup> leukocytes, and proteolytic cleavage releases the soluble domain that confers fractalkine its chemotactic properties (5). Importantly, CX3CR1 distinguished a monocyte subset in peripheral blood so-called resident, phenotypically identified as LFA-1<sup>+</sup>, L-Sel<sup>−</sup>, Ly6C<sup>−</sup>, CCR2<sup>−</sup>, CX3CR1<sup>+</sup>, whereas CCR2, the MCP-1 receptor (MCP-1 or CCL2), marked “inflammatory” monocytes LFA-1<sup>+</sup>, L-Sel<sup>+</sup>, Ly6C<sup>+</sup>, CCR2<sup>+</sup>, and CX3CR1<sup>−</sup> (13–15). Circulating resident monocytes patrol healthy tissues through long-range crawling on the healthy endothelium (13), playing a critical role in immune surveillance. In contrast, CCR2<sup>−/−</sup>CX3CR1<sup>−/−</sup>Ly6C<sup>+</sup> monocytes are typically found in inflamed tissues (16). The generation of CX3CR1/GFP knockin mice, in which the CX3CR1 was disrupted by insertion of GFP, clarified the expression pattern and has facilitated the tracking of CX3CR1<sup>+</sup> leukocytes in vivo. In the naive CNS of Cx3cr1<sup>−/−</sup> and Cx3cr1<sup>−/−</sup>GFP mice, CX3CR1 transcription unit was exclusively active in microglial cells, as GFP expression was undetectable in neurons, NG2<sup>+</sup> glia, and GFAP<sup>+</sup> astrocytes (12).

Because of the lack of appropriate anti-CCR2 Abs, most CCR2 expression analyses have been limited to mRNA levels (17). It has been proposed that microglia activation during inflammatory settings upregulate CCR2 (18, 19). However, microglial CCR2 protein expression has been challenging to prove in situ. The recent generation of red fluorescent protein (RFP)-CCR2 knockin mice has provided the tools to potentially fractionate CX3CR1 and CCR2 activation in microglial and peripheral monocytes and study their trafficking, molecular signatures, and biological functions (20). CCR2-RFP mice showed an accurate correlation of CCR2 and RFP expression in blood monocytes both at the protein and mRNA levels. Characterization of Ccr2<sup>−/−</sup>GFP mice revealed that in response to thioglycolate, CCR2 was required for in vivo migration of monocytes to the inflamed peritoneum. In mice with...
experimental autoimmune encephalomyelitis (EAE), CCR2 was critical for efficient accumulation of LyC6loCCR2hi monocytes. Interestingly, during CNS inflammation, monocytes that infiltrate the CNS maintained their signature chemokine receptor profiles as found in circulating monocytes (20). Importantly, microglia in the adult naïve and inflamed CNS were found as C3XR1hiCCR2h, using the described Cx3cr1GFP/Ccr2RFP reporter mice. CNS-infiltrating monocytes appeared as CCR2hiCX3CR1hi or CCR2loCX3CR1lo. Notably, Cx3cr1 expression by microglia was significantly higher when compared with blood C3XR1hi monocytes.

Given the fact that C3XR1 is predominantly expressed by “resident tissue monocytes,” we sought to investigate C3XR1 and CCR2 transcription unit activation patterns in microglial precursors as they colonize the developing CNS. For this, CCR2-RFP and C3XR1-GFP mice were crossed and Cx3cr1GFP/Ccr2RFP embryos analyzed at various stages during embryonic development. The results indicate that C3XR1 is active in early microglial precursors and sustained throughout adulthood. In contrast, RFP as reporter of activation of the CCR2 transcription unit was not significantly detected in developing microglia. Therefore, we propose C3XR1 and CCR2 as markers to easily differentiate infiltrating macrophages from resident fluorescent or activated microglia in tissue sections and by flow cytometric analyses. Importantly, the results support the contribution of the yolk sac as the source of microglial precursors. These results bring potential applications to monitor neuronal–glial interactions throughout development and to further the understanding of microglial/monocyte/macrophage and dendritic cell plasticity in vivo.

Materials and Methods

Mice

Cx3cr1GFP/RFP and C3XR1GFP/GFP mice were crossed at the Biological Resources Unit, Cleveland Clinic and the Laboratory Animal Resources Unit at the University of Texas at San Antonio. Timed pregnancies were set up to isolate embryos at embryonic stages 8.5–13.5. All experiments were performed in accordance with National Institutes of Health guidelines and approved by the Cleveland Clinic and the University of Texas at San Antonio Institutional Animal Care and Use Committees.

Tissue processing

Female mice were sacrificed by CO2 asphyxiation, and embryos dissected and kept in cold PBS. A small tissue (~1 mm) biopsy was taken from the caudal part of the embryo for genotyping purposes. Embryos (embryonic day [E]8.5–E9.5) were fixed in 4% PFA overnight (ON), and whole embryos were permeabilized ON in 3% Triton X-100. Alternatively, after fixation, embryos (>E10.5) were transferred sequentially to 15, 20, and 25% sucrose solutions and 10-μm cryosections were obtained, or 60-μm free-floating sections were collected by sectioning embryos on a sliding microtome (12).

Generation of bone marrow chimeric mouse

Recipient Cx3cr1GFP/Ccr2RFP CD45.2 mice (5–6 wk old) irradiated with a dose of 900 rad were reconstituted with 20 × 106 cells isolated from C57BL/6 CD45.1 donor mice as previously described (21). Six weeks after reconstitution, mice were bled via the submandibular vein, heparinized blood was lysed, and efficiency of reconstitution was determined by flow cytometry using anti-CD45.2 and anti-CD45.1 Abs. Chimeric mice were allowed to reconstitute for 10 wk after bone marrow transfer before EAE induction.

Microscopic analysis

Whole embryos or selected sections were rinsed in 1× PBS, covered with 2 ml of a solution containing 450 nM DAPI in PBS, and incubated for 10 min at room temperature. Excess DAPI was absorbed on paper towels and slides rinsed in PBS. To visualize the vasculature, tissues were permeabilized ON in 3% Triton in PBS and incubated at 4°C for 2 d with biotin-conjugated isocitrate-B4 (IB4). Streptavidin-Cy5 was used to detect the bound biotin-IB4. DAPI-counterstained tissues were visualized under a fluorescent microscope. Confocal images were obtained as projections of 29-μm-thick sections in the z-axis scanned at 1.5-μm steps. In some experiments, brain tissues were dissected from adult mice at peak of EAE disease and tissues sections incubated at 4°C with 7-4 Ab (Cedarlane, Burlington, ON, Canada), and imaged at 40× magnification on a Zeiss 510 confocal microscope as previously described (20).

EAE induction

Active EAE was induced by s.c. immunization with myelin oligodendrocyte glycoprotein 35–55 peptides in CFA as previously described (22). Mice were weighed and examined daily for EAE symptoms and scored as follows: 0, no signs of neurologic disease; 1, lack of tail tone; 2, abnormal gait, hind-limb weakness; 2.5, partial hind-limb paralysis; 3, complete hind-limb paralysis; 3.5, ascending paralysis; 4, tetraplegia; and 5, death. Mice were sacrificed when they reached a score of 2.5–3.0 (21). Brain tissues were obtained from PFA perfused mice and free-floating 30-μm tissues obtained after cryoprotection and confocal images obtained.

Isolation of brain mononuclear cells and flow cytometry

For flow cytometry analyses, perfused brain and spinal cord tissues were dissected and mononuclear cells separated over discontinuous 70/30 Percoll gradients as previously described (23), and cellular pellets were resuspended in cell staining buffer (Biologend, San Diego, CA). Blood was collected from the submandibular vein; RBCs were depleted by hypotonic lysis and washed in staining buffer. Isolated cells were incubated on ice for 5 min with anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen) to block FcRs and then incubated on ice for 30 min with a mix of fluorochrome-conjugated anti-mouse Abs: CD45.1-Pacific blue (clone A20; eBioscience), CD45.2-allophycocyanin (clone 104; eBioscience) and CD11b-PerCP (clone M1/70; Biologend), or CD45.1-PE-Cy7 (clone A20; eBioscience), CD45.2-allophycocyanin (clone 104; eBioscience), CD11b-PerCP (clone M1/70; Biologend), and 1-AF-E Pacific blue clone (clone M5/114.15.2). After washes, cells were resuspended in 2% paraformaldehyde and acquired in an LSRII (BD Biosciences, Franklin Lakes, NJ).

Results

CX3CR1-GFP reporter expression colocalizes to early microglial cells that colonize the developing CNS

Whole mounted embryos during E8.5–E10.5 embryonic stages were dissected as they easily displayed the overall mental map of the embryo and allowed grossly localization of CX3CR1-green/CCR2-red cells based on landmark histological structures. Untermurbed embryos at E8.5 stage showed clusters of double-positive CX3CR1GFP/CCR2-RFP cells colocalizing to the gut region and sparsely visualized throughout the embryo (Fig. 1A–C). At stage E8.5–E9.0 (turned embryo), cells revealing fluorescence intensity appeared as double positive within tracks in the developing somites (Fig. 1D–F) and also present in neural tissues, within the telencephalic vesicle (Fig. 1G–I). IB4 (Fig. 1; purple) was used to highlight the vasculature but also allowed the visualization of cells with features of developing microglia that colonize the neural tissue appearing as CX3CR1GFP (Fig. 1G, asterisks). The exact origin of these cells is not clear. However, visualization of yolk sac tissues at E9.5 revealed the presence of sparsely located GFP+ cells with large cell bodies, suggesting that a population of CX3CR1+ cells develops in the embryonic circulation (Supplemental Fig. 1). Double-positive cells were also found in fore and caudal regions of the embryo in close proximity to the peritoneal cavity colocalizing to the midline dorsal aorta with tracks of cells distributing throughout the developing embryo (Supplemental Fig. 1).

To closely monitor the morphology and neural colonization of CX3CR1+ cells, we analyzed cross sections (Fig. 2) and sagittal slices (Supplemental Fig. 2) from E9.5–E10.5 tissues. Double-positive cells were found in both posterior and anterior ends of the neural tube (Fig. 2), mostly confined to the surface ectoderm (SE) and tightly opposed to the outer layer of the neuroepithelium. A distinct population of CX3CR1-GFP+ and CCR2-RFP+ cells was visualized in the junction of the SE and neuroepithelium (Fig. 2).
CX3CR1, but not CCR2, is expressed by developing microglial cells

Fig. 3 shows a sagittal section at stage E11.5 highlighting IB4+ vasculature, with presence of double-positive cells inside blood vessels. Scarce CX3CR1 single-positive cells localized to the developing cortical tissue without direct association with vasculature. These CX3CR1+ cells displayed amoeboid morphology typical of early microglial precursors (Fig. 3). At stage E11.5, the neural tissue revealed thicker neopallial cortex regions, and mixed populations of cells showing various degrees of CX3CR1-GFP and CCR2-RFP intensities were visualized. Clusters of small/round cells appearing as CX3CR1-GFP Dim/CCR2-RFPbright were detected in the SE or meningeal membranes (Figs. 3, 4). Interestingly, single-positive CX3CR1-GFPbright cells appeared localized within the neuroepithelium, migrating from the SE reaching the inner part of the developing cortex (Fig. 4). The number of CX3CR1-GFP+ cells expands dramatically at E11.5 stage, and they colonize not only the developing cortex but the optic vesicles and developing spinal cord (Supplemental Fig. 2).

Detailed visualization of E13.5 embryos revealed a significant expansion of CX3CR1+ cells detected throughout the whole embryo. E13.5 neural tissue showed a similar pattern to E10.5 stage, with more single GFP+ cells populating the developing CNS. The developing spinal cord is easily visualized at this stage, and neural CX3CR1+ cells showed the characteristic microglial phenotype with bigger cell bodies and extended processes, IBA-1 immunoreactivity, and lack of CCR2-RFP reporter expression (Supplemental Fig. 2).

The previous observations suggest a scenario in which the cells that first colonize neural tissues are double-positive CX3CR1/CCR2 and accumulate at the SE. Cells that remain in the SE,
meningeal areas, downregulate CX3CR1 and appear predominantly as CCR2⁺. Because of the fact that most cells in the SE are double positive, it is likely that cells entering the deep layers of the neuroepithelium downregulate CCR2 appearing as single-positive CX3CR1⁺. However, we do not rule out the possibility of discrete single-positive cells that reach the SE, proliferate, and create the population that colonizes and expands within the developing CNS tissue. Therefore, early stages of development (E8.5) showed: 1) cells with the capacity of becoming peripheral monocytes, and 2) cells that colonize the perivascular and cortical regions as CNS microglia. Both populations appeared distinct based on the activity of the CCR2 and CX3CR1 transcription units, respectively.

At postnatal day 0, CCR2 expression remained confined to a population of cells that localize to meningeal membranes and also visualized within blood vessels. All CCR2⁺ cells appeared as CX3CR1⁺. As the cortical tissue developed, migrating microglial cells that seeded parenchymal tissues exhibited a rapid expansion and appeared as single CX3CR1⁺ (Fig. 5). In the developed CNS, RFP⁺ cells appeared confined to meningeal areas and absent in brain parenchyma, indicating that neither astrocytic, microglial cells, oligodendroglial, nor neuronal cell types express significant amounts of CCR2 protein to be visualized by expression of the RFP reporter (Fig. 5).

These observations suggest that CX3CR1 and CCR2 expression is timely orchestrated during development. It has been established that neither CX3CR1 nor CCR2 are required for the development of microglia as knockout model, for both receptors show normal microglia in the healthy adult CNS. However, the tight regulation of both receptors provides a key phenotypic approach to characterize microglia in the healthy adult CNS. However, the tight regulation of both receptors shows that neither CX3CR1 nor CCR2 are required for the development of microglia as knockout model, for both receptors show normal microglia in the healthy adult CNS. However, the tight regulation of both receptors provides a key phenotypic approach to characterize microglia in the healthy adult CNS.

Adult microglia exhibit a CX3CR1-GFP⁺/CCR2-RFP⁻ expressing pattern, and microglia do not show significant CCR2⁻ RFP expression upon inflammation

To get insights into the CCR2 expression pattern in microglial cells during inflammation, we immunized Cx3cr1⁺GFP/Ccr2⁺RFP mice with myelin oligodendrocyte glycoprotein 35–55. CX3CR1 and CCR2 expression was analyzed by flow cytometry in brain leukocyte preparations. Results reveal that brain inflammatory lesions contain cells with distinct levels of CX3CR1 and CCR2 expression. Notably, microglia did not upregulate RFP expression, flow cytometry analyses distinctly showed CCR2⁻RFP expression in hematogenous CD45hi leukocytes, and CCR2⁻RFP signal was absent in the CD45⁺ microglial population (Fig. 6A, 6B). Two populations of CCR2⁻RFP⁺ cells are visualized, and we have previously characterized them by flow cytometry (20) and shown that CCR2 deficiency depletes inflammatory Ly6Chi monocytes. Importantly, absence of CCR2⁻Ly6ChiCX3CR1⁻ Ly6Chi monocytes delays EAE progression. To have a better understanding of CCR2 and CX3CR1 expression in myeloid cells, brain tissues were imaged, and CCR2⁻RFP and CX3CR1⁻GFP cells were visualized (Supplemental Fig. 3). In addition, we implemented a three-color imaging using the 7-4 marker that identifies Ly6C⁺ monocytes and neutrophils, but not NK or T cells. In the lesions, we observed activated microglial cells with shorter and thicker processes, and these cells clearly appear as CX3CR1⁻ single positive (Supplemental Fig. 3), whereas classical infiltrating monocytes appear CX3CR1⁺ or negative and CCR2⁺.
eral CD45.2 recipient cells that remained created a CD45.2 hi population of resident microglial cells (Fig. 6 R2 gate) was analyzed for CD45.2 expression to identify the (CD45.1+ cells; Fig. 6 by the massive infiltration of peripherally derived leukocytes CCR2+ cells (Fig. 6, R2 gate). Disease severity is evident (Figs. 6A–C and hippocampal (D–F) images show that parenchymal cells are mostly CX3CR1high (A, C, D, F; green) and fewer CCR2+ cells (A, B, D, E; red) remain localized in the meningeal region.

Analyses of skin and cardiac tissues (Supplemental Fig. 4) show that in naive tissues, long-lived macrophage populations appear CX3CR1lo/CCR2-RFP pop similar to the phenotype of resident microglial cells.

During EAE, microglial cells become activated, and markers become indistinguishable from infiltrating myeloid cells using conventional monoclonal. Therefore, we sought to clarify the relationship between CCR2, CX3CR1, and microglial cells using congenic bone marrow transfer in CX3CR1-GFP/CCR2-RFP recipient mice. For this, Cx3cr1GFP/Ccr2RFP double-heterozygous mice (in a CD45.2 congenic background) were reconstituted with congenic wild type CD45.1 bone marrow cells. Upon EAE induction, these chimeric mice would allow us to distinguish the CD45.1 infiltrating population from radioresistant CNS resident microglial population were 98±0.6% GFP and 107±18.8% RFP (n = 5 mice). Furthermore, the CD11b population, although activated based on MHC-II upregulation expression (Fig. 6f), appears with a CD45.2lo phenotype in flow cytometric analysis. Confocal images of brain tissues of chimeric mice confirmed that the activated microglial population as noted by the shorter processes and bigger cell bodies appear as CX3CR1-GFP single positive (Fig. 6J, arrows).

Discussion

Surveillant parenchymal microglial cells are extremely plastic and provide the first line of defense within the CNS. Resident microglial cells are morphologically and functionally distinct from other mononuclear CNS populations, such as perivascular macrophages, suprapendymal macrophages, epiplexus cells of the choroids plexus, and meningeal macrophages (24, 25). In the naive brain, microglia display small cell bodies with thin, long, and branched processes (1). Although microglial functions are intended to be protective, it is documented that dysregulated microglial responses lead to neurotoxicity in vitro and in vivo (12). Recent data have clearly shown that activation of microglia might be beneficial in some pathological settings. More specifically, absence of CX3CR1 in two different models of Alzheimer’s disease correlated to reduced β-amyloid deposition because of enhanced phagocytosis by activated microglia (26, 27). Upon activation, because of inflammation of neuronal damage, microglial cells undergo programmed molecular and morphological changes that include cytoskeleton rearrangements, that is, increases in cell body area exhibiting shorter and thicker processes that ultimately make amoeboid microglia phenotypically indistinguishable from peripheral macrophages.

Another challenging area regarding microglial biology relates to their origin, which has been a subject of controversy for many years. It is now well accepted that microglia arise from mesodermal progenitors that colonize the nervous tissues during embryonic development and fetal periods, and become fully established during postnatal life (28–31). However, the exact mechanisms that control the accumulation of microglial progenitors to the developing CNS are still enigmatic. Del Rio-Hortega in the 1910s classified microglia into two morphological distinct types, which are still current: ramified and amoeboid microglia (1). Ramified microglial cells are fully established postnatally, persist in the adult, and represent the surveying population that constantly scans the surrounding environment via extension and retraction of their processes to perceive activating or inhibitory signals. It is hypothesized that during development, amoeboid microglia are initially highly proliferative cells. Subsequently with a decrease in plasticity, microglial proliferation slows and the remaining cells differentiate into ramified microglia (32, 33). Supporting these initial findings, Juba in 1933 carried out the first comprehensive study of microglia in a series of human embryos 23–280 crown-rump length (measurement of the length of human embryos and fetuses from the top of the head [crown] to the bottom of the buttocks [rump]) (34, 35). He identified the various cell forms with developmental progression, ranging from amoeboid to mature branched varieties. Juba considered microglia to be derived from invading mesodermal elements because microglia-like cells were also detected within connective tissue of the head, which were closely related to embryonic vascular elements. Since the early 1930s, it has been maintained that microglia were “transformed

![CX3CR1 is confined to microglial cells during development, and expression is sustained throughout adulthood. Confocal images were obtained from brain tissues at P0 and counterstained with DAPI (blue). Cortical (A–C) and hippocampal (D–F) images show that parenchymal cells are mostly CX3CR1high (A, C, D, F; green) and fewer CCR2+ cells (A, B, D, E; red) remain localized in the meningeal region.](http://www.jimmunol.org/content/early/2017/07/26/jimmunol.1400811/Figure5.jpg)
“blood elements” or “wandering mesenchymal elements” that migrated into the nervous tissue from the lumen of blood vessels. Although recent studies have elegantly identified microglia as an ontogenically distinct population in the mononuclear phagocyte system (36), we still lack a precise lineage-restricted marker for microglial progenitors that is a key to clarify the debates as to the transformation of these cells in vivo.

The presence of CX3CR1⁺ cells in the yolk sac at E9.5–E11.3 stages of development is of great interest. The yolk sac is an important structure defined as the birthplace of hematopoietic cells in mammals where primitive macrophages and erythrocytes originate, and therefore likely to harbor microglial progenitors. It was proposed that microglia have a dual origin: first coming from the yolk sac macrophages during the nonvascularized prenatal stage, followed by a second engraftment from a population that represents a developmental and transitory form of fetal macrophage (derived from blood-borne precursors, possibly monocytes), which may be related to the amoeboid microglial population seen...
in the postnatal period in rodents (34). This hypothesis has been supported by other groups, showing that in the embryo, microglia originate from monocytes that enter the CNS before development of the blood–brain barrier (37, 38). However, our data support the notion that resident CNS tissue macrophages arise from yolk sac, and as suggested previously, tightly regulated molecular cues dictate the final localization.

This study also brings a novel contribution in the characterization of monocyte subsets. The long-standing limitation of differentiating microglia and peripheral macrophages in inflamed tissue sections has been partially addressed in chimeric mice and through flow cytometric analyses using Abs against various activation-associated cell surface markers (39–42). CX3CR1GFP mice have been widely used to monitor various aspects of monocyte biology and inflammatory reactions associated with heart disease, pain, and cancer (43). Although CX3CR1- and CCR2-deficient mice show normal patterns of adult microglia, it is uncertain whether the timely and organized targeting of early microglia precursors in the developing CNS is altered in the absence of CX3CR1 or CCR2; these aspects are currently under investigation. The chimeric mice generated in this study provided close to 97% reconstitution. However, the 3% that remained from recipient accounted for a small proportion (<3%) of nonmyeloid inflammatory cells (CD45.2hiCD11b−GFP reporter in CX3CR1 GFP mice represents a valuable marker to genitors, and its expression is sustained throughout adulthood. The results show that CX3CR1 is expressed in early microglial precursors in the developing central nervous system by microglial precursors. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local microglia precursors to the CNS and play a pathogenic role during autoimmune demyelinating disease. Blood 113: 3100–3117.

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Disclosures
The authors have no financial conflicts of interest.

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Acknowledgments
We thank Dr. Bruce Trapp (Cleveland Clinic) for providing the BIA-1 Ab, Jenny A. Garcia (University of Texas at San Antonio) for secretarial assistance, Difarnando Vanegas (University of Texas Health Science Center at San Antonio) for assistance on the processing of skin tissues, the Research Centers in Minority Institutions Confocal Core Facility and Advanced Imaging Center at the University of Texas at San Antonio, and Elsevier Academic Press for permission to reproduce the schematic of embryos from The Atlas of Mouse Development by M.H. Kaufmann.
Supplementary Figure 1.  

**Cx3cr1^{+/GFP}** positive myeloid precursors are visualized within yolk sac at E9.5 stage of development. Yolk sacs were dissected and evaluated for the presence of CX3CR1 and CCR2 expressing cells (A, merged image). Single positive CX3CR1 cells (B, green), are prominent and CCR2 expression was detected in double positive precursors (C, red). Crosssection of a representative embryo at stage E9.5 (C) shows the presence of double positive cells (D, merged image). Single positive CX3CR1 cells (E, green), are detected on the neural tube (arrow) and most cells appear as double positive (D, and F, red).
Supplementary Figure 2. *Cx3cr1*<sup>+/GFP</sup> positive myeloid precursors rapidly colonize CNS tissues. Sagittal sections at E10.5 revealed a rapid expansion of CX3CR1+ cells (A, merged
image; B, CX3CR1-green; C, CCR2-red), colonizing the developing cortex (A-C), spinal cord (D-F) and optic vesicles (G-I). These precursors exhibit features of microglia, as evident by their branched morphology, IB4-binding and immunoreactivity to IBA-1 (J, merged image, CX3CR1-GFP/CCR2-Red and IBA staining in blue). In sections A, and G, nuclei visualized with DAPI, CX3CR1-GFP channel shown in B, E and H and CCR2-Red in C, F, and I.
Supplementary Figure 3. Confocal analyses of monocyte subsets in brain lesions of EAE CX3CR1+/GFP CCR2+/RFP mice at peak disease. Merged CX3CR1-GFP (green) and CCR2-RFP (red) image (A) shows activated microglial cells (representative cell “1”), and classical infiltrating monocytes (cell “2”) as CX3CR1\textsuperscript{neg} /CCR2\textsuperscript{hi}, and cell “3” CX3CR1\textsuperscript{lo} /CCR2\textsuperscript{hi} are visualized in (B) CX3CR1-GFP channel and (C) CCR2-RFP channel. (D-F) Sections were obtained after staining with the 7-4 antibody. (D) Merged images of red/CCR2, green/CX3CR1 and blue/7-4. (E) Red/CCR2 and green/CX3CR1 and (F) blue/7-4 and red/CCR2. The majority of CCR2\textsuperscript{+} cells are 7-4\textsuperscript{+} and CX3CR1 negative, further supporting that they are classical infiltrating monocytes (representative cell “4”) and also abundant in the lesion are activated microglia (cell “5”). Scale bar (C-H), 25 μm.
Supplementary Figure 4. CCR2-RFP is not detected in resident CNS, skin or cardiac monocytes. Confocal images from (A) Brain, (B) heart and (C, D) skin tissues show lack of CCR2-RFP expression in resident long-lived monocyte populations. All microglial cells appear CX3CR1\(^{\text{GFP}}\) positive (A, arrows) and neurons stained with anti-Neu antibodies are visualized in blue. CX3CR1\(^{\text{GFP}}\) positive myeloid cells were also visualized in heart (B, arrows) and skin (C, D). Nuclei visualized with DAPI is shown in blue in (B-D).