RESEARCH ARTICLE

Maturation and Localization of Macrophages and Microglia During Infection with a Neurotropic Murine Coronavirus

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Abstract

Macrophages and microglia are critical in the acute inflammatory response and act as final effector cells of demyelination during chronic infection with the neutrotropic MHV-JHM strain of mouse hepatitis virus (MHV-JHM). Herein, we show that "immature" $F4/80^+Ly\text{-}6C^{hi}$ monocytes are the first cells, along with neutrophils, to enter the MHV-JHM-infected central nervous system (CNS). As the infection progresses, macrophages in the CNS down-regulate expression of Ly-6C and CD62L, consistent with maturation, and a higher frequency express CD11c, a marker for dendritic cells (DCs). Microglia also express CD11c during this phase of the infection. $CD11c^+$ macrophages in the infected CNS exhibit variable properties of immature antigen-presenting cells (APCs), with modestly increased CD40 and MHC expression, and equivalent potent antigen uptake when compared with $CD11c^-$ macrophages. Furthermore, $CD11c^+$ and $F4/80^+$ macrophages and microglia are localized to areas of demyelination, in some instances directly associated with damaged axons. These results suggest that chronic CNS infection results in the appearance of CD11c-expressing macrophages from the blood that exhibit properties of immature APCs, are closely associated with areas of demyelination, and may act as final effectors of myelin destruction.

INTRODUCTION

Several strains of the murine coronavirus, mouse hepatitis virus (MHV), cause acute and chronic neurological diseases in susceptible rodents (43, 44). Of these, the neurotropic MHV-JHM strain is widely studied, in large part because infection with this agent results in a chronic demyelinating disease with similarities to the human disease, multiple sclerosis (MS). Like MS, MHV-JHM-induced demyelination is characterized by extensive infiltration of lymphocytes and monocytes and is primarily immune mediated (23, 28, 29, 44, 51, 52).

Monocyte-derived macrophages are also involved in acute inflammation in MHV-JHM-infected mice. Early after infection, neutrophils and monocytes infiltrate the central nervous system (CNS), with neutrophils postulated to be critical in the breakdown of the blood–brain barrier (BBB) (53). Neutrophils and monocytes in the blood of uninfected animals are readily differentiated on the basis of size and granularity. However, in inflamed tissues, distinguishing these populations is more difficult (14, 45). Neutrophils were identified in the MHV-JHM-infected CNS at early times post infection (p.i.), using an antibody that recognizes both Ly-6C and Ly-6G (anti-Gr-1, mAb RB6-8C5) (24, 53). While this antibody is commonly used to identify neutrophils (30), it also stains monocytes/macrophages as these cells express Ly-6C. Antibodies recognizing Ly-6G more specifically detect neutrophils. Additionally, most tissue macrophages express F4/80 antigen (15). Therefore, surface staining with anti-F4/80 mAb with either anti-Ly-6G or Ly-6C mAb clearly distinguishes macrophage and neutrophil populations.

Identifying the source of infiltrating monocytes/macrophages is complicated by accumulating evidence that circulating monocytes, formerly considered to be a homogeneous population, are actually heterogeneous (14, 45). Sunderkötter et al observed that monocytes newly released from the bone marrow (BM) are $Ly\text{-}6C^{lo/-}$ and serve as precursors to $Ly\text{-}6C^{lo/-}$ cells (45). $Ly\text{-}6C^{hi}$ cells also enter sites of inflammation, including the brains of mice infected with *Listeria monocytogenes* (9). Although these studies have provided important insights into monocyte/macrophage differentiation, the fate of these tissue-infiltrating cells during chronic infection of the CNS has not been examined. $Ly\text{-}6C^{-/lo}$ monocytes in the blood or CNS resident microglia may be precursors to CD11c-expressing dendritic cells (DCs) (45). Although $CD11c^+$ cells in the CNS may contribute to the immunopathology of chronic viral infections and inflammatory diseases via their ability to present antigen (10, 11, 37), other $CD11c^+$ cells such as lung alveolar macrophages exhibit poor antigen presenting ability (21, 22, 49, 50). Thus, CD11c expression does not always correlate with increased antigen-presenting cell (APC) function. Notably, CD11c also functions in phagocytosis of apoptotic cells and in cell adhesion, and in this capacity may participate in myelin destruction in the MHV-JHM-infected CNS (27, 32, 41, 46).

Monocytes/macrophages and microglia may be further recruited, activated, or directed to areas of viral infection

concomitant with the arrival of antigen-specific T cells in the CNS. During persistent infection with the attenuated MHV-JHM variant J2.2v-1, viral antigen is concentrated in myelin-producing oligo-dendrocytes in the white matter of the spinal cord (36, 43). As T cells traffic to infected areas and encounter antigen, they produce IFN-γ and other proinflammatory cytokines. IFN-γ directly activatesmacrophages/microglia, inducing up-regulation of MHC molecules on microglia (2), and attracts additional phagocytic cells by inducing local production of chemokines such as CCL2 (MCP-1) (47). Furthermore, activated macrophages and microglia are present in areas of demyelination in all associated experimental models and in MS patients (19, 48) and are likely the final effector cells of myelin destruction. However, because these two popula-tions share many phenotypic characteristics, their relative localiza-tion in areas of demyelination has not been clearly defined.

Macrophages and microglia are involved in the initial host response to MHV-JHM and in the demyelinating process. Mice infected with the attenuated J2.2v-1 variant of MHV-JHM develop a mild encephalitis that evolves into a chronic demyelinating disease. This infectious model is useful for the study of late events in macrophage and microglial recruitment to areas of demyelina-tion in the virus-infected CNS. However, because of attenuated infection, it is more difficult to assess early leukocyte recruitment in J2.2v-1-infected mice. On the other hand, mice infected with the parental strain of MHV-JHM develop a fatal acute encephalitis, with rapid and extensive recruitment of neutrophils and monocytes to the site of infection. Herein, we use mice infected with these two strains of MHV-JHM to show that macrophage maturation within the CNS is similar but not identical to monocyte maturation in the blood. Furthermore, we show that a fraction of macrophages and microglia express CD11c, a DC marker and that both CD11c⁺ and CD11c⁻ cells are localized to areas of myelin destruction, suggest-ing that both serve as final effector cells in this process.

MATERIALS AND METHODS

Animals and virus

Pathogen-free 5- to 6-week-old C57BL/6 (B6) mice were pur-chased from the National Cancer Institute (Bethesda, MD, USA). Transgenic mice expressing green fluorescent protein (GFP) under the control of the β-actin promoter were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Virus was grown and titered on HeLa cells expressing the cellular receptor for MHV (HeLa-MHVR). To obtain mice with acute encephalitis, mice were intra-nasally inoculated with 6×10^4 plaque forming units of a neu-rovirulent strain of MHV (JHM.IA (34), termed MHV-JHM herein) in 12 μL. To obtain mice with demyelination, mice were inoculated intracranially with 1000 plaque forming units of the attenuated J2.2v-1 strain of MHV-JHM (a gift from Dr J. Fleming, University of Wisconsin, Madison, WI, USA) in 30 μL of DMEM media. All animal studies were approved by the University of Iowa Animal Care and Use Committee.

Antibodies

The following mAbs were used in flow cytometric analysis (all purchased from BD Bioscience, San Diego, CA, USA unless stated): fluoroscein isothiocyanate (FITC)-conjugated mouse anti-

mouse MHC class II (25-9-17); biotinylated and phycoercythrin (PE)-conjugated rat anti-F4/80 (cl A3-1 Caltag Laboratories, Burlingame, CA, USA); Peridinin-chlorophyll-protein complex (PerCP) and FITC-conjugated rat anti-Gr-1 (mAb RB6-8C5); FITC-conjugated anti-Ly6C (mAb AL-21); FITC-conjugated anti-Ly6G (mAb 1A8); PerCP, PE, FITC-conjugated rat anti-CD11b (mAb M1/70); biotinylated hamster anti-CD11c (mAb HL3); PerCP-conjugated rat anti-CD45 (mAb 30-F11); purified rat anti-FcγRIII/II Ab (mAb 2.4G2); biotinylated rat anti-CD43 (mAb S7) and PE-conjugated rat CD62L (mAb Mel-14), gifts from Dr. Morris Dailey at the University of Iowa; isotype control antibodies. For biotinylated mAbs, samples were incubated with streptavidin (SA)-RPE or SA-APC (Jackson ImmunoResearch, West Grove, PA, USA).

Preparation of leukocytes

Blood was collected in 1 mL of ACK lysis buffer and leukocytes counted after red blood cell lysis. CNS-derived leukocytes were isolated from B6 mice with acute encephalitis or subacute/chronic encephalomyelitis as previously described (3). Briefly, animals were killed and perfused with sterile phosphate-buffered saline. Brain tissues were mechanically homogenized using frosted glass slides. Cells were suspended in 30% Percoll (Pharmacia, Piscat-away, NJ, USA) and centrifuged at 800*g* at 4°C for 30 minutes. Percoll and lipid layers were aspirated and the cell pellet was washed twice and counted. Leukocytes in the CNS were identified by expression of CD45 using flow cytometry.

FACS analysis

For phenotypic analysis, cells derived from the blood and CNS were blocked with 2.4G2 and then incubated with specific mAbs or isotype controls. Of note, for dual detection of F4/80 and Ly6C, cells were incubated sequentially with anti-F4/80 mAb and anti-Ly6C mAb. Flow cytometry was performed on FACScan or LSRII flow cytometers (BD Biosciences, Mountain View, CA, USA).

Evaluation of antigen uptake

The ability of monocyte/macrophage cells to uptake antigen was evaluated by incubation of CNS isolated cells at 9 days p.i. with FITC-Dextran, MW 40 000 kDa (Molecular Probes, Eugene, OR, USA) for 1 h at 37°C or in control tubes at 4°C. After incubation with FITC-Dextran, CNS cells were surface stained for CD45, F4/80 and CD11c. These markers were detected in conjunction with FITC-Dextran uptake by flow cytometric analysis.

Generation of bone marrow chimeras

To generate mice with GFP⁺ monocyte/macrophages and GFP⁻ microglia, B6 mice were irradiated with a lethal dose of 1000 rads and reconstituted with $1–2 \times 10^6$ BM cells isolated from the femur and tibia of GFP⁺ donor mice. After resting for 6 weeks to allow hematopoeitic reconstitution, chimerism was verified in peripheral blood and spinal cords by FACS and confocal microscopic analy-sis, respectively. Naïve chimeric mice were also tested 16 weeks post-transfer for hematopoietic replacement (from GFP⁻ to GFP⁺) of microglia. Peripheral blood monocytes in naïve chimeric mice

were defined by flow cytometry as F4/80$^+$Ly-6C$^+$CD45^{hi} while CNS microglia were defined as F4/80$^+$Ly-6C$^-$CD45^{int}.

Immunofluorescence staining and confocal microscopy

Eight μm sections were prepared from 4% p-formaldehyde-fixed, snap frozen spinal cords. Sections were blocked with 10% horse serum in phosphate-buffered saline, prior to incubation with primary antibody that detected macrophages/microglia (biotinylated rat anti-F4/80 (CI:A3-1; Serotec, Oxford, England), 1:200), CD11c antigen [biotinylated hamster anti-mouse CD11c (HL3), 1:200], or demyelinated axons (mouse SMI-32, anti-non-phosphoneurofilament, 1:200; Sternberger Monoclonals, Lutherville, MD, USA) for 1 h at room temperature. After washing, sections were incubated with streptavidin (SA)-HRP (1:1000) and FITC or Cy5 conjugated donkey anti-mouse antibody (1:100, Jackson ImmunoResearch, West Grove, PA) for an additional hour. For SA-HRP treated samples, sections were amplified using TSA-Cy3 (Tyramide Signal Amplification, 1:100; Perkin-Elmer, Boston, MA, USA) for 5 minutes. Samples were examined with a Zeiss 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Statistics

A two-tailed unpaired Student's *t*-test was used to analyze differences in mean values between groups. All results are expressed as means ± SEM. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Migration of Ly-6C^{hi} monocytes and Ly-6G$^+$ neutrophils into the MHV-JHM-infected CNS during acute infection

Although neutrophils were previously identified as the predominant cell type entering the CNS at early times after MHV-JHM infection, these prior studies used an antibody that did not distinguish between neutrophils and macrophages/microglia (24, 53). To more definitively distinguish between monocyte and neutrophil populations, we therefore used anti-F4/80 mAb and anti-CD11b with either anti-Ly-6G or Ly-6C mAb (Figure 1). Neurovirulent MHV-JHM was used in these initial studies to ensure a robust neutrophil and monocyte infiltration into the CNS.

Leukocytes were harvested from the CNS at day 4 p.i. and stained with Gr-1, anti-F4/80, and anti-CD45 antibodies. We stained cells with Gr-1 mAb because it was used in prior studies of MHV-JHM-induced encephalitis (24, 53). Staining with these antibodies revealed the presence of several populations of cells (Figure 1A). Population R1 was identified as microglia (CD11b^{hi}CD45^{int}MHC class II^{lo}Ly-6G$^-$), population R2 as activated macrophages (CD11b^{hi}CD45^{hi}MHC class II^{int}Ly-6G$^-$) and population R3 as neutrophils (CD11b^{hi}CD45^{hi}MHC class-II$^-$Ly-6G^{hi}) (Figure 1B). These conclusions were also supported by microscopic examination of sorted cells, with populations R2 and R3 exhibiting the morphology of macrophages and neutrophils, respectively (Figure 1D).

Next, CNS-derived macrophages/microglia, defined as F4/80$^+$ (Figure 1A) were examined for Ly-6C and CD62L expression (Figure 1C, left panel), as these molecules are up-regulated on tissue infiltrating monocytes. We identified two major populations at day 4 p.i., differing in their expression of CD62L and Ly6C. The CD62L$^-$Ly-6C$^-$ population was CD45^{int}, while the CD62L$^+$Ly-6C^{hi} population was CD45^{hi}, identifying these populations as microglia and monocyte-derived macrophages, respectively (data not shown). Thus, blood-derived CD62L$^+$Ly-6C^{hi} monocytes are recruited to the virus-infected CNS at early times p.i. In addition, Ly-6C^{hi} monocyte-derived macrophages expressed variable levels of the DC marker CD11c (Figure 1C, middle panel), and were negative for CD43, which is up-regulated as monocytes mature in the blood (Figure 1C, right panel) (45).

Using Ly-6C and Ly-6G for identification of monocyte-derived macrophages and neutrophils, respectively, we enumerated the numbers isolated from the CNS of MHV-JHM-infected mice with acute encephalitis. Both cell types appeared in the CNS at 2 days p.i., albeit at low levels (Figure 1E). Neutrophils and macrophages increased in the CNS until day 5, at which time numbers of macrophages, but not neutrophils continued to increase until mice became moribund at day 6 p.i. Unlike a previous report, we detected a greater number of macrophages than neutrophils in the CNS at all time points (45).

Maturation of macrophages and microglia during persistent J2.2v-1 infection

During maturation, circulating monocytes down-regulate Ly-6C and CD62L and up-regulate CD11c and CD43 (45), Figure 2A–D, top panels). To determine whether the predominant Ly-6C^{hi} monocyte-derived macrophage population in the virus-infected CNS (Figure 1C) matured similarly during infection, we infected mice with the attenuated J2.2v-1 variant. Unlike MHV-JHM-infected mice, J2.2v-1-infected animals survive past 7 days p.i., and develop a persistent infection with signs of hindlimb paresis/paralysis (12). Thus, infection with J2.2v-1 enables the observation of monocyte recruitment and maturation in the CNS of mice with a persistent infection and chronic demyelinating disease. Leukocytes were prepared from the J2.2v-1-infected CNS at several days p.i. and analyzed by four-color flow cytometry. Initially, monocyte-derived macrophages (CD45^{hi}F4/80$^+$) were examined for expression of Ly-6C and either CD11c, CD62L, or CD43 (Figure 2). As in mice infected with MHV-JHM (Figure 1C), the majority of macrophages examined at early times p.i. exhibited a blood monocyte phenotype of Ly-6C^{hi}CD62L$^+$ (Figure 2C and F). By day 9 p.i., the frequency of Ly-6C^{hi}CD62L$^+$ cells diminished compared with 6 days p.i. and a greater proportion were Ly-6C$^-$CD62L$^-$ macrophages. By day 20 p.i., mice recovered from the acute infection and infectious virus was undetectable (13). The number of macrophages did not decrease appreciably at this time, but the majority were Ly-6C$^-$CD62L$^-$. Furthermore, CD43, which is up-regulated during the course of monocyte maturation in the blood (45), was not up-regulated in the J2.2v-1-infected CNS (Figure 2D). We also determined whether the expression of CD11c was up-regulated on Ly-6C^{lo} macrophages as occurs on Ly-6C$^{lo/-}$ blood monocytes. As shown in Figure 2, CD11c expression was consistently detected by day 6 p.i. and increased by 9–21 days p.i. (Figure 2B and E). These data indicate that monocytes are recruited during the early stages of
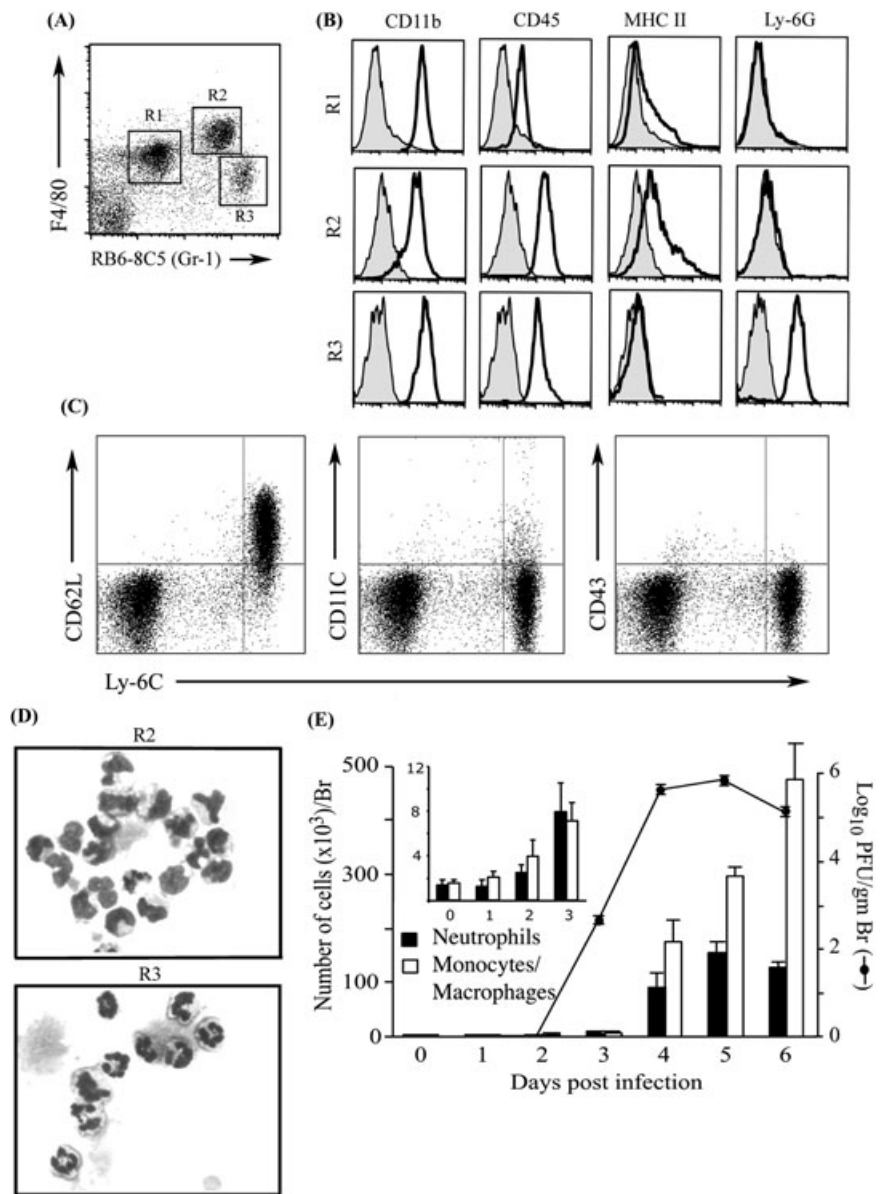
Figure 1. *Early recruitment of Ly-6C^hi monocytes and neutrophils into the MHV-JHM-infected CNS.* Leukocytes were prepared from mice intranasally inoculated with MHV-JHM at 4 days p.i. **A.** Cells were stained for F4/80 and Gr-1 antigen. Three distinctive populations were identified: R1—F4/80$^+$Gr-1$^-$; R2—F4/80$^+$Gr-1^{int}; R3—F4/80$^-$Gr-1^{hi}. **B.** The R1-R3 populations were examined for CD11b, CD45, MHC class II antigen or Ly-6G expression by flow cytometry. **C.** F4/80$^+$ cells were stained for Ly-6C and CD62L (left panel), CD11c (middle panel), or CD43 expression (right panel). Two major populations were apparent (Ly-6C^{hi}CD62L$^+$ and Ly-6C$^-$CD62L$^-$), with variable expression of CD11c. **D.** The R2 and R3 populations were identified morphologically as macrophages and neutrophils, respectively. **E.** CD45^{hi} leukocytes were prepared from the CNS of MHV-JHM-infected mice. Macrophages (CD45^{hi}F4/80$^+$Gr-1^{int}; open bar) and neutrophils (CD45^{hi}F4/80$^-$Gr-1^{hi}; closed bar) were both detected in the CNS at 2 days p.i. The inset depicts low-level relative recruitment with a smaller scale at days 0–3 p.i. By day 4 p.i., greater numbers of macrophages were present in the brain. Titers of virus in the infected brains of 3 mice were determined at the indicated times. Data are represented as mean ± SEM. Abbreviations: CNS = central nervous system; MHV-JHM = mouse hepatitis virus; p.i. = post infection; PFU = plaque forming units; Br = Brain.

infection and mature *in situ* at the site of inflammation. Less likely, these cells down-regulate markers of peripheral blood maturation coincident with entry into the CNS, but only at later times p.i.

To determine whether CD11c up-regulation in the MHV-infected CNS was confined to hematogenous macrophages, we investigated the expression of CD11c on microglia (identified as CD45^{int}F4/80$^+$Ly-6C$^-$, Figure 1A and B). While CD11c was not detected on microglia from infected animals at 3 days p.i., it was detectable at 6 days p.i. in mice infected with J2.2v-1 (Figure 2G). Surface CD11c staining appeared maximal at day 9 and gradually declined thereafter.

CD11c is considered a useful marker for DCs, a subset of APCs. Therefore, we next assessed whether CD11c$^+$F4/80$^+$ cells in the CNS exhibited a phenotype consistent with being a more mature APC than CD11c$^-$F4/80$^+$ cells. We examined expression of several surface molecules associated with DC maturation at 9 days p.i.

(Figure 3A and B). CD40, CD86 and MHC class I and II antigen were detected on all CD45^{hi}F4/80$^+$ cells. While the levels of CD86 expression were similar between CD11c$^+$ and CD11c$^-$ cells, CD40 and MHC class I and II antigen were expressed at increased levels on CD11c$^+$F4/80$^+$ cells compared with CD11c$^-$F4/80$^+$ cells. In addition to expression of stimulatory molecules, functional maturity in APCs correlates with decreased ability to uptake antigen. Surface expression of CD11c did not correlate with the ability of CD45^{hi}F4/80$^+$ cells to endocytose FITC-Dextran *ex vivo* when examined at 9 days p.i.; both populations exhibited potent antigen uptake (Figure 3C and D). In contrast, CD45^{hi}F4/80$^-$ cells did not uptake FITC-Dextran. Although CD45^{hi}CD11c$^+$F4/80$^+$ cells express relatively increased levels of stimulatory molecules compared with their CD11c$^-$ counterparts, the overall low level expressed by both subsets and their potent antigen uptake indicates that both cell types exhibit properties of immature APCs. Thus,
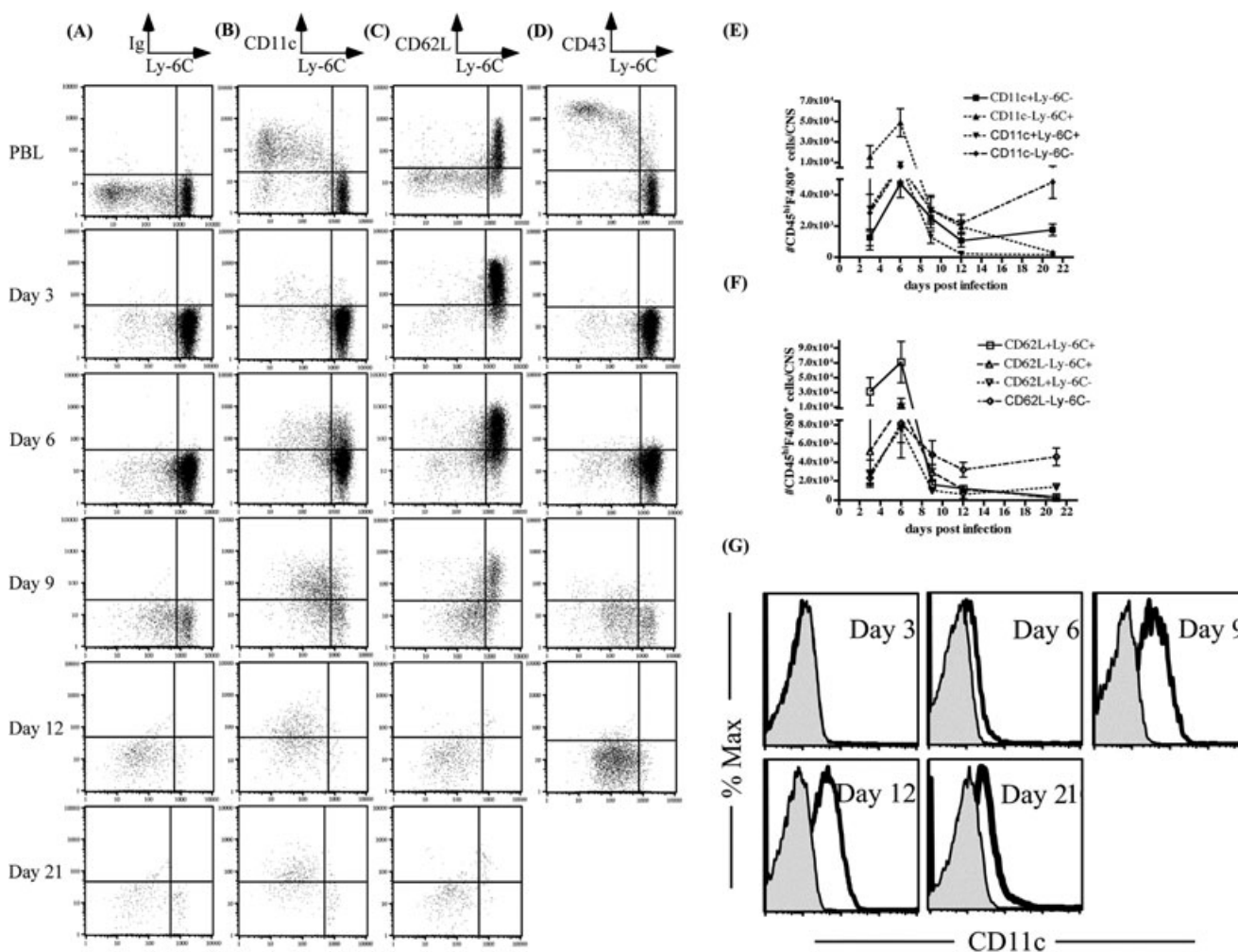
Figure 2. *Maturation of monocytes with variable CD11c expression in macrophages and microglia during persistent J2.2v-1 infection.* Leukocytes were prepared from the blood of naïve mice or from the infected CNS at the indicated days and stained for F4/80 and Ly-6C and CD11c, CD62L or CD43 antigen. CNS-derived cells were also stained for CD45. Monocytes were identified as F4/80+ and CNS-isolated macrophages as CD45hiF4/80+. (**A–D**) Representative flow cytometric analyses are depicted. Monocytes or macrophages were stained with isotype-matched control (**A**), anti-CD11c (**B**), anti-CD62L (**C**) or anti-CD43 (**D**) antibodies. (**E–H**) Kinetics of monocyte maturation in J2.2v-1-infected

mice. The total numbers (**E,F**) of CD45hiF4/80+ CNS macrophages are depicted as the mean ± SEM of 8–11 mice per time point. The total number of macrophages with or without Ly-6C expression is depicted in conjunction with CD11c expression (**E**) and CD62L expresssion (**F**). **G.** CD11c expression on microglia. Microglia were identified as CD45intF4/80+Ly6C−. Samples were stained with anti-CD11c (bold) and isotype-matched control mAb (shaded). Representative histograms of CD11c expression (bold line) are depicted at the indicated days p.i. (5–7 mice per group). Abbreviations: CNS = central nervous system; PBL = peripheral blood leukocytes; p.i. = post infection.

monocyte-derived macrophages may be distinguished from neutrophils and microglia by differential expression of the surface markers, Ly-6G, Ly-6C, CD45, F4/80 and CD62L (Table 1). Furthermore, macrophages and microglia express variable levels of CD11c during chronic J2.2v-1 infection.

Localization of CD11c+ and CD11c− macrophages/microglia in areas of demyelination

As CD11c+ and CD11c− cells are present in the MHV-JHM-infected CNS, and their differences in phenotype could reflect dif-

Table 1. Phenotype of inflammatory cell subsets in the central nervous system of mouse hepatitis virus-infected mice.

Surface marker	Neutrophils	Macrophages		Microglia
		Immature	Mature	
F4/80	−	++	++	++
CD45	++	++	++	+
Ly-6C	+	++	−	−
Ly-6G	++	−	−	−
CD62L	−	++	−	−
CD11c	−	+/−	+	+/−

ferences in their localization and thus their potential for myelin destruction, we determined the relative distribution of these cells in the white matter of the J2.2v-1-infected spinal cord, using antibodies that recognize F4/80 or CD11c in conjunction with an antibody (SMI-32) that detected non-phosphorylated neurofilament, a marker for demyelinated or damaged axons. We demonstrated previously that damaged axons were present in areas of demyelination in J2.2v-1-infected mice (7). As expected, F4/80+ cells, marking all macrophages and microglia, were concentrated in the white matter of the spinal cord, in areas of demyelination and many were located adjacent to SMI-32+ axons (Figure 4A and B). While fewer cells were CD11c+ when examined by confocal microscopy, their distribution within demyelinating lesions and proximity to damaged/demyelinated axons was indistinguishable from that of other F4/80+ cells (Figure 4C and D). Thus, the subset of macrophages/microglia-expressing CD11c+, like the total populations of these cells, is located in areas of demyelination, in some instances proximal to demyelinated axons in J2.2v-1-infected mice.

CD11c+ cells within areas of demyelination are of both monocytic and microglial origin

To determine whether cells at sites of demyelination were predominantly blood-derived macrophages or CNS-derived (microglia), we used BM chimeras in which hematogenous monocytes expressed GFP. We used these chimeric animals because, upon activation, microglia undergo morphological changes, from an initial ramified appearance to a morphology more similar to that of macrophages (38). Thus, microglia and macrophages are often difficult to distinguish in lesions of demyelination. For this purpose, recipient B6 were lethally irradiated and reconstituted with BM from mice in which all cells expressed GFP (33). At 6 weeks post BM transfer, F4/80+ PBMCs were GFP+ in the peripheral blood of chimeric mice, while F4/80+ microglia remained GFP− by both FACS analysis and confocal microscopy (Figure 5A–C). This phenotype was maintained in naïve chimeric mice at later time points, up to 16 weeks post transfer (Figure 5D and E).

To compare the localization of macrophages and microglia in mice with demyelinating disease, chimeric mice were infected with J2.2v-1 at 5–6 weeks post BM transfer. Brains and spinal cords were harvested at days 10–14 p.i. for flow cytometric and confocal microscopic analysis, respectively. Surface staining of cells from infected brains of chimeric mice indicated that F4/80+CD45hi macrophages were GFP+, whereas F4/80+CD45int microglia were GFP− (Figure 5F and G), as expected. Confocal analysis of spinal cord white matter from infected chimeric mice revealed macrophage/microglial infiltration with demyelination (detected by SMI-32 staining) apparent at days 10–14 (Figure 6A–D). Both GFP+ and GFP− cells expressing F4/80 (Figure 6A and B) or CD11c (Figure 6C and D) were present in areas of demyelination, in some instances in direct contact with demyelinated (SMI-32+) axons (arrows, Figure 6B), suggesting that both populations of CD11c+ and CD11c− macrophages and microglia participate in the demyelinating process.

DISCUSSION

Unlike previous studies, we show that monocytes, not neutrophils, are the predominant cell type to initially infiltrate the CNS

of MHV-JHM-infected mice. Our data suggest that these cells mature *in situ* during chronic virus infection. This process of maturation parallels monocyte maturation within the blood, as shown by down-regulation of Ly-6c and CD62L and up-regulation of CD11c. However, CD43, which is up-regulated during monocyte maturation in the blood does not occur on macrophages in the MHV-JHM-infected CNS. We also show that both blood-derived macrophages and microglia variably express a DC marker, CD11c, and that these CD11c+ cells exhibit properties of immature APCs. Finally, we demonstrate, using BM chimeras, that both macrophages and microglia are localized to areas of demyelination, independent of CD11c expression. Thus, they may play a direct role in myelin destruction in addition to potentially functioning as APCs.

The cells initially entering the CNS were defined as immature on the basis of elevated expression of Ly-6C and CD62L (45). CD62L is important for monocyte/macrophage recruitment and demyelination in the inflamed CNS of mice with experimental autoimmune encephalomyelitis (EAE), and may facilitate binding of these cells to myelin (16). Ly-6C is a member of a multigene family of GPI-anchored cell surface glycoproteins and is expressed variably on CD8 T-cell lymphocytes, monocytes, macrophages and endothelial cells (26, 40). Notably, it is expressed on brain endothelial cells, but not microglia, in the CNS of adult B6 mice (1). Its natural ligand has not yet been identified, but cross-linking of Ly-6C on CD8 T cells enhances adhesion to endothelium and homing (17, 25). Monocytes expressing Ly-6C at high levels preferentially migrate into sites of inflammation, suggesting that Ly-6C also has a role in homing (Figure 1). These cells matured as evidenced by down-regulation of CD62L and Ly-6C, with a sub-population exhibiting increased surface expression of the DC marker, CD11c (Figures 1C and 2). Differentiation of monocytes to DCs in the CNS is likely cytokine driven. TNF-α, an inflammatory cytokine that is up-regulated in the MHV-JHM-infected CNS (18), may contribute to DC differentiation by overriding IL-6-driven macrophage differentiation (5). Although it remains formally possible that Ly6C−/lo/CD11c+ monocytes directly enter the CNS, we detected predominantly Ly6ChiF4/80+ cells at early times p.i., in agreement with a previous report (45). Unlike mature monocytes in the blood, mature macrophages in the inflamed CNS did not express CD43 (Figures 1C and 2D). This lack of expression of CD43 suggests that mature monocytes in the blood, which are CD43+, did not directly migrate into the infected CNS, as, if they did so, mature macrophages in the CNS would be expected to express CD43. Therefore, it is likely that cells recruited to the CNS mature during the course of inflammation to express CD11c.

CD11c+F4/80+ cells in the MHV-infected CNS expressed higher levels of MHC class I and II antigen, and CD40, but not CD86, than did CD11c−F4/80+ cells (Figure 3A and B). However, F4/80+ macrophages did not differ in their ability to uptake antigen based on CD11c expression (Figure 3C and D). Because of their overall immature APC phenotype and lack of expression of CD43 [Figures 1C (right panel) and 2D], these CD11c+ macrophages may be less efficient as APCs than traditional DCs. CD43 regulates cell adhesion and is involved in cell activation (35). Ligation of CD43 results in DC maturation, increased cytokine production and enhanced ability to stimulate T-cell proliferation (6, 8). At present, it is not known whether the lack of up-regulation of CD43 on CD11c+

(A)

(B)

(C)

(D)
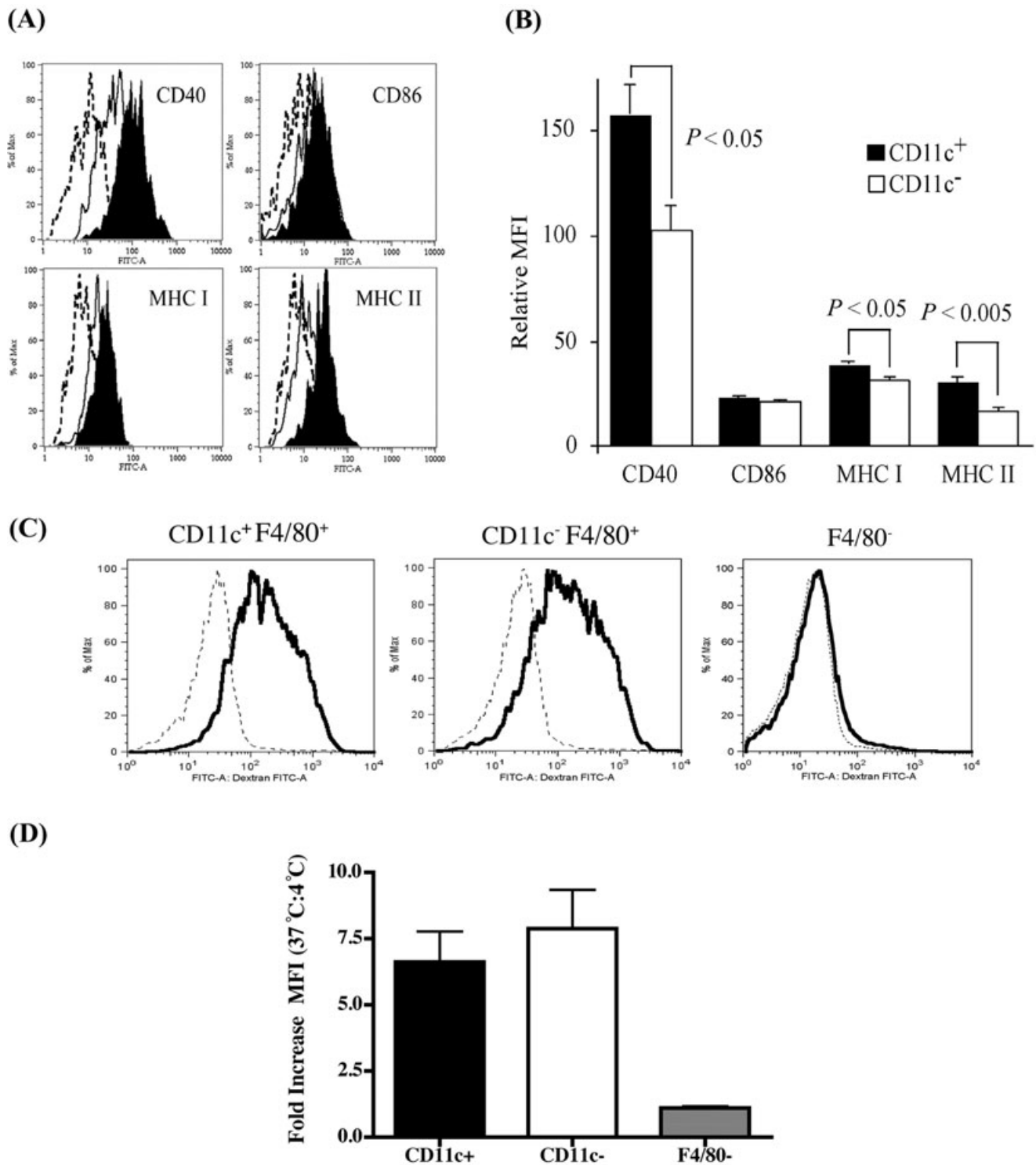
cells is unique to the MHV-infected CNS or whether it is a generally feature of macrophage maturation at sites of inflammation.

DCs are not present in the uninflamed parenchyma, but are detected in the meninges and choroid plexus in naïve mice (37). Consistent with this, influenza virus inoculated directly into the brain parenchyma did not elicit an antibody or T-cell response until

it spread to the cerebrospinal fluid, was transported to deep cervical lymph nodes and evoked an immune response (42). In addition to MHV-infected mice, CD11c$^+$F4/80$^+$ cells were also isolated from the CNS of animals with chronic toxoplasmosis or chronic EAE (10, 11, 37). These cells, considered DCs, expressed MHC class II antigen and co-stimulatory molecules such as CD40, CD54, CD80

Figure 3. *CD11c⁺ blood-derived macrophages in MHV-J2.2v-1-infected mice variably exhibit properties of immature APCs.* Leukocytes were prepared from the CNS of J2.2v-1-mice at 9 days p.i. and analyzed by flow cytometry. Macrophages were identified as CD45^{hi}F4/80⁺. **A.** Representative histograms of CD40, CD86 and MHC class I, and class II antigen expression on CD11c⁺ (filled) and CD11c⁻ (open) cells are indicated. Isotype-matched control antibodies are also shown (dashed line). **B.** MFI of CD40, CD86 and MHC class I and class II antigen are shown (3–4 mice per group; mean ± SEM). **C.** Representative histograms depicting FITC-Dextran uptake by CD11c⁺F4/80⁺ (left panel) and CD11c⁺F4/80⁻ cells (middle panel). F4/80- cells (right panel) are depicted as a negative control for uptake. Cells isolated from the CNS were incubated with FITC-Dextran at 4°C (dotted line) or 37°C (solid line) for 1 h, then analyzed by flow cytometry for FITC-Dextran uptake, and CD45, F4/80 and CD11c expression. **D.** Mean ± SEM of five mice per group of the fold increase in MFI of cells analyzed under optimal and control conditions for FITC-Dextran uptake (37°C:4°C). Abbreviations: APC = antigen-presenting cell; FITC = fluoroscein isothiocynate; MFI = mean fluorescence intensity.

and CD86 and produced IL-12 *ex vivo*. When compared with CD11c⁻CDllb⁺ macrophages, they more efficiently stimulated the proliferation of allogeneic and naïve TCR transgenic T cells, and mediated increased production of IFN-γ and IL-2 by naïve cells on exposure to antigen. However, our results show that CD11c⁺F4/80⁺ cells in the MHV-infected CNS exhibit characteristics of immature APCs, and therefore, may be less able to stimulate naïve T cells than mature DCs. Currently, only CD45^{hi}CD11c⁺F4/80⁻ cells in the CNS are known to exhibit professional APC functions uniquely attributed to DCs, such as cross-presentation, which is required for epitope spreading in mice with EAE (31). Importantly, this mature DC subset is found in very low frequencies in the CNS of MHV-JHM-infected mice, suggesting that CD11c⁺ cells in areas of demyelination also express F4/80. Previous reports suggest that DCs in the CNS may develop from either hematogenous macrophages or microglia (10, 11, 37, 39). While CD11c⁺F4/80⁺ and CD11c⁻F4/80⁺ cells in the MHV-JHM-infected CNS exhibited phenotypic differences, cell distributions in the spinal cord based on CD11c expression were indistinguishable (Figures 4 and 6). Both CD11c⁺ and F4/80⁺ cell types were observed in areas of demyelination, closely associated with damaged axons, an observation not previously reported. Furthermore, both of these populations were derived from hematogenous macrophages and from CNS resident microglia (Figures 5 and 6). Although CD11c⁺F4/80⁻ cells may also localize to areas of demyelination, they are present only at low frequency, making it unlikely that they represent a major subset of CD11c⁺ cells in the diseased spinal cord (unpublished data). As CD11c⁺F4/80⁺ cells retain the ability to uptake antigen (Figure 3C and D) and increase in frequency after the generation and contraction of T-cell responses (Figure 2B), these cells may function to
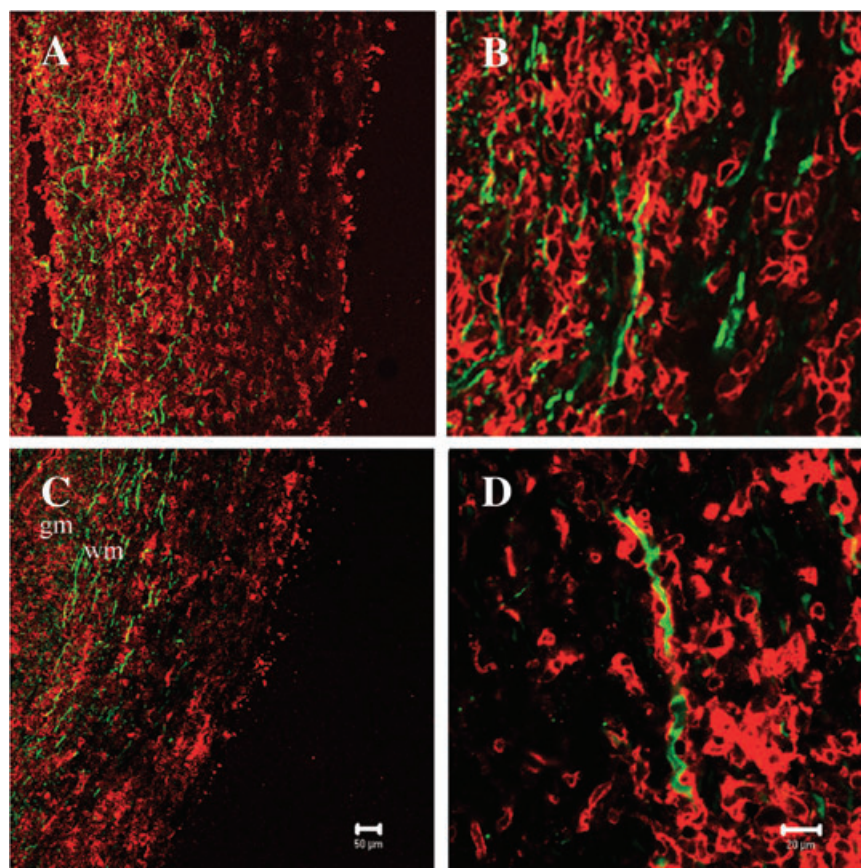

Figure 4. *F4/80⁺ and CD11c⁺ cells in infected mice are located adjacent to demyelinated axons.* Eight μm frozen sections of spinal cords were prepared from J2.2v-1-infected mice at 12 days p.i. and stained with anti-F4/80 (red, A and B) or anti-CD11c (red, C and D) mAbs. Damaged axons were detected using mAb SMI-32 (green). Four individual mice were analyzed with similar results. Note that F4/80⁺ and CD11c⁺ cells were both detected adjacent to damaged axons. Gray matter and white matter are labeled (**C**) as gm and wm, respectively.
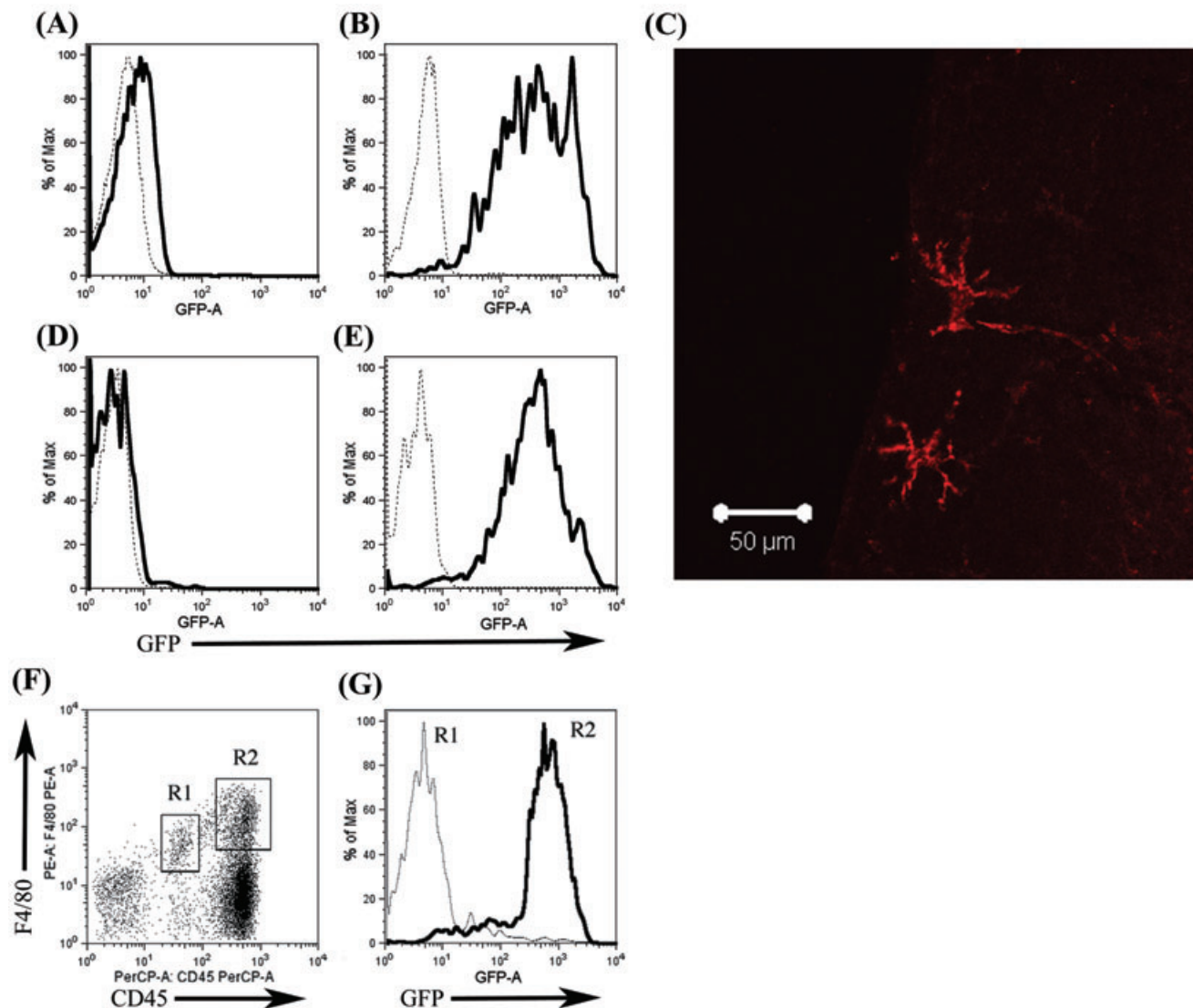
Figure 5. *Monocyte/macrophages are GFP⁺, while microglia are GFP⁻, in naïve and J2.2v-1-infected GFP⁺ donor/GFP⁻ recipient bone marrow chimeric B6 mice.* BM chimeras were created with mice expressing green fluorescent protein (GFP) as donors and lethally irradiated B6 mice as recipients. Chimerism was verified by flow cytometry of isolated CNS (**A,D**) and peripheral blood (**B,E**) monocytes, and by confocal microscopy of frozen spinal cord sections from naïve chimeric mice (**C**). GFP⁻ microglia (**A,C,D**) and GFP⁺ blood monocytes (**B,E**) were observed in naïve chimeric mice at 6 weeks (**A–C**) and 16 weeks (**D,E**) post transfer. (**A,B,D,E**) Histograms of GFP expression in chimeric mice are depicted by heavy solid lines, while cells from B6 control mice are depicted with dashed lines. **C.** Ramified GFP⁻ microglia were observed in frozen spinal cords of naïve chimeric mice by staining with anti-F4/80 (red). **F.** Representative flow cytometric dot plot of brain cells isolated 10 days p.i. from J2.2v-1-infected chimeric mice (infected 6 weeks post BM transfer). Macrophages (R2) are identified as CD45^hiF4/80⁺, while microglia (R1) are CD45^intF4/80⁺. **G.** Microglia from the CNS of infected animals were GFP⁻, while monocytes/macrophages were GFP⁺. Three to four mice in each group were analyzed with similar results. Abbreviations: CNS = central nervous system; BM = bone marrow.

stimulate CD8 T cells as well as playing a role in myelin destruction during the chronic phase of the infection.

Ly6C^hi monocytes, entered the MHV-JHM-infected CNS with the same kinetics as neutrophils (Figure 1). Although it is generally believed that neutrophil infiltration precedes monocyte recruitment to sites of inflammation, other studies suggest that monocytes are able to enter sites of inflammation in the absence of neutrophils (20). In MHV-JHM-infected mice, neutrophils were postulated to mediate BBB breakdown, based upon depletion of these cells with mAb RB6-8C5 (53), which recognizes both Ly-6C and the neutrophil-specific antigen, Ly-6G. Using antibodies that specifically recognized either Ly-6G or Ly-6C, we showed that Ly6C^hi monocytes, rather than neutrophils, were the most abundant cell type that initially infiltrated the MHV-JHM-infected CNS. Both monocytes/macrophages and neutrophils express pro-inflammatory cytokines, such as TNF-α, reactive
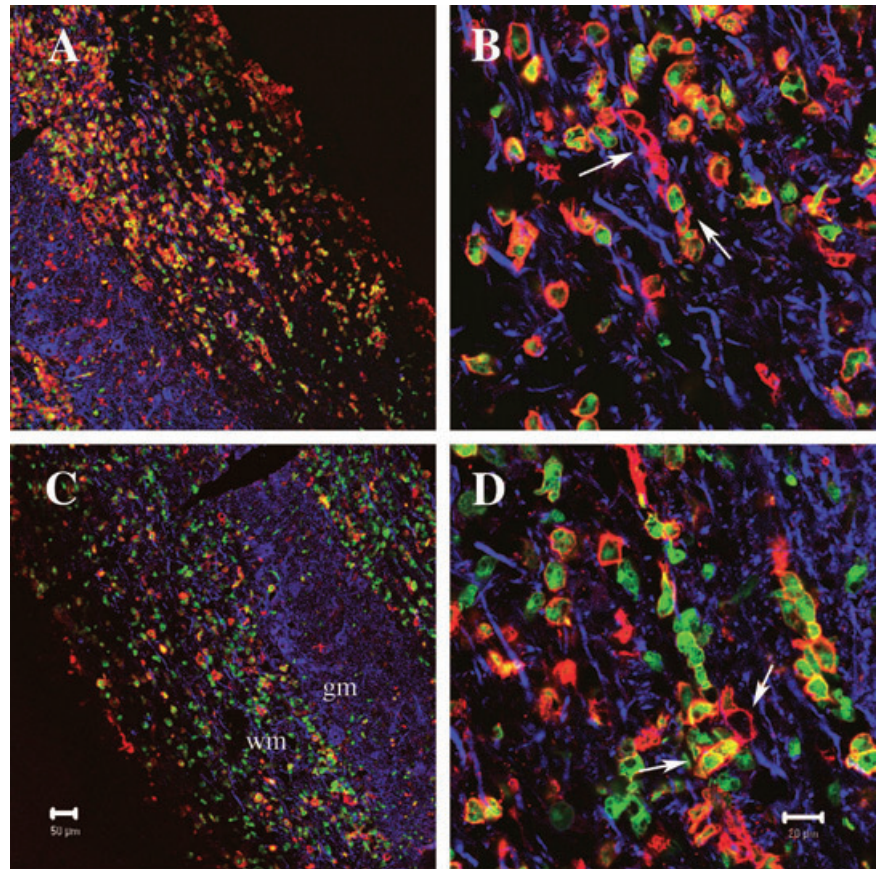
Figure 6. *CD11c⁺ and CD11c⁻ bone marrow-derived macrophages and CNS resident microglia are co-localized with demyelinated axons.* (**A–D**) 8 µm frozen sections of spinal cords were prepared from J2.2v-1-infected GFP BM chimeric mice at 10–14 days p.i and stained with anti-F4/80 (red, **A and B**) or anti-CD11c (red, **C and D**) mAbs. GFP⁺ BM derived cells appear green. Damaged axons were detected using mAb SMI-32 (blue). Four individual mice were analyzed with similar results. Note that F4/80⁺ and CD11c⁺ cells of both macrophage (GFP⁺) and microglial (GFP⁻) origin were detected in association with damaged axons (arrows, **B,D**). Areas of gray and white matter are labeled (**C**) as gm and wm, respectively. Abbreviation: CNS = central nervous system; GFP = green fluorescent protein; BM = bone marrow.

oxygen species, and metalloproteases, including MMP9, which function to degrade basement membrane and extracellular matrix components such as collagen and laminin (4). Thus, it is not possible at present to know which is most important in BBB breakdown because both are likely depleted by the RB6-8C5 antibody (53).

Collectively, these results show the dynamic nature of the monocyte/macrophage response to a viral infection of the CNS. They demonstrate that peripheral blood monocytes enter the inflamed CNS and modulate their expression of surface molecules associated with maturation. Along with CNS resident microglia, they up-regulate the DC marker CD11c, and ultimately migrate to areas of demyelination where they directly associate with demyelinated axons. The final effector cells of demyelinating disease are phenotypically heterogeneous, which may reflect different roles for subsets of these cells in the disease process.

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