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Microglial Recruitment, Activation, and Proliferation in Response to Primary Demyelination

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We have characterized the cellular response to demyelination/remyelination in the central nervous system using the toxin cuprizone, which causes reproducible demyelination in the corpus callosum. Microglia were distinguished from macrophages by relative CD45 expression (CD45^{dim}) using flow cytometry. Their expansion occurred rapidly and substantially outnumbered infiltrating macrophages and T cells throughout the course of cuprizone treatment. We used bromodeoxyuridine incorporation and bone marrow chimeras to show that both proliferation and immigration from blood accounted for increased microglial numbers. Microglia adopted an activated phenotype during demyelination, up-regulating major histocompatibility class I and B7.2/CD86. A subpopulation of CD45^{dim-high} microglia that expressed reduced levels of CD11b emerged during demyelination. These microglia expressed CD11c and were potent antigen-presenting cells *in vitro*. T cells were recruited to the demyelinated corpus callosum but did not appear to be activated. Our study highlights the role of microglia as a heterogeneous population of cells in primary demyelination, with the capacity to present antigen, proliferate, and migrate into demyelinated areas. (Am J Pathol 2007; 170:1713–1724; DOI: 10.2353/ajpath.2007.060783)

Microglia, central nervous system (CNS)-resident bone marrow-derived cells, and infiltrating macrophages are thought to play a pivotal role in the response to demyelination and the pathogenesis of multiple sclerosis (MS).¹ In the CNS of mice with experimental autoimmune encephalomyelitis (EAE), a widely used mouse model of MS, microglia proliferate and, similar to macrophages, up-regulate expression of molecules needed for efficient antigen presen-

tation, such as major histocompatibility (MHC) class I and II, B7.2/CD86, B7.1/CD80, and CD40.^{1–5} Although CNS-infiltrating and/or -associated macrophages and dendritic cells have been considered to be more efficient antigen-presenting cells *in vitro*,^{6–8} microglia can also be effective as antigen-presenting cells after demyelination in mouse models of MS.^{2,3} In EAE, microglia were found to be activated in the CNS before the onset of symptoms and before macrophage and lymphocyte entry into the CNS.⁴ Furthermore, blocking microglial response in the CNS of mice with EAE led to amelioration of symptoms.⁹

Although MS, like EAE, may be initiated by autoreactive lymphocytes migrating from the periphery, lymphocyte recruitment and activation can also follow primary demyelination in the CNS.^{10–12} Therefore, it is important to understand the response of microglia, macrophages, and lymphocytes to primary demyelination in the CNS. To accomplish this, we have used a model of primary demyelination, induced in adult mice by the copper chelator cuprizone. Treatment with cuprizone, administered as a feed additive, induces demyelination in the corpus callosum of C57BL/6 mice, and withdrawal of the toxin allows remyelination.^{13–15} The model is considered immune-independent because RAG^{−/−} mice show no difference in demyelination or remyelination.¹⁶ Cuprizone-induced demyelination also does not promote blood brain barrier break down.^{17–19}

Previous immunohistochemical studies of cuprizone-treated mice demonstrated increased response of microglia and/or macrophages in the demyelinated corpus callosum.^{13,20–22} These studies were unable to differentiate between the two cell types because there is overlap of surface marker expression, and activated cells share morphology. However, microglia can be distinguished from macrophages by their differential expression of CD45.^{7,23,24} To differentiate microglia from macrophages

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on the basis of this distinction and to quantify the cell types from the demyelinated corpus callosum, we have used flow cytometry. We show that in response to cuprizone treatment, microglia, macrophages, and T cells accumulate within the demyelinated corpus callosum, with microglia constituting the majority among these cells. Our data indicate that during primary demyelination, microglial cells increase in number through proliferation as well as by recruitment of progenitors from blood. Furthermore, we identify a unique population of activated microglia during cuprizone treatment that expresses CD11c and has antigen-presenting capability.

Materials and Methods

Cuprizone Treatment

Cuprizone chow was prepared by adding 0.2% cuprizone by weight (Sigma, Oakville, ON, Canada) to milled Purina chow (Harlan Teklad, Madison, WI). To induce demyelination, mice were fed cuprizone chow for up to 6 weeks. Remyelination was induced in mice that had been treated for 6 weeks with 0.2% cuprizone by placing the mice back on normal food for 3 weeks. Unless otherwise specified, 8-week-old female C57BL/6 mice, purchased from The Jackson Laboratory (Bar Harbor, ME), were used in these experiments. To investigate effects of CCL2-CCR2 signaling, CCL2-deficient mice (10 generations C57BL/6; The Jackson Laboratory²⁵) and CCR2-deficient mice (F2 C57BL/6 $\times$ 129/Ola, bred in our facility²⁶) were treated with cuprizone. B6129P F2/J mice purchased from The Jackson Laboratory were used as background controls in studies with CCR2-deficient mice. Mouse handling and experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care as approved by the McGill University Animal Care Committee.

Bone Marrow Chimeras

Twenty 6- to 8-week-old C57BL/6 Ly5.1 female mice (CD45.2) were irradiated and reconstituted with bone marrow from congenic C57BL/6 Ly5.2 mice (CD45.1) (The Jackson Laboratory) as previously described.²⁷ After 7 weeks, mice were placed onto a 0.2% cuprizone diet and sacrificed after 3 or 4.5 weeks of treatment. Chimeric mice that were not treated with cuprizone were also sacrificed at equivalent time points. Chimerism was assessed by flow cytometric analysis of CD45 isoform expression in blood. Flow cytometric analysis of blood showed that between 10 to 11.5 weeks after reconstitution only $3.9 \pm 0.3\%$ (mean $\pm$ SEM) of the circulating blood cells were host derived (CD45.2-positive), indicating successful reconstitution.

Immunohistochemical Analysis and Myelin Staining

Mice were anesthetized with sodium pentobarbital (Somnotol) (120 mg/kg) (MTC Pharmaceuticals, Cambridge, ON, Canada) by intraperitoneal injection in 150 μ l of

(0.9%) saline. Mice were then perfused intracardially with 20 ml of ice-cold phosphate-buffered saline (PBS). The brains were removed and fixed overnight in 4% paraformaldehyde (Sigma) followed by overnight incubation in 20% sucrose and then mounted in OCT (Canemco, Montreal, QC, Canada) and frozen in cryomolds (Canemco). Tissues were stored at -80°C and then sectioned on a cryostat (Microm; Scientific Instruments, Montreal, QC, Canada). Eight- μ m coronal sections were taken between bregma 0.38 and 0.98 mm. Slides were stained with Luxol fast blue and cresyl violet to show myelin and nuclei. For immunohistochemistry, slides were fixed in 4% paraformaldehyde and then permeabilized in a PBS/1% Triton X-100 solution for 30 minutes followed by blocking in 20% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 20% normal goat serum (Jackson ImmunoResearch, West Grove, PA), 80% rat anti-mouse FcR (24G2; American Type Culture Collection, Rockville, MD) hybridoma supernatant and 0.01% sodium azide for 1 hour. The primary antibodies used were as follows: monoclonal rat anti-mouse CD11b (clone 5C6; Serotec, Toronto, ON, Canada) at 1/150, polyclonal rabbit anti-mouse laminin at 1/50 (Cedarlane, Hornby, ON, Canada), and fluorescein isothiocyanate-conjugated monoclonal hamster anti-mouse CD11c (BD Pharmingen, Oakville, ON, Canada) at 1/100. Sections were then incubated with secondary antibodies (if appropriate) conjugated to either Alexa Fluor 555 or Alexa Fluor 488 (Invitrogen), stained with Hoechst 33258 stain (Invitrogen) and mounted with Prolong-Gold (Invitrogen). For all staining procedures, controls were performed with no primary antibodies, serum controls (for polyclonal antibodies), and isotype controls for monoclonal antibodies.

Luxol Fast Blue Quantification

To analyze demyelination in the corpus callosum, coronal sections stained with Luxol fast blue were analyzed using an Olympus BH-2 light microscope (Tokyo, Japan). The qualitative scoring scheme assigned 4 for an unmanipulated mouse (having normal myelin levels), whereas a fully demyelinated mouse was given a score of 0. Sections were scored by an independent individual who was blinded to the study design.

Flow Cytometry

For flow cytometry analysis, microdissected corpus callosa were dissociated by forcing through a 70- μ m sieve (BD Pharmingen) with a syringe plunger. The cell suspension was then processed as previously described²⁸ and stained with various combinations of antibodies or isotype controls, obtained from BD Pharmingen unless otherwise specified (Table 1). Cells stained with biotinylated antibodies were incubated with streptavidin-fluorochrome conjugates (BD Pharmingen). Samples were run on a FACScan (BD Pharmingen) or a FACSCalibur (BD Pharmingen). Samples were gated using forward scatter/side scatter to exclude dead cells. Data were analyzed using Cell Quest software (BD Pharmingen).

Table 1. Antibodies Used for Flow Cytometry

Antibody	Clone
Rat anti-mouse CD11b	M1/70
Hamster anti-mouse CD11c	HL3
Rat anti-mouse CD4	GK1.5
Rat anti-mouse CD19	1D3
Rat anti-mouse CD40	3/23
Rat anti-mouse CD44	IM7
Rat anti-mouse CD45	30-F11
Rat anti-mouse CD45R (B220)	RA3-6B2
Mouse anti-mouse CD45.1	A20
Mouse anti-mouse CD45.2	104
Hamster anti-mouse CD69	H1.2F3
Rat anti-mouse CD8	53-6.7
Rat anti-mouse B7.1 /CD80	16-10A1
Rat anti-mouse B7.2/CD86	GL1
Mouse anti-mouse H2-K ^b	AF6-88.5
Mouse anti-mouse I-A ^b	25-9-17
Hamster anti-mouse TCR β chain	H57-597
Hamster IgG1	G235-2356
Mouse IgG _{2a}	G155-178
Mouse IgG _{2a} (BioLegend)	MOPC-173
Rat IgG _{2a}	R35-95
Rat IgG _{2b}	A95-1

T-Cell Proliferation

Eight-week-old female C57BL/6 mice were immunized on each side at the base of the tail with 100 μ g of myelin

oligodendrocyte glycoprotein (MOG) peptide 35-55 (Sheldon Biotechnology Center, Montreal, QC, Canada) emulsified in complete Freund's adjuvant (Fisher, Montreal, QC, Canada) containing 500 μ g of heat-killed *Mycobacterium tuberculosis* (H37RA; Difco, Detroit, MI). Mice were sacrificed on day 8 after immunization, and the lymph nodes were collected into ice-cold Hanks' balanced salt solution. Lymph nodes were dissociated in Hanks' balanced salt solution by forcing through a 70- μ m sieve (BD Pharmingen). Negative selection of T cells was then performed using Dynal mouse T-cell-negative isolation according to the manufacturer's specifications (Invitrogen). After negative selection, 6×10^5 T cells were cultured in flat-bottomed microwells in a 200- μ l final volume with 50 μ g/ml MOG p35-55 and 1.2×10^6 CD11c-positive or CD11c-negative microglia (CD45^{dim}, CD11b-positive), which had been sorted (FACSaria; BD Pharmingen) from pooled microdissected corpus callosa of either 6-week cuprizone-treated or unmanipulated mice. T cells or microglia alone in MOG 35-55 or medium were cultured as controls. Cells were maintained at 37°C in 5% CO₂ in a humidified atmosphere. Ten μ l of [³H]thymidine (0.5 μ C) (MP Biomedicals, Aurora, OH) was added to the cultures during the last 18 hours, and the cultures were harvested after 72 hours and incorporated radioactivity measured by scintillation counting.²⁹

Figure 1. Cuprizone treatment induces demyelination and increases cellularity in the corpus callosum. Luxol fast blue staining of coronal brain sections from unmanipulated mice (**A**), 6-week cuprizone-treated mice (**B**), and mice treated with cuprizone for 6 weeks followed by 3 weeks of recovery (6 + 3* weeks) (**C**) revealed demyelination in 6-week treated mice and remyelination in recovered animals. **D**: Quantification of the Luxol fast blue stains showed that animals treated for 3 weeks with cuprizone had incomplete demyelination, whereas after 6 weeks of treatment, demyelination was more complete. Six weeks of cuprizone treatment followed by 3 weeks of recovery showed myelination scores returning to those found in unmanipulated animals. **E**: Flow cytometry of corpus callosa from mice during cuprizone treatment showed changes in the number of live-gated cells recovered, which peaked at 4.5 weeks. Significance of data was analyzed using a one-way analysis of variance with a Dunnett posthoc test. * $P < 0.05$, ** $P < 0.01$.

Microglia Preparation for Cell Sorting

Mice were anesthetized with Somnotol as described above and then perfused intracardially with 20 ml of ice-cold PBS. The corpus callosa were then dissected from each animal, pooled, and dissociated in minimal essential medium with penicillin and streptomycin. The cells were centrifuged at $423 \times g$ for 10 minutes at 4°C . Percoll (37%; GE Healthcare, Baie d'Urfe, QC, Canada) was then layered over the cells, and the microglia were harvested as previously described.³⁰ The cells were then incubated with fluorescence-activated cell sorting (FACS) block [2% fetal bovine serum, 0.01% azide, and 50 $\mu\text{g/ml}$ hamster IgG (Bio/Can Scientific, Mississauga, ON, Canada) in 24G2 supernatant], and stained with the appropriate antibodies.

Bromodeoxyuridine (BrdU) Incorporation

Mice undergoing cuprizone treatment or unmanipulated mice received an intraperitoneal injection of 100 μl of BrdU (1 mg/ml) (Sigma) dissolved in sterile saline daily from days 22 to 27. Cuprizone treatment was continued until day 32 (4.5 weeks). Corpus callosa were dissected and analyzed by FACS for BrdU incorporation using a BrdU flow kit as per the manufacturer's instructions (BD Pharmingen). A second group of mice received daily intraperitoneal injections of BrdU from day 27 of cuprizone treatment until sacrifice on day 32 (4.5 weeks).

Results

Cuprizone Treatment Increases Cellularity in the Demyelinated Corpus Callosum

Demyelination occurred in female C57BL/6 mice treated with 0.2% cuprizone, as previously reported.^{15,31,32} After 3 weeks, animals showed varying levels of demyelination, as assessed by Luxol fast blue staining, and demyelination was complete after 6 weeks (Figure 1, B and D). Mice treated for 6 weeks with cuprizone followed by 3 weeks of feeding with normal food (6 + 3*) showed remyelination in the corpus callosum, with myelination scores returning to those of unmanipulated mice (Figure 1, C and D). Cuprizone treatment has been reported to increase cellularity in the demyelinated corpus callosum, despite a loss of oligodendrocytes.¹³ We confirmed that cellularity was enhanced in the corpus callosum of cuprizone-treated mice and that cellularity decreased after remyelination (Figure 1, A–C). To quantify changes in cellularity, we used flow cytometry. Cell suspensions from isolated corpus callosa were acquired and gated on forward scatter/side scatter to exclude dead cells and debris from these analyses. Significant increases in cell numbers were observed in the corpus callosum after 3, 4.5, and 6 weeks of cuprizone treatment (Figure 1E). This represented an increase from $120,200 \pm 14,900$ (mean $\pm$ SEM) cells in unmanipulated corpus callosum to $235,570 \pm 19,560$ at 3 weeks, which then increased to $532,140 \pm 93,890$ at the time of peak response (4.5

Figure 2. Flow cytometric analysis of CD45- and CD11b-positive cells isolated from corpus callosa. Corpus callosa were microdissected from animals treated with cuprizone at 0, 1, 3, 4.5, and 6 weeks as well as from mice undergoing recovery (6 + 3*). Dissociated corpus callosa were analyzed by FACS to quantify the number of microglia and macrophages. Both microglia (A) and macrophages (B) significantly increased in number during cuprizone treatment compared with the number found in unmanipulated mice. Each data point represents the number of cells from one mouse. Data were analyzed using a one-way analysis of variance with a Dunnett posthoc test. ** $P < 0.01$.

weeks). Cell numbers dropped by half at 6 weeks and then approached basal levels during the remyelination phase. Subsequent analyses showed that ~ 15 to 35% of these cells expressed CD45.

Macrophage/Microglial Response in the Demyelinated Corpus Callosum

Relative CD45 levels can be used to distinguish infiltrating macrophages (CD11b-positive, CD45^{high}) from parenchymal microglia (CD11b-positive, CD45^{dim}).^{7,23,24} We evaluated the relative dynamics of the microglial and macrophage response by assessing the total numbers of CD11b-positive cells that expressed low or high levels of CD45, respectively, using flow cytometry.

The majority of CD11b-positive cells expressed low levels of CD45 (CD45^{dim}), which identifies parenchymal microglia. The number of microglia increased during cuprizone treatment, peaking at 4.5 weeks when there were $109,590 \pm 6,770$ microglia per corpus callosum (Figure

Figure 3. Expression of markers of activation on microglia at 4.5 weeks of cuprizone treatment. Microglia were analyzed for expression of activation markers using flow cytometry at either 0 weeks (gray histograms) or 4.5 weeks (black histograms) after cuprizone treatment. For analysis, samples were gated on CD11b-positive CD45^{dim} cells for microglia. The mean fluorescence intensity was examined for B7.2/CD86 (A), B7.1/CD80 (B), MHC class I (C), MHC class II (G), and CD40 (H). Isotype controls for each histogram are shown in white. The percentage of microglia expressing each marker was also examined for B7.2/CD86 (D), B7.1/CD80 (E), and MHC class I (F). Significance of data was analyzed using a two-tailed unpaired Student's *t*-test. **P* < 0.05, ****P* < 0.001.

2A). Microglia comprised 99.5% of the CD11b-positive cells at 4.5 weeks. During recovery (6 + 3*), the number of microglia subsided to levels observed in unmanipulated mice.

Very few (less than 100) CD11b-positive cells expressed the high levels of CD45 (CD45^{high}) that identify blood-derived macrophages in the corpus callosum of unmanipulated mice (Figure 2B). There was a significant increase in the number of macrophages by 4.5 weeks, when there were 520 ± 120 macrophages in the demyelinated corpus callosum. The number of macrophages then declined by 6 weeks but remained elevated compared with untreated mice. Macrophage numbers decreased to lower levels during remyelination.

Cuprizone Treatment Induces Microglial Activation

At 4.5 weeks, the peak of microglial expansion, we found a significant increase in microglial expression of B7.2/CD86 and MHC class I (filled black histograms) compared with unmanipulated mice (gray histograms) (Figure 3, A and C). At 4.5 weeks, the mean fluorescent intensity of B7.2/CD86 expression on microglia was 103.7 ± 1.5 compared with 57.5 ± 0.5 at 0 weeks. For MHC class I, the mean fluorescent intensity at 4.5 weeks

was 240.5 ± 15.3 compared with 73.5 ± 4.6 at 0 weeks. Mean fluorescent intensity for B7.1/CD80 increased slightly but was not statistically significant (Figure 3B). However, the proportions of microglia expressing B7.2/CD86, B7.1/CD80, and MHC class I were significantly greater after 4.5 weeks of cuprizone treatment than in unmanipulated mice (0 weeks) (Figure 3, D–F). There was no change in basal MHC class II or CD40 expression, compared with that on microglial cells from unmanipulated mice (Figure 3, G and H).

A Subpopulation of Microglia Express CD11c

Throughout the course of cuprizone treatment, a subpopulation of microglia emerged that expressed CD11c (Figure 4A, black dots). The proportion of microglia expressing CD11c was significantly increased during cuprizone treatment, from $1.1 \pm 0.1\%$ in controls to $4.9 \pm 0.8\%$ at 3 weeks (Figure 4B). At 4.5 weeks, the peak of total microglial expansion, $14.3 \pm 0.8\%$ of microglia were CD11c-positive, whereas at 6 weeks $39.0 \pm 2.3\%$ were positive. After 6 weeks of cuprizone treatment, the CD11c-positive microglia fell within a subpopulation of microglia that had significantly reduced CD11b expression and elevated CD45 expression (although remaining CD45^{dim}), as compared with CD11c-negative microglia (Figure 4, A and C). Some macrophages also expressed

Figure 4. A distinct population of microglia express CD11c. **A:** Throughout the course of cuprizone treatment, the proportion of CD11c-positive microglia increased in the corpus callosum. These cells formed a distinct cluster defined by CD45 and CD11b staining. FACS plots in **A** show only the CD45-positive (dim + high) cells. CD11c-positive cells are represented by **black dots**. **B:** The percentage of microglia expressing CD11c increased during cuprizone treatment with a maximum at 6 weeks. **C:** At 6 weeks of treatment, CD11c-positive microglia had lower CD11b levels and higher CD45 levels than CD11c-negative microglia. **D:** CD11c-positive microglia from cuprizone-treated mice at 4.5 weeks had significantly higher expression of MHC class I, B7.2/CD86, and B7.1/CD80 as compared with CD11c-negative microglia. Significance of data in **B** was analyzed using a one-way analysis of variance with a Dunnett posthoc test. Other panels were analyzed using a two-tailed unpaired Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

CD11c. A higher proportion of CD11c-positive microglia expressed either MHC class I, B7.2/CD86, or B7.1/CD80 at 4.5 weeks compared with CD11c-negative microglia at this time point (Figure 4D). As with the total microglial population, MHC II and CD40 were not detectably elevated above basal levels on the CD11c-expressing microglia (data not shown).

Histological staining showed that CD11c-positive cells were detected throughout the corpus callosum of mice fed cuprizone for 6 weeks (Figure 5, A and B). CD11c-positive cells were rarely detected in sections from untreated mice (Figure 5C). CD11c-positive cells were restricted to the demyelinated corpus callosum and were not detected in other, nondemyelinated regions of the brain (data not shown). Immunohistochemistry for CD11b showed co-localization with CD11c-positive cells, confirming the flow cytometry results (Figure 5, D–F). Double staining for laminin and CD11c showed that the CD11c-positive cells were within the parenchyma of the CNS and not associated with blood vessels (Figure 5G).

Recruitment from the Blood and Proliferation Contribute to Microglial Expansion during Cuprizone Treatment

We created bone marrow chimeras to investigate the source of the microglia that accumulated during cuprizone-induced demyelination. Lethally irradiated

C57BL/6 (CD45.2) mice were reconstituted with bone marrow from congenic C57BL/6 (CD45.1) mice. Bone marrow-derived CD45.1-positive cells were quantified in the corpus callosum of untreated mice or mice fed cuprizone for 3 or 4.5 weeks. A small increase in bone marrow-derived immigrant microglial cells occurred after 3 weeks when $2.5 \pm 1.0\%$ of the CD45^{dim} microglial population were CD45.1-positive (Figure 6A). There was a significant increase by 4.5 weeks, with $29.2 \pm 4.8\%$ of the CD45^{dim} microglial population expressing CD45.1. Thus, immigration from blood contributed to the microglial population in the demyelinating corpus callosum.

Only a minor subset of CD11c-positive microglia expressed CD45.1 at 3 weeks ($1.7 \pm 0.9\%$). By 4.5 weeks, a slightly greater proportion of CD11c-expressing microglia were CD45.1-positive ($8.2 \pm 1.5\%$), indicating that they had recently immigrated from blood. In parallel stainings, most CD11c-positive microglia were found to express CD45.2 at 3 and 4.5 weeks ($88.5 \pm 8.5\%$ and $94.1 \pm 0.9\%$, respectively), consistent with their being resident microglial cells. By contrast, more than 70% of CD11c-positive CD45^{high} macrophages were CD45.1-positive, indicating their recent immigration from blood. The need for down-regulation of CD45 in transition to CD45^{dim} microglia as well as the relative paucity of CD11c-positive compared with CD11c-negative microglia leaves open that immigrant cells expressing this marker could be underestimated.

Figure 5. CD11c-positive microglia accumulate in the corpus callosum after cuprizone treatment. Immunohistochemistry for CD11c (green) counterstained with Hoechst (blue) in coronal sections showed CD11c-positive cells in 6-week cuprizone-treated mice (**A**) but not in unmanipulated animals (**C**). **B:** Higher magnification of the CD11c-positive cells in the corpus callosum of a cuprizone-treated mouse. Double staining for CD11c (**D**, green) and CD11b (**E**, red) showing co-localization (yellow, **F**). Double staining for laminin (red) to visualize blood vessels (**G**) and CD11c (green) showed that the CD11c-positive cells are not blood vessel-associated. Original magnifications: $\times 400$ (**A**, **C**); $\times 1000$ (**B**).

We also investigated whether proliferation contributed to microglial expansion between 3 and 4.5 weeks. Mice were injected with BrdU for 5 days starting at day 22 or day 27 of cuprizone treatment. Mice were then sacrificed at day 32 (4.5 weeks), and cells that had incorporated BrdU were detected by flow cytometry. Close to 8% of microglia from cuprizone-treated mice had incorporated BrdU at 4.5 weeks ($7.5 \pm 1.1\%$ and $7.7 \pm 2.1\%$, respectively) (Figure 6B). A greater proportion ($11.1 \pm 3.3\%$) of CD11c-positive microglia were BrdU-positive in mice treated days 22 to 27, whereas $6.4 \pm 1.4\%$ of CD11c-positive microglia were BrdU-positive after treatment on days 22 to 32. The kinetics of the BrdU experiments probably underestimates total proliferation, and these data indicate that proliferation contributes significantly to microglial expansion in the demyelinated corpus callosum. Expansion of CD11c-positive microglia also involved proliferation.

CCR2 Contributes to the Increase in CD11c-Positive Microglia during Cuprizone Treatment

mRNA for a number of chemokines, including CCL2 was rapidly up-regulated in the CNS of mice treated with cuprizone (data not shown). It has been proposed that CCL2-CCR2 signaling recruits microglial progenitors to the

CNS.^{33,34} Total numbers of microglial cells were similar in CCL2-deficient mice and C57BL/6 control mice at 3 and 6 weeks after cuprizone treatment, whereas macrophage numbers were significantly reduced (Figure 7, B and D). This selective impairment of macrophage recruitment but not microglial expansion was also seen in CCR2-deficient mice. Indeed, macrophage recruitment was significantly blocked in CCR2-deficient mice after 3, 4.5, 6, and 6 + 3* weeks of cuprizone treatment, compared with background matched B6.129 controls (F2), whereas no significant changes in total microglial numbers were observed (Figure 7, A and C). We confirmed that mice lacking CCR2 and B6.129 controls responded similarly to cuprizone by demyelination at 6 weeks (data not shown).

The fold increase in CD11c-expressing microglia was transiently reduced at 4.5 weeks of cuprizone treatment in CCR2^{-/-} mice compared with B6.129 controls (Figure 7E). This effect was no longer seen at 6 weeks of cuprizone treatment, but after 3 weeks of remyelination, CCR2-deficient mice had fewer CD11c-positive microglia. No changes in CD11c-expressing microglia were noted in CCL2-deficient mice at either 3 weeks or 6 weeks (data not shown). Therefore, chemokine signaling may also play a role in generation or maintenance of CD11c-positive microglia after 4.5 weeks of cuprizone treatment and during recovery.

Figure 6. Microglia increase in the corpus callosum during cuprizone treatment because of recruitment from the blood as well as proliferation. Flow cytometry was used to enumerate blood-derived (CD45.1-positive) microglia in corpus callosa from bone marrow chimeras treated with cuprizone. **A:** The percentage of CD45.1-positive microglia in untreated chimeras and in chimeras treated with cuprizone for 3 and 4.5 weeks. **B:** BrdU treatment of cuprizone-treated mice showed that at 4.5 weeks microglia were proliferating. Corpus callosa from cuprizone-treated mice injected with BrdU for 5 days between either 22 to 27 days or 27 to 32 days were isolated for flow cytometry. The graph shows the number of microglia that had incorporated BrdU at either 4.5 weeks of treatments or 0 weeks. Data were analyzed using a one-way analysis of variance with a Bonferroni posthoc test. ** $P < 0.01$, *** $P < 0.001$.

CD11c-Positive Microglia Are Potent Antigen-Presenting Cells

Previous reports have indicated that CD11c-expressing cells in the CNS are potent antigen-presenting cells.^{8,35} To investigate the antigen-presenting capacity of the CD11c-positive microglia, we performed proliferation assays using FACS-sorted microglia as antigen-presenting cells. MOG-reactive T cells isolated from the lymph nodes of MOG/complete Freund's adjuvant-primed mice were co-cultured either with CD11c-positive microglia that were sorted from pooled corpus callosa taken after 6

Figure 7. CCR2 contributes to the increase in CD11c-positive microglia during cuprizone treatment. Total microglial (**A, B**) and macrophage (**C, D**) numbers were assessed in CCR2-deficient mice, CCL2-deficient mice, and wild-type controls by FACS. C57BL/6 mice were used as controls in experiments with CCL2-deficient mice, whereas B6.129 mice (F2) were used to control for genetic background in studies with CCR2-deficient mice. **E:** CCR2^{-/-} mice (open circles) had fewer CD11c-positive microglia at 4.5 weeks of cuprizone treatment and after recovery (6 + 3*) compared with B6.129 controls (filled circles) at the same time points. Significance of data at each time point was analyzed using a two-tailed unpaired Student's *t*-test. * $P < 0.05$, ** $P < 0.01$.

weeks of cuprizone treatment or with CD11c-negative microglia sorted from unmanipulated or cuprizone-treated mice. No T-cell responses were observed without addition of MOG peptide, indicating that there was no presentation of endogenously acquired MOG antigen. However, when co-cultured with CD11c-positive or CD11c-negative microglia and with MOG peptide, T-cell proliferation was induced (Figure 8). CD11c-positive microglia stimulated approximately three times greater T-cell response than CD11c-negative cells, showing enhanced capacity for antigen presentation.

T Cells Are Recruited to the Demyelinated Corpus Callosum

We assessed TCR β -positive T-cell entry to the corpus callosum by flow cytometry (Figure 9). T-cell numbers

Figure 8. Antigen-presenting capacity of CD11c-positive microglia versus CD11c-negative microglia. Proliferative response of MOG-specific T cells to MOG p35-55 presented by FACS-sorted microglial subpopulations was measured by [^3H]thymidine incorporation. Microglia were plated in a 1:2 ratio to T cells. Results are shown as mean stimulation index in response to CD11c-positive (white) and CD11c-negative (black) microglial cells from 6-week cuprizone-treated corpus callosum and to CD11c-negative microglia from unmanipulated corpus callosum (black). Significance of differences was analyzed using a one-way analysis of variance with a Bonferroni posthoc test. * $P < 0.05$, ** $P < 0.01$.

increased during cuprizone treatment, with levels being highest after 6 weeks of cuprizone treatment when there were 1630 ± 290 T cells. After 3 weeks of recovery, T-cell numbers declined to lower levels. Analysis of T-cell subsets demonstrated that the proportion of CD4 T cells steadily declined throughout cuprizone treatment. CD4-positive T cells accounted for $48.4 \pm 6.7\%$ of the T-cell population at 3 weeks. This dropped to $41.4 \pm 3.2\%$ by 4.5 weeks. By 6 weeks, the CD4/CD8 ratio showed a predominance of CD8 T cells with $63.5 \pm 7.7\%$ of the TCR β -positive cells being CD8-positive. The activation markers CD44 and CD69 were not up-regulated on TCR β -positive T cells (data not shown). There were almost no B cells present in the corpus callosum at 6 weeks using FACS (data not shown).

Discussion

We demonstrate that in response to primary demyelination induced by the toxin cuprizone, microglia become activated, migrate to, and proliferate within the demyelinated corpus callosum. The microglial population became heterogeneous, and a CD11c-positive subpopulation emerged. T cells also infiltrated, and there was a minor contribution to gross cellularity from macrophages. These proinflammatory changes tended to resolve on withdrawal of toxin.

The microglial and macrophage response was maximal at 4.5 weeks, with fewer of either cell type at 1, 3, and 6 weeks of treatment as well as 3 weeks after cuprizone withdrawal. In contrast to an earlier report in which macrophages constituted up to 20% of the CD11b-positive population at 6 weeks,¹⁸ our data indicate that at 6 weeks

Figure 9. T cells infiltrate the demyelinated corpus callosum. The T-cell response to demyelination and remyelination was measured in 0-, 1-, 3-, 4.5-, and 6-week treated mice as well as from mice treated for 6 weeks with cuprizone followed by 3 weeks of recovery (6 + 3*). T cells were identified as TCR β -positive CD45^{high} cells. Data were analyzed using a one-way analysis of variance with a Dunnett posthoc test * $P < 0.05$, ** $P < 0.01$.

macrophages constituted only $\sim 0.5\%$ of the CD11b population. The aforementioned study used pooled whole brains for flow cytometry analysis, whereas our study used individual microdissected corpus callosa, and it may be that macrophages deriving from other brain regions contributed to their results. The macrophage response to cuprizone-induced demyelination therefore resembles the macrophage infiltration seen after axonal injury^{28,36} rather than the massive macrophage infiltration seen in MOG-induced EAE in C57BL/6 mice.^{1,29} The large increase in microglial numbers between 3 to 4.5 weeks of cuprizone treatment was in part attributable to recruitment of microglial progenitors from blood as well as from microglial proliferation. Both mechanisms of microglial expansion occur in the unmanipulated mouse CNS, albeit to a lesser extent.³⁷ Between 3 and 4.5 weeks of treatment, $\sim 7.6\%$ of the microglia in the corpus callosum had proliferated, which is greater than the rate of microglial proliferation reported in the CNS of mice with EAE³⁵ but less than what was observed in the acutely injured CNS.³⁸ The signals that initiate microglial proliferation are unknown but may involve innate signaling pathways, as we have observed in other systems.³⁶ At 4.5 weeks, although the blood brain barrier remains intact during cuprizone treatment,¹⁷⁻¹⁹ $\sim 30\%$ of the microglia were recent immigrants from the blood. The environmental cues necessary to recruit microglial progenitors from the blood during demyelination are unclear but may include chemokines or cytokines that are produced after cuprizone treatment.³⁹ Our data demonstrate that CCL2-CCR2 signaling is not the critical cue driving this response because microglial numbers were similar in knockout and wild-type animals during demyelination. Microglia originating from bone marrow precursors have been shown to engraft the CNS after injury to the adult mouse CNS.^{33,38,40,41} Here, we show that influx of microglia progenitors from blood to the corpus callosum is a major contributor to the increase in microglial numbers during primary demyelination.

At the peak of gliosis at 4.5 weeks, microglia from cuprizone-treated mice expressed increased levels of MHC class I, CD11c, and B7.2/CD86, indicating activation in response to cuprizone treatment. The expression of such activation-associated molecules by microglia is similar to the expression profile of microglia seen in other demyelinating models.^{1,2,4,7,42} Interestingly, microglia did not up-regulate MHC class II. This may reflect lack of specific cytokine production such as interferon- γ , which is known to up-regulate MHC class II expression on microglia.^{43–45} Activated microglia in mixed glial cultures have also been shown to down-regulate MHC class II expression while up-regulating levels of co-stimulatory molecules such as B7.2/CD86.⁴⁶ Unlike Mana and colleagues⁴⁷ who detected interferon- γ mRNA in microdissected cuprizone-demyelinated corpus callosum from C57BL/6 mice, we did not detect interferon- γ mRNA by real-time polymerase chain reaction (PCR) analysis at any time (not shown). Although basal MHC class II levels were not up-regulated on microglia, CD11c-positive and CD11c-negative microglia presented antigen to T cells isolated from mice immunized with MOG in complete Freund's adjuvant, usually considered a MHC II-dependent response. This probably reflects that baseline MHC II levels were sufficient for T-cell recognition—very few MHC class II molecules are required to initiate T-cell activation.^{48,49}

The presence of CD11c-expressing microglia in the CNS after primary demyelination complements studies from mice with EAE,⁴ in the ischemic CNS,⁵⁰ as well as in canine distemper lesions⁴² and Toxoplasma encephalitis,⁵¹ in which a proportion of microglia were found to be CD11c-positive. In our study, CD11c-positive microglia expressed higher levels of MHC class I, B7.2/CD86, and B7.1/CD80 than CD11c-negative microglia, in accordance with the notion that CD11c-expressing cells in the CNS are potent antigen-presenting cells. We find that CD11c-expressing microglia are better antigen-presenting cells *in vitro* compared with CD11c-negative microglia. Previous studies have shown that CNS-associated CD45^{high} CD11b-positive cells expressing CD11c were potent antigen-presenting cells,^{8,52} but these studies did not specifically examine the antigen-presenting capacity of CD11c-expressing microglia. Other studies examining the antigen-presenting capacity of CD11c-positive cells in the CNS examined CD11b-positive populations in the CNS regardless of their CD45 levels.^{35,51}

The origin of the CD11c subpopulation of microglia is of interest. Migration of microglia expressing CD11c into the demyelinated corpus callosum from other areas of the CNS seems unlikely since there were few CD11c-positive cells outside the corpus callosum (data not shown). Nevertheless, our data do not exclude that CD11c-positive microglia arise through proliferative expansion of a small pre-existing pool, either in or entering the corpus callosum. Indeed, a significant proportion of the CD11c-positive cells in the corpus callosum had incorporated BrdU at 4.5 weeks, supporting that proliferation may contribute to the expansion of this subpopulation. Fischer and colleagues⁵¹ reported that CD11c-negative microglia

from adult mice could be induced *in vitro* to differentiate into CD11c-positive microglia with potent antigen-presenting capability, in the absence of proliferation—these cells did not express the plasmacytoid dendritic cell markers CD8 α or DEC205.³⁵ It is possible that in our *in vivo* system, CD11c-negative microglia give rise to CD11c-positive cells, which then expand through proliferation. Our data also suggest that microglia arise by immigration from blood, as has been shown by others. CD11c has been shown to be expressed by microglia that had recently immigrated from the blood.³⁴ In our system, only a minority of CD11c-positive microglia were recent immigrants from blood. We did find immigrating cells that were CD11c-positive, but most of these cells expressed high levels of CD45, consistent with their being macrophages and/or dendritic cells, not CD45^{dim} microglial cells. Ponoromev and colleagues⁴ found that in the CNS of mice with EAE there were two different populations of CD11c-positive cells, one that was CNS resident and the other bone marrow-derived. Our results also suggest a role for chemokine signaling in the generation and/or maintenance of the CD11c-positive microglia. The fold increase in CD11c-expressing microglia was transiently reduced in CCR2^{-/-} mice at 4.5 weeks as well as after 3 weeks of remyelination.

The contribution of activated microglia to demyelination and remyelination is unclear. Activated microglia may phagocytose debris and produce cytokines/growth factors that stimulate oligodendrocyte precursor cells but can also release cytotoxins such as reactive oxygen species that can damage oligodendrocytes. Pasquini and colleagues⁵³ investigated the role of the microglial response by administering minocycline to mice undergoing cuprizone treatment. This decreased the number of microglia and prevented demyelination. The role of specific microglial subsets needs further examination. When microglia activated by aggregated β -amyloid were induced to express CD11c, microglial-mediated inhibition of neurogenesis was counteracted *in vitro*.⁵⁴ Recently immigrating microglia were found to be better phagocytes than resident microglial cells in a mouse model of Alzheimer's disease.⁵⁵

We consider it unlikely that the antigen-presenting capability that CD11c-positive microglia show *in vitro* plays any role in cuprizone demyelination. RAG^{-/-} mice showed no differences in either the extent or kinetics of cuprizone-induced demyelination or remyelination,¹⁶ indicating no role for T cells. Consistent with this, we found that T cells that infiltrated the demyelinated corpus callosum did not show an activated phenotype. The conditions required for activation of T cells in this system remain to be identified.

The response to primary demyelination in the CNS induced by cuprizone involves the recruitment of T cells, macrophages, and microglia to the demyelinated tissue. The CNS microenvironment likely controls interactions between these cell types. Our study has implications for diseases like MS in which activated microglia and T cells are associated to demyelinating lesions.

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