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The Kinetics of Myelin Antigen Uptake by Myeloid Cells in the Central Nervous System during Experimental Autoimmune Encephalomyelitis

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Induction of experimental autoimmune encephalomyelitis (EAE) in susceptible animals requires reactivation of encephalitogenic CD4+ T cells by APCs in the CNS. However, it has remained unresolved from where APCs in the CNS acquire myelin Ag for T cell activation and under which conditions, that is, whether only during EAE or also in the naive CNS. In this study, we investigated the kinetics of myelin Ag uptake by CNS APCs during EAE and in the naive CNS. Our results show that during EAE CX3CR1+ CD11b+ microglia were the first APCs in the CNS to contain myelin Ag upon induction of disease, albeit in very small numbers. Dendritic cells (DCs) arrived in the CNS in sizable numbers significantly later (day 5 postimmunization), without detectable myelin Ag, but acquired it by day 7 postimmunization. Furthermore, a sharp increase in neuroantigen-containing DCs coincided with the onset of EAE symptoms. Importantly, in naive mice a low but consistent number of microglia contained myelin Ag, suggesting release by oligodendrocytes under steady state conditions. Although microglia isolated from naïve brain and spinal cord did not elicit a strong CD4+ T cell response in vitro, myelin Ag-containing microglia may still play a local role in modulating encephalitogenic CD4+ T cell responses in early EAE prior to the arrival of other professional APCs, such as DCs. Finally, newly arriving DCs in the CNS not yet loaded with myelin Ag before the onset of EAE may be a potential therapeutic target. The Journal of Immunology, 2013, 191: 5848–5857.

Multiple sclerosis (MS) is the most common autoimmune disease of the CNS, affecting > 400,000 people in the United States alone (1). Hallmarks of the disease are focal demyelinated lesions and transection of axons, believed to be mediated by infiltrating inflammatory cells, including CD4+ and CD8+ T cells, B cells, and APCs such as dendritic cells (DCs) and macrophages (2–5).

Autoreactive CD4+ T cells are thought to be important contributors to the disease process (6). To mediate disease, myelin-reactive CD4+ T cells have to migrate to the CNS, where they encounter their cognate myelin Ag presented by MHC class II molecules on APCs and become activated and exert their effector functions (7, 8). Along these lines, it has been shown that impaired expression of MHC class II molecules in the CNS abrogates experimental autoimmune encephalomyelitis (EAE), as does depletion of APCs (9–11). Potential APCs found in the CNS during EAE and MS are tissue-resident microglia as well as infiltrating DCs, macrophages, B cells, and neutrophils (12–14). Parenchymal microglia rapidly upregulate MHC class II molecules in response to a variety of inflammatory stimuli, suggesting that they may play a role in Ag presentation in the CNS (15–17); however many current molecular markers used to identify microglia are also present on peripheral macrophages, which makes it difficult to ascertain their origin during CNS inflammation. CNS-resident astrocytes have been shown in vitro to upregulate MHC class II molecules and present Ag to T cells (18); however this has not been demonstrated in vivo. In addition, they do not upregulate the costimulatory molecules CD80 or CD86 (19), and therefore may be unable to activate T cells. It has been shown that infiltrating DCs are critical for Ag presentation to encephalitogenic T cells in EAE (11, 17, 20). Recently, it has been suggested that during the early stages of EAE, myelin Ag presentation takes place in the subarachnoid space and leptomeninges (21). Presumably, this Ag presentation would require the translocation of myelin Ag from oligodendrocytes/myelinated axonal membranes to the perivascular spaces.

Oligodendrocytes represent the most likely source of myelin Ag presented by APCs to CD4+ T cells in MS and its animal model, EAE, as they are the CNS-resident cells responsible for myelination by extending their own membranes around neuronal axons (22). However, oligodendrocytes themselves do not express MHC class II molecules and thus cannot directly present myelin Ag to CD4+ T cells (23–26). Therefore, an important unanswered question is from where and under which conditions myelin Ag is translocated to APCs in the CNS for Ag presentation and the activation of pathogenic CD4+ T cells. Furthermore, little is known about the kinetics and cellular distribution of myelin Ag uptake by APCs in the naive CNS or during disease conditions.

In this article, we show that myelin Ag was constitutively present in a small number of CNS-resident microglia in naïve mice. Upon induction of EAE, CD4+ T cells, DCs, neutrophils, and macrophages...
infiltrated the CNS in increasing numbers. Myelin Ag was not detected in DCs infiltrating the CNS before day 5 after induction of EAE, whereas as a small percentage of microglia contained myelin Ag already by day 1 after immunization. Onset of EAE coincided with a sudden spike in the number of infiltrating DCs and macrophages in the CNS, the majority of which contained myelin Ag. In contrast, disease remission was paralleled by a strong decline in CNS APCs associated with myelin Ag. Our data suggest that myelin uptake by CNS APCs is a dynamic process shaped by inflammatory cells during the course of disease.

Materials and Methods

Animals, immunization, and EAE scoring

Female 6- to 8-wk-old C57BL/6 and SJL mice were obtained from The Jackson Laboratory. The 2D2 transgenic (tg) mice, DRB1*15:01 tg mice, CX3CR1<sup>Cre</sup><sup>+</sup> mice, and HLA-DR2 tg shiverer (Mbp<sup>−/−</sup>) mice were bred in-house, and all animals were maintained in pathogen-free conditions under the guidelines established by the Institutional Animal Care and Use Committee at the University of Texas at San Antonio. Mice were fed and watered ad libitum. To induce EAE, 6- to 8-wk-old mice were immunized s.c. with 200 μg proteolipid protein (PLP<sub>39–151</sub>) in CFA containing 5 mg/ml Mycobacterium tuberculosis. Peritoneal exudate cells (PEC) (day 7) was given i.p. on day 0 and day 2 relative to immunization. Clinical scores were monitored daily and assigned a score based on the following symptoms: 0, no clinical disease; 1, flaccid tail; 2, partial hind limb paralysis; 3 total hind limb paralysis; 4, frontal and hind limb paralysis; 5, moribund or dead. For adoptive transfer, donor SJL mice were immunized s.c. with 200 μg proteolipid protein (PLP<sub>39–151</sub>) emulsified 1:1 in CFA containing 5 mg/ml PLP<sub>139–151</sub> Ag and 20 ng/ml IL-23, and put into a 37˚C incubator overnight. To induce EAE, 6- to 8-wk-old mice were immunized i.p. into each recipient SJL mouse.

Immunofluorescence staining, Abs, and quantification

Abs to myelin basic protein (MBP, 7H11), MOG, and PLP were purchased from Neuronics. Fluorochrome-conjugated Abs to CD4 (GK1.5), CD11b (M1/70), CD11c (N418), CD19 (1D3), DEC205 (205xekte), and I-A/I-E (MHci class II, NIMR-4, M5/114/152 or 10-3.6 as haplotype-appropriate) were purchased from eBioscience. Ly-6G (1A8) was purchased from BD Biosciences. DAPI was purchased from Sigma-Aldrich. Immunofluorescent staining was performed as follows: Murine brains and spinal cords were obtained in designated time points, placed in OCT freezing compound and frozen at −80˚C. Tissue was cut into 4- to 6-μm-thick transverse sections and staggered onto glass slides. Alternatively, tissue was cut into 20- to 60-μm-thick transverse sections and placed in fixative solution in 24-well non-tissue culture–treated plates (Fisher), then placed on slides following the staining procedure. Sections were fixed (fixation buffer, eBioscience) on ice, then permeabilized (permeabilization buffer, eBioscience) and blocked with appropriate serum prior to staining. Purified Primary Abs were allowed to incubate on sections overnight at 4˚C in a closed, humid chamber. Secondary Abs and directly labeled primary Abs were then added for an hour at room temperature in a closed, humid chamber. Between incubations, slides were rinsed with PBS, PBS + 0.05% Tween 20 (PBST) or PBS+0.1% Triton-X, depending on the Ag of interest. Coverslips were mounted with ProLong Gold Antifade Reagent (Invitrogen) and slides were allowed to dry and cure overnight at room temperature in the dark. Images were acquired on a Zeiss LSM510 confocal laser scanning microscope, using Zen 2009 (Zeiss) acquisition software. Image analysis was performed using Imaris 3D/4D software (BD Biosciences).

In vitro titration and detection of myelin Ag by confocal microscopy

For in vitro titration of myelin Ags, naive splenocytes were obtained from 6- to 8-wk-old C57BL/6 mice and processed into single-cell suspensions. CD11c<sup>+</sup> DCs were positively selected for using magnetic separation on autoMACS (Miltenyi Biotec, 94.1% purity). In addition, single-cell splenocyte suspensions from 2D2 tg mice were enriched for MOG<sub>35–55</sub> Peptide-reactive CD4<sup>+</sup> T cells, using negative selection on autoMACS (Miltenyi Biotec, 95.3% purity). CD11c<sup>+</sup> and CD4<sup>+</sup> cells were cultured overnight with increasing concentrations of recombinant rat MOG (rMOG) protein alone or in combination with 100 ng/ml LPS. Cells were spread onto positively charged “plus” slides (Fisher) at a concentration of 1 × 10<sup>5</sup> cells/ml and stained according to the protocol described in immunofluorescence staining section. Slides were imaged with a Zeiss LSM510 confocal laser scanning microscope and analyzed using Imaris software.

Reconstructing neuroantigen loading in three dimensions

The three-dimensional structure of APCs in the CNS was reconstructed from confocal laser scanning microscopy z-stack images taken 0.2–0.3 μm apart from 1.2 to 59.6 μm in depth, using Imaris software, depending on the thickness of the CNS tissue being imaged. Quantification of the absolute number of APCs within a defined region of interest was enabled using the fact that Imaris can recognize the center of mass in each nucleus. The number of nuclei present in each 3D stacked image that could be radially expanded to coloculate with APC cell surface markers within 5–10 μm in >75% of all possible directions were counted as APCs, using Imaris 7.2 software. To distinguish myelin Ag that was associated with an APC, including myelin Ag colocalizing to the cell surface, from Ag in the extracellular microenvironment, APCs were digitally enclosed using the reconstructed 3D surface (Supplemental Fig. 2).

Cytokine ELISPOT

Cytokine ELISPOT assays were performed as described previously (27). Briefly, ELISPOT plates (Multiscreen IP; Millipore) were coated with 1 μg/ml IFN-γ-specific (AN-18; eBioscience) or IL-17–specific capture mAb (17F5; Bio X Cell) diluted in PBS. The plates were blocked with 1% BSA in PBS for 1 h at room temperature and then washed four times with PBS. Cells were added with or without Ag and incubated for 24 h at 37˚C. The plates were washed three times with PBS and four times with PBS, and IFN-γ–specific biotinylated detection mAb (R4-6A2; eBioscience) or IL-17–specific biotinylated detection mAb (TC11-81-14; BioLegend) was added and allowed to incubate overnight. The plate was washed four times with PBS and incubated with streptavidin–alkaline phosphatase (Invitrogen). Cytokine spots were visualized by 5-bromo-4-chloro-3-indolyl phosphate/NBT phosphate/NBT phosphate substrate (Kirkegaard & Perry Laboratories). Image analysis of ELISPOT assays was performed on a Series 2 ImmunoSpot analyzer and software (Cellular Technology), as described previously (28). In brief, digitized images of individual wells of the ELISPOT plates were analyzed for cytokine spots, based on the comparison of experimental (containing T cells and APC with Ag) and control wells (T cells and APC, no Ag). After separation of spots that were touching or partially overlapped, nonspecific noise was gated out by applying spot size and circularity analysis as additional criteria. Spots that fell within the accepted criteria were highlighted and counted.

Flow cytometry and cell sorting

Spleen, lymph nodes, brain, and/or spinal cord tissues were removed from naive mice following cardiac perfusion or EAE mice without perfusion, as previously described (29). Single-cell suspensions were obtained from tissue by mechanical isolation. Extracellular myelin removal was performed for all brain and spinal cord suspensions according to the manufacturer’s instructions. (Miltenyi Biotec). CD11b<sup>+</sup> microglia were sorted from a BD FACSAria II (BD Biosciences) at a concentration of 1 × 10<sup>6</sup> cells/ml and stained with 107 cells/ml in DMEM containing 10% FCS and 1% L-glutamine, placed into a 24-well culture plate with 10 in injected i.p. into each recipient SJL mouse.

Statistical analysis

Statistical evaluation was performed using JMP SAS software. Comparison across groups of infiltrating and MBP-associated cells was evaluated using ANOVA. T cell activation was assessed using a two-tailed Student t test. A p value ≤ 0.05 was considered significant.
Results
Distribution of potential APCs and MHC class II in the CNS during EAE and in naive mice

It has remained unresolved which APCs are most critical for presentation of neuroantigen to CD4+ T cells for induction of EAE, and whether myelin Ag is presented in the naive CNS and by which APCs. To begin to address these questions, we investigated the presence of cells with the potential to present myelin Ag based on myeloid lineage and expression of MHC class II molecules in the CNS of naive mice and animals with EAE.

EAE was induced in C57BL/6 mice with MOG35–55 peptide, and animals were evaluated for clinical disease as described (2). In parallel, a group of age- and gender-matched C57BL/6 mice was maintained unimmunized. The distribution of potential APCs and other inflammatory cells was examined in brain tissue slices of these mice by H&E (Fig. 1A, 1H) and immunofluorescence (Fig. 1B–G; I–N) staining at peak EAE [usually about day 21 postimmunization (p.i.)]. As expected, inflammatory infiltrates containing large numbers of CD4+ T cells were detected in the CNS of C57BL/6 mice (Fig. 1B versus 1I). CD11c+ DCs were abundantly present and clustered in CNS lesions in mice with EAE, whereas they were exceedingly rare in the CNS of naive mice and found contained only within the meninges (Fig. 1C, 1J). Large clusters of CD11b+ cells, which included microglia and infiltrating macrophages, were found in the highest numbers during EAE (Fig. 1D). They were distributed evenly and occurred in lower numbers mostly throughout the parenchymal white matter in the CNS of naive mice and represent the resident microglial population (Fig. 1K). Similarly, neutrophils (Ly-6G+) were virtually absent from the CNS of naive mice, whereas they were detected in low numbers in mice with EAE (Fig. 1E, 1L). CD19+ B cells were not detected in notable numbers in the CNS of naive mice with EAE in this study (Fig. 1F, 1M). Finally, expression of MHC class II molecules was upregulated in inflammatory lesions in the CNS of mice with EAE, whereas it was rarely detected in the CNS of naive mice (Fig. 1G, 1N), and only on CD11b+ microglia.

Thus, activated microglia/macrophages and DCs were the predominant potential APCs in CNS lesions in the MOG35–55 peptide–induced EAE model in our studies. In contrast, microglia were the only potential APC identified in the CNS parenchyma of naive mice.

Visualizing uptake of neuroantigen by myeloid cells in the CNS during EAE

Next, we asked which myeloid cells contained myelin Ag during EAE and whether it was presented on MHC II molecules for T cell activation. To address this issue, EAE was induced in C57BL/6 mice with MOG35–55 peptide or via adoptive transfer of PLP139–151–reactive T cells in SJL mice, as previously described (30). CNS tissue was obtained at the peak of disease and analyzed by confocal microscopy for colocalization of MBP, MOG, or PLP with myeloid cells.

Shown in Fig. 2A–C are representative confocal images of MBP, MOG, or PLP expression in the CNS of naive mice. Specificity of staining was confirmed using CNS tissue from MBP-deficient shiverer mice, which, as expected, did not show MBP staining (Fig. 2D). Importantly, all three myelin Ag colocalized with DCs and microglia/macrophages in EAE mice (Fig. 2E–H, arrows) and were detected within DCs and macrophages/microglia (Fig. 2I). A similar kinetic of colocalization of myelin Ags (e.g., MBP, MOG, PLP) with DCs and microglia was observed when EAE was induced by adoptive transfer of PLP139–151–specific T cells into SJL mice (Supplemental Fig. 1). Consistent with our earlier results, infiltrating CD11b+ inflammatory macrophages acquired myelin Ag (e.g., PLP) only upon entering the parenchymal white matter at the onset of EAE (day 7; Supplemental Fig. 1A). As shown in Fig. 2I, MBP was clearly detectable within APCs at high magnification (×100; MBP+) and could easily be distinguished from APCs that did not contain MBP in internal slices of z-stacks made from each lesion area (Fig. 2I, MBP+; Supplemental Fig. 3). Only a small percentage of neutrophils in the CNS showed staining for MBP or MOG (not shown), arguing against a major role for these phagocytic cells in the presentation of myelin Ag during EAE.

To determine if myelin Ag detected in CNS APCs was processed and loaded onto MHC II molecules for Ag presentation to T cells, we took advantage of the MK16 Ab, which specifically recognizes the MBP85–99 peptide bound to HLA-DR2 molecules (31). EAE was induced in HLA-DR2b (DRB1*15:01) tg mice with MOG35–55 peptide, and CNS tissue was obtained at the peak of disease for confocal imaging. Staining of brain tissue from HLA-DR2b tg mice with EAE with anti-MBP and anti-CD11c and -CD11b mAb confirmed that MBP colocalized with APCs in a pattern similar to that observed in C57BL/6 mice (Fig. 2J). Importantly, MK16 staining colocalized to APCs (Fig. 2J, large arrow) and overlapped with the

FIGURE 1. Detection of inflammatory cells and MHC class II expression in the CNS. Brains were obtained from EAE mice at the peak of clinical EAE (day 21 p.i., top row) and from naive mice (bottom row). (A and H) H&E staining of an inflammatory perivascular focus in CNS. Confocal images of immunofluorescence staining (green) for (B) CD4+ T cells; (C and J) CD11c+ DCs; (D and K) CD11b+ microglia/infiltrating macrophages; (E and L) Ly-6G+ neutrophils; (F and M) CD19+ B cells; and (G and N) MHC II+ cells. Cell nuclei are stained with DAPI. As expected, only microglia were present in significant numbers in the naive parenchyma (K), whereas other cell types were found only in meninges prior to immunization (I, J, and L). (F and M) CD19+ B cells were not detected in the CNS of naive or EAE mice. (G and N) IF staining for MHC class II molecule shows increased expression during EAE, whereas expression is low in the naive brain. Scale bars, 50 μm.
MBP\(^{+}\) staining (Fig. 2J, small arrow, box). In contrast, no MK16 staining above background levels was detected in HLA-DR2b tg shiverer mice (MBP\(^{2/2}\)) or HLA-DR4 tg mice with EAE (not shown), confirming the specificity of MK16 staining. Finally, CD4\(^{+}\) T cells (small arrow) were found adjacent to CNS APCs containing MBP, consistent with Ag-presenting function by these cells (Fig. 2K).

Taken together, the results show that myelin Ag is primarily taken up and presented by CD11b\(^{+}\) microglia/infiltrating macrophages and CD11c\(^{+}\) DCs during EAE. The data suggest that the contribution of other potential APCs to neuroantigen presentation in the CNS is minor during the clinical disease course.

Detection of myelin Ag in APCs by confocal microscopy corresponds to the T cell activation threshold for cytokine production

We showed that myelin Ag, for example, MBP and MOG, were detected by confocal microscopy in several APC subsets in the CNS over the course of EAE. However, it remained unresolved how sensitive this technique was; for example, was myelin Ag detected by confocal microscopy in CNS APCs at levels that could be considered sufficient for the activation of encephalitogenic T cells?

To begin addressing this issue, we developed a semiquantitative in vitro assay that replicated confocal imaging of myelin Ag in CNS tissue. Splenocytes were pulsed with recombinant rat MOG\(_{1-125}\) protein, and cell spreads were prepared for confocal imaging to determine the concentration of MOG that could be detected in APCs. In parallel, MOG-specific 2D2 T cells were incubated with MOG\(_{1-125}\) protein-pulsed splenocytes to determine by cytokine ELISPOT assay for IFN-\(\gamma\), IL-17, and GM-CSF at what Ag concentrations T cell responses were induced.

The results show that MOG protein could be detected internalized within cells by internal slices of z-stacks taken by confocal microscopy starting at 0.01 \(\mu\)g/ml of Ag (Fig. 3A, arrows). Importantly, as determined by cytokine ELISPOT assay, this concentration of MOG protein was suboptimal for the activation of MOG\(_{35-55}\)-specific T cells for the production of IFN-\(\gamma\), IL-17, and GM-CSF (Fig. 3B). At 1 \(\mu\)g/ml of Ag, similarly strong results were detected by confocal microscopy as well as by cytokine ELISPOT assay (Fig. 3A, 3B).

**FIGURE 2.** Myelin Ag localization to myeloid cells in naive and EAE CNS. (A) MBP, (B) MOG, and (C) PLP staining in naive mice cerebellum showing the characteristic staining pattern for white matter myelin tracts. (D) MBP staining in naive MBP\(^{2/2}\) shiverer mice confirms specificity of immunofluorescence staining. (E–H) Myelin Ag and APCs colocalize at lesions during peak of disease (arrows). (I) APCs containing MBP (MBP\(^{+}\)) can be distinguished from those without (MBP\(^{-}\)) in internal z-stack slices. (J) MK16 Ab colocalizes with MBP at APCs. (K) CD4\(^{+}\) T cells (arrow) in close proximity to MBP\(^{+}\) APCs in and around lesions. Scale bars, 50 \(\mu\)m (A–D, K), 20 \(\mu\)m (E–H), and 5 \(\mu\)m (I, J).
In sum, the results suggested that confocal microscopy was a highly sensitive technique for detection of myelin Ag internalization by APCs. Because myelin Ag was detected in vitro by confocal microscopy in APCs at concentrations below the optimal T cell activation threshold, the data suggest that this technique could similarly detect myelin Ag in CNS APCs at low concentrations, conceivably at concentrations that were not sufficient to induce optimal activation of encephalitogenic T cells in situ.

**Myelin Ag is first detected in microglia in the CNS during EAE**

Next, we asked whether APCs found in the CNS during EAE constitutively contained myelin Ag for presentation to T cells and, if not, at what time point after induction of disease it was acquired.

To address this issue, EAE was induced in C57BL/6 mice with MOG\textsubscript{35–55} peptide as described (30). Representative animals were sacrificed daily starting 1 d after immunization, and CNS tissue sections were obtained for confocal microscopy analysis and quantification of CD4\textsuperscript{+} T cells and various APC populations present. Quantification was performed for the total number of CD4\textsuperscript{+} T cells, as well as APCs containing MBP as a surrogate marker for myelin Ag uptake, as described in Materials and Methods by Imaris image analysis software and Supplemental Fig. 2.

The results show that CD4\textsuperscript{+} T cells infiltrated the CNS in substantial numbers as early as day 5 following immunization, and a rapid rise in CD4\textsuperscript{+} T cells coincided with the onset of EAE about day 11 (Fig. 4A, 4B). The number of CD4\textsuperscript{+} T cells further increased until the peak of EAE, occurring about day 21, followed by a dramatic decrease in numbers upon remission by day 23 (Fig. 4A, 4B). A similar kinetic was observed for CD11c\textsuperscript{+} DCs (Fig. 4C). Consistent with previous reports, DCs appeared first in the meningeal areas and several days later became detectable in the perivascular spaces and surrounding tissues (data not shown) (21).

Of interest, DCs detected in the CNS until day 5 did not contain MBP (Fig. 4C), MOG, or PLP (data not shown), indicating that these cells had not acquired myelin Ag until after their arrival in the CNS. Similar results were observed upon induction of passive EAE via adoptive transfer (Supplemental Fig. 1). However, by day 7, ∼70–80% of DCs contained MBP, and this percentage remained relatively stable throughout the course of the disease.

The number of CD11b\textsuperscript{+} microglia/infiltrating macrophages substantially increased at about day 13 following the onset of EAE, and, similarly to CD4\textsuperscript{+} T cells and DCs, cell numbers peaked with clinical disease, at approximately day 21 (Fig. 4D).

Parallel to the decline in clinical disease symptoms beginning about day 23, the number of DCs decreased substantially, falling to levels nearly three times less than at disease peak. Although the absolute number of DCs and microglia/macrophages decreased during the remission of EAE, the proportion of myelin-associated DCs and microglia/macrophages remained consistently high. By day 35 the number of DCs present in the CNS of EAE mice was negligible (Fig. 4C).

As shown in Fig. 4E, Ly-6G\textsuperscript{+} neutrophils were also detected in the CNS ∼5 d following induction of EAE. As with DCs and microglia/macrophages, the numbers of neutrophils also peaked at the height of clinical EAE. However, although the number of neutrophils increased, overall they were dramatically lower in numbers compared with DCs or CD11b\textsuperscript{+} microglia. Of note, ≤25% of neutrophils contained myelin Ag over the course of EAE (Fig. 4E), in strong contrast to DCs and microglia/infiltrating macrophages (Fig. 4C, 4D). Also of note, astrocyte numbers increased, and colocalized to lesions at peak EAE and later time points; however, GFAP\textsuperscript{+} cells were not found to contain myelin Ag (not shown).

CD11b\textsuperscript{+} cells in the CNS include CNS-resident microglia and infiltrating macrophages from the immune periphery. Thus, to specifically dissect the time point at which peripheral macrophages infiltrated the CNS, we used the well-characterized CX3CR1\textsuperscript{GFP\textsuperscript{+}} mouse to visualize CNS-resident microglial cells, as previously reported (32–34). CD11b\textsuperscript{+}GFP\textsuperscript{+} (CX3CR1\textsuperscript{+}) microglia were observed throughout early EAE (Fig. 5A, 5B), some of which were also MBP\textsuperscript{+} (Fig. 5C, small arrows), whereas CD11b\textsuperscript{+}GFP\textsuperscript{+} (CX3CR1\textsuperscript{+}) infiltrating macrophages were not detected until day 4 p.i. (Fig. 5F). Myelin Ag could not be detected in infiltrating macrophages for several days after entering the CNS, as was similar for DCs. Of note, CNS-resident microglia and infiltrating macrophages were constitutively contained myelin Ag for presentation to T cells, and, similarly to CD4\textsuperscript{+} T cells and DCs, cell numbers peaked with clinical disease, at approximately day 21. However, although the number of neutrophils increased, overall they were dramatically lower in numbers compared with DCs or CD11b\textsuperscript{+} microglia. Of note, ≤25% of neutrophils contained myelin Ag over the course of EAE (Fig. 4E), in strong contrast to DCs and microglia/infiltrating macrophages (Fig. 4C, 4D). Also of note, astrocyte numbers increased, and colocalized to lesions at peak EAE and later time points; however, GFAP\textsuperscript{+} cells were not found to contain myelin Ag (not shown).

**FIGURE 3.** MOG detection by confocal microscopy corresponds to the activation threshold of pathogenic T cells. CD11c\textsuperscript{+} cells from wild-type B6 mice and CD4\textsuperscript{+} cells from 2D2 TCR-tg mice were sorted by FACS and cocultured in a 1:1 ratio with increasing concentrations of rMOG\textsubscript{1–125} protein. (A) Detection of increasing levels of rMOG in CD11c\textsuperscript{+} DCs (arrows) by confocal microscopy. Shown is an internal slice from z-stack depicting inside of cells. Scale bars, 20 μm. (B) Detection of MOG-specific T cell responses by cytokine ELISPOT assay upon incubation with DCs pulsed with increasing concentrations of rMOG protein. Shown is mean ± SD of number of cytokine spots over three separate experiments with three replicates each.
morphologically distinct, with CX3CR1 +CD11b+ cells having long, ramified processes (Fig. 5A) and CX3CR1 ^2 CD11b+ cells being more rounded with larger cell bodies (Fig. 5F) until the onset of clinical symptoms, when they became indistinguishable by morphology alone.

Taken together, the data showed that the number of CNS-resident microglia, as well as infiltrating macrophages and DCs containing myelin Ag, strongly increased after the onset of EAE. The observation that a small percentage of microglia contained myelin Ag already by day 1 after immunization, whereas DCs appeared to first migrate to the CNS and subsequently acquire myelin Ag, may have important implications for EAE pathogenesis.

Only rare microglia in the CNS of naive mice contain myelin Ags

Our results showed that myelin Ag was detected in microglia as early as day 1 after immunization for EAE (Fig. 4D). The presence of myelin Ag this early after induction of EAE in the absence of other inflammatory cells, such as T cells or DCs, raised the question whether microglia and/or other APCs in the CNS of naive mice

FIGURE 4. Myelin Ag is first detected in microglia in the CNS during EAE. C57BL/6 mice were immunized with MOG35–55 peptide for induction of EAE, as previously described. Animals were monitored, and the disease was scored daily. (A) Representative mice were scored for EAE and sacrificed as indicated over the course of EAE (n = 3 per day), and brain tissue was frozen and sectioned. Tissue sections were stained as indicated and evaluated by confocal microscopy analysis for (B) CD4+ T cells, (C) CD11c+, (D) CD11b+, or (E) Ly-6G+ cells. Cells in (C)–(E) were also stained with anti-MBP mAb. Numbers shown represent mean ± SD; ANOVA, p = 0.002.

FIGURE 5. Peripheral macrophages infiltrate the CNS by day 4 of EAE. CX3CR1 ^ GFP mice were immunized with MOG35–55 peptide for induction of EAE and scored for disease as previously described. Confocal microscopy analysis for expression of CX3CR1, CD11b, and MBP shows (A) CX3CR1 (green); (B) CD11b (pink); and (C) merge A + B. CX3CR1+CD11b+ cells are indicated by large arrows, of which a small percentage are also MBP+ (small arrows), and are found primarily in the corpus callosum (green box), whereas (D–F) CX3CR1 ^2 CD11b+ cells (F, large arrow) are only detectable by day 4 p.i. in swollen meninges (red box) and do not contain MBP Ag. Scale bars, 10 μm.

EAE day 4 p.i.
Myelin Ag Uptake by CNS APCs During EAE

Microglia isolated from the naive CNS do not activate myelin-reactive T cells in the absence of exogenous Ag

Because a small number of microglia in the CNS of naive mice contained myelin Ag, the question arose whether these cells could activate myelin-specific T cells. Alternatively, and not mutually exclusive, these cells could function in a regulatory role during development of EAE. To go about answering this question, microglia were isolated from the CNS of naive mice by FACS (>96% purity) and tested with or without exogenous myelin Ag for their potential to activate neuroantigen-reactive T cells.

The results show that microglia isolated from the brains of naive C57BL/6 mice induced significant production of IFN-γ or IL-17 by MOG35–55 peptide–reactive CD4+ T cells only when exogenous MOG peptide was added (Fig. 7). Microglia-induced T cell responses (Fig. 7, middle and right sets of bars) were substantially lower, compared with responses induced by spleen APCs (Fig. 7, left set of bars). Preactivation of microglia with LPS enhanced T cell responses (Fig. 7, microglia, right versus middle set of bars) but did not result in significant T cell cytokine production in the absence of MOG35–55 peptide (not shown).

Overall, the data indicate that microglia in naive CNS are inefficient at activating neuroantigen-reactive T cells.

Discussion

In this study we investigate the kinetics of myelin Ag uptake by potential CNS APCs over the course of EAE and in the naive CNS. Our results show that CNS-resident microglia were the first APCs to contain myelin Ag after the induction of EAE, as early as 1 d after immunization. In contrast, peripheral macrophages and DCs appeared in substantial numbers later during the course of EAE. Of note, DCs or infiltrating macrophages arriving in the CNS did not contain myelin Ag, and it took several days before Ag became detectable. Other potential APCs, such as astrocytes or neutrophils, were not significantly loaded with myelin Ag throughout the course of EAE.

In the naive brain, as expected, and early after induction of EAE, a substantial number of microglia were present. In contrast, DCs were only rarely observed in the CNS of naive mice, and, when present, found predominantly in the meninges, whereas in EAE they arrived in the parenchyma approximately by day 5 relative to immunization. Although the critical role of DCs for the induction of EAE has been appreciated (9, 10), it has not been resolved whether DCs or other APCs are critical for providing presentation of myelin Ag to encephalitogenic T cells for the initiation and/or propagation of EAE. Along these lines, it is noteworthy that ~8% of microglia in the brain contained MBP as early as 1 d after induction of EAE. In strong contrast, infiltrating DCs and neutrophils did not appear to contain myelin Ag during the first days after induction of EAE. CD11b+CX3CR1+ infiltrating macrophages were detected in the EAE CNS as early as day 4 p.i. and, like other infiltrating cells, did not contain myelin Ag initially.

GFAP+ astrocytes were present in the naive CNS but did not colocalize to any of the myelin Ag tested either before or after immunization.
induction of EAE (not shown). However, we observed many astrocytes surrounding lesions at peak and later time points, as has been previously reported (35), consistent with an important role in the disease process.

Neutrophils represent a relatively small percentage of the cells infiltrating the CNS during EAE. It has been reported that these cells can take up and present Ag via MHC class II molecules under certain conditions (36) and could conceivably play a role in promoting T cell responses. Therefore, we determined the number of myelin-containing neutrophils in our model. Of note, neutrophils infiltrated the CNS very early during EAE; however, they acquired myelin Ag only several days later, and the number of Ag-containing neutrophils remained consistently low over the course of EAE, arguing against a significant role for the cells in promoting T cell responses via Ag presentation.

At the onset of clinical EAE symptoms (day 11), the number of DCs and microglia infiltrating macrophages in the CNS increased dramatically. At this time point, the majority of DCs and microglia contained myelin Ag (70–80%). In striking contrast, although the number of Ly-6G+ neutrophils in the CNS also increased until the appearance in the CNS, as has been suggested previously (39).

After this point, the role of microglia in promoting EAE may become secondary to that of DCs and possibly shift to other functions, such as removal of myelin debris to limit CNS damage.

The role of astrocytes in EAE has remained controversial. Some studies have suggested a role for astrocytes in promoting EAE, whereas work by Zipp and others has suggested a regulatory role for this cell population (30–52). Our studies did not detect myelin Ag in astrocytes. Therefore, the results argue against a critical role for astrocytes in Ag presentation to encephalitogenic T cells. We have not fully addressed the possibility that myelin peptides were bound externally to MHC II expressed by astrocytes, but it must be noted that the expression of MHC II by astrocytes in vivo in the CNS is controversial (53, 54).

Novel information provided by our studies concerns the distribution of APCs and loading of these cells with myelin Ag in the brains of naive mice. In the naive brain, we observed that the level of MHC class II expression was relatively low and colocalized with microglia. DCs and neutrophils were only rarely detected, and if so, then only in the meninges of the naive brain and without containing myelin Ag. Similarly, in naive mice, astrocytes did not constitutively express MHC class II, and we observed no colocalization of astrocytes with myelin Ag.

Importantly, 3–4% of microglia in the brains of naive mice contained myelin Ag, and in a very small number of mice the percentage of myelin-containing microglia was ≤ 20% of the cells. This finding contrasted with the lack of myelin Ag detected in rarely observed DCs. These observations raise several questions: 1) What is the mechanism whereby myelin Ag moves from myelin sheaths to microglia in the naive CNS? Presumably, this mechanism is not dependent on inflammatory mediators in the absence of EAE. 2) Does myelin Ag presented by microglia in the naive CNS play a role for the activation of encephalitogenic T cells arriving in the brain after being activated in the immune periphery? Or, does uptake of myelin Ag by resting microglia in the naive CNS represent phagocytosis and removal of myelin debris as a housekeeping function and serve to prevent undesired autoimmune pathology? In this view, myelin uptake by microglia in the absence of inflammatory stimuli may not be conducive to
T cell activation and may rather maintain T cell tolerance. Answering these questions will require future mechanistic studies; however, insights gained could potentially lead to novel approaches to preventing Ag presentation to autoreactive T cells in MS patients. Our findings suggest that upon induction of EAE, myelin Ag containing microglia may be the APCs initially encountered by eosophagelitotic T cells in the CNS. Conceivably, this encounter could result in the activation of these T cells and could promote subsequent recruitment of DCs and additional autoreactive T cells into the CNS. Once a certain threshold of DCs and myelin-reactive T cells has accumulated in the CNS, overt clinical disease may ensue. At this point, DCs may be requisite and sufficient for promotion of disease. Should DCs not arrive in sufficient numbers or be functionally compromised in the CNS to take over further activation from microglia, as shown by Becher and colleagues (11), the disease process may not develop.

Finally, we propose that uptake of myelin Ag within the CNS and uptake outside the CNS (e.g., cervical draining lymph nodes) are not mutually exclusive concepts but may go together in a sequential fashion. In the EAE model, we favor a view in which myelin Ag is first taken up by CNS APCs, then presented to myelin-specific T cells, resulting in reactivation, cytokine production, and enhanced demyelination and subsequent drainage of myelin to the periphery, for example, cervical draining lymph nodes. However, in certain situations myelin Ag may be released in the CNS (e.g., trauma) and drain to the periphery, where it may, under conditions of a “perfect storm”—such as a simultaneously occurring upper respiratory infection—result in the activation and priming of eosophagelitotic T cells. A better understanding of the cellular players and Ag presentation mechanisms may lead to novel therapeutic approaches for MS.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 3
**SUPPLEMENTAL FIGURE S1.** Adoptive transfer of PLP_{139-151}-reactive CD4^{+} T cells into recipient SJL mice show similar myelin Ag kinetics to active immunization model. (A) Infiltrating CD11b^{+} immune cells do not co-localize with myelin Ag (PLP^{+}) until associating with parenchymal white matter of the CNS (PLP^{+}) during onset of disease day 4 post-transfer (p.t.). (B-C) Most CD11b^{+} cells co-localize to myelin Ag at peak of disease (day 14 p.t.). Scale bars = 20 \mu m.

**SUPPLEMENTAL FIGURE S2.** Reconstructing neuroantigen uptake by myeloid cells in three dimensions. (A and D) An original confocal image of 30\mu m-thick brain tissue section from C57BL/6 Wt EAE mouse at disease peak (d21 p.i.) showing dendritic cells (DCs, CD11c) in green, myelin Ag (MBP) in red, and nuclei (DAPI) in blue in three dimensions. (B and E) three-dimensional reconstruction of DCs (DAPI^{+}CD11c^{+}) by Imaris software along with unaltered MBP from original confocal image. (C and F) The number of DCs (DAPI^{+}CD11c^{+}, blue dots) was determined by using Imaris software to grow seed points from center of individual nuclei to DC cell surface (CD11c) only if present (excludes nuclei with no CD11c^{+} co-staining). The number of MBP^{+} DCs (purple dots) was determined in Imaris for DCs with intracellular or cell surface MBP. A-C represents side view of tissue slice as mounted on slide and D-F is top-down view.

**SUPPLEMENTAL FIGURE S3.** MBP localization within APCs. 39 slice image gallery of 300 nm z stacks taken for Fig. 2I (* denotes slice 16 used for Fig. 2I) demonstrating MBP signal is internalized within MBP^{+} APC (labeled MBP^{+}, top box in each image slice) as it is only
present in interior cell slices #13-28, and that no MBP is associated with any slice of MBP APC (labeled MBP’, bottom box in each slice).