Brain Dendritic Cells and Macrophages/Microglia in Central Nervous System Inflammation[1]

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Microglia subpopulations were studied in mouse experimental autoimmune encephalomyelitis and toxoplasmic encephalitis. CNS inflammation was associated with the proliferation of CD11b$^+$ brain cells that exhibited the dendritic cell (DC) marker CD11c. These cells constituted up to 30% of the total CD11b$^+$ brain cell population. In both diseases CD11c$^+$ brain cells displayed the surface phenotype of myeloid DC and resided at perivascular and intraparenchymatic inflammatory sites. By lacking prominent phagocytic organelles, CD11c$^+$ cells from inflamed brain proved distinct from other microglia, but strikingly resembled bone marrow-derived DC and thus were identified as DC. This brain DC population comprised cells strongly secreting IL-12p70, whereas coisolated CD11c$^-$ microglia/brain macrophages predominantly produced TNF-α, GM-CSF, and NO. In comparison, the DC were more potent stimulators of naive or allogeneic T cell proliferation. Both DC and CD11c$^-$ microglia/macrophages from inflamed brain primed naive T cells from DO11.10 TCR transgenic mice for production of Th1 cytokines IFN-γ and IL-2. Resting microglia that had been purified from normal adult brain generated immature DC upon exposure to GM-CSF, while CD40 ligation triggered terminal maturation. Consistently, a functional maturation of brain DC was observed to occur following the onset of encephalitis. In conclusion, these findings indicate that in addition to inflammatory macrophage-like brain cells, intraparenchymatical DC exist in autoimmune and infectious encephalitis. These DC functionally mature upon disease onset and can differentiate from resident microglia. Their emergence, maturation, and prolonged activity within the brain might contribute to the chronicity of intracerebral Th1 responses. ***The Journal of Immunology*****, 2001, 166: 2717–2726.**

The CNS is susceptible to inflammatory diseases, although the healthy organ is immunologically quiescent, presumably due to the blood-brain barrier and the relative lack of intraparenchymatic leukocytes and MHC class II expression. The current concept of brain immunity is based on experimental evidence that activated T cells extravasate into the brain but undergo apoptosis upon contact with local APC or by other microenvironmental signals (1–3). An intracerebral T cell response is likely to be further inhibited by the antiproliferative effect of CNS lipids and the constitutive expression of TGF-β (4–7). Despite such limitations imposed on the immune system, vigorous inflammatory responses can rapidly develop within the brain, resulting in the recruitment of naive bystander T cells (8).

Experimental autoimmune encephalomyelitis (EAE)[3] is a murine model of autoimmune disease directed against CNS Ags. Disease is initiated in animals of susceptible strains by sensitization or the adoptive transfer of neural Ag, e.g., myelin basic protein-specific CD4$^+$ T cells leading to multiple inflammatory, often demyelinating, lesions in spinal cord and brain (reviewed in Ref. 9). A prototypic model of infectious CNS inflammation is murine toxoplasmic encephalitis (TE), which is induced by experimental infection with the intracellular parasite *Toxoplasma gondii*. While TE is transient in mice that are genetically resistant to the pathogen (e.g., BALB/c), susceptible mouse strains (e.g., C57BL/6) develop progressive lethal encephalitis during chronic infection (reviewed in Ref. 10). Common to EAE and TE is a type 1 CD4$^+$ T cell-mediated immunopathology, although it is still unclear how the intracerebral T cell response is polarized. In EAE, brain microglia that function as resident APC are thought to skew the cellular response, as they have been shown to preferentially activate Th1 cells (11). Besides microglia, other potential APC are brain macrophages and astrocytes (reviewed in Refs. 12 and 13) as, of course, are the dendritic cells (DC), which have been recently identified in the brains of BALB/c mice during chronic latent TE (14). These brain DC seem to be related phenotypically to macrophages/microglia.

Since myeloid DC progenitors are present in the newborn mouse brain (15) and mature upon challenge by *T. gondii* of glial cells in vitro (14), the emergence of brain DC in TE raises the question of whether these DC appear and expand as a direct consequence of the presence of the pathogen or as a result of the local inflammatory reaction. In the present study we address the hypothesis that CNS inflammation in general is associated with an intracerebral presence of functional DC. EAE as a noninfectious inflammatory CNS disease and progressive TE as a severe infectious encephalitis were examined for the presence and location of brain DC. Isolated DC were compared with coisolated microglia/brain macrophages with respect to their morphology, T cell stimulatory activity, and cytokine secretion profiles. Furthermore, we delineate a *Toxoplasma*-independent differentiation pathway that leads from resting microglia to functionally mature DC.

Materials and Methods

Animals and induction of EAE and TE

BALB/c and C57BL/6 mice were bred at the Tierversuchsanlage, University of Duesseldorf, from breeding stock supplied by Biological Research

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; BMNC, brain mononuclear cells; BrdU, 5-bromo-2′-deoxyuridine; DC, dendritic cell(s); SN, supernatant; TE, toxoplasmic encephalitis; CD40L, CD40 ligand.

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Laboratories (Fuellinsdorf, Switzerland). SJL/NBom mice were obtained from M&B (Ry, Denmark). Mice transgenic for TCR that recognize $OVA_{323–339}$ peptide in the context of I-A^d (DO11.10 TCRα/β transgenic mice) on a BALB/c background (16) were provided by Dr. M. Kopf (Basel Institute of Immunology, Basel, Switzerland). EAE was elicited in 8-wk-old female SJL mice according to the method of Miller and Karpus (17) by s.c. immunization with 180 μg of myelin basic protein 89–101 peptide (VHFFKNIVTPRTP; synthesized and HPLC purified by the Center for Biological and Medical Research, University of Duesseldorf) in CFA containing 200 μg of *Mycobacterium tuberculosis* H37Ra. In addition, mice were injected i.p. with 400 ng of pertussis toxin (Alexis, Lausen, Switzerland) on days 0 and 2. About 70% of the animals showed symptoms that corresponded to grades 0.5–2 (beginning tail paresis and additional hind limb paresis, respectively) in EAE scoring (17). Mice were analyzed at the peak of disease. Control animals treated with CFA and pertussis toxin were simultaneously analyzed. Female BALB/c or C57BL/6 mice were infected at 8–12 wk of age with the DX strain of *T. gondii* by i.p. injection with three cysts prepared as previously described (14). Infection was serologically verified 2–3 wk later. Unless otherwise stated, *T. gondii*-infected mice were analyzed between days 28 and 42 postinfection when TE was histologically apparent.

Bromodeoxyuridine (BrdU) labeling

For pulse-labeling of proliferating cells in vivo, mice were injected i.p. with 1.2 mg of BrdU (Roche, Mannheim, Germany) 12 h before preparation of brain cells.

Immunohistochemistry

Mice were transcardially perfused with ice-cold PBS. The brains were excised, mounted in OCT compound (Sakura, Zoeterwoude, The Netherlands), snap-frozen in 2-methylbutane, and stored at −70°C. Frozen sections, 5 μm thick, were stained with anti-CD11c mAb N418 (Endogen, Cambridge, MA), anti-TCRβ mAb H57-597 (PharMingen, San Diego, CA), or hamster isotype control mAb G235-2356 (PharMingen) as detailed previously (14). Ab binding was detected using biotinylated goat anti-hamster IgG (The Jackson Laboratory, Bar Harbor, ME) and peroxidase-conjugated avidin-biotin complex (Vector Elite ABC kit, Vector, Burlingame, CA). Sections were developed using the 3,3′-diaminobenzidine substrate kit (Vector) and were counterstained with hematoxylin.

Preparation of microglia and DC populations

Brains were removed from perfused mice, minced, passed through an 18-gauge needle, and digested by incubation with 250 μg/ml collagenase/dispase and 250 μg/ml DNase I (Roche) at 37°C for 45 min each. The resulting cell suspensions were fractionated on 30/60% Percoll gradients at 1000 × *g* for 25 min (18), and brain mononuclear cells (BMNC) were collected from the interface. Due to the low number of BMNC obtained, cells from 3–12 mice/experiment were pooled. For isolation of DC or microglial cells, BMNC were depleted of T cells by treatment with anti-Thy1.2 mAb 30-H12 (PharMingen) and complement (low tox rabbit complement; Cedarlane, Hornby, Canada). Then, $CD11c^+$ cells were magnetically sorted by positive selection using N418-coated beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instruction, resulting in a >90% pure population as controlled by flow cytometry. Spleen DC were similarly isolated from normal splenocytes. DC-free microglia/brain macrophages were prepared from the residual $CD11c^-$ BMNC by positive selection using M1/70.15-coated magnetic beads (Miltenyi). In general, the resultant population contained >96% $CD11b^+$ and <0.5% $CD11c^+$ cells. For assays, all MACS-enriched cells were resuspended in phenol red-free DMEM with 10% FCS (containing <10 pg/ml LPS as determined by *Limulus* amebocyte lysate assay), 2 mM L-glutamine, and 50 μM 2-ME.

In vitro differentiation and activation of brain DC

Microglia prepared from normal adult BALB/c mice were plated at 2×10^5 cells/ml into low adherence six-well plates (Costar, Cambridge, MA) with medium containing 50 ng/ml murine GM-CSF (provided by Dr. F. Seiler, Behringwerke, Marburg, Germany) and cultivated for 4–6 days. Microglia were also harvested from GM-CSF-supplemented primary culture of newborn mouse brain (19). $CD11c^+$ cells were then immunomagnetically isolated, washed, and recultured for a further 24 h with GM-CSF. During that time, 10 μg/ml soluble CD40L and cross-linking Ab (Alexis) or 10 μg/ml LPS (*Escherichia coli* O55:B5; Difco, Detroit, MI) were added to some cultures to trigger DC terminal maturation.

Flow cytometry

Freshly prepared BMNC or cells isolated from brain cell culture were stained using the following Abs: biotinylated anti-CD11c mAb N418 (Endogen) or HL3 (PharMingen), FITC-labeled anti-F4/80 mAb CI:A3-1 and FITC-labeled anti-DEC-205 mAb NLDC-145 (Dianova, Hamburg, Germany), and PE- or FITC-labeled anti-CD11b mAb M1/70.15, FITC-labeled anti-CD8α mAb 53-6.7, and PE- or FITC-labeled anti-CD45 mAb 30-F11 (PharMingen). Species- and isotype-matched control Ab and FITC- or PE-conjugated streptavidin as secondary reagent were obtained from PharMingen. To block unspecific binding of primary Ab, cells were preincubated with anti-FcγII/IIIR mAb 2.4G2 (PharMingen). Double staining of surface markers was performed according to standard procedures (18). Propidium iodide was added in the final wash to label dead cells, which were excluded from the analyses. BrdU incorporated into cellular DNA was detected by using the BrdU flow kit (PharMingen). After surface staining, cells were fixed and permeabilized, and DNA was denatured as detailed in the kit protocol. Cells were then incubated with FITC-labeled anti-BrdU mAb 3D4 (PharMingen). All samples were immediately measured on a FACSCalibur flow cytometer, 10^4 events were acquired, and data were analyzed with CellQuest software (Becton Dickinson, Heidelberg, Germany).

Phase contrast and electron microscopy

Freshly isolated $CD11c^+$ and $CD11c^-$ microglia/brain macrophages were incubated in culture medium for 6 h at 37°C to allow reconstitution of cell morphology. Light microscopy was performed on a Zeiss photomicroscope III (Zeiss, Jena, Germany). For standard electron microscopy, cells were fixed in Karnovsky's solution, postfixed in 2% osmium tetroxide, dehydrated in ethanol, and embedded in Durcupan ACM (Fluka, Neu-Ulm, Germany). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a Zeiss EM 109 transmission electron microscope.

Mixed leukocyte reaction

$CD11c^+$ splenocytes and BMNC, $CD11c^-/CD11b^+$ BMNC, or in vitro differentiated brain DC were tested as stimulators of allogeneic T cell responses by MLR. T cells were prepared from spleens of C57BL/6 mice by passing splenocytes through nylon wool and an Ab-coated T cell enrichment (R&D Systems, Minneapolis, MN) column. In A/2 microtiter wells containing 100 μl of IMDM with 5% FCS, glutamine, and antibiotics, 1.5×10^5 purified T cells (>99% $CD3^+$) were mixed with titrated numbers of gamma-irradiated APC of BALB/c origin. On day 3 test cultures were fed by adding 50 μl of medium. T cell proliferation was measured via [^{3}H]TdR incorporation during the last 18 h of a 5-day incubation.

Priming and activation of TCR transgenic T cells

To analyze T cell priming by brain APC, naive T cells purified from DO11.10 mice were exposed in primary stimulation culture to sorted $CD11c^+$ or $CD11c^-/CD11b^+$ BMNC from chronically *T. gondii*-infected BALB/c mice. $CD11c^+$ normal splenocytes that had been immunomagnetically depleted of $CD45R^+$ cells by negative selection using B220-coated microbeads (Miltenyi) served as reference APC. Naive T cells were purified from the spleens of 8-wk-old DO11.10 mice via nylon wool and a T cell enrichment column and subsequent sorting for $CD62L^{high}$ cells using MEL14-coated magnetic beads (Miltenyi). In a total volume of 1 ml (48-well plate), 5×10^5 DO11.10 T cells were coincubated with 5×10^4 irradiated APC as described above, but in medium containing 0.3 or 1 μM $OVA_{323–339}$ peptide (ISQAVHAAHAEINEAGR; synthesized and HPLC purified by the Center for Biological and Medical Research, Duesseldorf, Germany). On day 3 cultures were fed with 1 ng/ml IL-2. Subsequently, proliferating T cells were allowed to expand by transfer to 24-well plates. On day 6 T cell blasts were washed, and 2×10^5 cells/200 μl were plated using medium without IL-2 in microtiter wells precoated with anti-CD3ϵ mAb (1 μg/ml). Supernatants (SN) from restimulated T cells were collected at 24 h and were stored at −70°C until tested for cytokine content.

Cytokine assays and measurement of NO production

Freshly prepared brain cells were seeded into microtiter wells at a density of 2×10^5/200 μl in DMEM with 10% FCS. After 24 and 72 h of incubation, samples of the SN were collected. In SN from 24-h culture of brain cells or T cells, IL-1α, IL-10, IL-12p40, IL-12p70, TNF-α, GM-CSF, IFN-γ, IL-4, and IL-2 were quantified using specific sandwich ELISAs (Endogen, PharMingen, and R&D Systems) with recombinant mouse cytokines as standard. Assays had a minimum sensitivity of 5 (IL-10, IL-12p40, GM-CSF), 10 (IL-1α, IL-2), 20 (IL-4, IL-12p70, TNF-α), and 50

(IFN-γ) pg/ml, respectively. In 3-day SN from brain cell cultures, the concentration of nitrite indicating cellular NO production was determined photometrically with the Griess reagent and an $NaNO_2$ standard (20). The detection limit of this assay was 2.5 nM NO_2^-.

Statistics

The significance of differences was examined by Student *t* test, using Prism software (GraphPad, San Diego, CA), and $p < 0.05$ was considered significant.

Results

In CNS inflammation the number of microglial cells/brain macrophages increases, consistent with proliferation of CD11b$^+$ and CD11c$^+$ brain cells

Microgliosis is a sign of CNS inflammation (21). For a quantitative approach mononuclear cells were prepared from perfused normal and inflamed brain, and the percentage of CD11b$^+$ cells was determined by flow cytometry. As calculated from the BMNC number in pooled cell isolates, on the average, 3×10^5 CD11b$^+$ mononuclear cells were recovered per brain of normal adult mice (Table I). In EAE and TE as experimental models of brain inflammation, the total yield of BMNC increased, which is partially due to T cells infiltrating the CNS (data not shown). In parallel, the number of CD11b$^+$ cells increased, correlating with the severity of disease (Table I). Although the origin of the expanding cells remains unclear, evidence that a proliferation of CD11b$^+$ cells contributes to this increase was obtained by pulse-labeling in vivo with the thymidine analogue BrdU; while mononuclear cells from control brains showed no substantial DNA synthesis, those from encephalitic brains contained approximately 10% BrdU-labeled cells, which, by double staining, were identified as microglia and T cells. About 3% of all CD11b$^+$ BMNC had incorporated the BrdU label (Fig. 1*A*). In comparison, the proportion of BrdU-positive cells among CD3$^+$ brain cells was 6–7% (Fig. 1*B*). The detection of cycling microglia in EAE and TE confirms a previous observation in graft-vs-host disease (22). Collectively, these results indicate a proliferative reaction in a subset of microglia/macrophages from encephalitic brains.

As recently shown, DC emerge in the brains of BALB/c mice early in chronic latent toxoplasmosis (14). To test whether these cells proliferate, BMNC were stained for intracellular BrdU and surface CD11c. Flow cytometric analysis revealed that DC in the inflamed brain were cycling, as has been observed for microglia (Fig. 1*C*). Within the BrdU-positive gate approximately 60% of brain cells expressed the pan-DC surface marker CD11c (Fig. 1*D*). These data identify DC as a major population within the proliferating BMNC population during the onset of CNS inflammation.

In EAE and TE, CD11b$^+$ BMNC that coexpress CD11c appear at inflammatory sites and exhibit a comparable DC phenotype

To test whether CNS inflammation is generally associated with local expansion of DC, BMNC from autoimmune and severe infectious encephalitis were analyzed for the presence of DC. In brain cells from SJL mice during EAE as well as in those from C57BL/6 mice during progressive lethal TE, DC were identified within the CD11b$^+$ cell pool (Fig. 2*A*). While only minute numbers were detected among CD11b$^+$ mononuclear cells from normal brain, CD11b$^+$ cells from encephalitic brain contained significant numbers (up to 30%) of cells that proved similar to myeloid-related DC. Double stainings showed that CD11c$^+$ brain cells in EAE lacked CD8α and DEC-205, but coexpressed CD11b and F4/80 (Fig. 2*B*). By phenotype, the CD11c$^+$ BMNC in EAE thus correspond to those in TE (14) and further resemble normal mouse bone marrow-derived DC (23). In agreement with previous studies (24–26), CD45 was expressed at a low level on mononuclear cells from normal brain. By contrast, CD45 was markedly up-regulated on encephalitic BMNC (Fig. 2*C*). In an attempt to define levels of surface CD45 expression in CD11b$^+$ brain cell populations, BMNC from inflamed brain (progressive TE, wk 6 postinfection) were depleted of T cells and immunomagnetically sorted for CD11c$^+$ vs CD11c$^-$/CD11b$^+$ cells. Fig. 2*D* shows that the purified brain DC expressed CD45 at high and intermediate levels, while all CD11c$^-$ microglia/brain macrophages exhibited a CD45^{high} phenotype.

In EAE, brain DC colocalized at inflammatory foci with T cells, as detected by immunohistochemical staining of CD11c and TCR (Fig. 3*A–C*). Brain DC were found at perivascular and intraparenchymatic sites of inflammation. A comparable pattern in tissue distribution of brain DC was observed in progressive (Fig. 3*D*) and chronic latent TE (14). In immunized SJL mice, CD11c$^+$ brain cells were detected only when EAE was apparent. Local expansion of DC possibly starts with few cells dispersed in parenchymatic areas lacking substantial leukocyte infiltrates (Fig. 3*E*). In TE, a correlation between the number of brain DC and the severity of disease was evident. By comparison, significantly more brain DC

Table I. *Numbers of CD11b$^+$ and CD11c$^+$ mononuclear cells recovered from the brains of mice with different experimental encephalitides*

Strain	Disease	BMNC[a] (×10^6) CD11b$^+$	BMNC[a] (×10^6) CD11c$^+$
BALB/c	Transient TE[b]	2.13 ± 0.18	0.84 ± 0.14
C57BL/6	Progressive lethal TE[b]	5.75 ± 2.05	2.4 ± 0.98
SJL	EAE, mean score 0.5	0.62 ± 0.18	0.05 ± 0.02
	EAE, mean score 1.8	1.71 ± 0.27	0.56 ± 0.47

[a] Mononuclear cells were prepared from the brain as described in *Materials and Methods.* Numbers were calculated from the yield of isolated BMNC and the percentage of CD11b$^+$ or CD11c$^+$ BMNC as determined by flow cytometry. On average, 0.3×10^6 CD11b$^+$ and 0.015×10^6 CD11c$^+$ BMNC were obtained from the brains of normal or mock-immunized mice irrespective of the strain. Values are mean ± SD of two experiments with three to five mice each.

[b] At wk 4 postinfection.

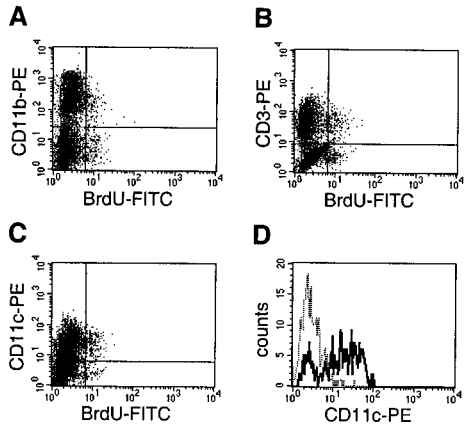

FIGURE 1. Proliferation of BMNC at the onset of CNS inflammation. On day 13 after infection with *T. gondii*, BALB/c mice were injected with 1.2 mg of BrdU. BMNC were prepared 12 h later and double stained as described in *Materials and Methods* for incorporated BrdU and surface expression of CD11b (*A*), CD3 (*B*), or CD11c (*C* and *D*). Quadrants were set according to staining with isotype-matched control mAbs. Analysis of BMNC from mice that were not treated with BrdU gave results similar to those obtained with the control mAb. *D*, Gated on BrdU-positive cells, the histogram shows the fluorescence intensity of CD11c staining (bold line) compared with staining with an isotype control mAb (dotted line).

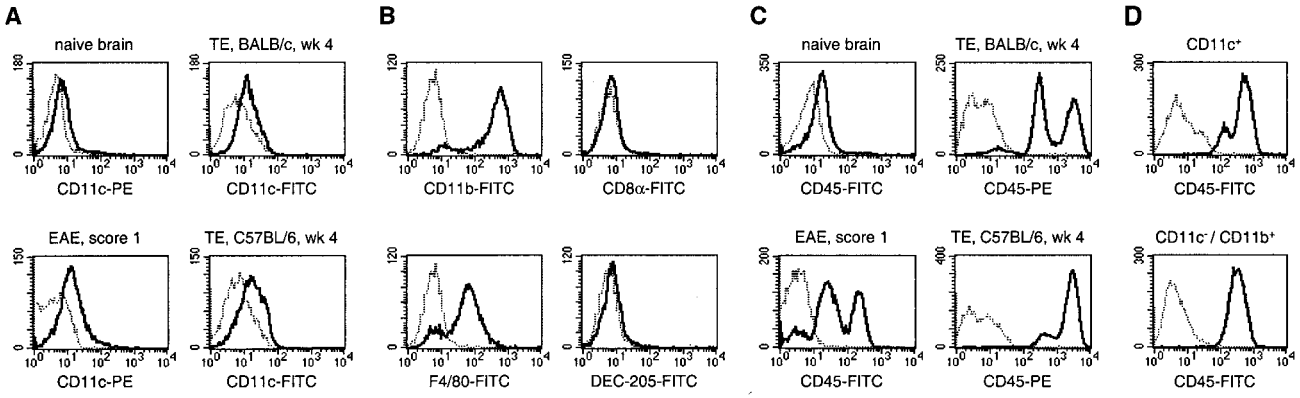

FIGURE 2. Phenotypic analyses of $CD11b^+$ and $CD11c^+$ mononuclear cells isolated from normal and encephalitic brain. Mononuclear cells were prepared from brains of normal mice, *T. gondii*-infected BALB/c and C57BL/6 mice (4 wk postinfection), and SJL mice with clinical signs of EAE (score of 1 or 2). Isolated BMNC were stained and analyzed for the expression of various surface molecules by flow cytometry. Histograms are representative of three to five experiments and show staining of specific mAb (bold line) and isotype-matched control mAb (dotted line). *A*, BMNC isolated from normal or encephalitic brain were double stained for CD11b and CD11c. Gated on cells expressing CD11b, histograms show the fluorescence intensity of CD11c staining. *B*, Phenotype of brain DC in EAE. BMNC isolated from SJL mice with EAE (score 2) were stained for DC with anti-CD11c mAb. In addition, cells were stained for the expression of CD11b, F4/80, CD8α, or DEC-205. Samples were gated on cells expressing CD11c. *C*, BMNC isolated from normal or encephalitic brain were stained for CD45. Note that the fluorescence intensity of staining with PE-labeled anti-CD45 mAb is stronger than that of staining with FITC-labeled anti-CD45 mAb. *D*, BMNC isolated from C57BL/6 mice at 4 wk postinfection were immunomagnetically sorted for $CD11c^+$ or $CD11c^-/CD11b^+$ cells and stained for CD45.

were isolated in progressive than in chronic latent disease (Table I). In BMNC from *T. gondii*-resistant BALB/c mice after recovery from TE and in BMNC from immunized, but asymptomatic, or mock-immunized SJL mice, few DC could be identified only by flow cytometry.

$CD11b^+$ $CD11c^-$ and double-positive BMNC (macrophages/microglia and DC) from encephalitic brain exhibit different morphologies and profiles in production of cytokines and NO

Given the parallel existence of dendritic and macrophage-like brain cells during CNS inflammation, both cell types were compared in shape and function with regard to their putative roles in local immunity. Freshly prepared $CD11c^+$ and $CD11c^-/CD11b^+$ mononuclear cells from encephalitic brain were incubated in medium with 10% FCS for 24 h to allow reconstitution of cell morphology. During this time, most $CD11c^+$ brain cells remained floating and exhibited features of DC such as spinous, typically not branched, processes. Electron microscopic examination revealed irregularly shaped nuclei, numerous mitochondria, and few electron-dense granules and phagocytic structures (Fig. 4, *A* and *B*). Thereby, $CD11c^+$ brain cells closely resemble bone marrow-derived DC (23). As a rule, the brain DC population contained some

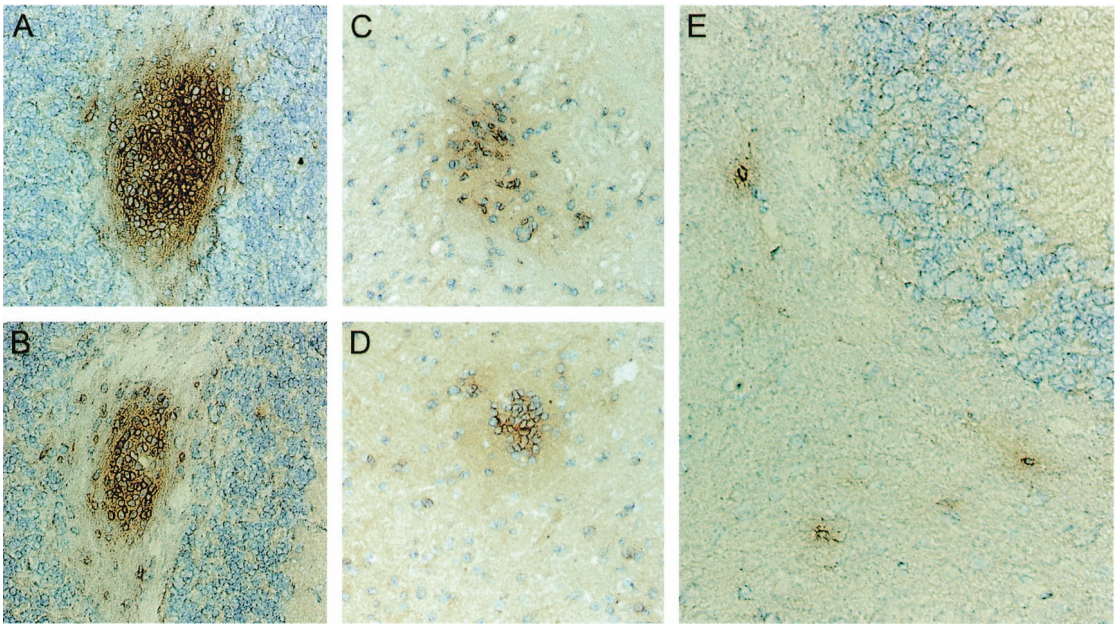

FIGURE 3. Immunohistochemical localization of DC in encephalitic brain. Brains of SJL mice with EAE (score of 1 or 2) (*A–C* and *E*) and of C57BL/6 mice 4 wk after infection with *T. gondii* (*D*) were prepared for cryoimmunohistochemistry. Sections were stained with anti-CD11c mAb (*A* and *C–E*) or anti-TCR mAb (*B*) as detailed in *Materials and Methods. A*, DC localized in an inflammatory focus in the cerebellum; *B*, T cells in an adjacent section. *C* and *D*, $CD11c^+$ cells in inflammatory foci in the cortical parenchyma. *E*, Occasional individual $CD11c^+$ cells in a contralateral cerebellum section from the same brain as that in *A*. Magnifications: *A–D*, ×160; *E*, ×250. Comparable staining was observed in two additional experiments; no staining was observed when using isotype-matched anti-TNP hamster mAb for a control.

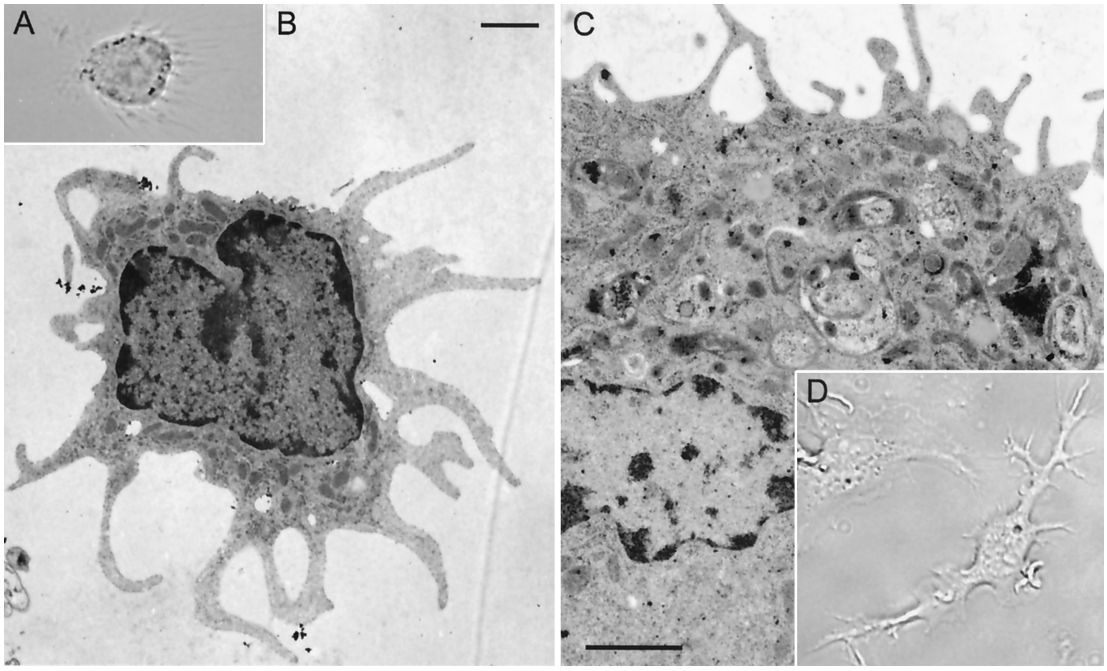

FIGURE 4. Morphology and ultrastructure of brain DC and CD11c$^-$ microglia/macrophages. Cells were isolated as detailed in *Materials and Methods* from the brains of C57BL/6 mice suffering from TE at wk 6 postinfection and analyzed within 10 h of cytokine-free culture with FCS. *A*, Illustration of a loosely adherent cell found to be enriched in the CD11c$^+$ population. *B*, Transmission electron micrograph of a cell from the same culture, showing few electron-dense granules and phagocytic structures and numerous mitochondria. Bar = 1 μm. *C* and *D*, Electron (*C*) and light micrograph (*D*) of cells from the coisolated CD11c$^-$/CD11b$^+$ BMNC population. Prominent phagosomes, vacuoles, and mitochondria and abundant rough endoplasmatic reticulum are shown. Bar = 1 μm.

mitotic cells. Coisolated CD11c$^-$/CD11b$^+$ BMNC became adherent, with extending pseudopodia and branched cytoplasmic protrusions. Ultrastructural characteristics included phagosomes, prominent vacuoles and mitochondria, and abundant rough endoplasmatic reticulum (Fig. 4, *C* and *D*). Comparative measurement of cytokine secretion ex vivo (Fig. 5) identified encephalitic brain DC to release predominantly IL-12p70. CD11c$^-$ inflammatory microglia/brain macrophages, by contrast, proved superior in secretion of TNF-α and GM-CSF and production of NO ($p < 0.005$). IL-1α and IL-10 were released by both cell populations; the higher levels at CD11c$^-$ microglia/macrophages were not significant ($p > 0.05$). These results characterize brain DC as inflammatory cells specialized in IL-12 secretion, whereas CD11c$^-$/CD11b$^+$ BMNC, by producing TNF-α and NO, resemble inflammatory macrophages.

DC and CD11c$^-$ microglia/macrophages from inflamed brain qualify distinct in induction of T cell proliferation, but similarly prime naive T cells for a Th1 response

To assess CD11c$^+$ brain DC and CD11c$^-$/CD11b$^+$ BMNC for their capacities to stimulate an alloreactive T cell response, cells coisolated from encephalitic brain were used in MLR. Titrated numbers of both APC types were cocultured, each with a fixed number of allogeneic T cells. Fig. 6*A* shows that DC derived from inflamed brain were potent stimulators, already inducing T cell proliferation at a ratio of 1 cell per 100 T cells. CD11c$^-$/CD11b$^+$ inflammatory BMNC proved about 4-fold less efficient. Similarly, CD11c$^+$ cells from normal adult BMNC were significantly superior in T cell stimulation ($p < 0.05$) compared with residual CD11c$^-$/CD11b$^+$ brain cells (Fig. 6*B*), thus indicating that potent stimulator cells were enriched. The level of T cell response induced by brain DC was comparable to that induced by spleen DC as reference stimulator cells and is consistent with previous studies that suggest that brain microglia comprise cells active in allo-MLR (26, 27).

During CNS inflammation, brain APC can encounter naive T cells that extravasate into the parenchyma (8). To address the question of how naive T cells are primed by brain DC and other microglia/brain macrophages, cells isolated from BALB/c mice suffering from TE were used to stimulate CD4$^+$/CD62L^{high} T cells from OVA TCR transgenic mice in the presence of $OVA_{323–339}$ peptide. T cell activation was strictly dependent on the presence of peptide. T cell expansion during the 6-day primary stimulation culture confirmed the superior capacity of brain DC to trigger naive T cell proliferation (data not shown). The cytokine secretion pattern of OVA TCR transgenic T cells primed with brain DC or CD11c$^-$ microglia/brain macrophages was determined after secondary stimulation with anti-CD3ϵ. T cells primed with brain DC as well as those primed with CD11c$^-$/CD11b$^+$ BMNC secreted high levels of IFN-γ and IL-2, minimal amounts of IL-4, and no IL-10 (Fig. 7). Taking into account that brain DC are superior in stimulating naive T cell proliferation, they can be considered the principal inducers of Th1 responses in inflamed CNS tissue.

Functional DC differentiate from resting microglia due to exposure to GM-CSF and CD40 ligation

Since microglia from adult mouse brain or brain cell primary culture share functional and phenotypic characteristics with immature DC (15, 26), we tested whether mature DC can differentiate from microglia. Resting microglia were prepared from adult mouse brain and depleted of CD11c$^+$ cells, resulting in 96–99% F4/80$^+$/CD45^{low} cells as controlled by flow cytometry (Fig. 8*A*). To stimulate differentiation to DC, isolated microglia were maintained with GM-CSF for 6 days. While a subpopulation of cells rapidly adhered by exhibiting a ramified morphology, a further population

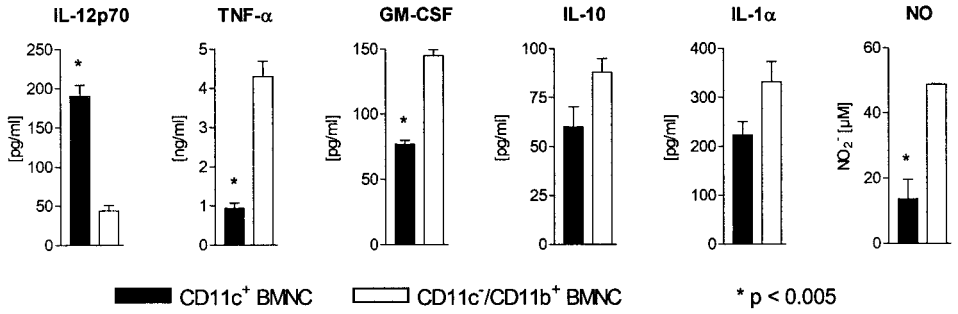

FIGURE 5. Production of cytokines and NO by DC and CD11c$^-$ microglia/macrophages from encephalitic brain. BMNC were prepared from *T. gondii*-infected C57BL/6 mice at peak encephalitis (at wk 5 postinfection) and cells pooled from five to eight animals were immunomagnetically sorted for CD11c$^+$ and CD11c$^-$/CD11b$^+$ cells. Secretion of IL-12p70, TNF-α, GM-CSF, IL-10, and IL-1α was tested by ELISA on aliquots of both populations incubated for 24 h ex vivo. NO production was determined via the release of nitrite during 72 h of incubation. Values are the mean ± SD from triplicate SN productions. Profiles shown are representative of at least three experiments.

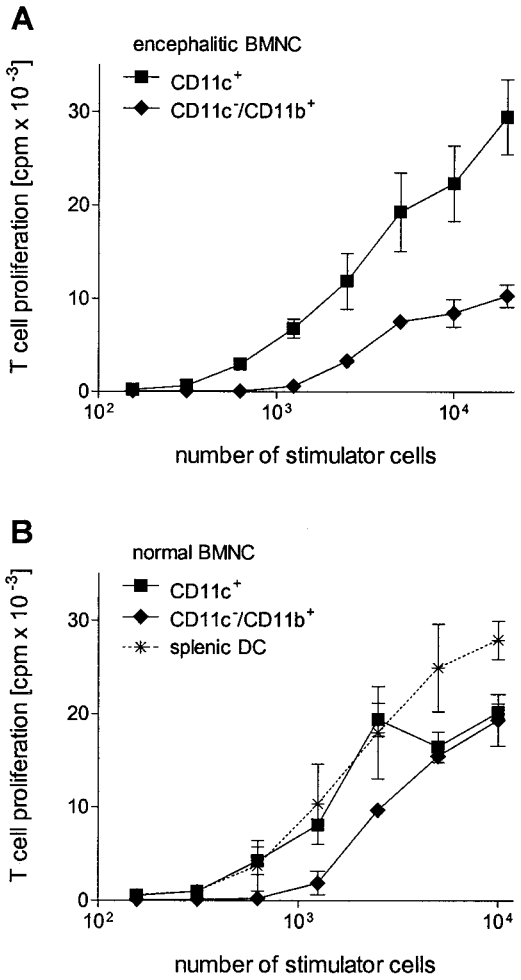

FIGURE 6. Brain DC are more potent stimulators of allogeneic T cell proliferation than are CD11c$^-$ microglia/brain macrophages. Varying numbers of CD11c$^+$ or CD11c$^-$/CD11b$^+$ BMNC isolated from BALB/c mice (H-2^d) were cocultured with 1.5×10^5 purified T cells from C57BL/6 mice (H-2^b). CD11c$^+$ cells from normal spleen served as reference stimulator cells. Proliferation was measured by [^{3}H]TdR uptake during the last 18 h of a 5-day incubation. Data are shown as the mean ± SD of triplicate test cultures. Background proliferation of stimulator cells or T cells alone was <160 cpm. Comparable results were obtained in four separate experiments. Test stimulator cells were isolated from inflamed brains of mice that had been infected for 5 wk with *T. gondii* (*A*) or from normal adult mouse brains (*B*).

of veiled or dendriform cells remained in suspension, often forming clusters (Fig. 8, *B* and *C*). At the concentration used to promote differentiation of brain DC, GM-CSF did not induce cellular proliferation, as assessed by [^{3}H]TdR incorporation (not shown). After 5 days of incubation with GM-CSF, approximately 30% of cells expressed CD11c (Fig. 8*D*) compared with <0.5% of cells in the initial population. Testing by MLR of sorted CD11c$^+$ cells revealed their allostimulatory capacity, although these microglia-born APC were significantly less potent than reference splenic DC. Engagement of CD40 by overnight incubation with a CD40 agonist, however, resulted in full competence to trigger maximum proliferation of alloreactive T cells (Fig. 8*E*). Similar results were obtained when using microglia derived from primary brain cell culture, although their basic stimulatory capacity was minimal compared with that of ex vivo microglia (Fig. 8*F*). Measurement of IL-12 secretion confirmed the activation of CD11c$^+$ microglia via CD40 ligation (Fig. 8*G*). These findings demonstrate a GM-CSF-dependent differentiation of microglia to DC and their functional maturation in response to CD40 signaling.

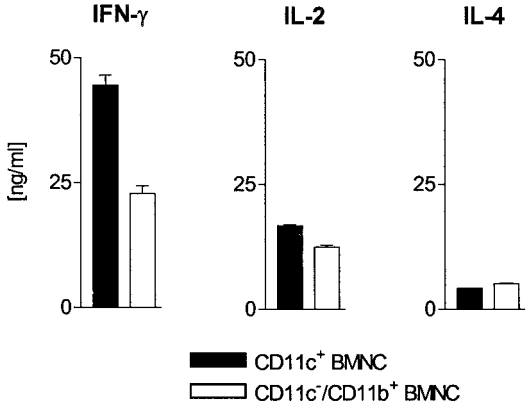

FIGURE 7. Priming by brain DC or CD11c$^-$ microglia/macrophages of OVA TCR transgenic T cells. Naive CD4$^+$/CD62L^{high} T cells from DO11.10 mice were stimulated with OVA peptide and CD11c$^+$ or CD11c$^-$/CD11b$^+$ BMNC isolated from *T. gondii*-infected BALB/c mice at wk 5 postinfection. After 5 days of primary stimulation culture, T cells were restimulated with plate-bound anti-CD3ϵ for an additional 24 h. SN were assessed for IFN-γ, IL-2, IL-4, and IL-10 by ELISA. Values represent the mean ± SD of quadruplicate SN productions. With CD11c$^+$ normal spleen cells as primary stimulators, the IL-4 response amounted to 3.7 ± 0.14 ng/ml, while no IL-10 was detectable. This experiment was repeated five times, producing similar cytokine secretion patterns.

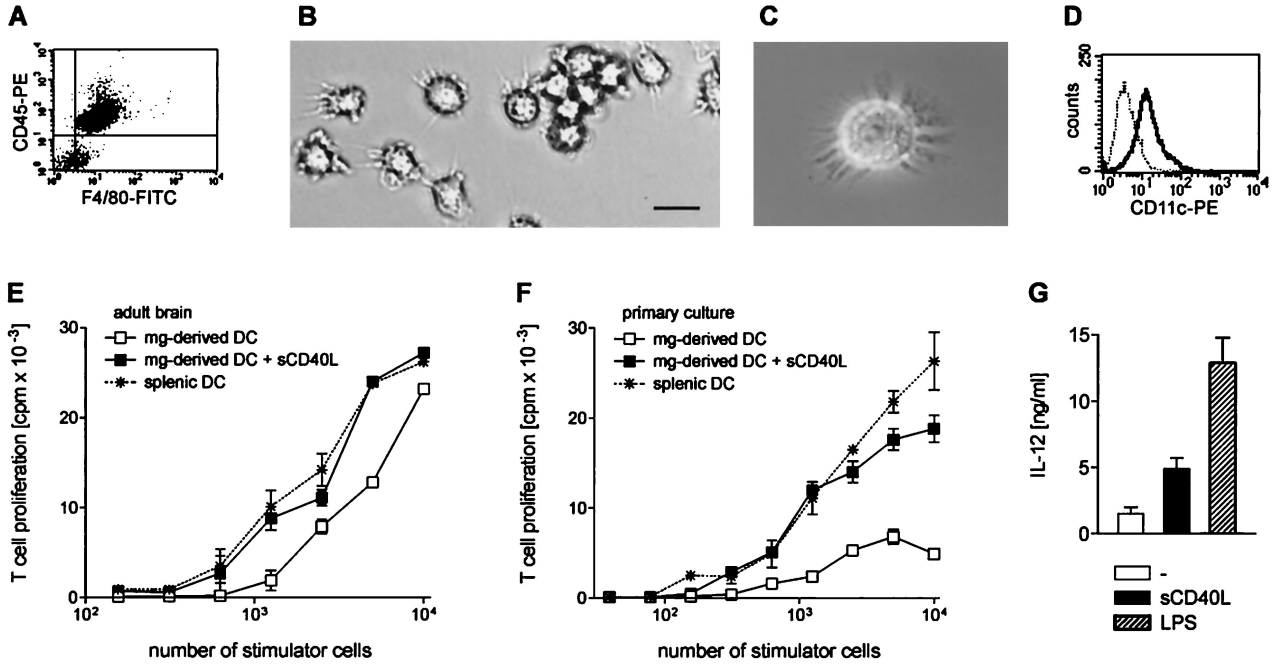

FIGURE 8. DC develop from resting microglia and functionally mature upon CD40 ligation. Cells were analyzed that had been derived from normal adult brain (*A–E*) or primary culture from neonatal brain (*F* and *G*). Data shown are representative of three independent experiments. *A*, Phenotypic characterization of microglia isolated from the CNS of BALB/c mice. Immediately after isolation of $CD11c^-/CD11b^+$ BMNC, cells were double stained for flow cytometric analysis with anti-CD45-PE and anti-F4/80-FITC mAb. Ninety-seven percent of cells were in the $F4/80^+/CD45^+$ gate. *B*, Micrograph showing a cluster of nonadherent dendriform cells that are enriched in 5-day culture of adult microglia with GM-CSF. Bar = 5 μm. *C*, A closeup phase-contrast micrograph illustrates the dendritic morphology. *D*, Surface expression of CD11c by adult microglia after 5-day culture ex vivo with GM-CSF. In flow cytometric analysis, cells were stained with anti-CD11c mAb N418 (solid line) or hamster isotype control Ab (dotted line). *E*, Allostimulatory capacity of adult microglia-derived DC. $CD11c^+$ cells were isolated from GM-CSF-cultured adult microglia (same cells as those analyzed in *A* and *D*) and subcultured for an additional 24 h with GM-CSF in the presence or the absence of additional soluble CD40L and cross-linking Ab (10 μg/ml each). In MLR, titrated numbers of these microglia-derived DC or reference splenic DC of BALB/c origin were incubated with 1.5×10^5 T cells of C57BL/6 origin. Proliferation was measured by $[^3H]$TdR uptake during the last 18 h of a 5-day incubation period. Data are shown as the mean ± SD of triplicate test cultures. Background proliferation of stimulator cells or T cells alone was <100 cpm. *F*, Allostimulatory capacity of newborn mouse microglia-derived DC. Analogous to the experimental design in *E*, $CD11c^+$ cells were isolated from GM-CSF-supplemented primary culture of newborn mouse brain and tested as stimulator cells in MLR. *G*, Secretion of IL-12 by microglia-derived DC. $CD11c^+$ cells were harvested from GM-CSF-supplemented primary brain cell culture and subcultured for 24 h with GM-CSF in the presence of soluble CD40L and cross-linking Ab (10 μg/ml each) or LPS (10 μg/ml) or without further stimulus. On aliquots of cells, the release of IL-12 during a subsequent 24 h of incubation was determined by ELISA.

Brain DC functionally mature during prolonged encephalitis

Based on our observation that resting microglia can acquire a DC phenotype and develop into functionally mature DC, we tested whether brain DC isolated during all stages of transient CNS inflammation vary in their maturational stage. Since CD11c is expressed on both immature and mature DC, sorting of $CD11c^+$ cells as a final step in the isolation procedure yields highly enriched DC regardless of their individual maturational stage. The relative proportions of fully matured DC among $CD11c^+$ BMNC isolated at different time points during transient TE were estimated by their allostimulatory capacities in MLR. Compared with cells from normal brains, DC prepared from the brains of BALB/c mice at the onset of TE (day 14 postinfection) showed a significantly ($p < 0.005$) elevated capacity to stimulate alloreactive T cell proliferation (Fig. 9). Maximum levels in T cell stimulation were measured on brain DC prepared during ongoing CNS inflammation (day 24 or 36 postinfection) or later when encephalitis in those mice resistant to infection with *T. gondii* was resolved (day 77 postinfection). These results indicate that a maturation of brain DC occurs in periods of apparent CNS inflammation and that functionally mature DC continue to be present in the brain after termination of a local inflammatory process.

Discussion

The mechanisms underlying the initiation and control of T cell responses in CNS inflammation are poorly understood. Resident brain cells, e.g., microglia (28), are undoubtedly involved as APC in the stimulation or reactivation of CNS-targeted T cells. However, it is unclear which cells initiate and amplify such responses before IFN-γ is produced and prime naive T cells entering the inflamed brain. The present data demonstrate in autoimmune and infectious encephalitis that CNS inflammation is associated with the local emergence of DC that perform these functions. Concomitant to microglia proliferation at disease onset, myeloid-related DC appear and expand at inflammatory sites within CNS tissue and functionally mature as disease progresses. Generation of DC from microglia, as proved ex vivo, may represent a brain-intrinsic mechanism by which the organ acquires full immunoreactivity upon inflammatory challenge and which may favor the chronicity of cellular responses in the CNS.

To date, DC have not been identified in the normal adult brain, whereas outside the blood-brain and meningeal barriers MHC class II^+ dendriform cells have been localized in the dura mater, leptomeninges, and choroid plexus (29, 30). An intraparenchymatic presence of DC was indicated by the detection of cells exhibiting MHC

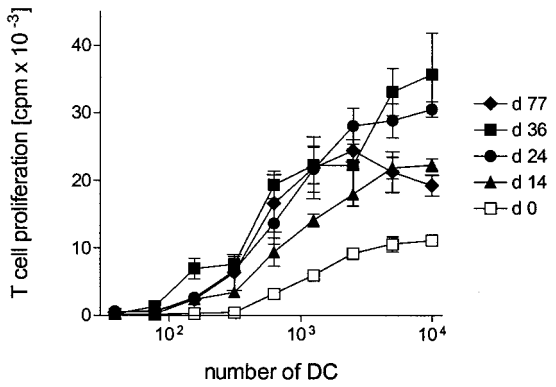

FIGURE 9. Brain DC functionally mature after the onset of encephalitis. BMNC were prepared from normal BALB/c mice and from animals at various time points during chronic latent TE (days (d) 14–77 postinfection). $CD11c^+$ cells were isolated from BMNC pooled from 2–10 mice/group and tested in one MLR experiment as stimulator cells against purified T cells of C57BL/6 origin. In MLR, titrated numbers of brain DC were incubated with 1.5×10^5 T cells, and proliferation was measured by $[^3H]$TdR uptake during the last 18 h. Data are shown as the mean ± SD of triplicate test cultures. Background proliferation of stimulator cells or T cells alone was <120 cpm. Comparable results were obtained in two independent experiments.

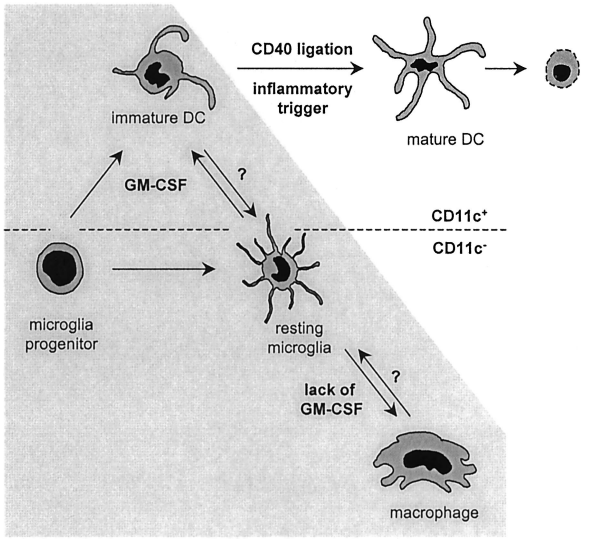

FIGURE 10. A model of mouse brain DC differentiation pathways. The development and function of cells on gray background are governed by astrocytic CSF-1.

class II or DC markers in delayed-type hypersensitivity and EAE lesions (31–33) and was evidenced on cell isolates in TE (14). Consistently, an intracerebral expression of the DC-specific MHC class II transactivator has recently been observed in mouse EAE (33). By immunohistochemistry in combination with ex vivo phenotyping and testing of brain cells for DC functions, we show here that activated myeloid DC accumulate at perivascular cuffs and parenchymatic foci in the brains of mice suffering from EAE or TE.

It is possible that blood-borne or meningeal DC are recruited by extravasation and via the perivascular space, respectively, to inflammatory sites within the CNS parenchyma. Alternatively, DC can differentiate from monocytes (34) that infiltrate the inflamed brain. Several lines of evidence support a third possibility, a GM-CSF-driven differentiation of brain DC from local, probably microglial, progenitors. It has been shown that the adult brain parenchyma harbors $CD11b^+$ myeloid precursors (35), and immature DC develop in primary culture with GM-CSF (15, 19). GM-CSF, which promotes the development of DC from bone marrow progenitors (23), is produced by glial cells upon exposure to inflammatory or microbial stimuli (36–38). Consistently, in EAE and TE, expression of GM-CSF is induced early during the onset of disease (39, 40). Intracerebral expression of CSF-1, as a cofactor required by immature brain DC for preserving GM-CSF-dependent APC function (15), is likewise up-regulated early in EAE (41). Furthermore, GM-CSF-triggered development of myeloid DC is accompanied by cellular proliferation (23), which agrees with the findings that most cycling cells from encephalitic brain were $CD11c^+$ and that mitotic cells were enriched in $CD11c^+$, but not in $CD11c^-/CD11b^+$, brain cell isolates. Finally, the progressive functional maturation of brain DC after the onset of encephalitis is compatible with a local development of DC. In contrast, an already advanced maturational stage, as would be expected for immigrating DC by virtue of their motility (42), would conflict with the early proliferative activity observed. Accordingly, in EAE single $CD11c^+$ parenchymatic cells were sometimes seen at a distance from T cell infiltrates, which renders a common influx of T cells and DC unlikely.

Evidence for a microglia differentiation pathway leading to brain DC is provided by the generation of potent allostimulatory $CD11c^+$ cells from normal resting microglia ex vivo. This development was independent of IFN-γ. The alteration of function and surface phenotype was detected on microglia regardless of whether they originated from normal adult brain or primary culture, thus excluding the possibility that the enzymatic extraction of brain cells might have influenced their functional potential. GM-CSF-induced expression of the pan-DC marker CD11c was accompanied by the acquisition of DC-like morphology and T cell-stimulatory activity; therefore, the cells were designated DC. In recent studies the GM-CSF-dependent differentiation step has been designated microglia activation or functional maturation. Exposure of a microglia cell line or adult microglia to GM-CSF induced up-regulation of CD80/B7.1 and MHC class II surface expression and of Ag-directed Th1 stimulatory capacity, respectively (25, 43). However, whether such GM-CSF-treated microglial cells exhibited DC markers was not analyzed in these studies. A model integrating the putative differentiation pathway(s) of brain DC and their relationship to the microglia/macrophage lineage is shown in Fig. 10. Upon trigger by GM-CSF, e.g., from reactive astroglia, microglial cells can differentiate via two steps into functional DC that finally die by apoptosis. Such a concept obtains support from the even tissue distribution of MHC class II^+ dendritic-like cells in rat EAE in the prefinal state (32) and the early emergence of $CD11c^+$ parenchymatic cells (Fig. 3*E*) and allows the migration of brain DC from intraparenchymatic sites also.

Despite the disparate etiology of both CNS disorders, brain DC in EAE and TE exhibit a similar $CD11c^+/CD11b^+/F4/80^+/DEC\text{-}205^-/CD8\alpha^-$ myeloid-related phenotype which argues for a general process causing the intracerebral presence of DC in CNS inflammation. Immunomagnetic sorting of T cell-depleted $CD11c^+$ and $CD11c^-/CD11b^+$ mononuclear cells from encephalitic brain allowed the separation of DC from other microglia/brain macrophages for comparative analyses ex vivo. Surprisingly, cells in both populations showed an intermediate to high expression of CD45; the proportion of $CD45^{high}$ cells correlated with the severity of disease and the total number of BMNC isolated. Assuming that low level expression of CD45 stably characterizes microglia, the fact that only $CD45^{high}$ cells are present in the $CD11c^-/$

$CD11b^+$ BMNC isolate from progressive TE might be due to the relative rarity of microglial cells among the exogenous-derived inflammatory macrophages and the preceding separation of $CD11c^+$ cells, which, indeed, contained considerable numbers of cells expressing CD45 at a low to intermediate level. Another explanation would be an up-regulation of CD45 on resident microglia under the conditions of progressive lethal TE. No correlation between the level of CD45 expression and the expression of CD11c was found on encephalitic BMNC.

With regard to cell morphology and ultrastructure, the brain DC strikingly resembled bone marrow (23) or liver-derived DC (44), whereas most inflammatory $CD11c^-$ microglia/brain macrophages exhibited features of other tissue macrophages. Distinctive immune functions were the secretion of IL-12p70 and efficient induction of proliferative T cell responses by brain DC, and the production of TNF-α, GM-CSF, and NO by $CD11c^-/CD11b^+$ BMNC, respectively. Based on these profiles, non-DC $CD11b^+$ brain cells seem to be predestined as amplifier and effector cells of CNS inflammation. Among them, microglia and brain macrophages can alternate in TNF-α production (45). By producing IL-10, inflammatory microglia/brain macrophages might provide a local counter-regulation of IL-12 effects (46–48). While in TE, NO is crucial for parasite inhibition (49), the role NO plays in CNS autoimmunity, mediating tissue damage or antagonizing autoaggressive cellular activity, is still debated (50).

On the other hand, brain DC functionally resemble (by their weak IL-10 production and strong Th1-inducing capacity) bone marrow-derived and splenic DC, but not liver-derived or Peyer's patch DC (51, 52), and thus must be considered initiators of Th1-mediated CNS immunopathology. The Th1-promoting activity of brain DC and their long-lasting presence even after resolution of a local inflammatory process suggest a key role in the exacerbation or maintenance of immune-mediated CNS diseases. As stimulators of naive T cells infiltrating the inflamed brain, these DC might be crucially involved in the phenomenon of epitope spreading during chronic T cell-mediated autoaggression against CNS Ags. Whether microglia are primed in situ to differentiation into the Th1-inducing DC type by IFN-γ, as has been demonstrated on human monocyte-derived DC (53), or by another, possibly tissue-specific, mechanism remains an issue relevant for future brain DC-targeted immune intervention.

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