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Induction of inhibitory central nervous system-derived and stimulatory blood-derived dendritic cells suggests a dual role for granulocyte-macrophage colony-stimulating factor in central nervous system inflammation

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The mononuclear phagocyte system, particularly dendritic cells, plays several pivotal roles in the development of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis. Here, we demonstrate that functionally distinct dendritic cell subpopulations are present in the central nervous system during experimental autoimmune encephalomyelitis. At peak experimental autoimmune encephalomyelitis, the majority of dendritic cells consisted of a CD11b$^+$F4/80$^+$ inflammatory dendritic cell subtype. Both granulocyte-macrophage colony-stimulating factor and chemokine (C–C motif) ligand 2 were previously suggested to recruit 'inflammatory' monocyte-derived dendritic cells to the central nervous system during experimental autoimmune encephalomyelitis. We show that intra-cerebral production of granulocyte-macrophage colony-stimulating factor leading to chemokine (C–C motif) ligand 2 induction and attraction of chemokine (C–C motif) receptor 2-positive precursors suffices to recruit dendritic cell populations identical to those observed in experimental autoimmune encephalomyelitis into the central nervous system of healthy mice. This does not occur with fms-like tyrosine kinase-3-ligand treatment. Both during experimental autoimmune encephalomyelitis and upon intra-cerebral granulocyte-macrophage colony-stimulating factor production, all myeloid dendritic cells, lymphoid dendritic cells and periphery-derived inflammatory dendritic cells stimulated T cell proliferation, whereas inflammatory dendritic cells that differentiated from central nervous system precursors inhibited T cell activation and pro-inflammatory cytokine production. Despite the capacity of granulocyte-macrophage colony-stimulating factor to induce central nervous system-derived inhibitory inflammatory dendritic cells, the administration of granulocyte-macrophage

colony-stimulating factor into mice with experimental autoimmune encephalomyelitis resulted in exacerbated disease. Granulocyte-macrophage colony-stimulating factor thus has a dual role in the central nervous system: it directs both central nervous system-derived dendritic cells towards an inhibitory phenotype and recruits peripheral dendritic cells exhibiting pro-inflammatory functions.

Introduction

Monocytes, macrophages and dendritic cells constitute the so-called mononuclear phagocyte system and are increasingly recognized as a highly plastic continuum of cells able to adopt many different phenotypes involved in the initiation and regulation of immune responses, as well as in inflammation, healing and tissue remodelling (Gordon and Taylor, 2005; Shortman and Naik, 2007; Auffray *et al.*, 2009). Macrophage colony-stimulating factor (M-CSF), fms-like tyrosine kinase-3 ligand (FL) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are known to be essential both for the steady state differentiation of monocytes, macrophages and dendritic cells and for the generation of their precursors (Gordon and Taylor, 2005; Shortman and Naik, 2007; Auffray *et al.*, 2009). Using receptor-deficient animals, fms-like tyrosine kinase-3 (Flt3) was shown to be essential for the steady state generation of all types of dendritic cells whereas the GM-CSF receptor was found to play a minor role in this respect (Waskow *et al.*, 2008). However, using single- and double-deficient ligand-knockout mice, it has been demonstrated that in steady state conditions GM-CSF and FL act in a concerted manner on the generation of both macrophage/dendritic cell progenitors and most dendritic cell subsets (Kingston *et al.*, 2009).

In contrast to steady state conditions, GM-CSF is thought to be the major player in modulation of the mononuclear phagocyte system during inflammatory conditions (Gordon and Taylor, 2005; Shortman and Naik, 2007; Auffray *et al.*, 2009). Both in systemic and central nervous system (CNS) inflammation, GM-CSF expression is markedly up-regulated (Fiehn *et al.*, 1992; Perrella *et al.*, 1993; Selig and Nothdurft, 1995) and leads to an increase in circulating Ly6C/Gr-1$^+$ inflammatory monocytes, which can develop into 'inflammatory' dendritic cells upon endothelial transmigration into the inflamed tissue (Geissmann *et al.*, 2003; Serbina and Pamer, 2006; Shortman and Naik, 2007; King *et al.*, 2009). These 'inflammatory' dendritic cells appear to be related to tumour necrosis factor-α and inducible nitric oxide synthase producing dendritic cells identified in CNS autoimmunity and infection (Serbina *et al.*, 2003; Xu *et al.*, 2007; Dogan *et al.*, 2008; King *et al.*, 2009; Mildner *et al.*, 2009).

The role of mononuclear phagocyte system cells in CNS autoimmune inflammation, i.e. in multiple sclerosis and its prototypic animal model experimental autoimmune encephalomyelitis (EAE) (Steinman, 1999), has been studied intensively in recent years (Becher *et al.*, 2006; McMahon *et al.*, 2006). Microglia and astrocytes were initially regarded as local antigen presenting cells of the CNS (Fontana *et al.*, 1984; Frei *et al.*, 1987). However, the identification of dendritic cells in the CNS of patients with multiple sclerosis (Pashenkov *et al.*, 2001; Plumb *et al.*, 2003) and of animals with EAE (Matyszak and Perry, 1996; Suter *et al.*, 2000) fostered numerous recent studies suggesting that macrophages and dendritic cells that enter the CNS by crossing the blood–brain barrier are the important antigen presenting cells for restimulation and full activation of myelin-specific T cells *in situ* (Fischer and Reichmann, 2001; Greter *et al.*, 2005; McMahon *et al.*, 2005; Bailey *et al.*, 2007; Deshpande *et al.*, 2007; Ifergan *et al.*, 2008; King *et al.*, 2009). However, in addition to such disease promoting dendritic cells, potentially disease mitigating CNS-dendritic cells have also been described to be present in EAE (Suter *et al.*, 2003; Deshpande *et al.*, 2007; Bailey-Bucktrout *et al.*, 2008).

GM-CSF has been demonstrated to be crucial for the development of CNS autoimmune inflammation (McQualter *et al.*, 2001; Ponomarev *et al.*, 2007). GM-CSF-deficient mice are resistant to EAE development and this resistance can be reversed by intra-peritoneal application of GM-CSF. Conversely, mice treated peripherally with recombinant GM-CSF develop an exacerbated disease (McQualter *et al.*, 2001). Notably, a disease enhancing effect by amplifying dendritic cell numbers has also been reported for the haematopoietic growth factor FL (Greter *et al.*, 2005). Using an *in vitro* model of the human blood–brain barrier, the Prat group showed that activated endothelia produce GM-CSF, which induces transmigration of CD14$^+$ monocytes and their differentiation into dendritic cells (Ifergan *et al.*, 2008). Very recently King *et al.* (2009) showed that circulating GM-CSF leads to mobilization of Ly6C$^+$ inflammatory monocytes from the bone marrow, which enter the CNS during EAE and develop into dendritic cells. Interestingly, Mildner *et al.* (2009) illustrated that these Ly6C$^+$ inflammatory monocytes require chemokine (C–C motif) receptor 2 (CCR2) signalling to reach the CNS. Both of these murine *in vivo* studies showed that inflammatory monocytes are crucially involved in the evolution of the disease. However, despite the recent progress in understanding the role of individual components of the mononuclear phagocyte system in EAE, the cellular and functional complexity of the mononuclear phagocyte system in CNS autoimmunity remains poorly understood, as is the role of the dendritic cell growth factors GM-CSF and FL in the regulation

of this cellular network. We therefore set out to study the role of GM-CSF and FL in the recruitment, differentiation and function of various dendritic cell subsets in EAE within the target organ of this prototypic autoimmune disease.

In the present report, we show that the presence of GM-CSF but not of Flt3-ligand within the CNS is sufficient to recruit dendritic cells that are phenotypically and functionally identical to dendritic cells found within the CNS during EAE. We found that intra-cerebrally produced GM-CSF exerted its function in part by the induction of local chemokine (C–C motif) ligand 2 (CCL2) expression and the recruitment of CCR2$^+$ blood-derived dendritic cell precursors. Both in EAE and after intra-cerebral GM-CSF production, the predominant dendritic cell populations are stimulatory blood-derived CD11b$^+$ myeloid and CD11b$^+$F4/80$^+$ inflammatory dendritic cells. In addition, we identified a CNS-derived CD11b$^+$F4/80$^+$ dendritic cell population that is able to inhibit T cell proliferation and limit pro-inflammatory cytokine production. However, despite the induction of inhibitory dendritic cells by GM-CSF, enhanced GM-CSF expression in the CNS during EAE resulted in a more severe disease outcome, suggesting that the 'anti-inflammatory' property of GM-CSF to induce inhibitory CNS-derived dendritic cells is overshadowed by its 'pro-inflammatory' effect on periphery-derived dendritic cells.

Materials and methods

Mice

Female C57BL/6 and Balb/c mice (10- to 12-week-old) were purchased from Harlan Laboratories (Horst, the Netherland) or RCC (Füllinsdorf, Switzerland). Flt3-ligand-deficient animals (FL$^{-/-}$) were obtained from Mark Suter, Institute of Virology, University of Zurich. CD45.1 and CD45.2 C57BL/6 mice were used to generate congenic bone marrow chimeras. Recipient CD45.2 animals were irradiated with 1200 rad in a split dose within a 24 h period and reconstituted by injecting 5×10^6 CD45.1 bone marrow cells intravenously. More than 95% of the blood leukocytes in the chimeras were of donor origin after 8 weeks of reconstitution. All experiments were performed with the permission of the Cantonal Veterinary Office of Zurich, Switzerland.

Growth factor treatment *in vivo*

The wild-type, GM-CSF-secreting and FL-secreting B16-F10 cell lines were kindly provided by Professor Nicolas Mach (University Hospital Geneva, Switzerland). The cells were cultured up to 80% confluency in 10% Dulbecco's modified Eagle's medium (Invitrogen). After harvesting with 0.25% EDTA/trypsin-solution (Biological Industries) and re-suspension in Hank's balanced salt solution at a concentration of $5 \times 10^5/30\,\mu l$, the cells were growth arrested by irradiation with a dose of 5500 rad. $30\,\mu l$ of the cell suspension were injected subcortically into the right frontal hemisphere (2 mm lateral of bregma, 2 mm deep) of C57BL/6 mice.

Microglia cultures

The brains of 2-day-old C57BL/6 newborns were isolated and passed through a 70-μm cell strainer. The cells from 2.5 brains were cultured in 20% foetal calf serum/Dulbecco's modified Eagle's medium in 150 cm^2 flasks for 6 days. The medium was then changed to 10% Dulbecco's modified Eagle's medium containing 20 ng/ml M-CSF or 40 ng/ml GM-CSF. After 5 days, cultures were shaken on a horizontal shaker. Non-adherent cells were harvested and prepared for flow cytometry or *in vitro* proliferation assays.

Induction and assessment of experimental autoimmune encephalomyelitis

Mice were immunized on Days 0 and 7 by two subcutaneous injections into the right and left flanks of a 1:1 emulsion of 100 μg/100 μl myelin oligodendrocyte glycoprotein (MOG)$_{35-55}$ peptide (ANAWA, Wangen, Switzerland) in phosphate buffered saline and 100 μl complete Freund's adjuvant (DIFCO, Detroit, USA). Pertussis toxin (List Biological Laboratories, Campbell, USA) (300 ng) was administered intraperitoneally at Days 0 and 2. Clinical EAE scores were assessed as follows: 0 = no clinical symptoms; 0.5 = partial limp tail; 1 = complete limp tail; 1.5 = limp tail and hind limb weakness; 2 = partial hind limb paralysis; 2.5 = complete unilateral hind limb paralysis; 3 = complete bilateral hind limb paralysis; 3.5 = complete bilateral hind limb paralysis and partial forelimb paralysis; 4 = paralysis of fore and hind limbs (euthanized); 5 = death.

Preparation of mononuclear cells present in the CNS

For brain and spinal cord isolation, mice were sacrificed using CO$_2$ and perfused with Ringer solution (Braun Medical, Sempach, Switzerland). The tissues were minced and digested for 30 min at 37°C in Hank's balanced salt solution containing 50 μg/ml DNaseI (Roche) and 100 μg/ml collagenase/dispase (Roche) for flow cytometry or 0.25% trypsin (Invitrogen) for cell sorting. The digestion was quenched by adding 10% foetal calf serum (Omnilab, Mettmenstetten, Switzerland) on ice. The tissue was passed through a 100 μm mesh, pelleted, resuspended in 30% Percoll (GE Healthcare) and centrifuged at 15 500 r.p.m. for 30 min at 4°C. The myelin layer was removed and the mononuclear cells accumulated in the intermediate phase were collected.

Flow cytometry and cell sorting

Flow cytometry was done with a CyFlow Space (Partec, Münster, Germany). Cell sorting was performed with a FACSAria (BD Bioscience, USA). The following antibodies were used: CD45-allophycocyanin (APC), CD45.1-fluorescein isothiocyanate (FITC), CD45.2-FITC, CD11b-biotinylated, CD11c-phycoerythrin (PE), F4/80-FITC or APC, streptavidin-APC-Cy7 or streptavidin-PacificBlue. 7-Aminoactinomycin D (7AAD) was used to exclude dead cells.

Histology

Organs were embedded in Jung tissue freezing medium (Leica Instruments GmbH, Nussloch, Germany) and frozen on a metal plate chilled by dry ice. Tissue sections of 6 μm thickness were cut in a cryostat and fixed in 2% paraformaldehyde/phosphate buffered saline for 5 min. Sections were incubated with biotin or FITC labelled antibody against CD45.1, CD45.2, CD11b, CD11c and F4/80. The binding of primary antibodies was revealed by streptavidin–horseradish peroxidase and anti-FITC–horseradish peroxidase, respectively (NEN/

PerkinElmer, Vienna, Austria), using cy5-, tetramethylrhodamin- and FITC-coupled tyramine as substrate. Fluorescence microscopy was done on a Leica LX.

Proliferation and inhibition assays

Proliferation and inhibition assays were performed as described previously (Suter *et al.*, 2003). Briefly, mice were sacrificed using CO_2, spleens of naïve Balb/c mice were passed through a 100 μm mesh and digested with DNaseI. After erythrocyte lysis, $CD4^+$ T cells were selected using CELLection Biotin Binder beads from Invitrogen. 1×10^5 $CD4^+$ T cells were cultured with titrating numbers of irradiated antigen presenting cells in 96-well plates. In the inhibition assay, 1×10^4 irradiated mature bone marrow-dendritic cells were added to the T cell/antigen presenting cell co-cultures. Mature bone marrow-dendritic cells were prepared as described (Suter *et al.*, 2003). After 2 days, the cells were pulsed with 1 μCi [^{3}H]thymidine (GE Healthcare) and analysed with a Wallac MicroBeta counter.

Reverse transcriptase polymerase chain reaction

RNA samples were obtained by homogenizing spinal cord tissues in TRIZOL according to the manufacturer's protocol (Invitrogen). Complementary DNA was synthesized using random hexamers (Roche) and Moloney murine leukaemia virus reverse transcriptase (Roche). TaqMan analysis was done with an ABI PRISM 7700 detection system (PE-Applied Biosystems) using the PE-TaqMan Master Mix and GM-CSF or FL primer probes purchased from Applied Biosystems. The 18s ribosomal RNA was used for normalization. RNA expression was calculated as $2^{-\Delta\Delta ct}$ value relative to the average of the pre-onset values. Each sample was measured in triplicate.

Enzyme-linked immunosorbent assay

Tissue samples were homogenized in an isolation buffer containing 50 mM Tris, 5 mM EDTA and 150 mM NaCl. After adding 1% NP40 (Fluka) for 30 min, samples were pelleted and supernatants collected for enzyme-linked immunosorbent assay (ELISA) (R&DSystem). Protein content was determined by the bicinchoninic acid assay reaction (Thermo-Pierce, Lausanne, Switzerland).

Enzyme-linked immunosorbent spot

Spleens of immunized animals (d12) were passed through a 100 μm mesh and digested with 50 μg/ml DNaseI (Roche). After erythrocyte-lysis, $CD4^+$ T cells were purified using CELLection Biotin Binder beads from Invitrogen. T cells and antigen presenting cells were co-cultured overnight in a 1:10 ratio. The following day, 2×10^4 T cells were cultured in either anti-interleukin (IL)-17 or anti-gamma-interferon (IFN-γ) pre-coated enzyme-linked immunosorbent spot-plates in the presence of 25 μg/ml $MOG_{35–55}$. After 20 h, plates were washed and stained by adding biotinylated antibodies, streptavidin–alkaline phosphatase and the substrate solution as described in the manufacturer's protocol (Mabtech, Sweden).

Statistical analysis

Statistical significances were assessed using the unpaired two-tailed Student *t*-test.

Results

Dendritic cells present in the CNS during EAE consist of three functionally distinct subpopulations: lymphoid, myloid and inflammatory dendritic cells

Dendritic cells present in the CNS remain poorly characterized both during steady state and in inflammation. On one hand, dendritic cells that occur in the inflamed CNS have been shown to stimulate T cell proliferation (Fischer and Reichmann, 2001; Greter *et al.*, 2005; Bailey *et al.*, 2007; Deshpande *et al.*, 2007). On the other hand, it has also been demonstrated that dendritic cells present in the CNS during the acute phase of EAE can inhibit T cell proliferation (Suter *et al.*, 2003; Deshpande *et al.*, 2007). We therefore performed a thorough analysis of the phenotypic and functional properties of CNS associated dendritic cells. Mononuclear cells isolated from the acutely diseased CNS of $MOG_{35–55}$ immunized mice were analysed by flow cytometry for the expression of markers previously found on dendritic cells (Vremec *et al.*, 2000; Fischer and Reichmann, 2001; O'Keeffe *et al.*, 2002). Gating on $CD45^{hi}CD11c^+$ cells revealed the presence of $21 \pm 4.7\%$ dendritic cells among total mononuclear cells (Fig. 1A). Unexpectedly, a majority of these cells expressed F4/80, the murine homologue of epidermal growth factor-like module containing mucin-like hormone receptor (EMR) 1, which has previously been reported to be expressed on a subpopulation of dendritic cells (Pulendran *et al.*, 1997; Vremec *et al.*, 2000). We found only very low numbers of $CD4^+$ or $CD8^+$ dendritic cells (data not shown). Microglia ($CD45^{int}$ $CD11b^{int}$ $F4/80^{int}$ $CD11c^{lo-neg}$) are not contained within the dendritic cell gate (Supplementary Fig. 1).

On the basis of these findings, we defined three dendritic cell subpopulations: a minor fraction ($6.1 \pm 2.9\%$) of $CD11b^-F4/80^-$ lymphoid dendritic cells, an intermediate fraction ($18 \pm 6.0\%$) of cells expressing high levels of CD11b (myeloid dendritic cells) and a major population ($73 \pm 9.7\%$) of $CD11b^+F4/80^+$ inflammatory dendritic cells (Fig. 1A). To characterize the three dendritic cell subsets further, we performed additional flow cytometric analysis to document the expression of activation markers and co-stimulatory molecules, including major histocompatibility complex II, CD80, CD86, CD40 and several macrophage and dendritic cell markers (Supplementary Fig. 2). Interestingly, no major difference in activation status was evident between lymphoid, myeloid and inflammatory dendritic cells. However, we found a panel of 'typical' macrophage and dendritic cell markers to be differentially expressed on the three dendritic cell populations. The majority of these markers are receptors involved in antigen capture and have been previously reported to be expressed by both dendritic cell and macrophage subsets. Interestingly, the M-CSF receptor (CD115) was not expressed on either of the dendritic cells, indicating that no monocytes are present in the CNS at peak EAE stage.
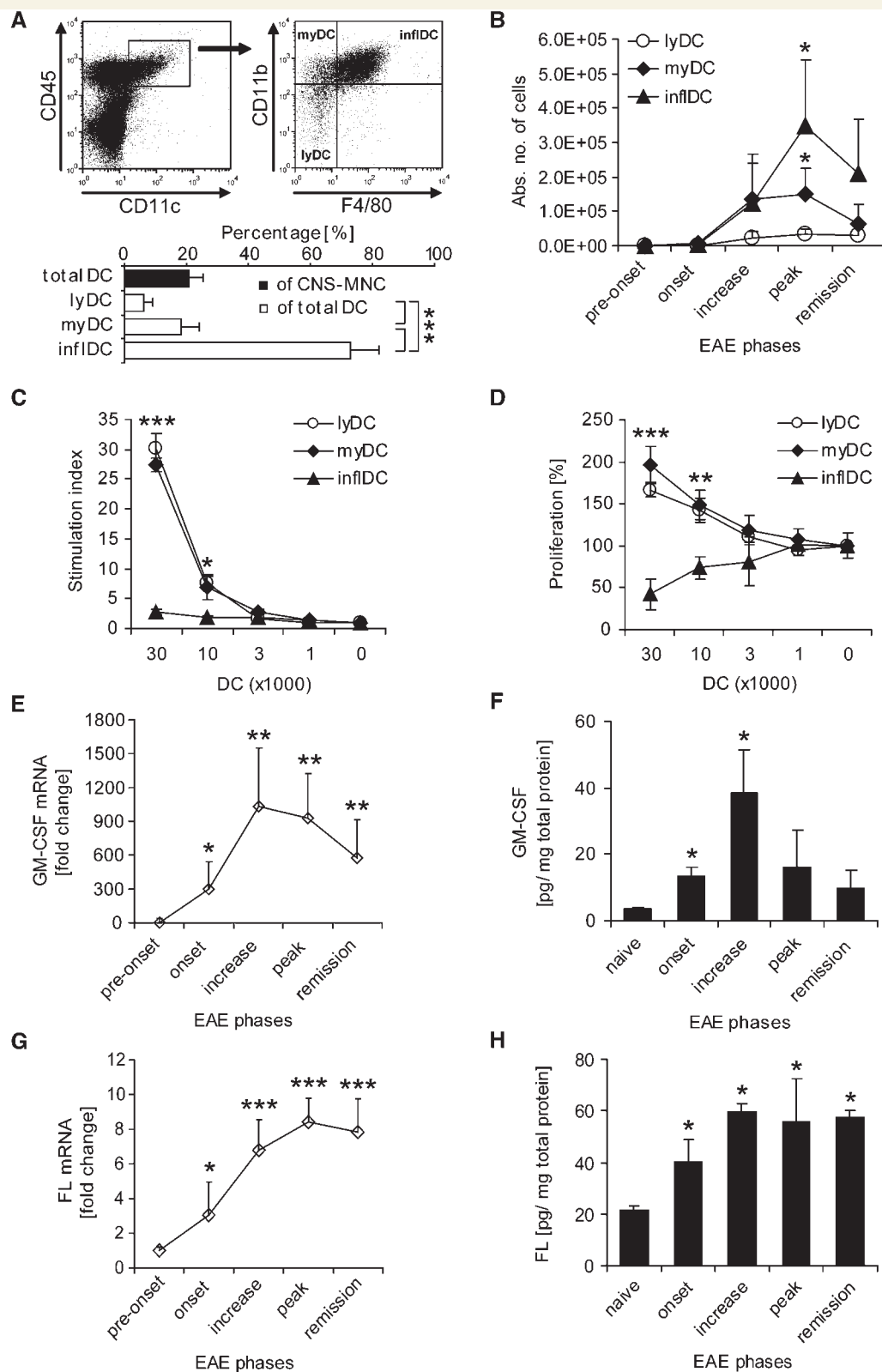
Figure 1 Functionally distinct dendritic cell (DC) subpopulations exist in the CNS during EAE. C57BL/6 mice were immunized with MOG$_{35-55}$ peptide in complete Freund's adjuvant and Pertussis toxin. (**A**) CNS-mononuclear cells (MNC) were prepared at peak EAE (score 3) and analysed by flow cytometry. The left plot is gated on 7-aminoactinomycin D-negative total CNS-mononuclear cells and the right plot is gated on CD45$^+$CD11c$^+$ dendritic cells, as indicated. Dendritic cells were separated into subpopulations according to their expression of CD11b and F4/80. The bar graph (**A**, *bottom*) shows the mean from 17 experiments of total dendritic cells gated on CNS-mononuclear cells and lymphoid, myeloid and inflammatory dendritic cells gated on total dendritic cells as indicated in the dot plots. For all comparisons among the subpopulations, $P < 0.001$. (**B**) The composition of the dendritic cell subsets in the CNS was assessed during EAE development: pre-onset (Day 10–11), onset (Day 12–14), increase (Day 14–16), peak (Day 17–18) and remission (Day 19–21).

(Continued)

The relative proportions of lymphoid, myeloid and inflammatory dendritic cells changed during EAE development. The numbers of lymphoid dendritic cells increased slightly and continuously during disease progression, but remained at low levels. In contrast, the numbers of myeloid and inflammatory dendritic cells reached a maximum during the aggravation phase and peak disease, respectively, and decreased thereafter (Fig. 1B). To investigate the respective abilities of lymphoid, myeloid and inflammatory dendritic cells to activate T cells, we performed allogeneic proliferation assays with naïve CD4$^+$ T cells and dendritic cells purified by flow cytometry from the EAE-CNS. Interestingly, despite comparable expression of major histocompatibility complex II and co-stimulatory molecules, lymphoid and myeloid dendritic cells stimulated T cell proliferation much more strongly than inflammatory dendritic cells (Fig. 1C). Furthermore, inflammatory dendritic cells were found to inhibit the proliferation of T cells stimulated by mature bone marrow-derived dendritic cells (Fig. 1D).

The haematopoietic growth factor FL is known to promote the development of lymphoid and myeloid dendritic cells. In contrast, GM-CSF predominantly stimulates the differentiation of myeloid dendritic cells and has been suggested to induce CD11b$^+$ F4/80$^+$Ly6C$^+$ 'inflammatory' dendritic cells (Daro *et al.*, 2000; Karsunky *et al.*, 2003; Pulendran *et al.*, 1999; Shortman and Naik, 2007). As both CD11b$^-$ and CD11b$^+$ dendritic cells accumulate in the EAE-CNS, we examined whether GM-CSF and FL expression in the CNS correlated with the appearance of individual dendritic cell subsets. GM-CSF mRNA and protein expression were highest during the increasing phase of EAE (Fig. 1E and F). In contrast, FL expression increased continuously during EAE development, reaching a plateau from the increase/exacerbation phase onwards (Fig. 1G and H). These results therefore suggest that increased GM-CSF expression correlates with the accumulation of CD11b$^+$ myeloid and inflammatory dendritic cells in the CNS during EAE. These dendritic cell populations play differential roles in activating T cells and might thus have differential effects on the regulation of EAE.

Intra-cerebrally expressed GM-CSF promotes an accumulation of dendritic cells as found in EAE

As the expression of GM-CSF in the CNS correlates with the appearance of myeloid and inflammatory dendritic cells, we determined whether increased GM-CSF levels *per se* would be sufficient to promote the recruitment of these dendritic cell subsets into the CNS. Irradiated GM-CSF-producing B16 cells, or the parental control cell line, were injected subcortically into the right frontal hemisphere of naïve mice. To define the optimal time point for dendritic cell accumulation, CNS-mononuclear cells were isolated and analysed by flow cytometry after 3, 7 and 10 days. Dendritic cell numbers in the CNS attained a maximum 7 days after intra-cerebral administration of the GM-CSF-producing cells (Supplementary Fig. 4). This time point was therefore chosen for further experiments. GM-CSF expression was highest in the brain but increased levels were also observed in the spinal cord, spleen and serum (Fig. 2A).

Compared to naïve mice, animals injected with the control cells showed only a slight non-significant increase in dendritic cells (Fig. 2B). However, GM-CSF expression in the CNS led to a markedly enhanced recruitment of dendritic cells. Dendritic cell populations were increased in the CNS and spleen and exhibited a similar composition, with myeloid dendritic cells being the most abundant, followed by inflammatory cells (Fig. 2B). In contrast, although the concentration of FL in the brain resulting from intra-cerebral injection of FL-producing cells was much higher than the GM-CSF levels (data not shown), the numbers of dendritic cells in both the CNS and spleen were lower and consisted mainly of lymphoid dendritic cells (Supplementary Fig. 5). These results are consistent with earlier reports on the *in vitro* and *in vivo* effects of GM-CSF and FL on bone marrow cells and blood-derived monocytes (Sallusto and Lanzavecchia, 1994; Daro *et al.*, 2000; Maraskovsky *et al.*, 2000; O'Keeffe *et al.*, 2002).

It was recently suggested that GM-CSF might increase dendritic cells by mobilizing GM-CSF-receptor$^+$ monocytic precursors from the bone marrow (King *et al.*, 2009). The fact that intra-cerebral expression of GM-CSF caused a systemic increase in GM-CSF (Fig. 2A) would be consistent with the proposition that intra-cerebrally produced GM-CSF recruits bone marrow-derived precursor cells from the periphery into the CNS. In addition, the recruitment of dendritic cells/monocytes into the autoimmune-inflamed CNS has been shown to depend on the recruitment of circulating CCR2$^+$ monocytes by CNS-derived CCL2 (Fife *et al.*, 2000; Dogan *et al.*, 2008; Mildner *et al.*, 2009). Moreover, a number of cell types (lymphocytes, macrophages, astrocytes and endothelial cells) were found to produce CCL2 in EAE or *in vitro* upon GM-CSF stimulation (Steube *et al.*, 1999; Mahad and

Figure 1 Continued

The cell numbers per CNS at each time point represent the mean of 6–10 animals. (**C** and **D**) CNS-dendritic cell subpopulations at peak EAE were sorted by flow cytometry. Experiments were repeated at least 6 times with similar results. (**C**) 10^5 naïve allogeneic CD4$^+$ T cells were cultured with titrating numbers of dendritic cells. After 2 days, cells were pulsed with [^{3}H]thymidine for another 16 h. Results are expressed as a stimulation index calculated as the ratio of mean counts per minute for T cells co-cultured with dendritic cells relative to T cells alone. (**D**) The 10^5 naive allogeneic CD4$^+$ T cells were cultured together with stimulatory *in vitro* generated mature bone marrow-dendritic cells and titrating numbers of the indicated EAE-dendritic cell subsets. After 2 days, cells were pulsed with [^{3}H]thymidine for another 16 h. T cell proliferation stimulated by mature bone marrow-dendritic cells was set at 100%. (**E–H**) GM-CSF and FL expression levels during EAE development were analysed by quantitative reverse transcriptase PCR (**E** and **G**) or ELISA (**F** and **H**). Spinal cord samples of at least three animals per EAE phase were homogenized and prepared as described in the 'Materials and methods' section. The quantitative reverse transcriptase PCR data were expressed relative to pre-onset levels. Experiments were performed twice with similar results. *$P<0.05$, **$P<0.01$, ***$P<0.001$; **A** and **B** comparing all dendritic cell subsets to each other, **C** and **D** comparing lymphoid and myeloid to inflammatory dendritic cells, **E–H** comparison to pre-onset or naïve values. lyDC = lymphoid dendritic cell; myDC = myeloid dendritic cell; inflDC = inflammatory dendritic cell.
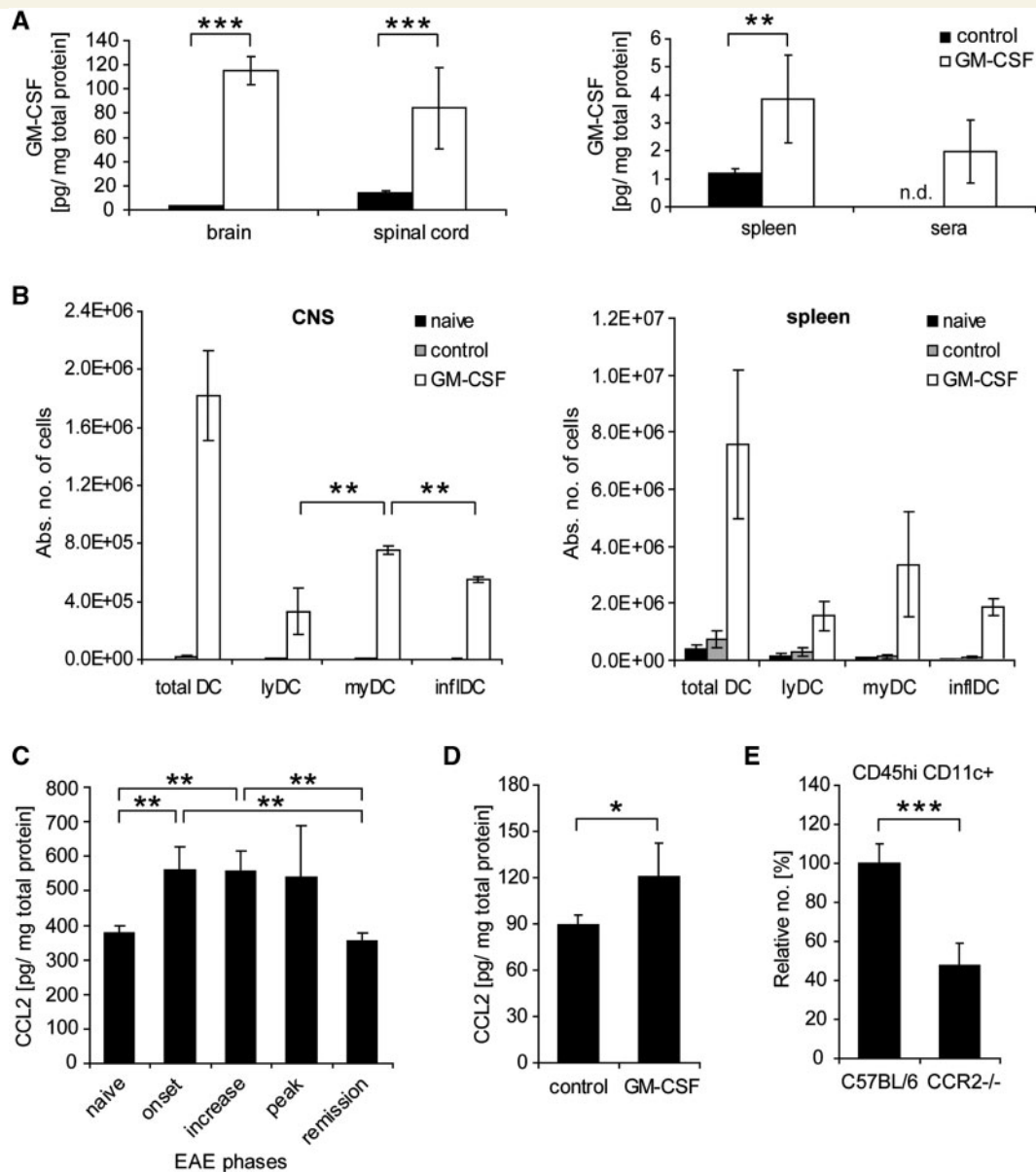
Figure 2 GM-CSF initiates an accumulation of lymphoid, myeloid and inflammatory dendritic cells. Irradiated control or GM-CSF-producing B16-F10 cells (5×10^5) were injected intra-cerebrally into naïve C57BL/6 mice. (**A**) The CNS, spleen and serum were collected 7 days after intra-cerebral administration and GM-CSF levels were assessed by ELISA (n.d., not detectable). At least four mice per group were analysed. (**B**) CNS-mononuclear cells and splenocytes from GM-CSF-treated, untreated and control mice were isolated at Day 7. The amount of the indicated dendritic cell subpopulations were analysed by flow cytometry as shown in Fig. 1. Data are representative for three independent experiments with three mice per group. (**C**) The CCL2 levels in spinal cords from MOG-immunized mice were measured by ELISA at different time points of disease progression. (**D**) Brain samples from naïve mice that had received either GM-CSF-producing or control cells for 7 days were analysed by ELISA. Data represent the mean of four animals per group. (**E**) Irradiated GM-CSF-producing B16-F10 cells (5×10^5) were injected intra-cerebrally into C57BL/6 and CCR2-deficient mice. Seven days after administration, CNS-mononuclear cells were prepared, and CD45$^+$CD11c$^+$ dendritic cells were quantified by flow cytometry. The mean of three animals is shown per group. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. lyDC = lymphoid dendritic cell; myDC = myeloid dendritic cell; inflDC = inflammatory dendritic cell.

Ransohoff, 2003; Xu *et al.*, 2007; Deshmane *et al.*, 2009). Furthermore, CCL2 deficiency affected EAE development only when targeted to the CNS (Dogan *et al.*, 2008). We therefore determined whether GM-CSF induces CCL2 expression in the brain. CCL2 was significantly up-regulated in the brain of mice with EAE (Fig. 2C) and of naïve mice after intra-cerebral GM-CSF application compared to control treated animals (Fig. 2D). Furthermore, CCR2-deficient mice treated intra-cerebrally with GM-CSF showed significantly reduced numbers of dendritic cells in the CNS (Fig. 2E). Thus, GM-CSF may play

an important role in both CCL2-mediated attraction of dendritic cells/monocytes and direct mobilization of precursors from the bone marrow.

Intra-cerebral GM-CSF administration results in the differentiation of functionally distinct dendritic cell subsets

Since we were able to identify both stimulatory and inhibitory CNS-dendritic cells during the acute phase of EAE (Fig. 1D), we determined whether GM-CSF can induce the recruitment of these functionally distinct dendritic cell populations in the CNS on its own. Lymphoid, myeloid and inflammatory dendritic cells isolated from the GM-CSF-treated CNS showed T cell stimulatory capacities comparable to the corresponding EAE-derived dendritic cell subsets. Lymphoid and myeloid dendritic cells stimulated CD4$^+$ T cell proliferation much more strongly than inflammatory dendritic cells (Fig. 3A). Furthermore, GM-CSF-elicited inflammatory dendritic cells inhibited T cell proliferation as seen with EAE-CNS-derived inflammatory dendritic cells (Figs 1D and 3B). This differential function of GM-CSF-driven dendritic cell subsets was also reflected in their ability to trigger cytokine production by T cells. To mimic the *in vivo* situation of the inflamed CNS, we used previously MOG-primed T cells for this assay. Production of the pro-inflammatory cytokines INF-γ and IL17 by encephalitogenic T cells was stimulated significantly less efficiently by inflammatory dendritic cells than by stimulatory lymphoid and myeloid dendritic cells (Fig. 3C and D).

To address whether these functional characteristics are specific to GM-CSF-elicited CNS-dendritic cells, we tested the functionality of CNS-dendritic cell subsets enriched after FL administration. While FL-induced lymphoid dendritic cells proved to be strongly stimulatory in an allogeneic proliferation assay, myeloid and inflammatory dendritic cells were neither stimulatory nor inhibitory in this setting (Supplementary Fig. 6). In summary, intra-cerebral GM-CSF, but not FL, recruits functionally distinct dendritic cell subsets showing characteristics similar to those of EAE-dendritic cells.

CNS-derived but not blood-derived inflammatory dendritic cells exhibit an inhibitory phenotype

Experiments with bone marrow-chimeric mice revealed that at peak EAE, around 20% of the dendritic cells present in the CNS are derived from CNS-resident cells (Ponomarev *et al*., 2005*b*). Using a similar approach, we investigated the origin of the functionally distinct lymphoid, myeloid and inflammatory dendritic cells. Bone marrow-chimeric mice were generated by reconstitution of lethally irradiated CD45.2$^+$ C57BL/6 mice with bone marrow from congenic CD45.1$^+$ mice. After two months of reconstitution, we induced EAE in these chimeras and examined the appearance of CNS-resident and peripheral dendritic cells in the CNS at peak disease. In agreement with previous publications (Ponomarev *et al*., 2005*b*), we found a ratio of CNS- versus periphery-derived dendritic cells of 1:4 (Fig. 4C). A hundred percent of CD11b^{int} microglia cells were CD45.2$^+$ (data not shown). Dendritic cell subset analysis demonstrated that the vast majority of CNS-derived dendritic cells (CD45.2$^+$CD11c$^+$) were inflammatory (Fig. 4B). In contrast, CD45.1$^+$CD11c$^+$ donor-derived dendritic cells consisted of lymphoid (3 ± 3.4%), myeloid (19 ± 7.4%) and inflammatory cells (78 ± 7.4%) (Fig. 4C).

Since total inflammatory dendritic cells exhibited an inhibitory phenotype (Fig. 1D) we assessed the relative inhibitory potential of CNS- and blood-derived inflammatory dendritic cells. CD45.2$^+$ and CD45.1$^+$ inflammatory dendritic cells were isolated from chimeric EAE diseased animals and tested in the inhibition assay. CNS-derived CD45.2$^+$ inflammatory dendritic cells inhibited T cell proliferation whereas blood-derived CD45.1$^+$ inflammatory dendritic cells did not either stimulate or inhibit proliferation (Fig. 4D). Thus, only inflammatory dendritic cells derived from CNS-resident precursors show an inhibitory phenotype. Interestingly, CNS- and periphery-derived inflammatory dendritic cells show no differences with respect to the expression of activation markers or co-stimulatory molecules (Supplementary Fig. 7).

We next investigated whether the same functionally distinct dendritic cell subsets could be induced by GM-CSF. Growth arrested GM-CSF-producing cells (5 × 10^5) were injected into naïve congenic bone marrow chimera. CNS-mononuclear cells were isolated after 7 days, and both CNS- and blood-derived lymphoid, myeloid and inflammatory dendritic cells were quantified. Approximately 97% of the dendritic cells were donor derived, with a main population of myeloid dendritic cells (Fig. 4E and F). The majority of the CNS-dendritic cells did thus not differentiate from CNS precursors but entered the CNS from the periphery. Among the CD45.1$^+$ donor-derived cells, the distribution of lymphoid, myeloid and inflammatory dendritic cells was similar to that of total CNS-dendritic cells in non-chimeric mice (Figs 2B and 4G). Interestingly, as seen in EAE, most of the CNS-derived dendritic cells were of the inflammatory dendritic cell phenotype (Fig. 4C and G). The absolute number of CNS-derived inflammatory dendritic cells obtained by GM-CSF treatment was approximately the same as in EAE (data not shown).

We also measured the ability of GM-CSF-elicited dendritic cells to restimulate primed encephalitogenic CD4$^+$ T cells to produce IFN-γ and IL17. Blood-derived myeloid dendritic cells induced the highest number of IFN-γ and IL17 producing cells (Fig. 4H and I). Intriguingly, the presence of CNS-derived inflammatory dendritic cells in the co-cultures did not trigger an increase in the production of IFN-γ or IL17 compared with T cells cultured in the absence of dendritic cells. In summary, with respect to cell numbers, the effect of intra-cerebral GM-CSF on the recruitment of peripheral dendritic cells dominates over local dendritic cell differentiation. However, the functionality of these GM-CSF-elicited dendritic cell subsets is identical to that of CNS-dendritic cells in EAE animals. Whereas myeloid, and blood-derived inflammatory, dendritic cells exhibit pro-inflammatory functions, CNS-derived inflammatory dendritic cells instead appear to down-modulate T cell activation. Thus, GM-CSF treatment of the CNS closely reproduces the pattern of dendritic cell recruitment that is observed in EAE.
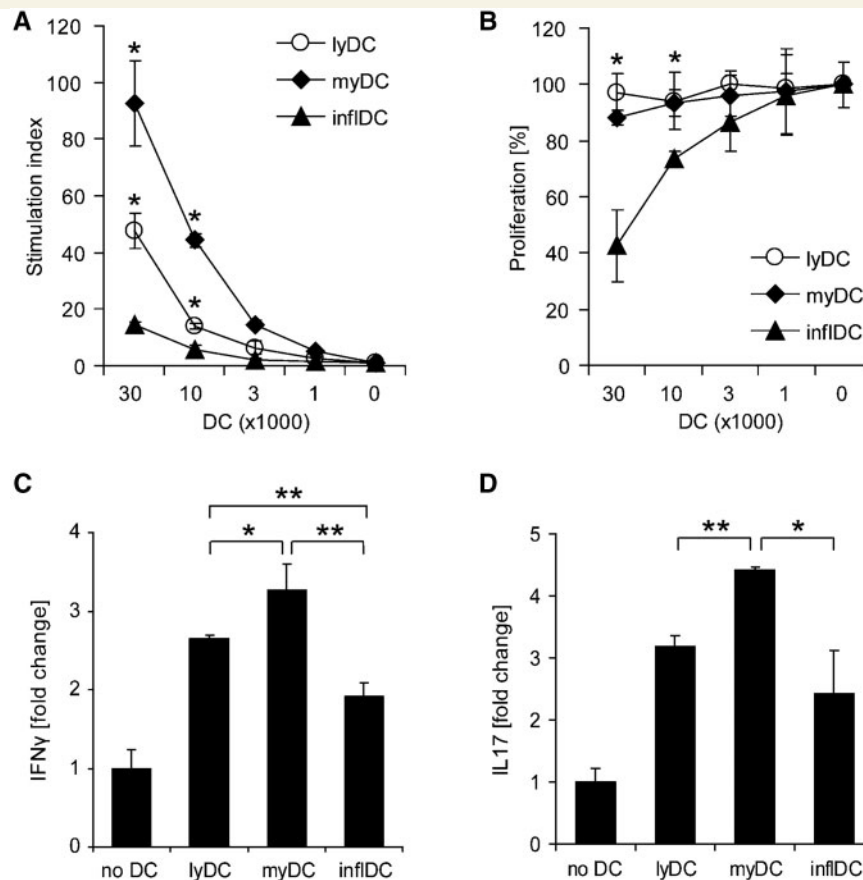
Figure 3 GM-CSF-recruited lymphoid, myeloid and inflammatory dendritic cells show different functions. Naïve C57BL/6 mice were treated intra-cerebrally with GM-CSF as described in the 'Materials and methods' section. After one week, CNS-dendritic cells were sorted by flow cytometry and their stimulatory capacity was tested. (**A** and **B**) Proliferation and inhibition assays were performed as described in Fig. 1. (**A**) CD4^{+} Balb/c T cells were co-cultured with titrating numbers of GM-CSF-dendritic cells. After 2 days, cells were pulsed with [3H]thymidine for another 16 h. (**B**) CD4^{+} T cells purified from Balb/c mice were co-cultured for 2 days with mature bone marrow-dendritic cells and titrating numbers of GM-CSF-dendritic cells. Proliferation was assessed by [3H]thymidine incorporation. The T cell proliferation induced by mature bone marrow-dendritic cells alone was set at 100%. The data represent two experiments. (**C** and **D**) EAE-primed CD4^{+} T cells were cultured with the indicated dendritic cell subsets in a 1:10 ratio. After 16 h, T cells were harvested and cultured on anti-IFN-γ (**C**) or anti-IL17 (**D**) pre-coated enzyme-linked immunosorbent spot-plates in the presence of 25 µg/ml MOG$_{35-55}$ peptide. Graphs show the fold change in spot numbers. Experiments were performed twice with similar results. *$P < 0.05$, **$P < 0.005$; **A** and **B** comparing lymphoid and myeloid to inflammatory dendritic cells. lyDC = lymphoid dendritic cell; myDC = myeloid dendritic cell; inflDC = inflammatory dendritic cell.

Subpopulations of dendritic cells present in the CNS show a characteristic location

The localization of dendritic cells in the naive CNS is restricted to the choroid plexus and the perivascular space (Matyszak and Perry, 1996; Suter et al., 2000; Fischer and Reichmann, 2001). To study whether the functionally different dendritic cell subpopulations are located at strategical sites within the inflamed CNS, we induced EAE in congenic bone marrow-chimeric animals (CD45.1→CD45.2) and studied at peak disease the location of the dendritic cells using triple-immunofluorescence microscopy. The analyses showed that the majority of the dendritic cells are located in perivascular cuffs in the white matter and in meningeal

infiltrates (Fig. 5B, F, O and S; Supplementary Table 1). The majority of dendritic cells at these sites are CD45.1[+] and thus are periphery derived (Fig. 5C, G, P and T). In contrast, hardly any CD45.2[+] CNS-derived dendritic cells are found in the meninges and perivascular cuffs (Fig. 5D, H, Q and U). In the white matter parenchyma an intriguing predominance of periphery derived CD45.1[+] inflammatory dendritic cells was evident (closed arrow heads in Fig. 5C and G). In contrast, periphery-derived F4/80[−] CD45.1[+] lymphoid or myeloid dendritic cells were rarely found at this site (open arrow head in Fig. 5C). The latter cells can also be identified as F4/80[−]CD45.2[−] bright red cells (open arrow head in Fig. 5H). CNS-derived CD45.2[+] inflammatory dendritic cells were almost exclusively found in the white matter parenchyma (arrows, CD45.2[+] in Fig. 5D and CD45.1[−] in Fig. 5G). Interestingly, although less dendritic cells are found in the grey
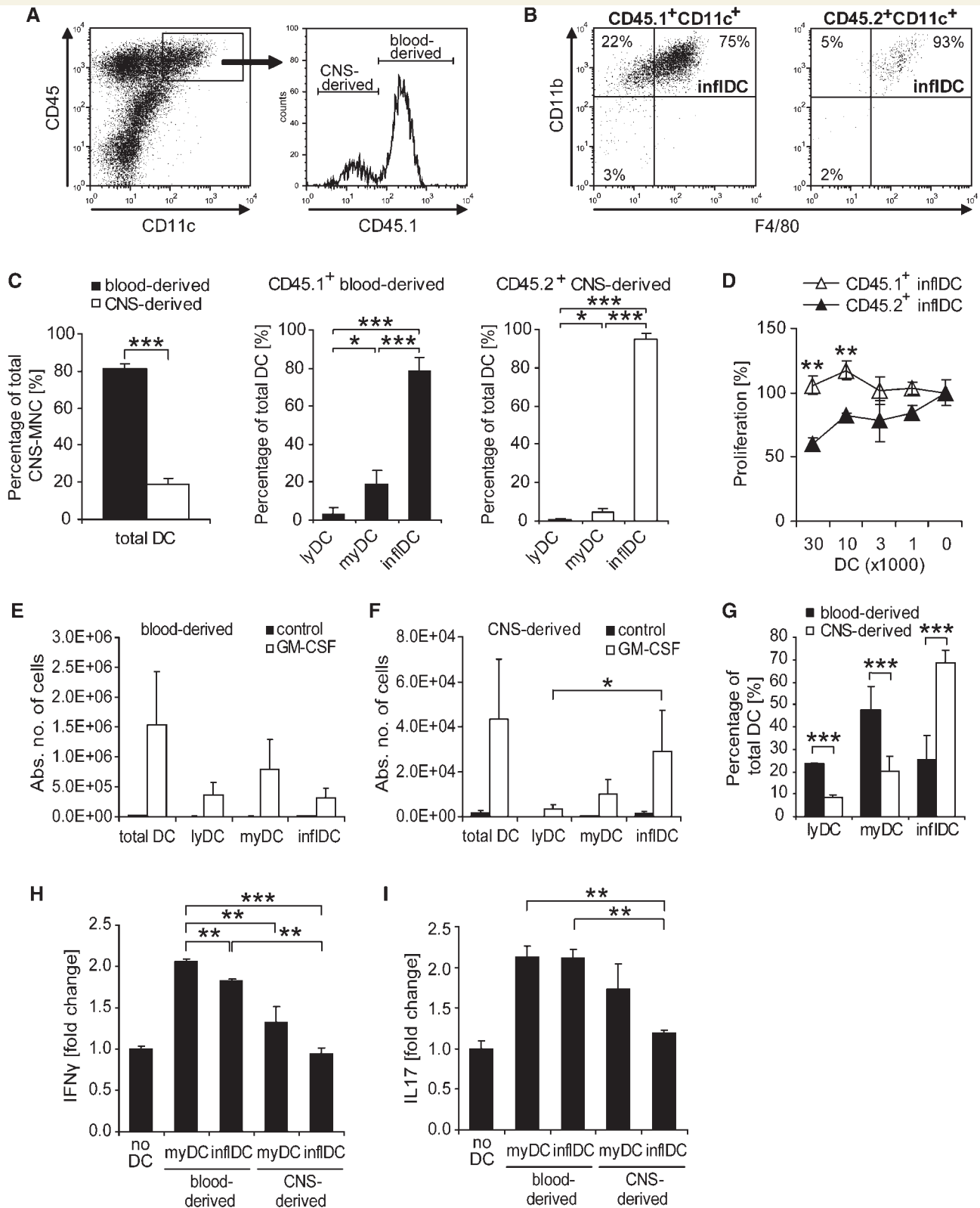
Figure 4 CNS-derived inflammatory dendritic cells inhibit T cell proliferation and pro-inflammatory cytokine production. Dendritic cells present in the CNS were analysed from a pool of chimeric animals at peak EAE (score 3) or 7 days after GM-CSF administration, respectively. (**A**) The chimerism of dendritic cells in the EAE-CNS was analysed by gating on CD11c^{+} cells in total CNS-mononuclear cells (dot plot) and CD45.1^{+} expression (histogram). (**B**) CD11b and F4/80 expression by CD45.1^{+}CD11c^{+} periphery-derived dendritic cells (dot plot left) and CD45.2^{+}CD11c^{+} CNS-derived dendritic cells (dot plot right) were analysed by flow cytometry. (**C**) Bar graphs show the mean of four experiments. Total dendritic cells (left) were expressed as percentage of CNS-mononuclear cells as indicated in panel **A**. The mean

(Continued)

matter parenchyma, peripheral CD45.1[+] inflammatory dendritic cells were readily identified there (closed arrow head in Fig. 5L) as well as peripheral macrophages and microglia in contrast to peripheral F4/80[−]CD45.1[+] lymphoid/myeloid dendritic cells. In summary, peripheral lymphoid and myeloid dendritic cells are preferentially found in the meninges and perivascular cuffs, the sites of invasion, whereas peripheral inflammatory dendritic cells are conspicuous in the parenchyma of both the white and grey matter. In contrast, CNS-derived inflammatory dendritic cells are almost exclusively found in the white matter parenchyma.

GM-CSF directs CNS precursors towards an inhibitory dendritic cell phenotype

To study whether microglia cells could be the CNS precursors giving rise to inhibitory F4/80^{+} dendritic cells, we treated primary microglia cultures with either GM-CSF or the control cytokine M-CSF. After 5 days, the cultures were harvested, and cells were analysed by flow cytometry or used as antigen presenting cells in allogeneic proliferation assays. Differentiation of microglia into dendritic cells was assessed by examining CD11c expression and upregulation of CD45. Of the GM-CSF-treated microglia, $11.2 \pm 4.8\%$ developed into dendritic cells, whereas almost no CD11c expressing cells were observed in untreated or M-CSF-treated cultures (Fig. 6A). Interestingly, CD45^{hi} cells expressed 2-fold higher levels of F4/80 in GM-CSF-treated cultures than in the control cultures (Table 1). No differences were detectable in the expression levels of major histocompatibility complex II, CD40, CD80 or CD86 (Table 1). Next, we assessed their ability to inhibit T cell proliferation in a manner similar to CNS-derived inflammatory dendritic cells. We indeed found that GM-CSF-treated microglia were inhibitory, whereas untreated or M-CSF-treated microglia slightly enhanced T cell proliferation (Fig. 6B). GM-CSF thus induces the differentiation of inhibitory CD11c^{+} F4/80^{+} cells in microglia cultures. This inhibitory phenotype is similar to that of the CNS-derived inflammatory dendritic cell subpopulation found in EAE and after intra-cerebral GM-CSF treatment.

Intra-cerebral GM-CSF leads to an overall exacerbation of EAE

The finding that GM-CSF induced the differentiation of inhibitory dendritic cells present in the CNS suggested that it might play a hitherto unrecognized anti-inflammatory role in CNS-directed immune responses, such as EAE. Previous reports had suggested a pro-inflammatory impact of GM-CSF in EAE. GM-CSF-deficient mice were found to be resistant to EAE, and mice treated with rGM-CSF exhibited an accelerated EAE onset and a slight exacerbation of clinical disease (McQualter *et al.*, 2001; Ponomarev *et al.*, 2007; King *et al.*, 2009). It should be pointed out, however, that GM-CSF was administered systemically in these studies. To evaluate whether intra-cerebral GM-CSF administration might modulate EAE differently from systemically administered GM-CSF, we treated EAE-diseased mice intra-cerebrally with GM-CSF-producing cells. The cells were administered at Days 4 or 11 after the first immunization with MOG$_{35-55}$ peptide, which would elicit maximal dendritic cell numbers 7 days later, during disease onset or the peak phase, respectively. Animals in which the onset phase was modulated developed disease 2 days earlier than the control group (Fig. 7A). Animals in which the peak phase was modulated developed significantly exacerbated disease (Fig. 7B). In contrast, EAE-diseased mice that received FL-producing cells at Days 4 or 11 did not show any significant differences in disease development (Fig. 7C). Furthermore, FL-deficient mice developed EAE almost normally (Fig. 7D) while GM-CSF-deficient animals are known to be protected from disease development (King *et al.*, 2009; McQualter *et al.*, 2001). Animals treated with FL at Days 4 or 11 showed no significant increase in dendritic cells present in the CNS compared to control EAE animals when analysed at either disease onset (Day 13) or early disease remission (Day 23) (Fig. 7G and H). However, in animals treated with GM-CSF, dendritic cells present in the CNS observed at disease onset were increased in numbers and showed the same distribution of lymphoid, myeloid and inflammatory dendritic cells as seen in periphery-derived dendritic cells present in the CNS populations of naïve GM-CSF-treated animals, with the main population being myeloid dendritic cells (Figs 4G and 7E). The composition of the dendritic cell populations in the CNS changed during the early remission phase: in both the GM-CSF-treated

Figure 4 Continued

of blood-derived (*middle*) or CNS-derived (*right*) lymphoid, myeloid and inflammatory dendritic cells was assessed gating on CD45.1^{+}CD11c^{+} or CD45.2^{+}CD11c^{+} cells as shown in panel **B**. (**D**) Purified periphery-derived inflammatory dendritic cells (CD45.1^{+} CD11c^{+}CD11b^{+}F4/80^{+}) and CNS-derived inflammatory dendritic cells (CD45.2^{+}CD11c^{+}CD11b^{+}F4/80^{+}) were co-cultured at indicated numbers with 10[5] naïve allogeneic T cells and 10[4] mature bone marrow-dendritic cells. Proliferation was assessed by [^{3}H]thymidine incorporation. Data are representative for three experiments. (**E** and **F**) Seven days after GM-CSF administration, the composition of blood-derived (**E**) and CNS-derived (**F**) lymphoid, myeloid and inflammatory dendritic cells in the CNS of chimeric mice was analysed by flow cytometry. Experiments were performed twice. (**G**) The bar graph displays the appearance of blood- and CNS-derived lymphoid, myeloid and inflammatory dendritic cells as percentage of total dendritic cells of two experiments. (**H** and **I**) Blood-derived and CNS-derived myeloid and inflammatory dendritic cells were sorted by flow cytometry and co-cultured overnight with EAE-primed T cells. T cells were harvested and cultured on anti-IFN-γ (**H**) or anti-IL17 (**I**) pre-coated enzyme-linked immunosorbent spot-plates in the presence of 25 µg/ml MOG$_{35-55}$ peptide. Graphs show the fold change in spot numbers. Data are representative for two independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$; **D** comparison of CD45.1[+] inflammatory dendritic cells to CD45.2[+] inflammatory dendritic cells. lyDC = lymphoid dendritic cell; myDC = myeloid dendritic cell; inflDC = inflammatory dendritic cell.
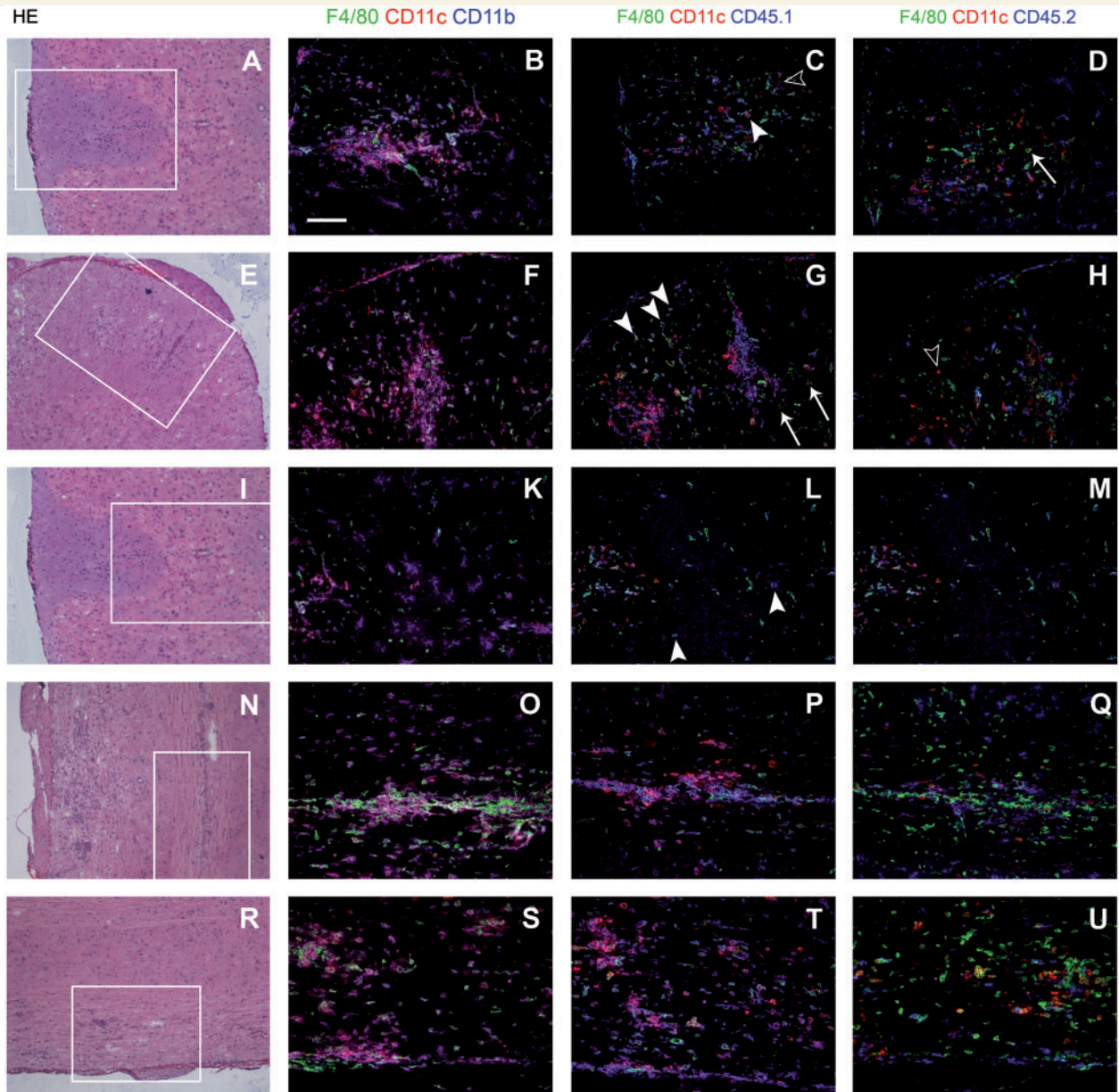
Figure 5 CNS-derived dendritic cell subpopulations show a characteristic location. EAE was induced in congenic bone marrow-chimeric animals (CD45.1→CD45.2). Cryo-histology was prepared at peak clinical stage. Consecutive sections were stained either with haematoxylin and eosin (HE) or with combinations of antibodies to F4/80 (green), CD11c (red) and either CD11b, CD45.1 or CD45.2 (blue), respectively. The white frame in the haematoxylin and eosin stains indicates the detail region shown in the fluorescent immunohistology. (**A–M**) Cross-sections of upper spinal cord, (**N–U**) longitudinal spinal cord sections, (**A–D**) dorsal funiculus, (**E–H**) lateral funiculus, (**I–M**) grey matter commisure, (**N–Q**) ventral commissure, (**R–U**) ventral part of lateral funiculus. The scale bar for all immunohistology is shown in **B** and corresponds to 100 µm. Open arrow heads indicate peripheral $CD45.1^+CD45.2^-$ stimulatory dendritic cells (lymphoid/myeloid). Closed arrow heads indicate peripheral $CD45.1^+CD45.2^-$ inflammatory dendritic cells. Arrows indicate CNS-derived $CD45.1^-CD45.2^+$ inflammatory dendritic cells. Colour split pictures are provided in Supplementary Fig. 8A and B.

mice and the control group, we observed mainly inflammatory dendritic cells (Fig. 7F). The fact that there is a significant change in the composition of the dendritic cell populations in the GM-CSF group over time (Fig. 7E and F; dendritic cell ratio myeloid:inflammatory 1.17 ± 0.13 and 0.65 ± 0.29 for analysis at Day 13 and 23, respectively; $P < 0.05$) suggests that GM-CSF boosts the naturally occurring shift as shown in Fig. 1B. Notably, the change in dendritic cell populations over time when GM-CSF was applied during EAE (Fig. 7E and F) is reminiscent of the different pattern of periphery- versus CNS-derived dendritic cells observed after GM-CSF treatment of naïve bone marrow chimera (Fig. 4G). Thus, the induction of anti-inflammatory CNS-derived dendritic cells by GM-CSF might be important during the remission phase of EAE, while the impact of GM-CSF on the recruitment of stimulatory blood-derived dendritic cell subsets dominates during the initiation phase of the disease.
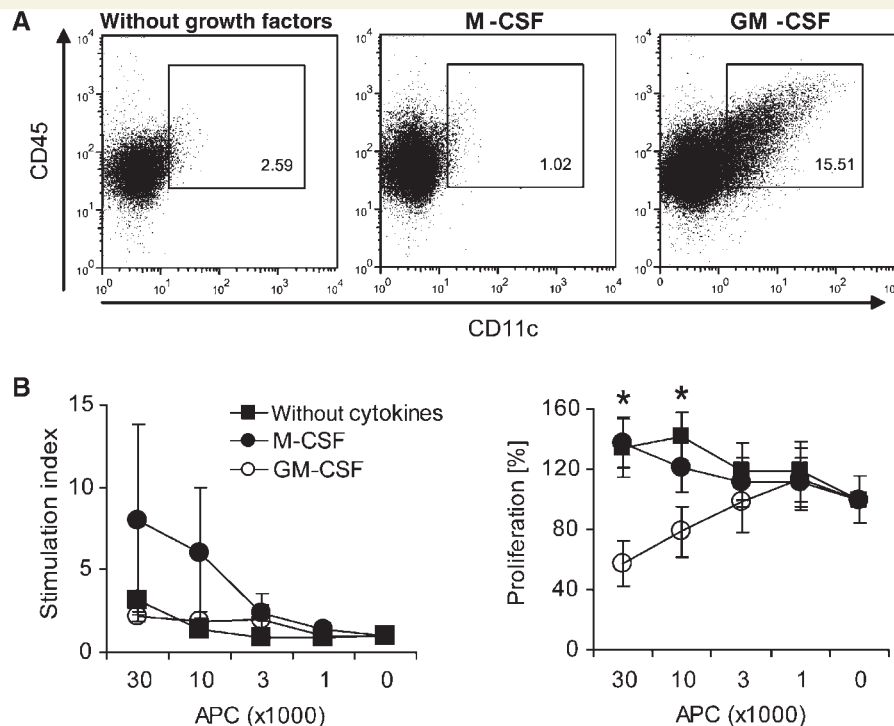
Figure 6 GM-CSF-treated microglia exhibit an inhibitory inflammatory dendritic cell-like phenotype. Primary microglia cells were cultured for 5 days without growth factors (w/o) or with either GM-CSF (40 ng/ml) or M-CSF (20 ng/ml). (**A**) The differentiation of CNS-resident cells into dendritic cells was assessed by determining the percentage of CD45hiCD11c+ cells in total FSChiCD11b+ cells. (**B**) The inhibitory capacity of untreated, M-CSF- and GM-CSF-treated microglia was tested by performing proliferation and inhibition assays as detailed in Fig. 1. Data represent the mean of four independent experiments (means ± SEM). *$P < 0.005$ comparing GM-CSF-treatment versus untreated and M-CSF treatment, respectively. APC = antigen presenting cell.

Table 1 Differentiation of CNS precursor cells upon GM-CSF treatment

	Dendritic cells[a] (%)	F4/80[b]	Major histocompatibility complex II[b]	CD40[b]	CD80[b]	CD86[b]
Untreated	1.6 ± 0.8	6.5 ± 1.1	4.0 ± 0.5	4.0 ± 0.5	177 ± 125	114 ± 66
M-CSF	0.6 ± 0.2	6.1 ± 1.2	3.9 ± 0.02	3.9 ± 0.04	146 ± 20	129 ± 36
GM-CSF	11.2 ± 4.8	12.8 ± 1.7	4.5 ± 0.7	4.2 ± 0.3	128 ± 53	131 ± 60

Microglia cultures were treated with M-CSF or GM-CSF, or were left untreated. After 5 days cells were harvested and stained for CD45, CD11c, CD11b, F4/80 and the indicated activation markers.
a The presence of dendritic cells (CD45hiCD11c+) is given as percentage of FSChiCD11b+ cells (three independent experiments).
b Values represent mean fluorescence intensity of the respective activation markers with gating on FSChiCD45hi populations (three independent experiments).

Discussion

Here, we show that although both FL and GM-CSF levels are increased in the CNS during EAE, only GM-CSF expression in the naïve CNS reproduces the accumulation of dendritic cell populations similar to the ones observed in EAE (Figs 1–3). Appearance of the characteristic F4/80+ dendritic cell subset can be explained by GM-CSF-induced recruitment of inflammation-associated dendritic cells or 'inflammatory' dendritic cells (Geissmann et al., 2003; Shortman and Naik, 2007; King et al., 2009). The periphery-derived inflammatory dendritic cells are CD11bhiF4/80+ and are thus likely to correspond to the 'inflammatory monocyte'-derived dendritic cells described in EAE by King et al. (2009) and Mildner et al. (2009), and are probably also present in the tumour

necrosis factor-α and inducible nitric oxide synthase-producing myeloid dendritic cell population described by Dogan et al. (2008). Interestingly, we found higher tumour necrosis factor-α levels in T cell:dendritic cell co-cultures in the presence of inflammatory compared with lymphoid or myeloid dendritic cells, further suggesting that the periphery-derived inflammatory dendritic cells are related to these previously described inflammatory dendritic cell populations (data not shown). In addition, we found that GM-CSF also induces CNS-resident precursors such as microglia cells to differentiate into local inflammatory dendritic cells (Figs 4 and 6). The latter cells thus represent tissue-derived GM-CSF-induced inflammatory dendritic cells. The increase in F4/80+ dendritic cells in the inflamed CNS is characteristic for the site of inflammation, as the numbers of F4/80+ dendritic
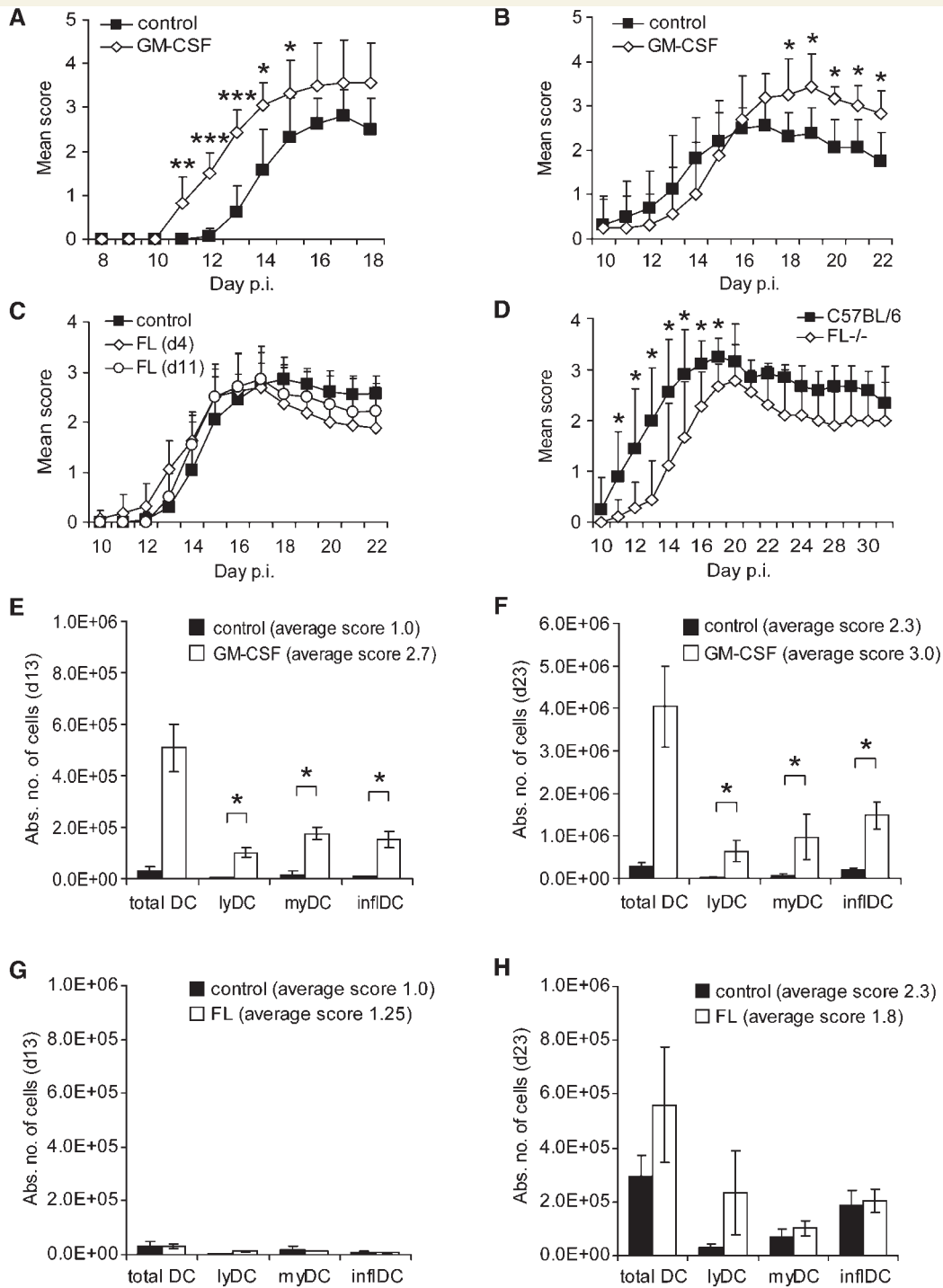
Figure 7 The impact of GM-CSF on stimulatory dendritic cells dominates over its role in the induction of inhibitory dendritic cells. C57BL/6 mice were immunized with MOG$_{35-55}$ peptide, treated with GM-CSF-producing, FL-producing or control cells at Days 4 (**A** and **C**) or 11 (**B** and **C**), and clinical scores were assessed as described in 'Materials and methods'. Experiments were done twice with eight mice per group and experiment. (**D**) FL-deficient or C57BL/6 mice were immunized with MOG$_{35-55}$ peptide (9–10 mice per group). (**E–H**) Mononuclear cells from brain tissue (**E** and **G**) or the CNS (**F** and **H**) were isolated and analysed by flow cytometry for the composition of lymphoid, myeloid and inflammatory dendritic cells. Mice that had received GM-CSF- or FL-producing cells for modulation of the onset phase were analysed at Day 13 (**E** and **G**). Mice treated with GM-CSF or FL for modulation of the peak phase were analysed during early disease remission at Day 23 (**F** and **H**). The data show the mean of three animals per group and time point. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$; **A–D** comparison to control series). lyDC = lymphoid dendritic cell; myDC = myeloid dendritic cell; inflDC = inflammatory dendritic cell; p.i. = post immunization.

cells in the periphery (spleen) of EAE animals is low and these cells are not inhibitory (data not shown).

Interestingly, we found that the different dendritic cell subsets present in the CNS vary in their ability to stimulate T cells. The CD11b$^+$F4/80$^+$ inflammatory dendritic cells inhibit T cell proliferation and pro-inflammatory cytokine production whereas CD11b$^-$ lymphoid dendritic cells and CD11b$^+$F4/80$^-$ myeloid dendritic cells stimulate T cell proliferation and the production of IFN-γ and IL17 by encephalitogenic T cells (Figs 1 and 3). Intriguingly, previous reports on 'inflammatory' dendritic cells also describe them as poor antigen presenting cells (Shortman and Naik, 2007; Varol et al., 2009) and to be dispensable for T cell priming in L. monocytogenes infection (Serbina et al., 2003). Also interesting are the findings that after alum immunization, inflammatory monocytes develop into dendritic cells and induce a T-helper cell 2 response (Kool et al., 2008). These examples, numerous other related reports, and our own study show that the cells of the mononuclear phagocyte system, monocytes and their descendants (tissue macrophages and dendritic cells), are highly plastic and can develop into cells of various phenotypes and functions depending on the stimuli (cytokines, microbial compounds and tissue) they encounter (Serbina et al., 2003; Gordon and Taylor, 2005; Shortman and Naik, 2007; Kool et al., 2008; Auffray et al., 2009; Merad and Manz, 2009; Varol et al., 2009). FL has been reported to induce dendritic cell accumulation in the CNS and enhance EAE severity when administered intraperitoneally in the form of recombinant human FL (Greter et al., 2005). We found that intra-cerebral treatment with murine FL does not have a major influence on dendritic cell recruitment in the CNS or EAE development (Fig. 7). Although FL treatment of naïve mice results in an increased number of dendritic cells present in the CNS, in comparison to GM-CSF-elicited dendritic cells these numbers remain significantly lower (Fig. 2 and Supplementary Fig. 5). We did not find increased levels of FL in the sera, spleen or lymph nodes after intra-cerebral treatment (data not shown), suggesting that the growth factor acts locally. The different consequences of intraperitoneal versus intra-cerebral FL administration suggest that FL exerts different functions in the periphery and CNS. Moreover, the species origin of the FL used influences the quality and quantity of the dendritic cell response and thus might play a role on the experimental outcome as well (O'Keeffe et al., 2002).

The era of GM-CSF as a crucial cytokine in CNS autoimmune inflammation began with the studies of McQualter et al. (2001), showing complete protection of GM-CSF-deficient animals after EAE induction and recovery of susceptibility after administration of recombinant GM-CSF. In addition, Ponomarev et al. (2007) showed that T cells from GM-CSF-deficient mice cannot induce EAE. More recently it was shown with a series of elegant experiments that systemically applied GM-CSF mobilizes Ly6C$^+$ circulating monocytes, which are able to migrate to the inflamed CNS and subsequently differentiate into dendritic cells (King et al., 2009). However, these experiments did not provide an explanation of how GM-CSF could lead to the recruitment of monocytes and dendritic cells into the CNS in EAE nor did they analyse different subpopulations of dendritic cells. Recently it has been found that intra-cerebro-ventricularly infused GM-CSF attracts

stimulatory dendritic cells into the CNS of naïve animals (Mausberg et al., 2009). This study did not, however, investigate subpopulations of dendritic cells, which is an important caveat because our results show that CNS-dendritic cells differ in their function and origin. Moreover, the discrepancy with our findings might be due to the methodology applied. GM-CSF injection into the ventricles might just reach the meninges and thus only induce attraction of periphery-derived dendritic cells while intra-parenchymal production of GM-CSF also substantially acts on microglia.

Interestingly, numerous publications have shown the essential implication of CCL2 and its receptor CCR2 in the development of EAE and other inflammatory diseases (Fife et al., 2000; Huang et al., 2001; Dogan et al., 2008). CNS-derived CCL2 is required for full-blown EAE and for recruitment of CD11b$^+$ tumour necrosis factor-α and inducible nitric oxide synthase producing dendritic cells (Dogan et al., 2008), suggesting that these comprise inflammatory monocyte-derived dendritic cells as defined by Shortman and Naik (2007). A recent detailed analysis on the role of CCR2 in EAE showed that CCR2 expression on the cells of the mononuclear phagocyte system, but not on lymphocytes or endothelial cells, is required for full-blown EAE (Mildner et al., 2009). In light of these findings, we wondered whether GM-CSF leads to recruitment of inflammatory monocytes and dendritic cells by induction of CCL2. Indeed, we found that CCL2 is expressed in the CNS both during EAE and after intra-cerebral GM-CSF expression in naïve animals (Fig. 2). Furthermore, the recruitment of dendritic cells by GM-CSF is partially dependent on CCR2 signalling. The sources producing CCL2 in response to GM-CSF remain to be determined. However, numerous cell types have been shown to produce CCL2 (Deshmane et al., 2009) and at least human monocytes are able to do so upon GM-CSF stimulation (Steube et al., 1999). Collectively, our results thus suggest that GM-CSF produced in the inflamed CNS induces local production of CCL2, which in turn attracts CCR2$^+$ monocytes to the CNS. However, at least part of the recruitment of dendritic cells to the CNS after intra-cerebral GM-CSF administration could be due to increased GM-CSF levels in the periphery (Fig. 2A and B), which may lead to mobilization of dendritic cell precursors directly from the bone marrow.

Dendritic cells do not only migrate into the CNS from the periphery, but also differentiate locally within the CNS. Several research groups have shown that GM-CSF induces the differentiation of dendritic cells from resident microglia cells (Fischer and Reichmann, 2001; Santambrogio et al., 2001; Ponomarev et al., 2005a). Using bone marrow chimeras, we found that CNS-derived inflammatory dendritic cells inhibit T cell activation whereas periphery-derived inflammatory dendritic cells, as well as lymphoid dendritic cells and myeloid dendritic cells, stimulate T cell proliferation and pro-inflammatory cytokine production in the CNS (Fig. 4). Moreover, both during EAE and in GM-CSF-treated naïve mice, the majority of CNS-derived dendritic cells are CD11b$^+$F4/80$^+$ inflammatory dendritic cells. The number of CNS-derived inflammatory dendritic cells induced by GM-CSF is virtually identical to that observed in EAE, suggesting that GM-CSF is required and sufficient for the development of an optimal number of inhibitory CNS-derived dendritic cells during EAE.

The fact that purified CNS-derived inflammatory dendritic cells, which should be enriched in the inhibitory cell population, do not mediate a stronger per cell inhibition compared to unseparated inflammatory dendritic cells (Figs 1D and 4D) might be due to an instructive effect of the inhibitory cells on the other dendritic cells, rendering the latter inhibitory as well (Supplementary Fig. 9). Strikingly, there is no difference between inflammatory dendritic cells of peripheral and CNS origin with respect to expression of the classical antigen presenting cell molecules major histocompatibility complex II, CD40, CD80 and CD86 (Supplementary Fig. 7). The difference in the functional abilities of these cells does therefore not arise from differences in the expression of these stimulatory molecules. The mechanism underlying the inhibitory function of inflammatory dendritic cell is under investigation in our laboratory.

We wondered whether the different dendritic cell populations identified here localize—from an immunological point of view—to functionally relevant sites within the CNS. To address this issue we performed triple-immunofluorescence microscopy on spinal cord sections of CD45.1→CD45.2 bone marrow-chimeric animals with EAE (Fig. 5 and Supplementary Table 1). The majority of CD45.1$^+$ peripheral dendritic cells were found in meningeal and perivascular cuffs, which are believed to be the entry sites of peripheral leukocytes into the CNS. Interestingly, the periphery-derived lymphoid and myeloid dendritic cells that we found to be strong T cell stimulators (Fig. 1C) were identified almost exclusively at these sites. Their positioning close to vessels and meninges may allow the efficient restimulation of infiltrating T cells. In contrast to lymphoid and myeloid dendritic cells, periphery-derived inflammatory dendritic cells can also be identified in the parenchyma of both the white and grey matter. The function of peripheral inflammatory dendritic cells in CNS inflammation is still unclear and needs further investigation. Monocyte-derived inflammatory dendritic cells might contribute to disease severity in the effector phase of EAE but might as well be involved in tissue repair and recovery from EAE (Mildner *et al.*, 2009). With respect to CNS-derived inflammatory dendritic cells, which are almost exclusively found in the white matter parenchyma, we speculate that these inhibitory dendritic cells prevent the inflammation from spreading throughout the parenchyma and thereby protect the vital neural network from immune-mediated damage.

To investigate the origin of GM-CSF-induced CNS-derived inflammatory dendritic cells further, we cultured neonatal microglia cells *in vitro* in the presence of recombinant GM-CSF. GM-CSF-treated microglia differentiate into dendritic cells, express F4/80 and, remarkably, inhibit T cell proliferation (Fig. 6). This suggests that GM-CSF can promote the differentiation of inhibitory CNS-derived inflammatory dendritic cells. A similar finding was reported in a recent *in vitro* study on human microglia (Lambert *et al.*, 2008). It is possible that microglia, as they are present in an 'immune privileged' site, have a higher capacity to differentiate into inhibitory dendritic cells than into stimulatory pro-inflammatory antigen presenting cells. Such a concept would be consistent with the notion that during the course of inflammation tissue-resident monocytes, which are derived from Ly6C$^-$CX3CR1$^+$ blood monocytes in steady state conditions (Geissmann *et al.*, 2003), can differentiate in non-lymphoid organs into M2 macrophages that initiate tissue remodelling and

immune deviation, for instance in the myocardium (Nahrendorf *et al.*, 2007). However, under steady state conditions not only Ly6C$^-$CX3CR1$^+$ but also Ly6C$^+$CX3CR1$^-$ monocytes can give rise to regulatory mononuclear phagocyte system cells, as shown for the tolerogenic dendritic cells of the gut (Coombes and Powrie, 2008; Varol *et al.*, 2009).

The induction of inhibitory CNS-derived dendritic cells by GM-CSF appears to be in contradiction with the findings that GM-CSF administered in the context of EAE enhanced disease severity (Fig. 7A and B) and that GM-CSF-deficient mice are protected from EAE development (McQualter *et al.*, 2001; Ponomarev *et al.*, 2007), which suggest that GM-CSF has mainly a pro-inflammatory role in EAE. However, our results indicate that the impact of GM-CSF on dendritic cell recruitment in the CNS during EAE depends on the time at which it is produced (Fig. 7E and F). It is thus likely that the relative impact of GM-CSF on the recruitment of stimulatory myeloid dendritic cells versus the development of inhibitory inflammatory dendritic cells depends on the time point of action in disease progression and hence the cytokine milieu in the CNS.

Taken together, our results show that dendritic cells present in the CNS of EAE-diseased mice comprise several phenotypically and functionally distinct dendritic cell subpopulations, including the new subset of inflammatory dendritic cells. The latter express F4/80 and are able to prevent T cell stimulation. These cells may therefore act as CNS regulatory dendritic cells implicated in EAE remission. Moreover, we found that GM-CSF administration results in accumulation of the same dendritic cell subsets present in the CNS as seen in the EAE-diseased CNS. Consequently, GM-CSF does not only have a 'pro-inflammatory' role, but also exerts an anti-inflammatory function in the 'immune privileged' CNS. However, despite the potential of GM-CSF to promote intra-cerebral dendritic cells that might act as disease modulators, our experiments show that intra-cerebral treatment with GM-CSF in an inflammatory condition such as multiple sclerosis is not indicated. Instead, the prevention of monocyte/dendritic cell influx into the CNS by neutralization of GM-CSF or of chemo-attractants such as CCL2 might present a new future therapy for CNS inflammation.

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Supplementary material

Supplementary material is available at *Brain* online.

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